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## **MC1R variants increased the risk of sporadic cutaneous melanoma in darker-pigmented Caucasians: a pooled-analysis from the M-SKIP project**

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## Abstract

The *MC1R* gene is a key regulator of skin pigmentation. We aimed to evaluate the association between *MC1R* variants and the risk of sporadic cutaneous melanoma (CM) within the M-SKIP project, an international pooled-analysis on *MC1R*, skin cancer and phenotypic characteristics. Data included 5,160 cases and 12,119 controls from 17 studies. We calculated a Summary Odds Ratio (SOR) for the association of each of the nine most studied *MC1R* variants and of variants combined with CM by using random-effects models. Stratified analysis by phenotypic characteristics were also performed.

Melanoma risk increased with presence of any of the main *MC1R* variants: the SOR for each variant ranged from 1.47 (95%CI: 1.17–1.84) for V60L to 2.74 (1.53–4.89) for D84E. Carriers of any *MC1R* variant had a 66% higher risk of developing melanoma compared to wild-type subjects (SOR; 95%CI: 1.66; 1.41–1.96), and the risk attributable to *MC1R* variants was 28%. When taking into account phenotypic characteristics, we found that *MC1R*-associated melanoma risk increased only for darker-pigmented Caucasians: SOR (95%CI) was 3.14 (2.06–4.80) for subjects with no freckles, no red hair and skin type III/IV.

Our study documents the important role of all the main *MC1R* variants in sporadic CM and suggests that they have a direct effect on melanoma risk, independently on the phenotypic characteristics of carriers. This is of particular importance for assessing preventive strategies, which may be directed to darker-pigmented Caucasians with *MC1R* variants as well as to lightly-pigmented, fair-skinned subjects.

## Keywords

melanocortin-1 receptor; melanoma; meta-analysis; genetic epidemiology

## Introduction

Starting from the 20<sup>th</sup> century, both incidence and mortality for cutaneous melanoma (CM) has increased among populations of European descent and the estimated annual melanoma incidence rate in the world is around 2.8 per 100.000.<sup>1</sup> Of the three most prevalent types of skin cancer, melanoma is the most lethal, with a five-year survival rate for metastatic melanoma of 11%.<sup>2</sup> Because of melanoma's high incidence and poor treatment outcome for metastatic disease, development of early detection and preventive strategies are of the highest importance.

Among risk factors for CM, epidemiological studies highlighted the role of sun exposure, and of personal characteristics, such as fair skin, light hair and eyes color, high number of melanocytic naevi, and family history of melanoma.<sup>3–5</sup> Twins studies estimated that genetic factors provide a contribution to CM risk of 18–55%, and familial melanoma was estimated to account for approximately 10% of new cases.<sup>6</sup>

The melanocortin-1-receptor gene (*MC1R*, MIM#155555) is the most common low risk susceptibility gene for melanoma and a key regulator of skin pigmentation. It is located on chromosome 16q24.3 and encodes for a seven pass transmembrane G-protein coupled-receptor of 317 amino acids, positively coupled to adenylate cyclase to increase cAMP levels upon binding its endogenous ligand, the  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH).<sup>7</sup> Binding of  $\alpha$ -MSH to the functional *MC1R* on melanocytes stimulates the synthesis of eumelanin pigments<sup>8</sup> resulting in significant increases of the ratio of black/brown eumelanin to red/yellow pheomelanin pigments. While eumelanin has been shown to be photoprotective, pheomelanin is poorly photoprotective and may even contribute to cancer risk through the production of free radicals in response to UV exposure.<sup>9</sup> The *MC1R* gene locus is highly polymorphic in populations of European origins, with more than 80 variants identified.<sup>10</sup> *MC1R* variant alleles, resulting in amino acid substitutions that have been shown to reduce receptor function<sup>11–13</sup>, result in a quantitative shift of melanin synthesis from eumelanin to pheomelanin,<sup>7</sup> and determination of the so called “red hair color” (RHC) phenotype, characterized by the co-occurrence of fair skin, red hair, freckles and UV irradiation (UVR) sensitivity (poor tanning response and solar lentigines).

Several studies in different populations have reported that the risk of melanoma is higher among individuals who carry *MC1R* variant alleles. More recently, meta-analyses and genome-wide association studies (GWAS) confirmed this finding<sup>14–18</sup>. Although melanoma risk attributable to *MC1R* may arise through the determination of the tanning response of skin to UV light, some studies and a recent meta-analysis<sup>15</sup> observed that *MC1R*-associated CM relative risk was stronger in darkly-pigmented subjects and for those with lower levels of recreational sun exposure. These results suggest that *MC1R* variants may partly mediate their effect through biological pathways that are independent of pigmentation and UV exposure. In keeping with this possibility, wild type (WT) *MC1R* has been shown to trigger DNA repair mechanisms and antioxidant defenses in UVR-exposed melanocytes, while inactivated *MC1R*, resulting in production of pheomelanin, increases damage from reactive oxygen species, even in the absence of UV-exposure.<sup>19,20</sup> Those mechanisms may be of importance for at least some of the variant alleles.

Although the previous meta-analyses and GWAS gave reliable evidence of a role of *MC1R* in CM development, the lack of access to individual epidemiological information precluded in-depth investigations, including the assessment of the role of possible confounders, the estimation of melanoma risk according to different *MC1R* variants compared to WT subjects, and stratification for phenotypic characteristics. These investigations are in fact crucial for sporadic CM, which represents up to 95% of melanoma cases and is a complex and heterogeneous disease, probably the result of interactions between genetic, phenotypic and environmental factors.

The aim of this work is to evaluate the association between specific and combined *MC1R* variants and the risk of sporadic CM, and to evaluate whether risk estimates varied according to different phenotypic characteristics through a large multicenter pooled-analysis of individual data from the Melanocortin-1 receptor gene, SKin cancer and Phenotypic characteristics (M-SKIP) project.

## Material and methods

Data for the present analyses were gathered through the M-SKIP project. A description of the project was previously published.<sup>21</sup> Briefly, we searched for published and unpublished epidemiological studies on *MC1R* variants, sporadic CM, non-melanoma skin cancer (NMSC), and phenotypic characteristics associated with melanoma. Original individual data on participants in each identified study were requested from principal investigators. From May 2009 to December 2010, 43 investigators were contacted and 31 (72%) agreed to participate. Non-participant investigators were those who either did not reply to our invitation letter, were not able to retrieve the original dataset and/or were not interested in the project. More details are reported elsewhere<sup>21</sup>. Participant investigators sent their data along with a signed statement declaring that their original study was approved by an Ethics Committee and/or that study subjects provided a written consent to participate in the original study. Quality controls and data coding were performed, and the pooled database was created, including data on 7,806 CM cases, 3,151 NMSC cases and 14,875 controls.

For the purpose of the present study, we selected from the M-SKIP database all the melanoma case-control studies (N=17)<sup>15, 22–36</sup>, thus including data on 5,160 CM cases and 12,119 controls overall. Case-only or control-only studies, and studies on NMSC were excluded from the present analysis.

## Statistical analysis

In order to evaluate the representativeness of the M-SKIP dataset, the main study population characteristics reported in publications of non-participating investigators were extracted and compared with those of studies included in the pooled-analysis, by the Chi-Square test for categorical variables and by the Wilcoxon two-sample test for continuous variables. We also assessed possible participation bias by drawing funnel plots and by Egger's test. We verified the departure of frequencies of each *MC1R* variant from expectation under Hardy-Weinberg (HW) equilibrium by the Chi Square test in controls for each included study.

We calculated study-specific Odds Ratio (OR) with 95% Confidence Interval (CI) by applying logistic regression to the data from each study. Beyond *MC1R*, each model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, family history of melanoma, number of common total body naevi count and presence of atypical naevi. Because of different definitions of covariates between studies, particular attention was given to recoding and standardizing the variables in the M-SKIP database, as previously discussed.<sup>21</sup> For each study, we imputed missing data with multiple imputation models for variables with less than 20% of missing data, by using the iterative Markov chain Monte Carlo method. For each of the nine most studied *MC1R* variants (V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q,

D294H), we tested different inheritance models and found that the dominant model was the one with the lowest Akaike's Information Criterion for almost all the studies and variants, therefore we assumed this model of inheritance in the pooled-analyses. We performed two different variant-specific analyses: the first one included the 12 studies in which *MC1R* was sequenced, for which we could identify the WT subjects as reference category; the second one included all the 17 studies and used as reference category for each variant the subjects without that variant. For studies which sequenced the *MC1R* gene, beyond the *MC1R* variant-specific OR, we estimated the OR associated with the most frequently observed haplotypes. Haplotype frequencies for the nine most common variants were calculated with the EM algorithm using UNPHASED, version 3.1.7. Rare haplotypes (frequency < 0.005) were ignored. In addition, we took into account all the identified (common and rare) variants and calculated the OR for 1) carrying at least one *MC1R* variant compared to WT, 2) carrying just one and carrying 2 *MC1R* variants compared to WT. Finally, a *MC1R* score was calculated, based on classification of likely pathogenicity of variants using bioinformatics analysis as implemented by Davies et al.<sup>37</sup> briefly, the score was calculated by summing across the *MC1R* alleles, giving a value of 1 to “r” and 2 to “R” variants.

Following the two-stage analysis approach, we pooled study-specific OR with random-effects model, using the DerSimonian-Laird method. When there were more than one OR calculated in a single study (i.e. analysis by *MC1R* score), the correlation between the ORs was taken into account by using a multivariate approach previously described.<sup>38</sup> We evaluated homogeneity among study-specific estimates by the Q statistic and I-Square, which represents the percentage of total variation across studies that is attributable to heterogeneity rather than to chance. When a significant heterogeneity was detected, we performed meta-regression to assess the influence on Summary Odds Ratio (SOR) of different study features, such as publication year, study area, genotyping methodology, deviation from HW equilibrium, source of controls, and source of DNA. In order to evaluate the robustness of the results, we also examined changes in SOR after exclusion of specific studies, and we compared the pooled-OR obtained on the M-SKIP dataset with the meta-OR calculated by pooling risk estimates reported in studies from not-participating investigators. This latter meta-OR was also obtained with DerSimonian-Laird random-effects models.

We computed the attributable risk in the population for each of the nine *MC1R* variants listed above and for the presence of at least one *MC1R* variant by using the Miettinen's formula:  $(OR - 1 / OR) \times$  proportion of cases exposed, with the corresponding 95% CI.

Finally, in order to investigate whether the observed association between *MC1R* variants and melanoma varied according with different phenotypic characteristics, we performed stratified analysis. We defined subjects with RHC phenotype as those with either red hair color, freckles or high sun sensitivity (skin type I or II according to the Fitzpatrick classification), and as darker-pigmented individuals those subjects with none of the above phenotypic characteristics. The hypothesis of homogeneity of ORs among strata was tested by meta-regression models with random-effects and restricted maximum likelihood estimates, after the calculation of strata-specific OR in each study. The correlation between the ORs calculated in the same studies was taken into account by using the multivariate approach proposed by van Houwelingen et al.<sup>38</sup>

P-values <0.05 were considered statistically significant for all the tests but Q statistic, where p-values <0.10 were considered statistically significant. Both the false discovery rate approach and the more conservative Bonferroni correction were also used to take into account the problem of multiple comparisons. The analysis was carried out by using the software SAS (version 9.2) and STATA (version 11.2).

## Results

Studies included in our pooled-analysis did not differ from studies from not-participating investigators according to publication period, study area, phenotype assessment, source of controls, genotyping methodology, mean age of cases and controls, sex distribution of cases and controls.

Among the 17 studies included in the pooled-analysis, no deviation from HW equilibrium was observed for the following *MC1R* variants: V60L, D84E, V92M, I155T, and R163Q. Deviation from HW equilibrium was observed in one study<sup>34</sup> for the R142H variant, in four<sup>23,26,27,33</sup> for R151C variant, in two studies<sup>29,34</sup> for R160W variant, and in one study<sup>29</sup> for D294H variant.

Complete sequencing analysis of the *MC1R* coding region was performed in 12 studies<sup>15,22,24,26–28,30,31,33,35,36</sup>, while in the remaining five studies *MC1R* was genotyped by SNaPshot<sup>23,29,34</sup>, Restriction Fragment Length Polymorphisms<sup>25</sup> or allele discrimination assay.<sup>32</sup>

A description of the studies included in the pooled-analysis is presented in Table 1. Studies were published between 2001 and 2012; the majority were carried out in Europe (N=13, 76%), mainly in the southern countries (N=7, 41%), followed by USA (N=3, 18%) and Australia (N=1, 6%). In 13 studies (76%) healthy controls were recruited, while in the remaining four (24%) hospital controls were included. Overall, the average age of controls was higher than that of cases (62 versus 53 years), while the percentage of males was the same for both cases and controls (44%). Individual information on age, sex and family history of melanoma was available for each study, while further confounders differed between studies.

### Association between single *MC1R* variants and melanoma

The most prevalent *MC1R* variants in the M-SKIP database were nine: V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D294H. When we compared carriers of each variant with WT subjects using study-specific ORs and combined ORs, we found that CM risk significantly increased for carriers of any of the nine *MC1R* studied variants, with SOR (95% CI) ranging from 1.47 (1.17–1.84) for V60L to 2.74 (1.53–4.89) for D84E (Figure 1 and Table 2). After adjusting for multiple comparisons, all the p-values were still significant following the false discovery rate approach, while the association between I155T variant and melanoma was not confirmed after the more conservative Bonferroni correction (adjusted p-value: 0.08). The related attributable risk (AR) is presented in Figure 2. The highest AR was observed for R151 variant (8.9%), followed by R160W (8.3%) and V60L (8.2%) variants. In Table 2 we compared the results obtained in the 12 studies with WT as reference category



with those obtained in the whole set of 17 studies and using, as reference group for each variant, the subjects without that variant. For this latter analysis, a significant association with CM was observed for all the *MC1R* variants but V60L, I155T and R163Q. It should be noted that the reference group of this latter analysis included, for each studied variant, both WT and any other *MC1R* variant.

Funnel plots for each *MC1R* variant are presented in Supplemental Figure 1 for the whole set of studies. We found some evidence of participation bias for the R163Q variant, with a borderline p-value (0.05).

Significant heterogeneity among the 12 risk estimates with WT as reference category was found for V60L ( $I^2$ :50.9%, Q statistic p-value:0.02) and R142H ( $I^2$ :49.3%, Q statistic p-value:0.03) (Figure 1). Variability in publication year, study area, genotyping methodology, deviation from HW equilibrium, source of controls, and source of DNA did not seem to explain the observed heterogeneity. Sensitivity analyses showed that the heterogeneity may be attributable to single studies: when we excluded the studies that lied out of the corresponding funnel plots, we obtained similar pooled-ORs than the original analyses, but with no more evidence of heterogeneity among study-specific estimates. For the analysis with WT as reference category SORs (95%CI) increased to 1.54 (1.27–1.88) and 2.76 (1.71–4.45) for V60L (excluding<sup>26</sup>) and R142H (excluding<sup>15</sup>), respectively;  $I^2$  (Q statistic p-value) were, respectively, 33.6% (p=0.13) and 29.3%, (p=0.18).

Meta-ORs calculated for studies not included in the M-SKIP project were similar to those obtained from our pooled-analysis on the whole dataset for all but R151C variant, for which it was significantly higher than our pooled-OR (meta regression p-value=0.05).

### Association between combined *MC1R* variants and melanoma

SOR for the most common haplotypes were reported in Supplemental Table 1 for the 12 studies in which *MC1R* was sequenced. Results were similar to those observed for the single-variant analysis, with the higher SOR (3.05; 95%CI: 1.56–5.98) observed for the haplotype corresponding to a single mutation at rs1805006 (D84E).

We found that subjects carrying at least one *MC1R* variant had a significantly increased risk of CM, with SOR (95%CI) = 1.66 (1.41, 1.96) (Table 3). The risk attributable to any *MC1R* variant was 28.3%.

Individuals carrying just one *MC1R* variant had almost 40% increased risk of CM compared to WT homozygous subjects (SOR 1.41; 95%CI 1.07–1.87, Table 3), whereas carriers of two or more *MC1R* variants had more than a double risk of CM than WT subjects (SOR 2.51; 95%CI 1.83–3.44, Table 3).

A significant linear trend was observed for one point increase in *MC1R* score (per-point SOR 1.39; 95%CI 1.31–1.48, p-value <0.0001). SOR (95%CI) increased from 1.24 (0.90–1.72) to 1.69 (1.21–2.35) to 3.28 (2.22–4.87) and then slightly decrease to 3.12 (1.99–4.91) with increasing score from 1 to 4+ (Table 3).

## Analyses stratified by phenotypic characteristics

Results from analyses stratified according by freckles, hair color, skin type and their combination for any *MC1R* variant and for the nine most prevalent *MC1R* variants are reported in Table 4 for seven studies with WT as reference category and information on phenotypic characteristics. Stratified analysis for the most common haplotypes are reported in Supplemental Table 2.

For subjects with the RHC phenotype, the risk of CM was not independently predicted by having *MC1R* variants: SORs (95%CI) for carriers of any *MC1R* variant were 1.16 (0.79–1.72), 0.90 (0.23–3.58), 1.16 (0.73–1.85), and 1.16 (0.88–1.52) for subjects with freckles, red hair, skin type I/II, and any of the above phenotypic characteristics, respectively. On the contrary, darker-pigmented subjects presented a significantly higher risk of CM associated with *MC1R* variants: SORs (95%CI) for carriers of any *MC1R* variant were 2.39 (1.60–3.57), 1.70 (1.20–2.42), 1.89 (1.29–2.78) and 3.14 (2.06–4.80) for subjects with no freckles, no red hair, skin type III/IV, and all the above phenotypic characteristics, respectively. This trend was observed also when we analyzed each single variant and when we looked separately at the 5 southern European studies,<sup>24,26–28,36</sup> the USA study<sup>15</sup> and the northern European study.<sup>22</sup> The difference between the strata-specific ORs was statistically significant for the analysis on freckles, skin type and the combination of the three RHC phenotypic characteristics for any *MC1R* variant.

Further stratified analyses were performed for skin color and eye color (Supplemental Table 3). Also for these variables, a constant trend was observed, with higher SORs obtained for darker-pigmented subjects compared to lighter-pigmented individuals.

## Discussion

Our pooled-analysis identified a significant association with sporadic CM for all the most common *MC1R* variants compared to WT. An unbiased and precise estimate of the risk of melanoma in carriers of *MC1R* variants for different populations of European origins is provided for the first time by our group, since in previous large meta-analysis<sup>14–16,18</sup> SORs were systematically biased toward the null, due to the inclusion of carriers of other *MC1R* variants in the reference category for each variant analysis. The three RHC variants R151C, R160W, and D294H were previously associated with CM risk.<sup>14,16,18,22,24–26</sup> The association of D84E, R142H and I155T with CM was not always identified in single studies, probably because of the low frequency of these alleles, but it was confirmed by more powerful meta-analyses.<sup>14,16,18</sup>

An important question raised by these findings refers to the functional characteristics of mutant alleles accounting for their association with CM and for their different penetrance. The functional properties of WT *MC1R*, the most common RHC variants, and a few rarer variants have been addressed in several independent studies. WT *MC1R* triggers at least three major signal transduction pathways. The Gs protein-dependent activation of the cAMP pathway leads to induction of the master melanocyte transcription factor MITF and its downstream targets including the melanogenic enzymes.<sup>39</sup> Efficient activation of the cAMP pathway appears essential for the synthesis of eumelanins and for a normal tanning



response.<sup>40</sup> In addition, MC1R activation triggers two mitogen-activated protein kinase (MAPK) modules, one leading to activation of p38 kinase,<sup>41</sup> and the other to the extracellular signal-regulated kinases ERK1 and ERK2.<sup>42</sup> The functional outputs in melanocytes of these two related pathways are poorly understood. Interestingly, whereas p38 activation downstream of the MC1R depends on cAMP,<sup>41</sup> ERK activation in human melanocytic cells appears cAMP-independent.<sup>42</sup> Accordingly natural mutations in the *MC1R* gene may have different effects on functional coupling to the cAMP and ERK pathways, as confirmed by several studies.<sup>43,44</sup>

Several independent studies have firmly established that the major RHC alleles are loss-of-function forms with decreased cAMP signaling,<sup>11–13</sup> although they may retain a high efficacy for ERK activation.<sup>13,42,44</sup> For the D84E, R151C, I155T and R160W receptors impairment in cAMP coupling is largely accounted for by reduced cell surface expression. Conversely, the R142H and D294H variants show normal or even slightly increased cell surface expression,<sup>11</sup> and their loss-of-function phenotype is most likely related to an inability to properly undergo the agonist-induced transition to the active state and/or to impaired coupling to the Gs protein. In any case, inefficient or even absent activation of the cAMP pathway downstream of these forms is consistent with impaired eumelanogenesis, which may account for their frequent occurrence in individuals with red hair and fair skin. This suggests a pigmentary component for their contribution to CM development,<sup>14</sup> whereby tanning ability would be compromised in these individuals and production of photoprotective eumelanin would switch towards biosynthesis of photosensitizing pheomelanin.

On the other hand, previous meta-analyses<sup>14,16,18</sup> reported controversial results for the association of V60L, V92M and R163Q variants with CM. It is worthwhile to note that the SOR calculated in the present study for all the nine studied variants compared to WT were markedly higher than those reported in previous meta-analyses. These differences are probably attributable to a bias towards the null in meta-analyses, due to the fact that reference category for OR calculation often includes, for each studied variant, both WT and any other *MC1R* variant. Indeed, when we compared the results obtained in our pooled-analysis, we noted that SORs calculated with WT as the reference category were always higher than the corresponding ones obtained by a classical analysis based on presence/absence of each variant.

A marginal effect of the V92M substitution on the cell surface expression or the ability to activate the cAMP and ERK cascades has been reported.<sup>11,44</sup> Concerning R163Q, a selective decrease in ERK activation has been recently described<sup>13</sup> but its functional coupling to the cAMP pathway is normal or only slightly modified,<sup>45</sup> and this variant is highly frequent in Asian populations. V60L allele has been shown to display reduced cell surface expression with a corresponding impairment in cAMP activation.<sup>11,44</sup> In fact, its functional properties are comparable to those of the RHC forms R151C or R160W mentioned above.

When we analyzed *MC1R* variants combined, we observed a significantly higher risk of CM both for carriers of one and carriers of two or more variants compared to WT subjects. It has

been previously suggested that RHC alleles may act in a recessive manner and that one fully functional copy of *MC1R* may be sufficient to provide normal function;<sup>12</sup> however our results suggested that CM risk increased even for carriers of just one *MC1R* variant. When we assigned a score to each subject according with the number of R and r alleles, a dose-response relationship was noted, with SORs significantly higher with increasing score, as expected.

A very interesting result of our pooled-analysis was that we observed a significantly higher CM risk associated with *MC1R* variants only for darker-pigmented subjects of European origins, while carriers of *MC1R* variants with RHC phenotype had no increased risk of melanoma compared to non carriers. A similar result was observed in previous studies and meta-analysis,<sup>15,24</sup> which highlighted that the association between some *MC1R* variants and CM was stronger in subjects with dark hair, dark eyes, skin type III/IV, and in subjects who reported low recreational sun exposure. A recent meta-analysis on darker-pigmented Southern European populations<sup>46</sup> also showed that *MC1R* RHC variants were strong CM risk predictors. These results suggest that *MC1R* variants may mediate their effects, at least partially, through biological pathways that are independent on pigmentation, thus providing additional information about melanoma risk in people who would not be identified as high risk based on their phenotypic characteristics alone. Recent studies implicate *MC1R* signaling in a number of key biological pathways involved in cell cycle control,<sup>47</sup> apoptosis,<sup>48</sup> and activation of DNA repair mechanisms and antioxidant defenses.<sup>19</sup> Production of pheomelanin pigments seems associated with increased oxidative DNA damage compared with synthesis of eumelanins.<sup>49</sup> Further evidence for pheomelanin-associated increased cellular oxidative stress was obtained in studies of mice carrying a loss-of-function mutation of the *Mclr* gene. These mice have a yellowish pheomelanin hair mimicking the human red hair and show higher levels of lipid peroxides, a product of reactive oxygen species (ROS)-mediated lipid damage.<sup>20</sup> This study provided evidence in support of a melanogenetic effect independent of UV exposure. In addition, as discussed above, stimulation of *MC1R* also activates MAPK pathway and regulates target genes involved in inflammation through the NF-Kb pathway.<sup>50</sup> Thus, interpretation of the effect of *MC1R* alleles in melanoma beyond its role in pigmentation is complex.

A strength of the present pooled-analysis is the large sample size, which gives power to detect associations with relatively rare *MC1R* variants, and to perform stratified analysis by phenotypic characteristics at an individual, rather than at a population, level. Moreover, the international collaborative nature of the M-SKIP project makes it possible to assess the *MC1R*-related CM risk in various populations and ancestries, thus providing evidence that the estimated risk is robust and consistent in different geographical areas. As it was previously pointed out,<sup>18</sup> a source of heterogeneity between studies in meta-analysis was the choice of study comparator group, which is often characterized, for each variant, by people without that variant. This practice leads to underestimate the true risk of disease in meta-analysis, because *MC1R* variants are very common: 66% of our study population had at least one variant. Within our pooled-analysis we were able to compare each variant with WT in the 12 studies with *MC1R* sequenced, and we indeed obtained markedly higher SORs. Moreover we were able to study the combined effect of one or more *MC1R* variants

compared to WT subjects, and this would not be usually possible in a meta-analytic context, where the analysis of variants combined usually differed from study to study. Finally, we could take into account all the available confounders in our centralized statistical analysis, with a homogeneous plan of analysis and homogeneous definition of confounders.

Concerning the possible limitations of this study, one drawback is the exclusion of GWAS in the first phase of the project. This was done to avoid increasing the heterogeneity of the pooled estimate with a different approach. However GWAS may be included in the M-SKIP in a second step of the project. Unavailability of information on other genes in most studies prevents the analysis of possible gene-gene interactions. The interactions of other low-risk loci need to be taken into account for a full assessment of genetic susceptibility to CM. Other genes such as *SLC45A2*, *TYR*, *TYRP1*, *ASIP*, *OCA2*, *XRCC* and *GSTP1* may also contribute to skin cancer susceptibility. Because we carried out a retrospective pooled-analysis, we did not perform centralized sequencing. However, previous studies<sup>37</sup> have reported excellent concordance in sequencing data from different centres. Finally, differences in the assessment of sun exposure did not allow for the use of this variable in stratified analysis, although it remained possible to take it into account for the adjustment for confounders.

In conclusion our study remarks the important role of all the main *MC1R* variants in sporadic CM and suggests that they have a direct effect on melanoma risk, independently on the phenotypic characteristics of carriers. This is of particular importance for assessing preventive strategies, which may be directed to darker-pigmented individuals of European origins with *MC1R* variants as well as to lightly-pigmented, fair-skinned subjects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>α-MSH</b>	α-melanocyte-stimulating hormone
<b>CI</b>	confidence interval
<b>CM</b>	cutaneous melanoma
<b>ERK</b>	extracellular signal-regulated kinases
<b>GWAS</b>	genome-wide association studies
<b>HW</b>	Hardy-Weinberg
<b>MAPK</b>	mitogen-activated protein kinase
<b>MC1R</b>	melanocortin-1-receptor
<b>NMSC</b>	non-melanoma skin cancer
<b>OR</b>	odds ratio
<b>ROS</b>	reactive oxygen species
<b>RHC</b>	red hair color
<b>SOR</b>	summary odds ratio
<b>WT</b>	wild-type

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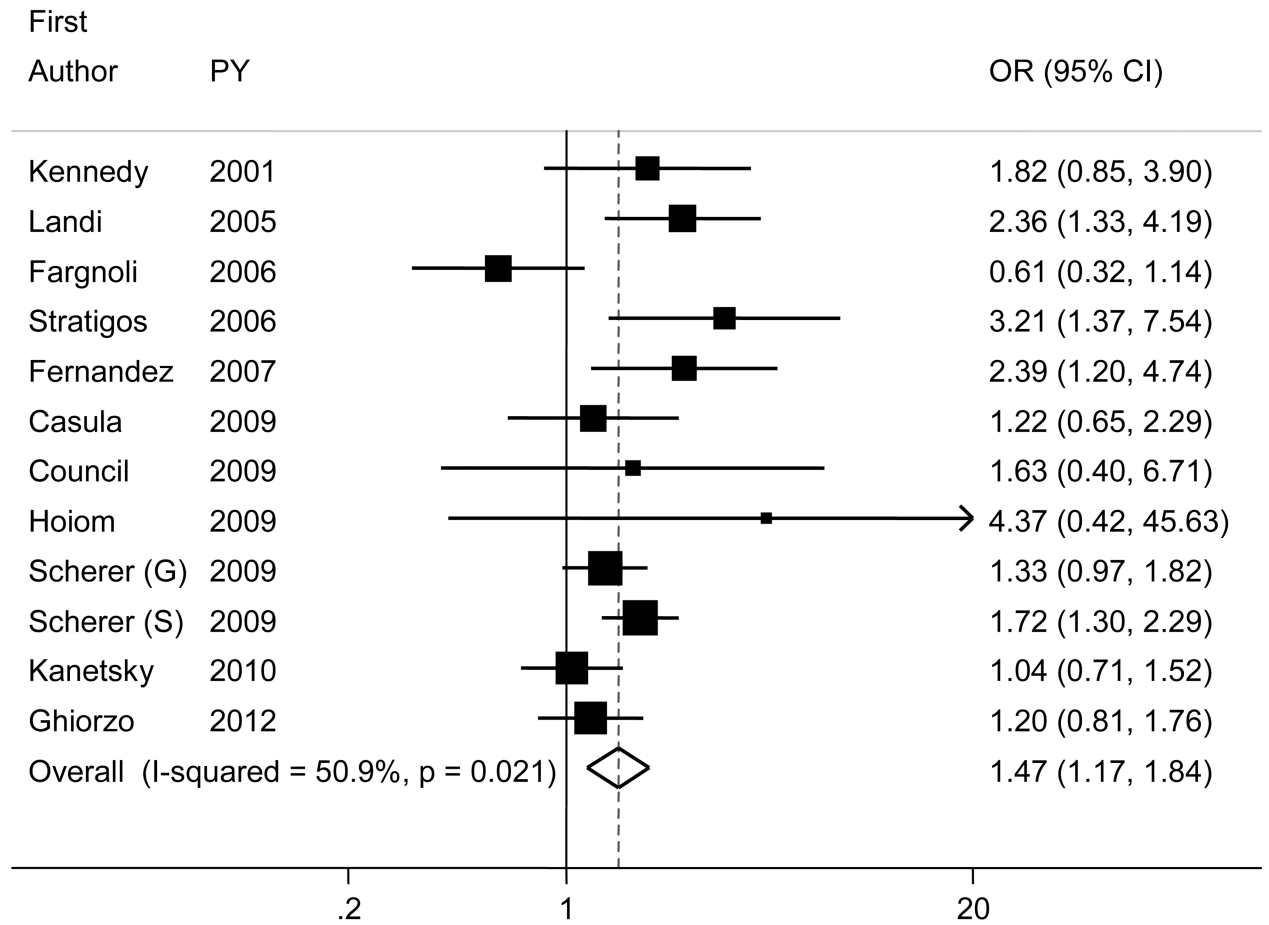
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(A)

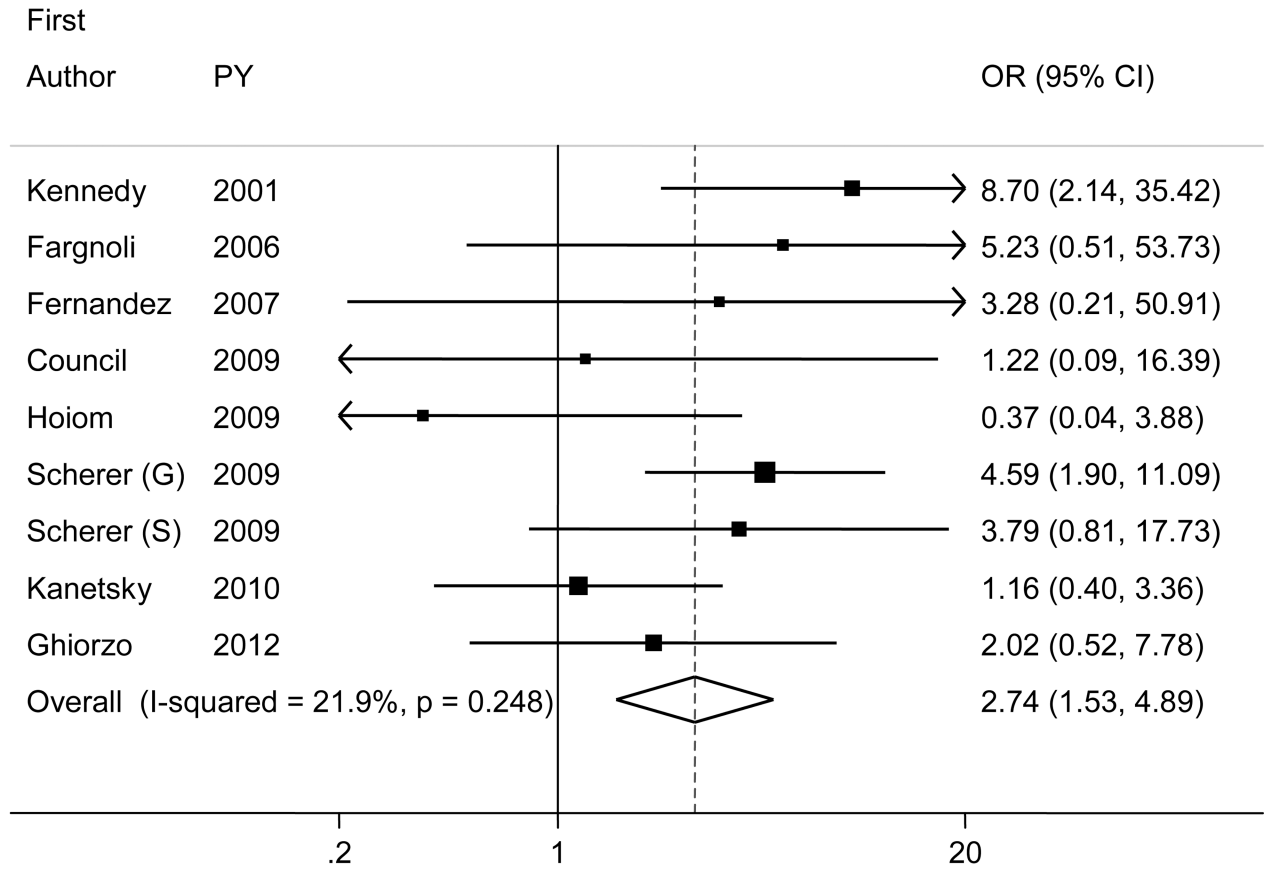
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G= German study; S= Spanish study

**(B)**

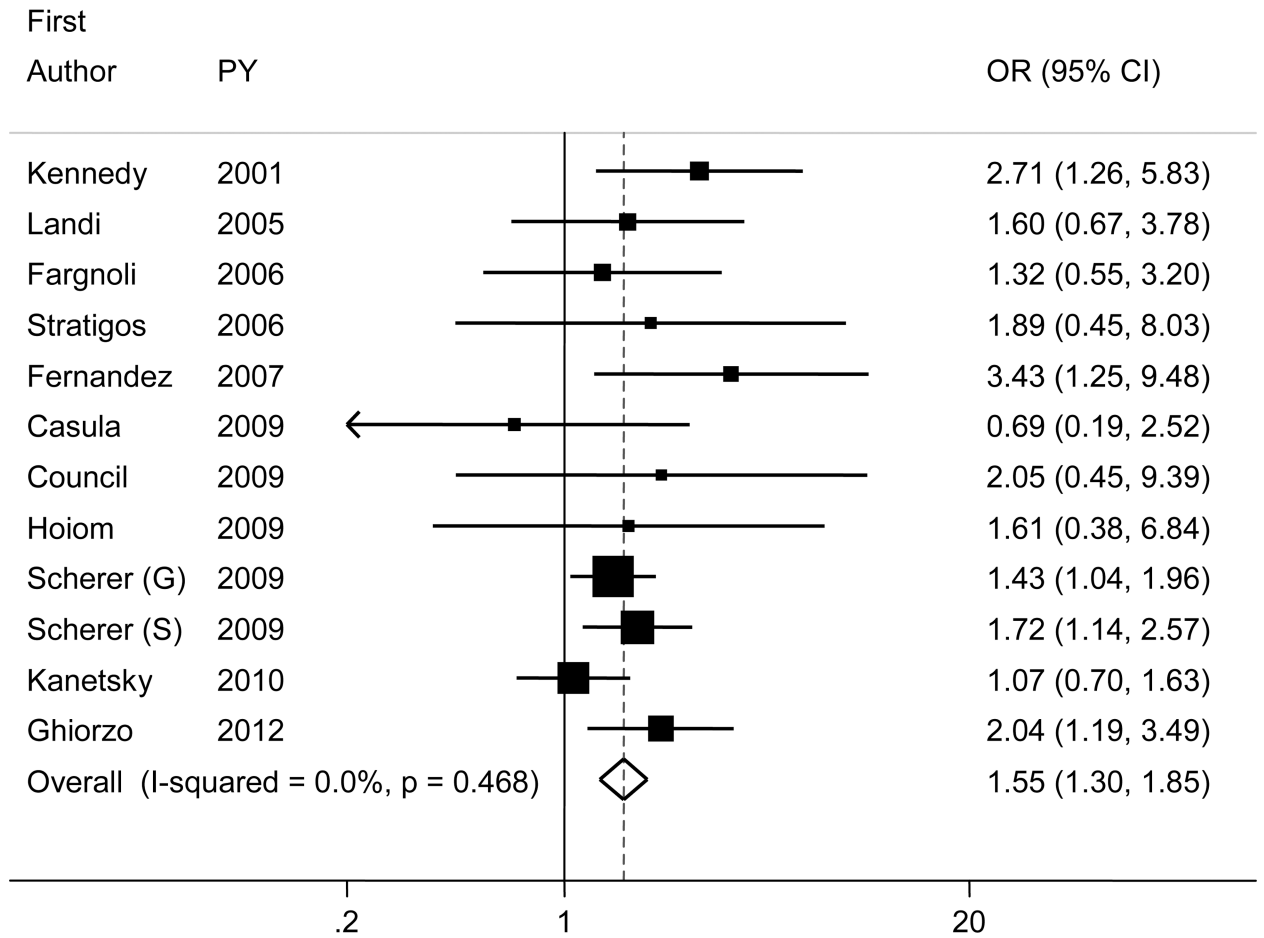
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(C)

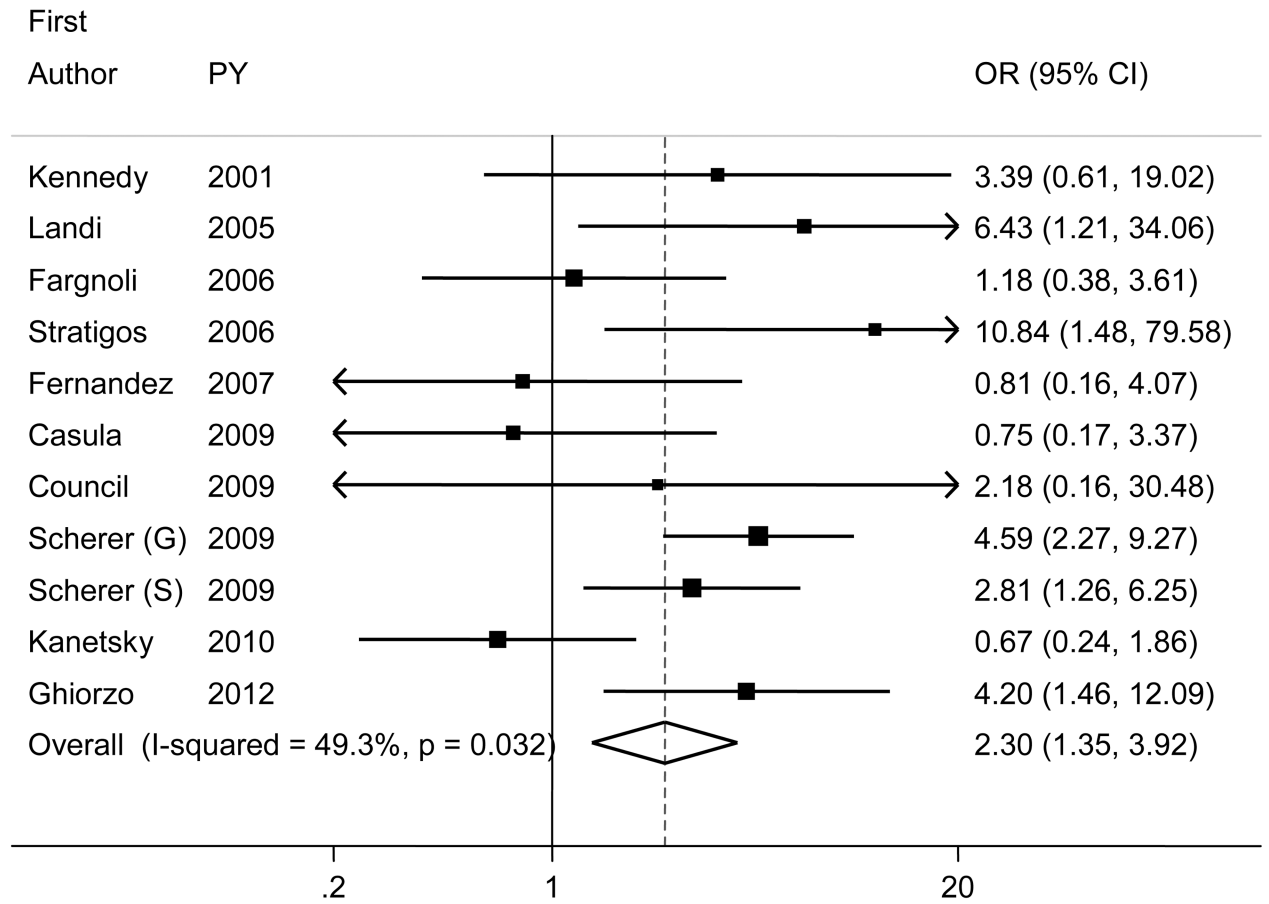
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G= German study; S= Spanish study

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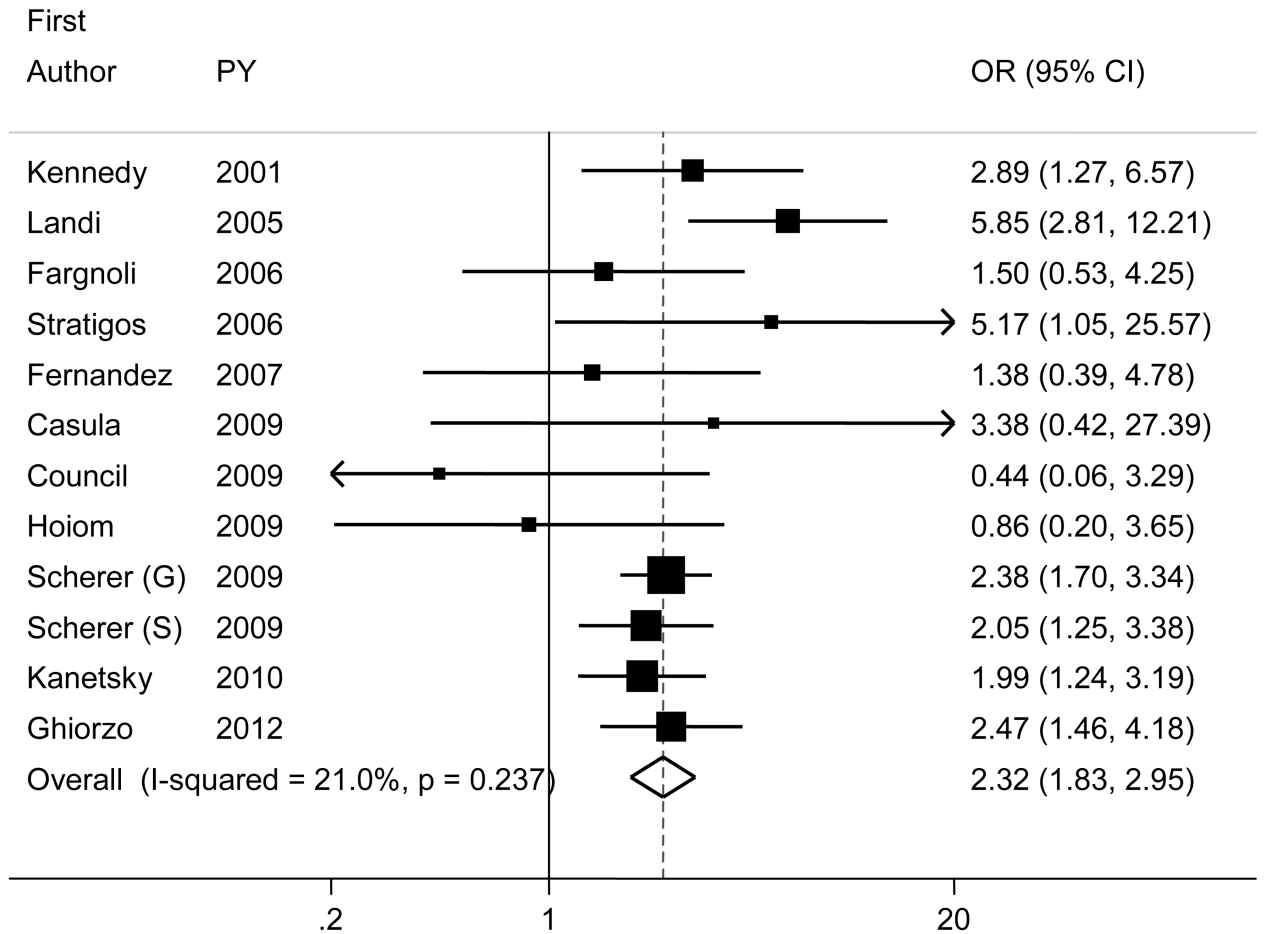
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G= German study; S= Spanish study

(E)

# R151C



G= German study; S= Spanish study

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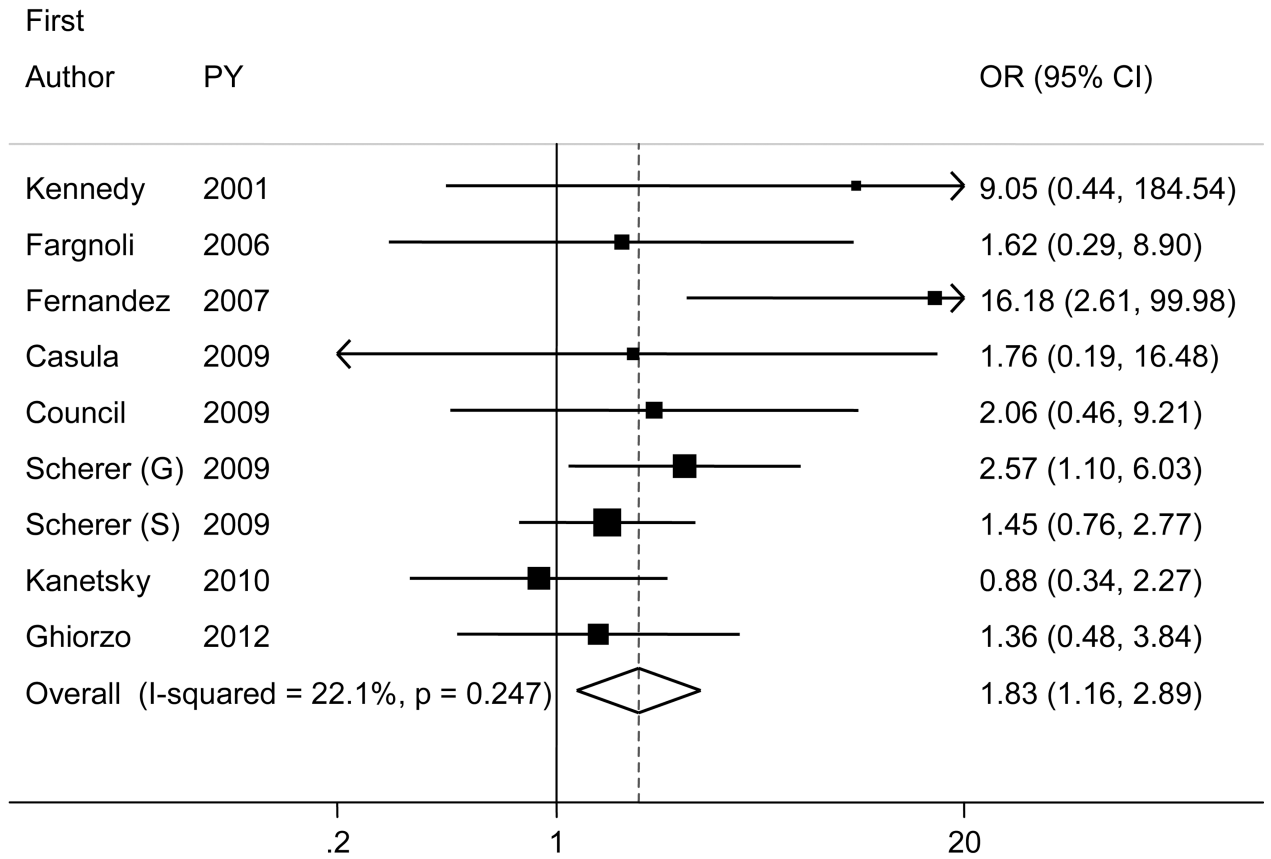
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(F)

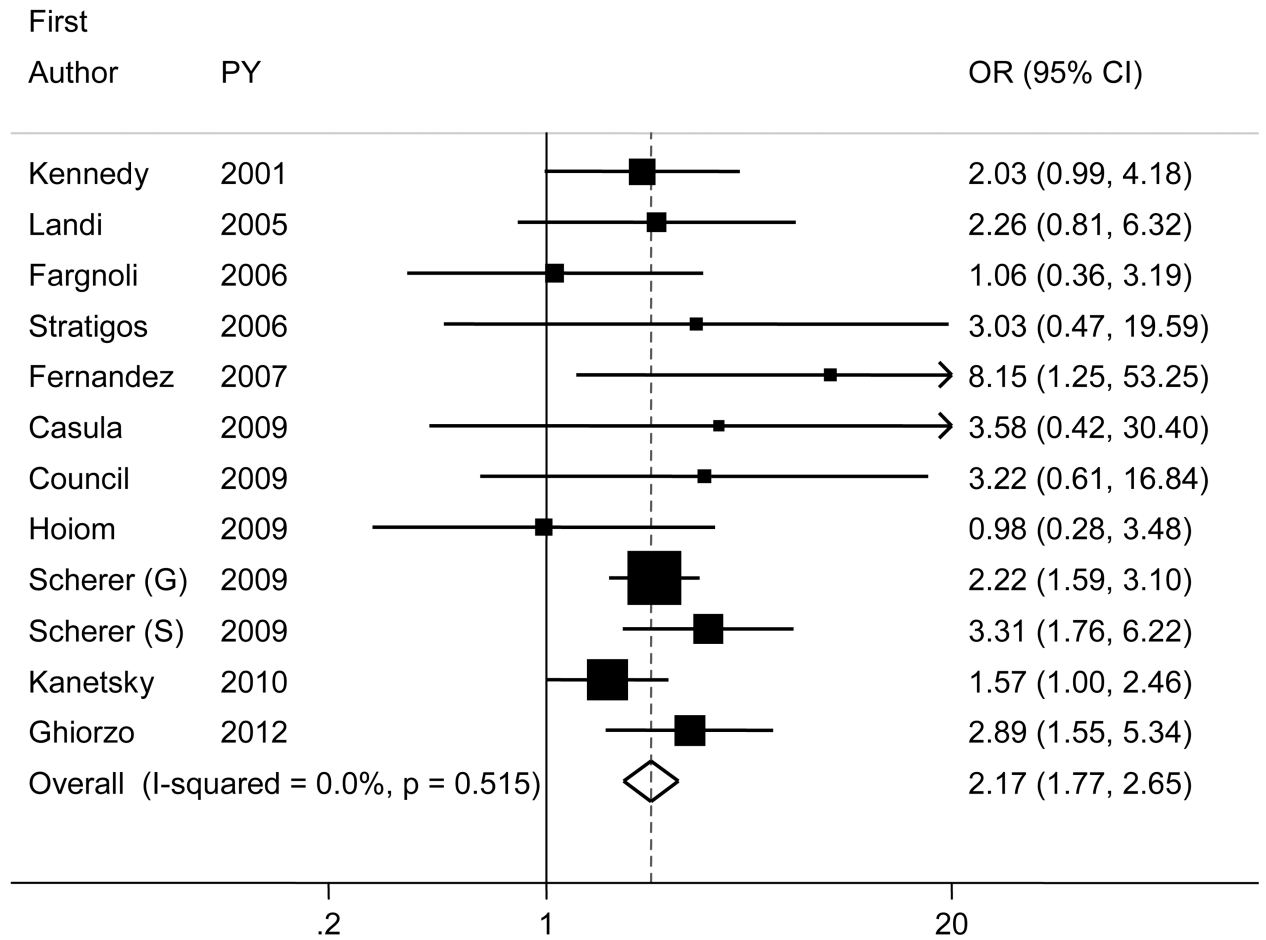
# I155T



G= German study; S= Spanish study

(G)

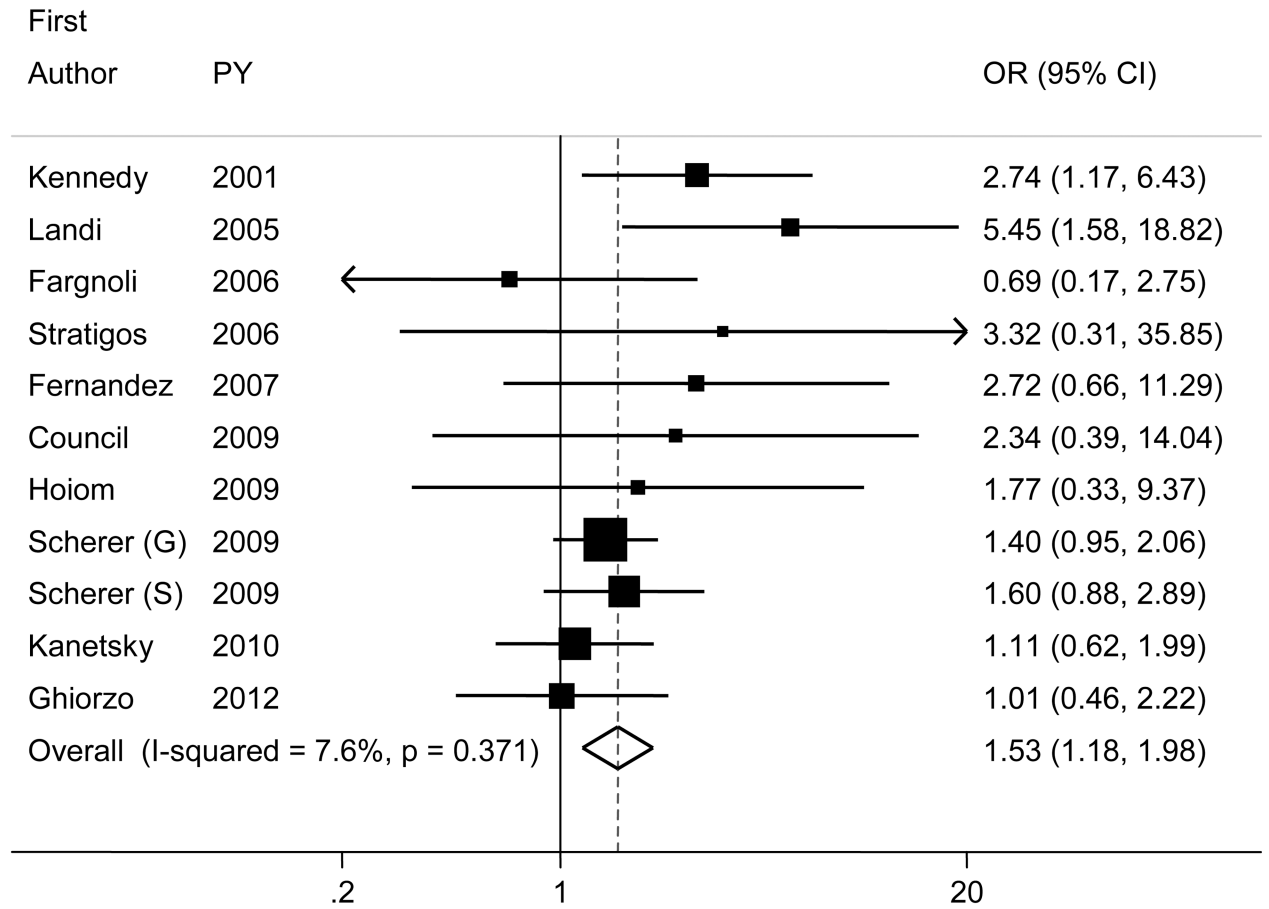
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G= German study; S= Spanish study

(H)

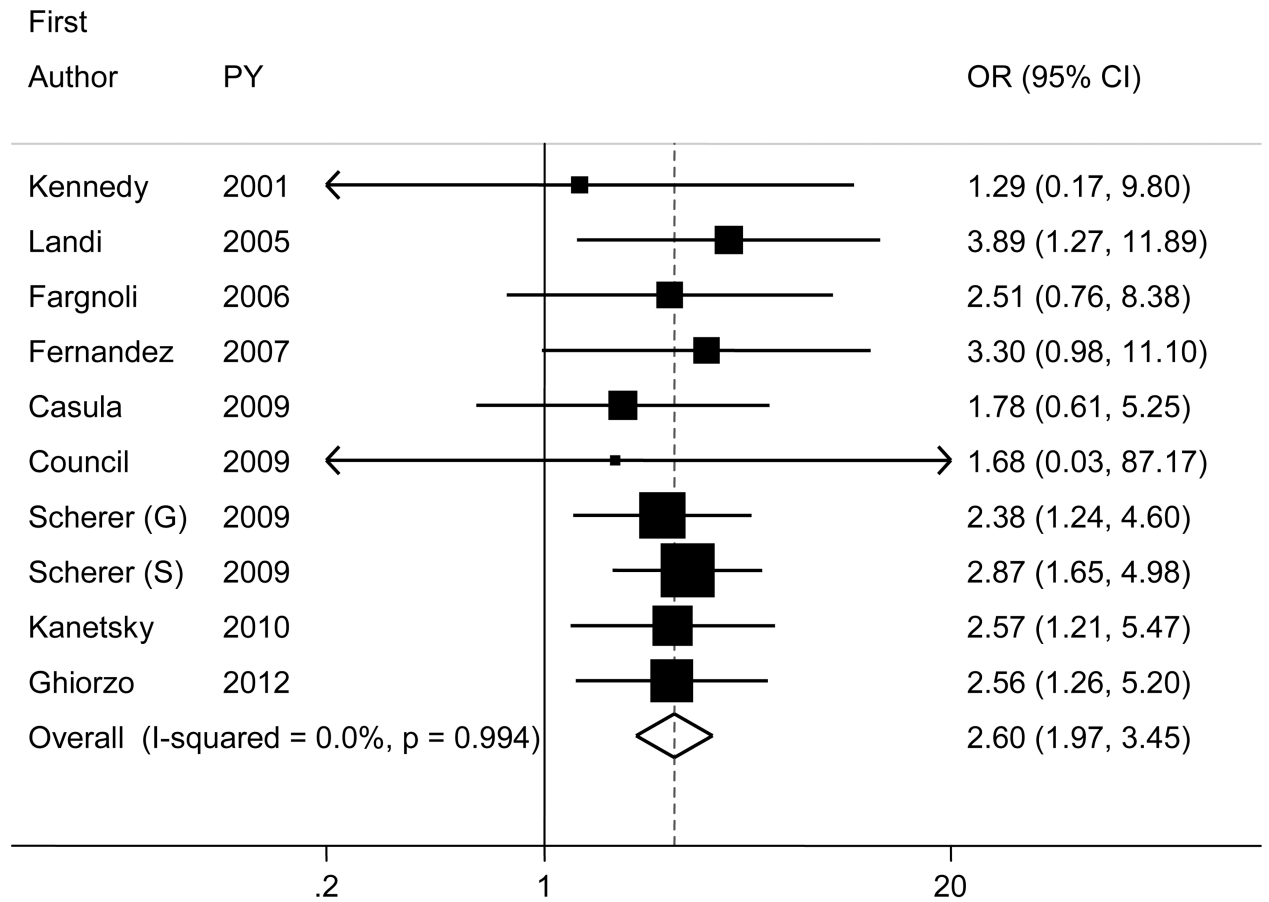
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G= German study; S= Spanish study

(I)

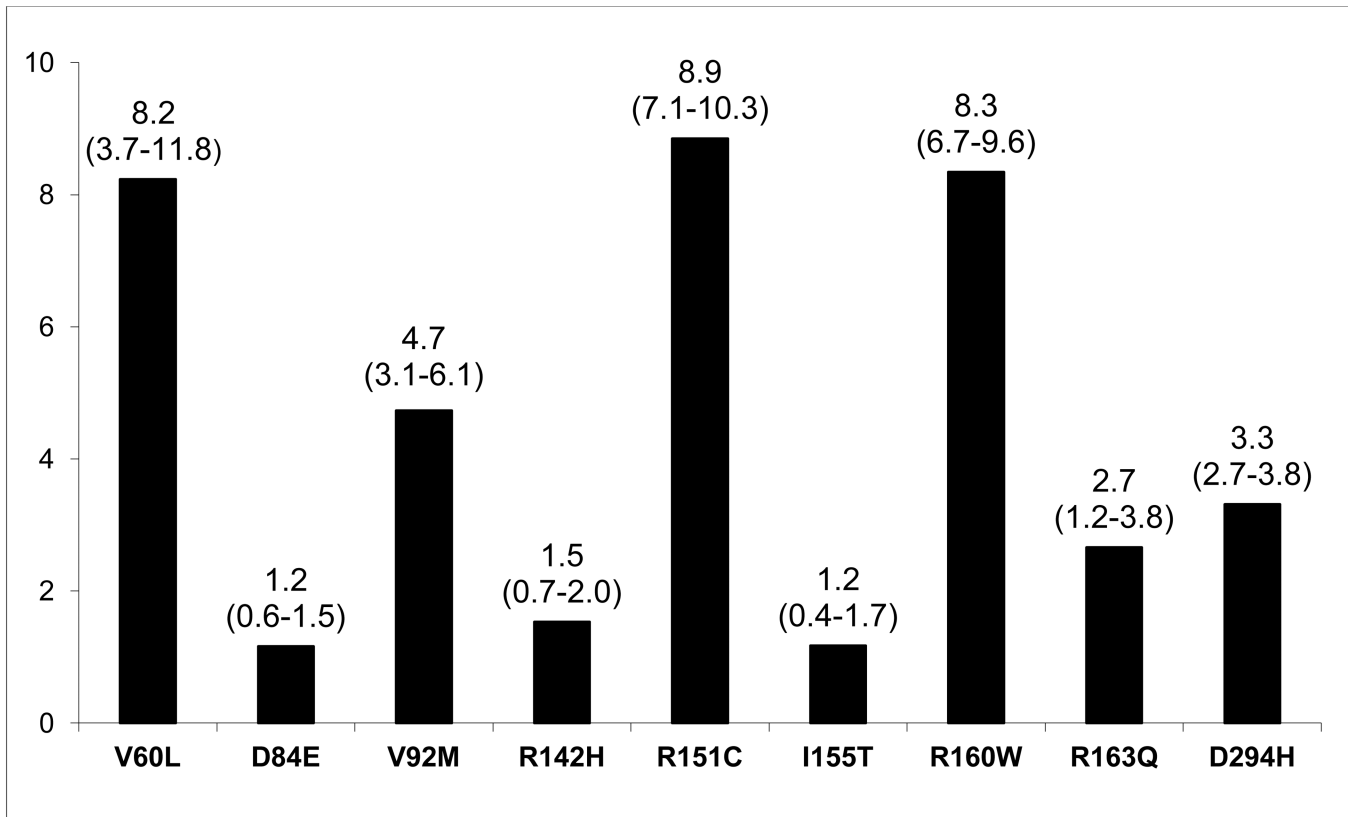
## D294H



G= German study; S= Spanish study

**Figure 1.**

Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between cutaneous melanoma and *MC1R* variants (A) V60L, (B) D84E, (C) V92M, (D) R142H, (E) R151C, (F) I155T, (G) R160W, (H) R163Q, (I) D294H. Reference category for each variant comprises WT subjects



\* Miettinen's formula (OR-1/OR x proportion of cases exposed).

**Figure 2.**

Attributable risks\* in the population for cutaneous melanoma according to each *MC1R* variant (percentages with 95% confidence intervals). Reference category for each variant comprises WT subjects

**Table 1**

Description of the 17 case-control studies included in the pooled-analysis

First Author	Publication year	Country	MC/R sequencing	Controls type <sup>a</sup>	N Cases/ N Controls	Mean Age (SD)		Percent of males		Available confounders <sup>b</sup>
						Cases	Controls	Cases	Controls	
Kennedy <sup>22</sup>	2001	The Netherlands	Yes	Hospital	115/378	49 (12)	58 (11)	37	42	Sun exposure, sunburns, common and atypical naevi
Dwyer <sup>23</sup>	2004	Australia	No	Healthy	159/290	44 (10)	44 (10)	41	46	Sun exposure, sunburns
Landi <sup>24</sup>	2005	Italy	Yes	Healthy	165/171	49 (15)	46 (13)	49	49	Sun exposure, sunburns
Debnia <sup>25</sup>	2006	Poland	No	Healthy	349/313	53 (14)	53 (13)	32	24	Sunburns
Fergnoli <sup>26</sup>	2006	Italy	Yes	Hospital	155/163	49 (14)	49 (14)	40	50	Sun exposure, sunburns, common and atypical naevi
Stratigos <sup>27</sup>	2006	Greece	Yes	Hospital	123/155	52 (16)	44 (15)	51	54	Sun exposure, sunburns, common and atypical naevi
Fernandez <sup>28</sup>	2007	Spain	Yes	Healthy	108/188	51 (15)	53 (14)	46	39	Sunburns, common naevi
Brudnik <sup>29</sup>	2009	Poland	No	Hospital	116/489	62 (14)	43 (19)	35	40	-
Casula <sup>30</sup>	2009	Italy	Yes	Healthy	259/75	49 (14)	61 (15)	48	27	-
Council <sup>31</sup>	2009	USA	Yes	Healthy	83/166	51 (15)	77 (7)	45	50	-
Nan <sup>32</sup>	2009	USA	No	Healthy	219/241	64 (8)	58 (7)	0	0	Sunburns
Hoiom <sup>33</sup>	2009	Sweden	Partially <sup>c</sup>	Healthy	675/477	53 (19)	42 (12)	47	64	-
Rotterdam Study <sup>34</sup>	2009	The Netherlands	No	Healthy	68/6,559	70 (8)	72 (9)	47	41	-
Scherer (G) <sup>35</sup>	2009	Germany	Yes	Healthy	512/1,064	58 (15)	54 (12)	56	56	-
Scherer (S) <sup>35</sup>	2009	Spain	Yes	Healthy	1,031/558	52 (16)	37 (12)	46	62	-
Kanetsky <sup>15</sup>	2010	USA	Yes	Healthy	769/325	49 (14)	48 (13)	49	43	Sun exposure, sunburns, atypical naevi
Ghiorzo <sup>36</sup>	2012	Italy	Yes	Healthy	254/507	52 (16)	51 (17)	50	47	Sunburns
<b>Total</b>					<b>5,160/12,119</b>	<b>53 (16)</b>	<b>62 (16)</b>	<b>44</b>	<b>44</b>	

G= German study; S= Spanish study

<sup>a</sup> Healthy controls are population controls, blood donors, friends or relatives of cases.

<sup>b</sup> Beyond age, sex and family history of melanoma, which were available in all the 17 studies. Sun exposure includes separate information on chronic and intermittent sun exposure.



*MC1R* sequencing performed on a subsample of 95 cases and 30 controls.

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Table 2

Allele frequency and Summary Odds Ratios (SOR) with 95% Confidence Intervals (CI) for the association between *MC1R* variants and cutaneous melanoma using two different reference categories.

<i>MC1R</i> variant	Allele frequency in controls (%)	1. Variant present vs WT controls			2. Variant present vs absent <sup>a</sup> controls		
		N studies	N cases/N controls	SOR (95%CI)	N studies	N cases/N controls	SOR (95%CI)
V60L	10.6	12	3,636/3,772	1.47 (1.17, 1.84)	17	5,111/11,854	1.07 (0.91–1.26)
D84E	0.4	9	3,089/3,371	2.74 (1.53, 4.89)	11	3,956/4,588	2.13 (1.44–3.17)
V92M	7.0	12	3,636/3,772	1.55 (1.30, 1.85)	14	4,577/4,948	1.15 (1.00–1.31)
R142H	0.6	11	3,571/3,742	2.30 (1.35, 3.92)	14	4,430/10,888	1.77 (1.14–2.75)
R151C	5.7	12	3,636/3,772	2.32 (1.83, 2.95)	17	5,146/11,943	1.62 (1.34–1.96)
I155T	0.8	9	3,283/3,416	1.83 (1.16, 2.89)	12	4,287/4,621	1.36 (0.97–1.90)
R160W	7.2	12	3,636/3,772	2.17 (1.77, 2.65)	17	5,144/11,968	1.74 (1.43–2.13)
R163Q	4.7	11	3,377/3,697	1.53 (1.18, 1.98)	15	4,734/11,739	1.10 (0.94–1.30)
D294H	1.3	10	3,448/3,587	2.60 (1.97, 3.45)	14	4,607/5,079	1.78 (1.40–2.28)

Note: In addition to *MC1R*, each study-specific logistic regression model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, family history of melanoma, number of common total body naevi count and presence of atypical naevi.

<sup>a</sup>For each *MC1R* variant, reference category included subjects either WT or carriers of any other *MC1R* variant.

**Table 3**

Summary Odds Ratios (SOR) with 95% Confidence Intervals (CI) for the association between combined *MC1R* variants and cutaneous melanoma, and heterogeneity estimates

Variant	N cases	N controls	SOR (95%CI)	O test p-value	I <sup>2</sup> for heterogeneity (%)
Wild-type	1,047	1,461	Reference	-	-
Any variant	2,589	2,311	1.66 (1.41–1.96)	0.10	36.8
1 variant	1,615	1,719	1.41 (1.07–1.87)	0.15	30.8
2+ variants	974	592	2.51 (1.83–3.44)	0.02	52.9
Score <sup>a</sup> 1	904	1,116	1.24 (0.90–1.72)	0.05	44.5
Score 2	955	834	1.69 (1.21–2.35)	0.44	0.6
Score 3	478	245	3.28 (2.22–4.87)	0.57	0
Score 4	252	116	3.12 (1.99–4.91)	0.02	53.1

Note: In addition to *MC1R*, each study-specific logistic regression model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, family history of melanoma, number of common total body naevi count and presence of atypical naevi.

<sup>a</sup>Score calculated as detailed in 37.

**Table 4**

Stratified analysis for *MC1R* variants and cutaneous melanoma association, according with freckles, hair color, skin type, and their combination. Reference category for each variant are WT subjects

Variant	Phenotypic characteristic	Strata	N studies (N cases/ N controls)	OR (95%CI)	P-value <sup>a</sup>
Any <i>MC1R</i> variant	Freckles	Any	4 (951/498)	1.16 (0.79–1.72)	0.003
		None	4 (326/655)	2.39 (1.60–3.57)	
	Hair color	Red	3 (114/40)	0.90 (0.23–3.58)	0.36
		Other	6 (1,424/1,621)	1.70 (1.20–2.42)	
V60L	Skin type	I/II	6 (689/567)	1.16 (0.73–1.85)	0.03
		III/IV	6 (832/1,111)	1.89 (1.29–2.78)	
	Freckles-red hair-skin type I/II	Any	4 (1,102/934)	1.16 (0.88–1.52)	<0.0001
		None	4 (197/444)	3.14 (2.06–4.80)	
	Freckles	Any	4 (951/498)	1.00 (0.63–1.56)	0.01
		None	4 (326/655)	2.00 (1.25–3.19)	
	Hair color	Red	2 (111/35)	0.59 (0.09–3.63)	0.33
		Other	6 (1,424/1,621)	1.44 (0.94–2.19)	
	Skin type	I/II	6 (689/567)	0.72 (0.37–1.38)	0.002
		III/IV	6 (832/1,111)	1.79 (1.03–3.12)	
D84E	Freckles-red hair-skin type I/II	Any	4 (1,102/934)	0.95 (0.70–1.30)	0.0008
		None	4 (197/444)	2.51 (1.54–4.07)	
	Freckles	Any	2 (806/341)	0.87 (0.33–2.28)	0.04
		None	2 (207/485)	5.23 (1.29–21.24)	
	Hair color	Red	2 (111/35)	1.25 (0.09–17.23)	0.45
		Other	4 (1,160/1,308)	3.39 (1.02–11.30)	
	Skin type	I/II	4 (577/481)	1.23 (0.32–4.67)	0.41
		III/IV	4 (666/876)	3.12 (0.33–29.13)	
	Freckles-red hair-skin type I/II	Any	3 (988/849)	1.56 (0.49–4.93)	0.51
		None	1 (26/167)	4.13 (0.25–67.39)	
V92M	Freckles	Any	4 (951/498)	0.87 (0.59–1.29)	0.0002
		None	4 (326/655)	2.36 (1.41–3.95)	
Hair color	Red	1 (94/19)	0.94 (0.09–9.78)	0.66	

Variant	Phenotypic characteristic	Strata	N studies (N cases/ N controls)	OR (95%CI)	P-value <sup>a</sup>
R142H	Skin type	Other	6 (1,424/1,621)	1.61 (1.12–2.29)	0.27
		I/II	6 (689/567)	1.21 (0.69–2.10)	
	Freckles-red hair-skin type I/II	III/IV	6 (832/1,111)	1.71 (1.10–2.66)	0.005
		Any	4 (1,102/934)	1.04 (0.74–1.47)	
		None	4 (197/444)	3.13 (1.59–6.14)	
		Any	3 (855/425)	1.93 (0.35–10.55)	
	Freckles	None	2 (208/289)	2.40 (0.31–18.36)	0.85
		Red	2 (111/35)	0.35 (0.03–4.55)	
		Other	5 (1,318/1,263)	3.03 (0.86–10.69)	
		I/II	6 (689/567)	1.76 (0.59–5.24)	
III/IV		5 (796/908)	2.24 (0.77–6.49)		
Any		4 (1,102/934)	1.84 (0.50–6.83)		
R151C	Skin type	None	2 (135/217)	3.75 (0.44–32.02)	0.50
		Any	4 (951/498)	1.67 (1.00–2.80)	
	Freckles-red hair-skin type I/II	None	4 (326/655)	4.27 (2.25–8.10)	0.12
		Red	3 (114/40)	0.85 (0.20–3.67)	
		Other	6 (1,424/1,621)	2.75 (1.90–3.97)	
		I/II	6 (689/567)	1.60 (0.95–2.70)	
	Freckles	III/IV	6 (832/1,111)	3.05 (1.89–4.93)	0.01
		Any	4 (1,102/934)	1.77 (1.26–2.49)	
		None	4 (197/444)	5.55 (2.47–12.48)	
		Any	2 (806/341)	0.58 (0.25–1.35)	
None		2 (207/485)	2.11 (0.43–10.46)		
Red		1 (94/19)	0.14 (0.01–2.52)		
II55T	Skin type	Other	4 (1,160/1,308)	1.25 (0.63–2.48)	0.15
		I/II	3 (498/306)	0.52 (0.18–1.46)	
	Freckles-red hair-skin type I/II	III/IV	2 (563/565)	1.29 (0.45–3.70)	0.23
		Any	2 (899/639)	0.76 (0.34–1.67)	
		None	2 (120/191)	1.83 (0.15–22.80)	
		Any	4 (951/498)	1.21 (0.79–1.84)	
	Freckles	None	4 (326/655)	3.72 (2.09–6.63)	0.0002

Variant	Phenotypic characteristic	Strata	N studies (N cases/ N controls)	OR (95%CI)	P-value <sup>a</sup>
	Hair color	Red	2 (111/35)	0.85 (0.15–4.68)	0.39
		Other	6 (1,424/1,621)	1.82 (1.33–2.48)	
	Skin type	I/II	6 (689/567)	1.24 (0.70–2.19)	0.10
		III/IV	5 (776/1,023)	2.15 (1.31–3.53)	
R163Q	Freckles-red hair-skin type I/II	Any	4 (1,102/934)	1.27 (0.88–1.84)	0.0003
		None	4 (197/444)	6.69 (2.92–15.35)	
	Freckles	Any	4 (951/498)	1.45 (0.55–3.77)	0.37
		None	4 (326/655)	2.31 (0.93–5.74)	
D294H	Hair color	Red	0 (0/0)	nc	nc
		Other	6 (1,424/1,621)	1.70 (0.93–3.14)	
	Skin type	I/II	4 (577/481)	1.66 (0.87–3.16)	0.51
		III/IV	6 (832/1,111)	1.24 (0.56–2.78)	
	Freckles-red hair-skin type I/II	Any	4 (1,102/934)	1.56 (0.64–3.80)	0.33
		None	4 (197/444)	2.60 (0.86–7.89)	
	Freckles	Any	4 (951/498)	1.64 (0.92–2.91)	0.04
		None	2 (208/289)	6.36 (2.00–20.21)	
	Hair color	Red	2 (111/35)	1.78 (0.22–14.52)	0.73
		Other	5 (1,315/1,474)	2.59 (1.64–4.11)	
	Skin type	I/II	5 (632/503)	0.97 (0.49–1.92)	0.001
		III/IV	4 (740/820)	4.57 (2.45–8.53)	
	Freckles-red hair-skin type I/II	Any	4 (1,102/934)	1.61 (0.96–2.68)	0.02
		None	2 (135/217)	14.36 (2.55–80.89)	

nc=not calculable; OR= Odds Ratio; CI=Confidence Intervals

Note: In addition to *MCI/R*, each study-specific logistic regression model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, family history of melanoma, number of common total body naevi count and presence of atypical naevi.

<sup>a</sup> Overall p-value for any significant difference among strata-specific ORs.