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MC1R gene variants and non-melanoma skin cancer: a pooled-analysis from the M-SKIP project

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Background: The melanocortin-1-receptor (*MC1R*) gene regulates human pigmentation and is highly polymorphic in populations of European origins. The aims of this study were to evaluate the association between *MC1R* variants and the risk of non-melanoma skin cancer (NMSC), and to investigate whether risk estimates differed by phenotypic characteristics.

Methods: Data on 3527 NMSC cases and 9391 controls were gathered through the M-SKIP Project, an international pooled-analysis on *MC1R*, skin cancer and phenotypic characteristics. We calculated summary odds ratios (SOR) with random-effect models, and performed stratified analyses.

Results: Subjects carrying at least one *MC1R* variant had an increased risk of NMSC overall, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC): SOR (95%CI) were 1.48 (1.24–1.76), 1.39 (1.15–1.69) and 1.61 (1.35–1.91), respectively. All of the investigated variants showed positive associations with NMSC, with consistent significant results obtained for V60L, D84E, V92M, R151C, R160W, R163Q and D294H: SOR (95%CI) ranged from 1.42 (1.19–1.70) for V60L to 2.66 (1.06–6.65) for D84E variant. In stratified analysis, there was no consistent pattern of association between *MC1R* and NMSC by skin type, but we consistently observed higher SORs for subjects without red hair.

Conclusions: Our pooled-analysis highlighted a role of *MC1R* variants in NMSC development and suggested an effect modification by red hair colour phenotype.

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Non-melanoma skin cancers (NMSC) are the most common malignancies in fair-skinned populations with a continuing increase in incidence during recent decades (Levi *et al*, 2001; Bath-Hextall *et al*, 2007; Flohil *et al*, 2011; Lomas *et al*, 2012). According to the estimates of the American Cancer Society, more than two million NMSC are diagnosed annually in the US (Housman *et al*, 2003; Rogers *et al*, 2010; American Cancer Society, 2012). In 1992, among US Medicare beneficiaries, NMSC ranked among the top five most costly cancers to treat (Housman *et al*, 2003). Moreover, from 1992 to 2006 in the same population, there was a 77% increase in the total number of skin-cancer-related procedures (~94% NMSC (Rogers *et al*, 2010)). The vast majority of NMSC are basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) with a BCC/SCC incidence ratio in immunocompetent patients of 4 : 1. BCC is the most common cancer in populations of European origin and accounts for 29% of all cancers (DePinho, 2000), although it is less likely to be lethal and rarely metastasises. Previous studies identified solar UV irradiation, fair skin, red hair and freckles as the most relevant risk factors for NMSC development (Rosso *et al*, 1996; Zanetti *et al*, 1996; IARC, 2012).

The melanocortin-1-receptor (*MC1R*) gene is involved in the genetics of human pigmentation. Binding of α-melanocyte-stimulating hormone (α-MSH) to MC1R stimulates the synthesis of melanin-activating adenylate cyclase enzyme, thereby elevating intracellular cyclic adenosine monophosphate (cAMP). Pigmentation is determined by regulation of the melanin proportion of photoprotective eumelanin and phaeomelanin, the latter being potentially mutagenic because it generates free radicals following UV exposure (Garcia-Borron *et al*, 2005).

MC1R is a highly polymorphic gene: more than 100 non-synonymous variants have been described to date (Garcia-Borron *et al*, 2005; Gerstenblith *et al*, 2007; Perez Oliva *et al*, 2009). Functional analysis of some of these variants revealed partial loss of the receptor's ability to stimulate cAMP pathway, leading to a quantitative shift of melanin synthesis from eumelanin to phaeomelanin (Duffy *et al*, 2004). Phaeomelanin is associated with the 'red hair colour' (RHC) phenotype, characterised by fair skin, red hair, freckles and sun sensitivity (solar lentigines and low tanning response) (Box *et al*, 1997). Variant alleles of the following six single nucleotide polymorphisms rs1805006 (D84E), rs11547464 (R142H), rs1805007 (R151C), rs1110400 (I155T), rs1805008 (R160W) and rs1805009 (D294H) were defined as 'R' alleles for their association with the RHC phenotype in population or familial association studies. The rs1805005 (V60L), rs2228479 (V92M) and rs885479 (R163Q) variants seem to have a lower association with RHC phenotype and have been designated as 'r' alleles (Garcia-Borron *et al*, 2005).

Previous studies reported that the risk of NMSC is higher among carriers of *MC1R* variants (Smith *et al*, 1998; Bastiaens *et al*, 2001; Kennedy *et al*, 2001; Han *et al*, 2006; Scherer and Kumar, 2010). However, it is not well known which variants are mostly associated with NMSC and whether the association completely depends on pigmentation characteristics.

The first aim of this study was to evaluate the association between specific and combined *MC1R* variants and the risk of NMSC through a large multicenter pooled-analysis of individual data from the melanocortin-1-receptor gene, skin cancer and phenotypic characteristics (M-SKIP) project. The second aim was to evaluate whether risk estimates differed by phenotypic characteristics.

MATERIALS AND METHODS

Data for the present analyses were gathered through the M-SKIP project, which was previously described (Raimondi *et al*, 2012). Briefly, we collected data from epidemiological studies on *MC1R* variants, sporadic cutaneous melanoma (CM), NMSC and phenotypic characteristics associated with skin cancer from 33 investigators who agreed to participate in the M-SKIP project. 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. We created a pooled database, including data on 8301 CM cases, 3542 NMSC cases and 15 589 controls.

For the present study, we identified in the M-SKIP database 8 independent case–control studies on NMSC (Kennedy *et al*, 2001; Dwyer *et al*, 2004; Scherer *et al*, 2008; Brudnik *et al*, 2009; Liu *et al*, 2009; Nan *et al*, 2009; Ferrucci *et al*, 2012; Andresen *et al*, 2013) that overall included data on 2587 BCC cases, 788 SCC cases, 152 cases with both BCC and SCC, and 9391 controls.

Statistical analysis. First, we compared population characteristics reported in publications of non-participant authors with those of studies included in our analysis, to assess the representativeness of our study population. Categorical and continuous variables were compared by the χ^2-test and by the Wilcoxon two-sample test, respectively. Small-study effects was graphically represented by funnel plots and formally assessed by Egger's test. We verified the departure of frequencies of each *MC1R* variant from expectation under Hardy–Weinberg (HW) equilibrium by the χ^2-test in controls for each included study.

We first pooled BCC and SCC together to evaluate the association between *MC1R* variants and NMSC risk overall, and then performed separate analyses to test the association of *MC1R* variants with BCC and SCC risk. For the first step, we considered all 3527 cases (2587 BCC, 788 SCC and 152 with both), while for the second step we first considered 2739 cases for BCC (2587 BCC and 152 with both) and then 940 cases for SCC (788 SCC and 152 with both). For all the analyses, controls are subjects free of any skin cancer.

We previously 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 (Pasquali *et al*, 2015). For each study, we calculated the odds ratio (OR) with 95% confidence interval (CI) of *MC1R* variants by applying logistic regression to the data. Beyond *MC1R*, each model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, and smoking status. Coding and standardisation of the variables in the M-SKIP database has been described elsewhere (Raimondi *et al*, 2012). For each study, we imputed missing data with multiple imputation models for variables with $<20\%$ of missing data, by using the iterative Markov chain Monte Carlo method, as previously described (Schafer, 1997). We choose this method because several data sets presented non-monotone missing data patterns and because it is robust to minor departures from the assumptions of multivariate normality (Schafer, 1997). For each imputation procedure, five data sets were generated, which was considered an adequate number for multiple imputation (Rubin, 1996). The results from the five logistic regression models applied to the imputed data sets were then combined for the inference with proc mianalyze (SAS software, Cary, NC, USA).

We performed the analysis using two different criteria to define the reference category for *MC1R*: the first one was applied to the four studies where *MC1R* was sequenced, and it used the wild-type (WT) subjects as a reference category for each variant; the second one was applied to the four studies where *MC1R* gene was not sequenced, and it used, for each study, subjects without any of the tested *MC1R* variants as a reference category for each variant. For this latter analysis, it should be noted that reference category

includes both WT and carriers of any *MC1R* variant, which was not specifically assessed in each original study (Table 1).

In all analyses, we took into account all the identified variants and calculated the OR for: (1) carrying at least one *MC1R* variant; (2) carrying just one *MC1R* variant; and (3) carrying two or more *MC1R* variants. Finally, a *MC1R* score was calculated, by summing across the *MC1R* alleles, giving a value of 1 to 'r' and 2 to 'R' variants. To calculate this score, we considered both common and rare variants and classified them as previously suggested (Davies *et al*, 2012).

Following the two-stage analysis approach, we pooled study-specific ORs using a random-effects model, implemented by the DerSimonian–Laird method. When there were more than one OR calculated in a single study (i.e., analysis by *MC1R* score), we took into account the correlation between the ORs by using the multivariate approach of van Houwelingen *et al* (2002). We evaluated homogeneity among study-specific estimates by the Q-statistic and I^2, which represents the percentage of total variation across studies that is attributable to heterogeneity rather than to chance. We considered that statistically significant heterogeneity existed when the P-value was $\leqslant 0.10$. When significant heterogeneity was revealed, we performed sensitivity analysis and meta-regression by year of publication of the study, geographic area where the study was carried out, *MC1R* genotyping methodology, deviation from HW equilibrium, type of controls and DNA source. To evaluate the robustness of the results, we also compared the pooled-OR obtained using the M-SKIP data set with the meta-OR calculated by pooling risk estimates reported in studies from not-participating investigators also using DerSimonian–Laird random-effects models.

We computed the attributable risk (AR) in the population for the presence of at least one *MC1R* variant and for the *MC1R* variants found to be statistically significantly associated with NMSC by using the Miettinen's formula: $(OR-1/OR)\times$ proportion of cases exposed, with the corresponding 95%CI.

Finally, we performed a stratified analysis, to investigate whether the observed association between *MC1R* variants and NMSC varied according with different phenotypic characteristics. Phenotypic characteristics were taken into account only in stratified analysis and were not considered as confounders in

Table 1. Description of the 8 case–control studies included in the pooled-analysis of BCC and SCC

					Mean age (s.d.)		Males (%)		
First author, publication year	**Country**	***MC1R* genotyping variables**	**Controls type**	***N* cases/ *N* controls**	**Cases**	**Controls**	**Cases**	**Controls**	**Available confounders[a]**
BCC									
Kennedy *et al*, 2001	The Netherlands	All	Hospital	341/378	62 (10)	58 (11)	54	42	Continuous and intermittent sun exposure, sunburns, smoking status
Dwyer *et al*, 2004	Australia	V60L D84E R151C R160W D294H	Population	157/290	44 (9)	44 (10)	48	46	Continuous and intermittent sun exposure, sunburns
Scherer *et al*, 2008	Hungary, Romania, Slovakia	All	Hospital	529/532	65 (10)	60 (12)	45	51	Intermittent sun exposure
Brudnik *et al*, 2009	Poland	V60L D84E V92M R142H R151C I155T R160W R163Q D294H	Hospital	110/489	68 (12)	43 (19)	43	40	—
Nan *et al*, 2009	USA	V60L V92M R151C I155T R160W R163Q D294H	Population	299/323	64 (7)	59 (7)	0	0	Sunburns
Rotterdam Study (Liu *et al*, 2009)	The Netherlands	V60L R142H R151C R160W R163Q	Population	927/6559	73 (8)	72 (9)	48	41	—
Ferrucci *et al*, 2012	USA	All	Hospital	376/383	35 (5)	35 (6)	32	30	Continuous and intermittent sun exposure, sunburns, smoking status
Total				2739/8954	62 (15)	66 (14)	40	40	
SCC									
Kennedy *et al*, 2001	The Netherlands	All	Hospital	151/378	66 (8)	58 (11)	66	42	Continuous and intermittent sun exposure, sunburns, smoking status
Dwyer *et al*, 2004	Australia	V60L D84E R151C R160W D294H	Population	144/290	50 (6)	44 (10)	54	46	Continuous and intermittent sun exposure, sunburns
Nan *et al*, 2009	USA	V60L V92M R151C I155T R160W R163Q D294H	Population	286/307	65 (7)	60 (7)	0	0	Sunburns
Rotterdam Study (Liu *et al*, 2009)	The Netherlands	V60L R142H R151C R160W R163Q	Population	272/6559	74 (8)	72 (9)	53	41	—
Andresen *et al*, 2013	Norway	All	Hospital[b]	87/130	56 (11)	63 (10)	63	62	—
Total				940/7664	65 (11)	69 (11)	40	40	

Abbreviations: BCC = basal cell carcinoma; MC1R = melanocortin-1-receptor; SCC = squamous cell carcinoma; s.d. = standard deviation.
[a]Beyond age and gender, which were available in all the studies.
[b]Controls are subjects with functional renal grafts at time of invitation.

previous analyses because they are likely in the pathway between MC1R and NMSC. Study-specific ORs were adjusted by age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, and smoking status, where available. 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 approach of van Houwelingen *et al* (2002).

The analysis was carried out using SAS (version 9.2, Cary, NC, USA) and STATA (version 11.2, Lakeway, TX, USA).

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.

Table 1 summarises the eight case–control studies included in the pooled analyses, four of which provided information on both BCC and SCC, three on BCC only and one on SCC only. The studies were published between 2001 and 2013 and the majority of them were carried out in Europe ($N=4$ out of 7 (57%) and $N=3$ out of 5 (60%), for BCC and SCC subgroups, respectively). For the BCC analysis, hospital controls were recruited in four studies (57%) and population controls in three studies (43%), while for the SCC analysis population controls were included in three studies (60%) and hospital controls in the remaining two studies (40%). All the studies included patients with histological confirmed diagnosis, except the Nurses Health Study (Nan *et al*, 2009) in which histological and self-reported diagnosis were collected. For this latter study, however, the validity of self-reported diagnosis was reported to be 90% (Nan *et al*, 2009). A complete sequencing analysis of the *MC1R* coding region was performed in three studies (43%) in BCC subgroup and in two studies (40%) in SCC subgroup. In general, cases were of a similar age, or slightly older than controls, and except for a study which only included women the sex distribution was similar between cases and controls. Individual information on age and sex were available for each study, but information on other potential confounders varied between studies. Among the eight included studies, 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 for R142H (Liu *et al*, 2009) and R151C (Dwyer *et al*, 2004), and in two studies for R160W (Brudnik *et al*, 2009; Liu *et al*, 2009) and D294H (Brudnik *et al*, 2009; Andresen *et al*, 2013). Further information on cases identification is presented in Supplementary Table S1.

Association between combined MC1R variants and NMSC. We found that subjects carrying any *MC1R* variant had a significantly increased risk of NMSC (Table 2) compared with subjects without any *MC1R* assessed variant. In more detail, carrying at least one *MC1R* variant increased the risk of NMSC overall, BCC and SCC: summary OR (SORs) (95%CI) were 1.48 (1.24–1.76), 1.39 (1.15–1.69) and 1.61 (1.35–1.91), respectively.

Carriers of two or more *MC1R* variants always presented higher SORs compared with subjects carrying one *MC1R* variant: SORs (95%CI) were 1.80 (1.49–2.17) for NMSC overall, 1.70 (1.36–2.12) for BCC and 2.10 (1.60–2.76) for SCC (Table 2).

Table 2. Summary odds ratios for the association between combined *MC1R* variants and non-melanoma skin cancer and heterogeneity estimates

	All studies				Sequenced studies only	
Variant	*N* studies (*N* cases/*N* controls)	SOR (95%CI)	*Q*-test *P*-value	I^2 (%)	*N* studies (*N* cases/*N* controls)	SOR (95%CI)
All						
Wild type[a]	8 (1162/4419)	Reference	—	—	4 (360/539)	Reference
Any variant	8 (2365/4972)	**1.48 (1.24–1.76)**	**0.01**	**60.5**	4 (1074/884)	**1.78 (1.50–2.11)**
1 variant	9 (1628/3942)	**1.40 (1.19–1.65)**	0.24	22.7	4 (670/650)	**1.54 (1.29–1.85)**
2+ variants	8 (737/1030)	**1.80 (1.49–2.17)**	**0.001**	**70.5**	4 (404/234)	**2.49 (1.99–3.12)**
Score[b] 1	8 (776/2043)	**1.24 (1.02–1.51)**	0.27	19.2	4 (350/371)	**1.41 (1.15–1.73)**
Score[b] 2	8 (1041/2183)	**1.61 (1.33–1.96)**	0.31	15.3	4 (422/354)	**1.81 (1.47–2.22)**
Score[b] 3	8 (351/439)	**1.93 (1.52–2.46)**	**0.001**	**69.5**	4 (199/111)	**2.68 (2.01–3.57)**
Score[b] ⩾4	8 (197/307)	**1.80 (1.37–2.38)**	**0.02**	**57.5**	4 (103/48)	**2.68 (1.81–3.96)**
BCC						
Wild type[a]	7 (937/4288)	Reference	—	—	3 (322/506)	Reference
Any variant	7 (1802/4666)	**1.39 (1.15–1.69)**	**0.01**	**63.6**	3 (924/787)	**1.75 (1.46–2.09)**
1 variant	7 (1244/3720)	**1.31 (1.08–1.60)**	0.21	28.6	3 (581/588)	**1.52 (1.26–1.83)**
2+ variants	7 (558/946)	**1.70 (1.36–2.12)**	**0.002**	**70.8**	3 (343/199)	**2.48 (1.96–3.15)**
Score[b] 1	7 (610/1925)	1.17 (0.94–1.46)	0.26	22.8	3 (308/344)	**1.39 (1.12–1.72)**
Score[b] 2	7 (786/2046)	**1.51 (1.21–1.87)**	0.27	20.5	3 (359/307)	**1.78 (1.43–2.21)**
Score[b] 3	7 (264/399)	**1.80 (1.37–2.36)**	**0.001**	**72.1**	3 (176/91)	**2.85 (2.10–3.86)**
Score[b] ⩾4	7 (142/296)	**1.62 (1.19–2.20)**	**0.07**	**48.7**	3 (81/45)	**2.36 (1.56–3.56)**
SCC						
Wild type[a]	5 (272/3783)	Reference	—	—	2 (45/175)	Reference
Any variant	5 (668/3881)	**1.61 (1.35–1.91)**	0.42	0	2 (192/333)	**2.17 (1.44–3.28)**
1 variant	5 (452/3124)	**1.55 (1.24–1.94)**	0.70	0	2 (113/237)	**1.89 (1.22–2.92)**
2+ variants	5 (216/757)	**2.10 (1.60–2.76)**	0.19	35.3	2 (79/96)	**2.80 (1.71–4.57)**
Score[b] 1	5 (190/1561)	1.23 (0.92–1.64)	0.59	0	2 (51/120)	1.51 (0.91–2.52)
Score[b] 2	5 (308/1755)	**1.94 (1.48–2.53)**	0.79	0	2 (80/145)	**2.28 (1.42–3.67)**
Score[b] 3	5 (105/318)	**2.28 (1.59–3.28)**	0.11	46.5	2 (34/49)	**2.49 (1.37–4.54)**
Score[b] ⩾4	5 (65/247)	**2.33 (1.50–3.61)**	0.10	48.2	2 (27/19)	**4.93 (2.28–10.64)**

Abbreviations: CI = confidence interval; MC1R = melanocortin-1-receptor; SOR = summary odds ratio. Note: significant ORs and *P*-values are in bold.
[a]For studies that did not sequence *MC1R* gene it includes both wild-type and carriers of any *MC1R* variant not specifically assessed in the original study.
[b]Score calculated as detailed in Davies *et al* (2012).

We observed a significant linear trend for one point increase in *MC1R* score for NMSC overall, BCC and SCC: per-point SOR (95%CI) were 1.25 (1.14–1.36), $P<0.0001$; 1.22 (1.11–1.34), $P<0.0001$ and 1.28 (1.17–1.41), $P<0.0001$, respectively (results not shown).

We then restricted the analysis only to the studies that sequenced the *MC1R* gene. ORs for these studies were always higher than ORs obtained both on the whole group of studies and on the studies with *MC1R* not sequenced ($P<0.0001$, results not shown).

Association between single MC1R variants and NMSC. The nine most prevalent *MC1R* variants in the M-SKIP database were: V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q and D294H. Table 3 and Supplementary Figure S1 present SORs for the association of each variant with NMSC in the whole group of eight studies, using as reference group for each variant the subjects without any *MC1R* assessed variant. Table 3 also presents results obtained by restricting the analysis only to the studies that sequenced the *MC1R* gene. We found positive associations between all the investigated *MC1R* variants and NMSC, with SORs always higher than 1.00 for both the whole group of studies and the sequenced studies. Particularly, in the whole group of studies, a statistically significant association with NMSC overall was found for all variants except R142H and I155T. Furthermore, we observed a significant association with BCC for six *MC1R* variants: V60L, D84E, V92M, R151C, R160W and D294H, and a significant association with SCC for six variants: V60L, V92M, R151C, I155T, R160W and D294H. Significant heterogeneity was found for 5 variants in the NMSC pooled analyses (D84E, R151C, I155T, R160W and R163Q) and for five variants in BCC (V60L, R151C, I155T, R160W and R163Q).

By meta-regression, we found that genotype methodology significantly affected the risk estimates for some analysed variants, probably due to different reference categories. Restricting the analysis only to the studies that sequenced the *MC1R* gene, ORs were almost always higher than ORs obtained both on the whole group of studies and on the studies with *MC1R* not sequenced ($P<0.0001$, results not shown). ORs significance was consistent for all *MC1R* variants with the only exception of the I155T that reached statistical significance for NMSC overall and BCC, while lost statistical power to confirm the association with SCC. Other

Table 3. Allele frequency, summary odds ratios for the association between single *MC1R* variants and non-melanoma skin cancer and heterogeneity estimates

			All studies				Sequenced studies only	
***MC1R* variant**	**NMSC**	**Allele frequency in controls (%)**	***N* studies (*N* cases/ *N* controls)**	**SOR (95%CI)[a]**	***Q*-test *P*-value**	**I^2 (%)**	***N* studies (*N* cases/ *N* controls)**	**SOR (95%CI)[a]**
V60L	All	9.7%	8 (3403/9129)	**1.42 (1.19–1.70)**	0.13	36.5	4 (1434/1423)	**1.73 (1.39–2.16)**
	BCC		7 (2664/8695)	**1.39 (1.12–1.72)**	**0.07**	**48.6**	3 (1246/1293)	**1.75 (1.39–2.20)**
	SCC		5 (886/7406)	**1.53 (1.22–1.93)**	0.67	0	2 (237/508)	**1.98 (1.16–3.38)**
D84E	All	0.5%	5 (1720/1703)	**2.66 (1.06–6.65)**	**0.07**	**53.1**	4 (1434/1423)	**3.16 (1.06–9.42)**
	BCC		4 (1396/1573)	**3.52 (1.49–8.31)**	0.16	42.2	3 (1246/1293)	**4.55 (1.75–11.82)**
	SCC		3 (375/788)	1.96 (0.45–8.55)	0.13	50.5	2 (237/508)	2.01 (0.18–22.76)
V92M	All	8.8%	6 (2125/2541)	**1.56 (1.23–1.97)**	0.20	30.4	4 (1434/1423)	**1.74 (1.38–2.20)**
	BCC		5 (1651/2105)	**1.46 (1.09–1.96)**	0.13	44.3	3 (1246/1293)	**1.74 (1.37–2.22)**
	SCC		3 (523/814)	**1.94 (1.32–2.85)**	0.85	0	2 (237/508)	**1.81 (1.05–3.10)**
R142H	All	0.6%	4 (2389/7469)	1.20 (0.74–1.94)	0.40	0	3 (1347/1293)	1.37 (0.66–2.84)
	BCC		4 (2123/7469)	1.12 (0.68–1.87)	0.41	0	3 (1246/1293)	1.29 (0.63–2.64)
	SCC		2 (412/6554)	1.59 (0.59–4.24)	0.83	0	1 (150/378)	1.87 (0.31–11.22)
R151C	All	6.0%	8 (3465/9229)	**1.99 (1.50–2.65)**	**0.002**	**67.7**	4 (1434/1423)	**2.57 (1.90–3.48)**
	BCC		7 (2698/8796)	**1.86 (1.35–2.56)**	**0.004**	**69.1**	3 (1246/1293)	**2.52 (1.68–3.77)**
	SCC		5 (915/7509)	**2.10 (1.53–2.87)**	0.21	31.3	2 (237/508)	**3.16 (1.82–5.50)**
I155T	All	1.0%	5 (2040/2408)	1.80 (0.87–3.72)	**0.06**	**52.0**	3 (1347/1293)	**2.38 (1.25–4.53)**
	BCC		5 (1655/2105)	1.54 (0.69–3.45)	**0.07**	**53.6**	3 (1246/1293)	**2.33 (1.23–4.44)**
	SCC		2 (434/681)	**4.60 (1.44–14.75)**	0.42	0	1 (150/378)	11.46 (0.93–140.38)
R160W	All	8.5%	8 (3475/9238)	**1.67 (1.37–2.05)**	**0.08**	**43.4**	4 (1434/1423)	**1.92 (1.52–2.43)**
	BCC		7 (2702/8805)	**1.61 (1.26–2.06)**	**0.03**	**55.9**	3 (1246/1293)	**1.89 (1.47–2.42)**
	SCC		5 (920/7515)	**1.97 (1.57–2.47)**	0.72	0	2 (237/508)	**2.66 (1.60–4.43)**
R163Q	All	4.9%	7 (3217/9097)	**1.50 (1.11–2.02)**	**0.05**	**51.0**	4 (1434/1423)	**1.93 (1.22–3.06)**
	BCC		6 (2574/8660)	1.39 (0.99–1.94)	**0.05**	**54.3**	3 (1246/1293)	1.69 (0.96–2.99)
	SCC		4 (792/7374)	1.37 (0.90–2.08)	0.24	28.6	2 (237/508)	1.85 (0.60–5.73)
D294H	All	1.2%	7 (2393/2816)	**2.06 (1.45–2.93)**	0.86	0	4 (1434/1423)	**2.37 (1.39–4.05)**
	BCC		6 (1788/2382)	**1.77 (1.17–2.68)**	0.97	0	3 (1246/1293)	**2.09 (1.17–3.72)**
	SCC		4 (656/1095)	**2.90 (1.70–4.96)**	0.39	0.6	2 (237/508)	**5.07 (1.79–14.36)**

Abbreviations: BCC = basal cell carcinoma; CI = confidence interval; MC1R = melanocortin-1-receptor; NMSC = non-melanoma skin cancer; SCC = squamous cell carcinoma; SOR = summary odds ratio. Note: significant ORs and *P*-values are in bold.

[a]For studies that did not sequence MC1R gene reference category includes both wild-type and carriers of any MC1R variant not specifically assessed in the original study. For studies that sequenced MC1R gene it includes only wild type.

possible sources of between-study heterogeneity, as publication year, study area, deviation from HW equilibrium, source of controls and source of DNA, seemed not to play a role to the observed heterogeneity. Otherwise, sensitivity analysis indicated that the heterogeneity may be attributable to single studies: when we excluded the studies that were outliers in the corresponding funnel plot, we obtained similar pooled-ORs than the original analysis, but with no more evidence of heterogeneity among study-specific estimates (results not shown).

Funnel plots for each *MC1R* variant are presented in Supplementary Figure S2 for the whole set of eight studies. We found suggestion of small-study effects for R151C and R160W variants, with *P*-values of 0.021 and 0.006, respectively.

Figure 1 presents AR for *MC1R* variants significantly associated with NMSC in the previous analysis. The highest AR for both BCC and SCC was observed for R151C (7.3% and 11.1%, respectively), followed by R160W (7.0% and 11.2%, respectively).

Meta-ORs calculated for studies not included in the M-SKIP project were similar to those obtained from our pooled-analysis for all variants (results not shown).

Analysis stratified by phenotypic characteristics. Table 4 presents SORs for the association between NMSC and any *MC1R* variant stratified by skin type, hair colour and freckles. For this analysis, subjects without any *MC1R* assessed variant were the reference group for each variant. We consistently observed higher SORs for the association between *MC1R* and NMSC for subjects without red hair and without freckles. For hair colour, the difference between SOR of red-haired and not red-haired subjects was statistically significant for SCC and borderline for NMSC overall ($P = 0.01$ and 0.06, respectively), indicating that *MC1R* is more important for subjects with darker hair colour than with red hair. Similarly, although not significant, subjects without freckles have greater *MC1R*-associated risk than subjects with freckles. Stratified analyses on the four studies that sequenced the *MC1R* gene were not feasible due to the limited sample size in each strata-specific analysis.

DISCUSSION

We found a statistically increased risk of NMSC for carriers of at least one *MC1R* variant, with slightly higher SOR observed for SCC than for BCC. Although these two kinds of tumours share many similarities, they present rather different incidence rates and aetiological factors. It has been suggested that neoplastic transformation of epithelial cells requires significantly less UV for BCC than for SCC (Rosso *et al*, 1996). Cumulative exposure to sunlight was indeed found to be the main risk factor for SCC, while intermittent sun exposure plays a major role in BCC development (IARC, 2012).

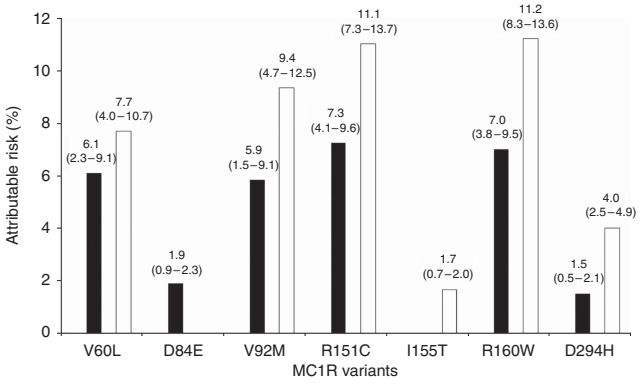

Figure 1. Attributable risks[a] with 95% confidence intervals in the population for non-melanoma skin cancer according to different *MC1R* variants[b] (percentages). Black bars represent BCC, white bars represent SCC. [a]Miettinen's formula (OR − 1/OR × proportion cases exposed). [b]Only variants significantly associated with BCC and/or SCC are represented.

We consistently observed significant *MC1R*-associated NMSC risk only for subjects without red hair, while in subjects with red hair *MC1R* seemed not to have an effect in addition to phenotype. Previous studies (Bastiaens *et al*, 2001; Liboutet *et al*, 2006; Scherer *et al*, 2008; Ferrucci *et al*, 2012; Andresen *et al*, 2013) also suggested that *MC1R* variants had an independent role in NMSC by phenotypic characteristics, and a similar finding has been observed for melanoma development (Pasquali *et al*, 2015). *MC1R* may therefore contribute to skin carcinogenesis through other mechanisms than pigmentation. *MC1R* signalling has been implicated in a number of key biological pathways involved in cell cycle control (April and Barsh, 2007), apoptosis (Hauser *et al*, 2006), and activation of DNA repair mechanisms and antioxidant defenses (Bohm *et al*, 2005; Kadekaro *et al*, 2010; Maresca *et al*, 2010; Kadekaro *et al*, 2012). In addition, stimulation of *MC1R* also activates the MAPK pathway and regulates target genes involved in inflammation through the NF-κb pathway (Wikberg *et al*, 2000). Finally, α-MSH affects proliferation and differentiation of both melanocytes and keratinocytes (Slominski *et al*, 1991).

Concerning individual variants, all of them showed a positive association with NMSC, with a consistent statistically significant association with either NMSC overall, BCC and SCC observed for V60L, V92M, R151C, R160W and D294H. Functional studies revealed that these variants resulted in inefficient or even absent activation of the cAMP pathway downstream. Specifically, V60L, R151C and R160W variants reduced cell-surface expression with a corresponding impairment in cAMP activation, while the loss-of-function phenotype of the D294H variant is probably due to inability to properly undergo the agonist-induced transition to the active state and/or to impaired coupling to the Gs protein (Schioth *et al*, 1999; Beaumont *et al*, 2007; Herraiz *et al*, 2012). Only a marginal effect of the V92M substitution on cell-surface expression or ability to activate the cAMP and ERK cascades has been reported (Beaumont *et al*, 2007; Herraiz *et al*, 2012).

The R163Q variant reached statistical significance only for the analysis of NMSC overall, while the D84E variant was associated with both NMSC overall and BCC. Furthermore, the I155T variant was associated only with SCC, although after restricting analysis to sequenced studies there was a suggestive association of I155T with BCC and NMSC overall. For D84E and I155T, receptor impairment in cAMP coupling is largely accounted by reduced cell-surface expression (Beaumont *et al*, 2007; Sanchez-Laorden *et al*, 2007, 2009). It is not clear whether our results are attributable to a specific role of the above-mentioned variants in the pathogenesis of each tumour type. This would need to be further investigated in functional studies focused on the carcinogenic mechanisms leading to BCC and SCC, respectively.

Finally, the R142 variant did not show a statistically significant association with NMSC, probably due to its low allele frequency (<1%) and, consequently, limited statistical power.

In previous studies, contradictory results were reported for the associations of the most common *MC1R* variants and NMSC (Smith *et al*, 1998; Jones *et al*, 1999; Bastiaens *et al*, 2001; Dwyer *et al*, 2004; Han *et al*, 2006; Liboutet *et al*, 2006; Scherer *et al*, 2008; Andresen *et al*, 2013), probably due to small sample size of single studies, especially for variants with relatively low allele frequencies.

To the best of our knowledge, our study has for the first time put together and meta-analysed results from different studies on *MC1R* and NMSC, thus providing powerful estimates of the association between single and combined *MC1R* variants and NMSC risk in populations living in different geographical areas.

Table 4. Stratified analysis for any *MC1R* variants and non-melanoma skin cancer association, according with skin type, hair colour and freckles

Phenotypic characteristic	Strata	MC1R variant	*N* studies (*N* cases/*N* controls)	SOR (95%CI)	*P*-value[a]
All					
Skin type	I/II	Wild type[b]	7 (224/279)	1.00 (reference)	0.29
		Any variant	7 (861/738)	**1.42 (1.12–1.80)**	
	III/IV	Wild type[b]	7 (382/736)	1.00 (reference)	
		Any variant	7 (872/972)	**1.66 (1.38–1.99)**	
Hair colour	Red	Wild type[b]	4 (17/31)	1.00 (reference)	0.06
		Any variant	4 (105/211)	0.67 (0.32–1.44)	
	Other	Wild type[b]	7 (971/4145)	1.00 (reference)	
		Any variant	7 (1796/4394)	**1.40 (1.18–1.66)**	
Freckles	Yes	Wild type[b]	3 (171/217)	1.00 (reference)	0.51
		Any variant	3 (545/428)	**1.52 (1.08–2.15)**	
	No	Wild type[b]	3 (122/190)	1.00 (reference)	
		Any variant	3 (275/214)	**1.79 (1.18–2.71)**	
BCC					
Skin type	I/II	Wild type[b]	6 (176/258)	1.00 (reference)	0.13
		Any variant	6 (642/654)	1.28 (0.97–1.69)	
	III/IV	Wild type[b]	6 (289/633)	1.00 (reference)	
		Any variant	6 (638/766)	**1.63 (1.31–2.05)**	
Hair colour	Red	Wild type[b]	4 (13/31)	1.00 (reference)	0.23
		Any variant	4 (90/211)	0.78 (0.33–1.83)	
	Other	Wild type[b]	6 (753/4020)	1.00 (reference)	
		Any variant	6 (1292/4118)	**1.31 (1.08–1.57)**	
Freckles	Yes	Wild type^	3 (116/217)	1.00 (reference)	0.66
		Any variant	3 (431/428)	**1.52 (1.04–2.23)**	
	No	Wild type^	3 (103/190)	1.00 (reference)	
		Any variant	3 (222/214)	**1.71 (1.09–2.67)**	
SCC					
Skin type	I/II	Wild type[b]	4 (54/129)	1.00 (reference)	0.41
		Any variant	4 (251/297)	**2.02 (1.34–3.06)**	
	III/IV	Wild type[b]	4 (96/271)	1.00 (reference)	
		Any variant	4 (245/385)	**1.62 (1.18–2.24)**	
Hair colour	Red	Wild type[b]	2 (6/27)	1.00 (reference)	**0.01**
		Any variant	2 (21/170)	0.34 (0.11–1.07)	
	Other	Wild type[b]	5 (263/3749)	1.00 (reference)	
		Any variant	5 (593/3662)	**1.59 (1.33–1.90)**	
Freckles	Yes	Wild type[b]	2 (58/137)	1.00 (reference)	0.11
		Any variant	2 (127/216)	1.31 (0.86–2.02)	
	No	Wild type[b]	1 (25/125)	1.00 (reference)	
		Any variant	1 (82/167)	**2.32 (1.33–4.03)**	

Abbreviations: BCC = basal cell carcinoma; CI = confidence interval; MC1R = melanocortin-1-receptor; SCC = squamous cell carcinoma; SOR = summary odds ratio. Note: significant ORs and *P*-values are in bold.

[a]Overall *P*-value for any significant difference among strata-specific ORs.

[b]Reference category for SORs is subjects without any of the assessed *MC1R* variants.

Moreover, the availability of individual data from each study allowed a stratified analysis by phenotypic characteristics to be performed, and thus the independent contribution of *MC1R* variants on NMSC risk to be assessed. We were also able to provide both separate risk estimates for BCC and SCC and a combined risk estimate for NMSC overall. A further strength is that we took into account in our centralised statistical analysis all the available confounders, with a homogeneous plan of analysis and homogeneous definition of co-variables. Our results might have an important impact on public health since *MC1R* variants may be considered, along with other epidemiological risk factors, in both primary and secondary prevention strategies for NMSC, the most common neoplasm in populations of European origin. The improved identification of at risk subjects might enable public-health messages and early diagnostic procedures to be targeted to the population at risk.

One limitation of our study is that *MC1R* gene sequencing was completed in only four out of the eight studies included in our pooled-analysis, and thus we were able to compare carriers of *MC1R* variants with WT subjects only in a small subset of studies. As was previously pointed out (Williams *et al*, 2011; Pasquali *et al*, 2015), the inclusion of some *MC1R* variants in the reference category for the analyses would lead to underestimate the true risk of disease, because *MC1R* variants are very common among populations of European origins (66–67% of our study population had at least one variant). We sought to overcome this problem in our analysis by excluding from the reference category all the *MC1R* variants that were specifically assessed in each study. Since the nine most common variants were examined in the majority of studies, the reference category would mainly include WT and rare variants that were observed in ~4% of the study subjects in the M-SKIP data set. We performed separate stratified analyses on subjects with and without red hair, skin type I/II and freckles, but unfortunately we could not compare subjects with and without any of these at risk phenotypic characteristics because they were collected jointly only in two studies (Kennedy *et al*, 2001; Ferrucci *et al*, 2012). Lack of availability of information on other genes in most studies prevented the analysis of possible gene–gene interactions. Other genes have been indeed involved in NMSC development and include pigmentation genes like *ASIP*, *TYR*, *TYRP1 OCA2*, *SLC*, *POMC* and *IRF4* (Nan *et al*, 2009; Scherer and Kumar, 2010), and, for BCC, the inactivating mutations in the *PTCH* gene (Liboutet *et al*, 2006). Since we carried out a retrospective pooled-analysis, we did not perform centralised

sequencing. However, previous studies (Harland *et al*, 2008; Davies *et al*, 2012) reported excellent concordance in sequencing data from different centres. Finally, differences in the assessment of sun exposure did not allow us to use this variable in stratified analysis, although it was possible to take it into account the adjustment for confounders.

In conclusion, our pooled-analysis provided evidence for a role of all the most common variants in NMSC development, with consistent significant association with NMSC overall found for the *MC1R* variants V60L, D84E, V92M, R151C, R160W, R163Q and D294H. Since the contribution of *MC1R* variants in addition to phenotype in NMSC risk was mainly observed in subjects with no red-hair or no freckles, prevention strategies involving avoidance of indoor and outdoor ultraviolet radiation should not only be recommended for fair skin phenotypes, and *MC1R* assessment may be tailored to darker-pigmented subjects.

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