ELECTRONIC LETTER

Melanocortin 1 receptor (*MC1R*) gene variants may increase the risk of melanoma in France independently of clinical risk factors and UV exposure

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Malignant melanoma (MM) is increasing in most Caucasian populations, the incidence doubling every 10 years.[1] Multiple phenotypic risk factors, including the number of melanocytic naevi (moles), freckling, dysplastic naevi, propensity to sunburn, and the number of severe sunburn episodes during youth, have been identified in the aetiology and pathogenesis of this disease.[2]

Approximately 10% of MM cases occur in kindreds, suggesting hereditary predisposition to melanoma, often in association with the atypical mole syndrome (AMS) phenotype. Germline mutations affecting two highly penetrant genes predisposing to melanoma, *CDKN2A* and *Cdk4*, have been associated with an increased risk for the development of familial cutaneous melanoma.[3–6] However, these mutations are found in only 30 to 40% of kindreds, indicating that other genes may predispose to MM.

In addition to the major predisposition to melanoma caused by these genes, polygenic inheritance determining the development of melanoma has been shown to depend upon polymorphisms located on genes controlling different cellular pathways such as DNA repair,[7] pigmentation,[8] and reactive oxygen detoxification.[9 10] Among these, loss of function variants of the human melanocortin 1 receptor gene (*MC1R*), which plays a crucial role in pigmentation,[11 12] seems important in determining MM risk.[8 13]

MC1R maps to chromosome 16q24.3 and encodes a G protein coupled receptor with seven transmembrane domains expressed on many cell types,[14] including melanocytes. *MC1R* is the receptor of two melanocortin peptides synthesised in the pituitary gland, alpha melanocyte stimulating hormone (α-MSH) and ACTH. These have the same affinity for *MC1R*, and are cleavage products of the large precursor peptide propiomelanocortin. Their binding to this receptor activates adenylate cyclase, increases intracellular c-AMP production, then leads to enhanced tyrosinase transcription and traduction, and, ultimately, to production of photoprotective eumelanin and melanocyte proliferation. Regulation of melanogenesis also depends on many paracrine factors, such as agouti signalling protein (ASP), an *MC1R* antagonist, and endothelin 1.[15 16] Recently, the physiological role of ASP in the regulation of human pigmentation has been suggested. This role may be through the association of a polymorphism, localised 25bp downstream of the TGA termination codon in the non coding exon 4 of the ASP gene, with dark hair and eye colours in a Caucasian population from Pennsylvania.[17]

Recent investigations have demonstrated that *MC1R* is highly polymorphic in the Caucasian population.[12 18 19] About 30 variants have been described, of which nine have been demonstrated to be loss of function variants.[20 21] Some of these variants (Val60Leu, Ile40Thr, Arg142His, Arg151Cys, Arg162Pro, Arg160Trp, and Asp294His) are unable to stimulate cAMP production as strongly as the wild type receptor in response to α-MSH stimulation,[22–26] whereas others (Val122Met) demonstrate a decreased α-MSH binding affinity.[25] Three *MC1R* variants alleles (Arg151Cys, Arg160Trp, and Asp294His) have been shown to be associated with the red hair and fair skin phenotype (RHC). RHC is characterised by fair pigmentation (fair skin, red hair, and freckles), and by sun sensitivity (poor tanning response and

Key points

- Variants causing loss of function of the melanocortin 1 receptor (*MC1R*) gene, a key control of human skin pigmentation, increase the risk of malignant melanoma (MM) in Queensland, the UK, and the Netherlands.
- The prevalence and role of *MC1R* variants in genetic predisposition to MM are not known in the French population; 108 patients with MM and 105 controls were investigated for these variants. The patients were divided into two categories: those suspected of genetic predisposition to MM but without *CDKN2A* or *Cdk4* germline mutations (79), and those with sporadic simple melanomas (29).
- *MC1R* variants causing loss of function were present in 68% of patients *v* 31% of controls ($p<0.0001$), confirming the role of *MC1R* in genetic predisposition to MM in the French population. Three frequent variants were significantly associated with MM risk: Val60Leu, Arg151Cys, and Arg160Trp. The risk persisted after stratification on clinical risk factors (skin colour and type, hair and eye colour, solar lentigines, and naevus count) and UV exposure parameters.
- The data showed that *MC1R* variants causing loss of function are a strong and independent melanoma risk factor in France.
- Assessment of *MC1R* status as well as of clinical risk factors could be useful in the identification of high risk groups which could then be targeted for prevention.

Abbreviations: MM, malignant melanoma; FAM, familial melanoma; MPM, multiple primary melanoma; NPIM, non-photo induced melanoma; ALM, acral lentiginous melanoma; *MC1R*, melanocortin1 receptor; α-MSH, alpha melanocyte stimulating hormone; PAR, population attributable risk; AMS, atypical mole syndrome; ASP, agouti signalling protein; RHC, red hair characteristics; NMSC, non-melanoma skin cancer

solar lentigines).[11 12 18 19 27 28] In addition, seven other alleles (Val60Leu, 86insA, Asp84Glu, Arg142His, Ile155Thr, 537insC, and His260Pro) may be statistically considered full or partial RHC causing alleles, as shown by genetic associations in populations or through inheritance of phenotype in families.[29]

MC1R variants have also been found to be associated with both MM and non-melanoma skin cancer (NMSC) risks.[8 30–34] Furthermore, *MC1R* variants appear to increase the penetrance of $p16^{INK4A}$ mutations in melanoma prone families.[35 36]

Despite these data, several questions remain to be elucidated. First, *MC1R* studies have been carried out only in Caucasian populations with RHC originating from North Europe; the relationship between *MC1R* variants and MM in other populations is still unknown. Secondly, at present it is not clear whether the MM risk attributed to *MC1R* variants is distinct from its effects on pigmentation characteristics.[8] Thirdly, the prevalence of *MC1R* variants in patients with a suspected hereditary predisposition to MM has only been investigated in Australian melanoma families.[8] Finally, the role of *MC1R* variants regarding UV irradiation has not yet been examined.

Therefore, we sought to examine the association between *MC1R* variants and MM risk in a French case control study, by comparing individuals who had histologically confirmed MM with controls without personal or family history of skin cancer. Additional specific aims of this study were to assess the prevalence of *MC1R* variants among patients suspected of genetic predisposition to MM, divided into subgroups: familial melanoma (FAM), multiple primary melanoma (MPM), melanoma associated with another cancer, melanoma arising before the age of 25 years, and non-photo induced melanoma (NPIM). The risk for MM from *MC1R* variants was estimated after adjustment for clinical risk factors (naevi count, skin types I and II, eye colour, fair hair and skin colours, lentigines) and UV exposure (particularly severe sunburns and high intermittent exposure).

MATERIALS AND METHODS

Study population

We recruited 108 patients with MM and 105 controls.

Of the MM patients, 29% were incident cases and 71% were prevalent cases. The median time between diagnosis and genetic investigation was 44.6 months. All participants were Caucasian and attended dermatology departments in one of three hospitals in Paris (France), namely Bichat Claude-Bernard, Percy, and Saint-Louis Hospitals. The study population consisted of patients aged from 20 to 80 years with histologically proved MM.

A total of 79 patients sharing features that might underlie genetic predisposition to MM, but without germline mutations in the *CDKN2A* or *Cdk4* genes, were enrolled according to five different criteria:

- sporadic multiple primary melanomas (MPMs), with at least 2 MMs at different sites
- familial melanomas (FAMs), with at least two cases of MM in first or second degree relatives
- MMs associated with another cancer
- MMs diagnosed before the age of 25 years
- MMs considered to be non-photo induced (NPIMs).

NPIMs included melanomas located on non-photo exposed sites, and subungual and acral lentiginous melanomas (ALMs). ALMs are considered to be a particular subtype of MM because, in contrast to other subtypes, they exclusively involve acral skin which is rarely exposed to sunlight, thus suggesting that ultraviolet irradiation is not a major factor in their development.[37 38] Six patients who carried *CDKN2A* germline mutations were not included (three with FAMs, one with MPM, one with MM before the age of 25 years, and one with NPIM).

Another group of 29 ''simple'' melanoma patients without any of the above criteria was also enrolled. Patients were not included if immune depressed (HIV or transplantation), or suffering from a genodermatosis predisposing to skin cancer (albinism, Gorlin's syndrome, or xeroderma pigmentosum).

A control group, without any personal or familial history of skin cancer, was enrolled for the comparison of *MC1R* allele frequencies. The subjects, aged from 20 to 80 years, were recruited in the same hospitals, as representative of the same demographic area as the patients with MM. Information concerning personal and familial details (parents and grandparents) and country of birth was also recorded. Non-Caucasians were not included in the study.

The Medical Ethics Committee (CCPPRB) approved the study protocol. Informed consent was obtained from all subjects.

Collection of data on risk factors for MM

All participants attended for a standardised personal interview and a total skin examination with a dermatologist, to collect data which was entered onto a preprinted examination sheet.

Information concerning skin (recorded as being either dark, medium, or light), eyes (classified as dark (brown) or light (blue, green, or grey)), and original hair colour (classified as red, blond, light brown, dark brown, or black) was collected. We also recorded naevus body count ($<$ or $\geqslant$50 naevi); presence of solar lentigines; and presence of AMS. AMS was recorded if 50 or more naevi were found, at least three being clinically atypical: 6 mm or more in diameter, variable pigmentation, and indistinct and irregular outline).[39] Skin type was assessed according to TB Fitzpatrick's classification (1988) as follows:

- I, always burns and never tans
- II, always burns and then tans
- III, always tans and sometimes burns
- IV, always tans and never burns.

In addition, for the MM group, location of lesions, age at diagnosis, and histopathological data were collected.

Assessment of UV exposure

A questionnaire was used to determine the history of severe sunburns before and after the age of 15 years, defined as erythema for more than 48 hours or blistering (scored yes/no). Intermittent sun exposure before and after the age of 15 years was defined as UV exposure during the holidays: beachside or sunny vacations were scored as strong exposures. Chronic exposition was evaluated throughout the year, and was scored as nil, light, medium, or strong. Sunscreen use was noted as never, sometimes, or always.

Detection of *MC1R* variants

Genomic DNA was isolated from peripheral blood leukocytes of all participants by routine methods. The *MC1R* coding sequence was amplified by PCR with two overlapping couples of primers: *MC1R*-F1–5′-CAG CAC CAT GAA CTA AGC AGG ACA CCT G -3′ and *MC1R*1-IR-5′-CCA GCA TAG CCA GGA AGA AGA CCA CGA G -3′, and *MC1R*-F2–5′-TGG GTG GCC AGT GTC GTC TTC AGC A -3′ and *MC1R*2-R-5′-AAG GGT CCG CGC TTC AAC ACT TTC AGA G -3′, (respective sizes of PCR products, 671 and 610 bp). The PCR reaction mixture comprised 150 ng genomic template DNA, 5 µl 10X PCR buffer, 25 mM $MgCl_2$, 5 µM each of dGTP, dTTP, dATP, and dCTP, 22.5 pmol each of PCR primer, and 2 U AmpliTaq (Perkin Elmer; Courtabocuf cedex, France), in a total volume

of 50 µl. Samples were denatured for six minutes at 96°C, and passed through 35 cycles of amplification, consisting of 30 seconds of denaturation at 95°C, 30 seconds of primer annealing at 60°C, one minute of elongation at 72°C, and final elongation for three minutes at 72°C. The amplifications were carried out in 0.5 ml tubes (Perkin Elmer).

Sequence analysis

DNA samples for sequencing were obtained by PCR as described above. Sequence analysis was performed on an ABI-Prism 3100 automated DNA sequencer using 10 ng PCR purified products and Big-Dye Terminator Cycle Sequencing kits (Perkin Elmer), according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were carried out using SAS software release 8.2 (SAS Institute, Cary, NC, USA). ANOVA or χ^2 analysis (Fisher's exact test when necessary) were used to compare clinical and genetic characteristics, as well as UV exposure parameters, between MM patients and controls.

For clinical analysis, the usual MM risk factors were compared between cases and controls.

For genetic analysis, the only *MC1R* polymorphisms retained were:

- those previously shown to be loss of function variants by functional studies[22–26 41 47]
- those associated with MM risk[8 13 30]
- those associated with RHC phenotype[29]
- those predicted to be possibly damaging by the Polyphen informatics program (http://tux.EMBL-Heidelberg.DE/ramensky/).

This approach was conservative and allowed us to include all patients and controls for the analysis. Pairwise linkage disequilibrium between the different *MC1R* polymorphisms was also studied, using the EH (Estimation of Haplotypes) program. First, univariate analyses were used to compare genetic risk factors (*MC1R* genotype) between MM patients and controls. Secondly, multiple logistic regression analysis (using a stepwise procedure) was performed to take into account potential confounders among clinical risk factors, such as skin type, eye and hair colour, number of naevi, AMS, dorsal lentigines, and UV exposure. Odds ratios (ORs) were calculated with 95% confidence intervals. All significance levels reported were two-sided and set at $p<0.05$.

Comparisons were carried out of age at onset between:

- MM patients carrying *MC1R* variants *v* MM patients that do not carry *MC1R* variants
- and MM patients carrying *MC1R* variants *v* MM patients carrying germline *CDKN2A* mutations.

Comparisons were performed using the ANOVA test.

The percentage population attributable risk (PAR%) associated with the most frequent *MC1R* variants was calculated as previously described.[48]

RESULTS

Composition of patient population

The final series for analysis comprised 213 participants: 108 patients with MM and 105 controls. The percentage of individuals that were born in France, with both parents born in France, was 70% in the patients' group, and 60% in the control group. Other places of birth included European countries (Switzerland, Germany, Greece, Spain, Portugal, Italy, Slovenia, Croatia, and Turkey) or the Maghreb (Tunisia, Algeria, Morocco). The 108 patients were categorised into two different groups.

The first group was composed of 79 patients suspected of genetic predisposition to MM, including the following pathologies:

- 18 FAMs, three of which harboured the typical AMS phenotype
- 17 sporadic MPMs
- 11 MMs with an additional cancer (two mammary adenocarcinomas, one papillary thyroid carcinoma, one prostate carcinoma, one uterine carcinoma, one carcinoma of the colon, four non-melanoma skin cancers, one meningioma)
- 20 MMs diagnosed before the age of 25 years
- 14 NPIMs (one on the anus, two on the colon, four on the buttocks, two on the genitals, one on the scalp, and four ALMs (three subungual and one on the sole of the foot)).

The second group comprised 28 simple sporadic MMs without any of the above pathologies.

Clinical characteristics

The clinical characteristics of all patients with MM and of control subjects are summarised in table 1. The strongest risk factors identified for MM were a mole count >50 ($p<0.0001$), presence of AMS ($p = 0.0004$), presence of fair skin ($p<0.0001$), and dorsal lentigines ($p<0.0001$). Other pigmentation characteristics (light eye and hair colours), as well as severe sunburns before and after the age of 15 years, were lower predictors of MM risk (table 1). In this sample, skin type I or II, although more frequent in MM patients, was not significantly associated with MM risk.

Ultraviolet exposure

Univariate analysis showed that severe sunburns before and after the age of 15 years ($p = 0.03$ and 0.05, respectively), intermittent exposure during holidays after the age of 15 years ($p = 0.03$), and absence of sunscreen use ($p = 0.04$) were associated with MM risk, as in previous studies (table 1). Multiple analyses, after controlling for other variables, confirmed this association for two parameters—absence of sunscreen use, and severe sunburns after the age of 15 years (data not shown). In this study, cumulative sun exposure, as determined by chronic exposure during the week during occupational activities, was not identified as a risk factor for MM (data not shown).

Frequency of *MC1R* variants and effect on MM risk

We identified 16 non-synonymous *MC1R* variant alleles, of which 12 have shown experimental and/or predicted loss of function effects, and/or have been previously associated with MM risk and/or RHC (see *Materials and Methods* and table 2). Among these, two previously unreported *MC1R* variants (Arg142Cys and Tyr298His) were observed, each in one (differing) MM patient.

Functional variants were much more common in the MM group, with 73/108 patients (67.6%) carrying at least one *MC1R* variant, compared with only 33/105 control subjects (31.4%) ($p<0.0001$, table 3). In addition, there was a gene dosage effect of *MC1R* variant on MM risk (ORs, respectively, 4.3 for one variant and 6.78 for two variants; Mantel Haenszel trend test $p<0.0001$).

Not all individual *MC1R* alleles conferred the same MM risk (table 2). Only three variants (Val60Leu, Arg151Cys, and Arg160Trp) were significantly more frequent in the MM group, with the strongest risk being for Arg151Cys (OR 6.26). The nine other *MC1R* variants (Val122Met, Arg142His, Arg142Cys, Ile155Thr, ins86, Ser83Pro, Asp84Glu, Asp294His, and Tyr298His) were not individually associated with MM

Table 1 Clinical characteristics and number of patients with melanoma and of controls

	Melanoma (108)	Controls (105)	p	OR
Gender				
Women	64.8 (70)	54.3 (57)	–	Reference
Men	35.2 (38)	45.7 (48)	0.12	0.6 (0.4–1.1)
Mean age at onset (SD) and range	44.5±17.3 (14–77)	*51 (20–93)	0.008	NA
Skin type				
III/IV	52.0 (53)	59.0 (62)	–	Reference
I/II	48.0 (49)	41.0 (43)	0.31	1.3 (0.8–2.4)
Skin colour				
Dark	6.9 (7)	27.6 (29)	–	Reference
Medium/light	93.1 (94)	72.4 (76)	<0.0.001	5.1 (2.1–12.3)
Hair colour				
Dark or dark brown	52.0 (51)	72.4 (76)	–	Reference
Blond, light brown, red	48.0 (47)	27.6 (29)	0.0028	2.4 (1.3–4.3)
Eye colour				
Dark	46.9 (46)	67.6 (71)	–	Reference
Light	53.1 (52)	32.4 (34)	0.003	3.4 (1.3–4.2)
Total naevi				
⩽50	53.5 (54)	93.3 (98)	–	Reference
>50	46.5 (47)	6.7 (7)	<0.0001	12.2 (5.2–28.8)
Atypical mole syndrome				
No	80.6 (83)	96.2 (101)	–	Reference
Yes	19.4 (20)	3.8 (4)	0.0004	6.1 (2.0–18.5)
Lentigines				
No	34.4 (32)	67.0 (69)	–	Reference
Yes	65.6 (61)	33.0 (34)	<0.0001	3.9 (2.1–7.0)
Sunburns with blistering at <15 years of age				
No	50.0 (49)	64.8 (68)	–	Reference
Yes	50.0 (49)	35.2 (37)	0.03	1.8 (1.0–3.2)
Sunburns with blistering at >15 years of age				
No	41.4 (41)	55.2 (58)	–	Reference
Yes	58.6 (58)	44.8 (47)	0.05	1.7 (1.0–3.0)
Holiday UV exposure at <15 years of age				
Low	11	21	–	2.0 (0.9–4.4)
High	87	84	0.09	Reference
Holiday UV exposure at >15 years of age				
Low	10	22	–	Reference
High	89	83	0.03	2.4 (1.1–5.3)
Sunscreen use				
Yes	15	28	–	Reference
No	75	68	0.04	2.1 (1.0–4.2)

NA, not applicable, *, median age; **OR,** odds ratios with 95% confidence intervals.

Table 2 Allelic frequencies of *MC1R* variants in patients with melanoma and in control subjects

	Patients (n = 216)	Controls (n = 210)	Odds ratios (95% CI)	p
***MC1R* consensus**	46.3 (100)	74.7 (157)	–	–
***MC1R* variants type 1**				
Val92Met	5.6 (12)	7.1 (15)	–	–
Arg163Gln	4.6 (10)	2.4 (5)	–	–
Thr95Met	1.4 (3)	0.9 (2)	–	–
Val180Ile	0 (0)	0.5 (1)	–	–
Total *MC1R* wt	57.9 (125)	85.7 (180)	1.0	Reference
***MC1R* variants type 2**				
Arg151Cys†§††	8.3 (18)	1.9 (4)	6.48 (2.14–19.61)	0.0002
Arg160Trp†§	7.9 (17)	2.4 (5)	4.73 (1.70–13.18)	0.0013
Val60Leu†	16.7 (36)	6.2 (13)	3.99 (2.03–7.82)	<0.0001
Asp294His†‡§††	3.7 (8)	1.4 (3)	3.84 (0.90–22.81)	0.058*
Arg142His†‡††	1.4 (3)	0 (0)	NA	0.07*
Arg142Cys††	0.5 (1)	0 (0)	NA	0.41
Ile155Thr††	0.9 (2)	0.9 (2)	1.44 (0.10–20.08)	1.0
Ser83Pro††	0.5 (1)	0 (0)	NA	0.41
Asp84Glu§††	1.4 (3)	0.9 (2)	2.16 (0.24–26.14)	0.41*
Tyr298His††	0.5 (1)	0 (0)	NA	0.41
Val122Met‡	0 (0)	0.5 (1)	NA	1.0*
Ins 86‡‡	0.5 (1)	0 (0)	NA	0.41
Total variants type 2	42.1 (91)	14.3 (30)	4.37 (2.73–7.00)	<0.0001

n, number; NA, not applicable.
ORs are indicated with 95% confidence interval (CI).
Type 1 variants may not modify *MC1R* function, as these variants have not been previously associated with melanoma nor tested in functional assays (see statistical analyses in *Material and Methods*). These variants are pooled with the *MC1R* consensus sequence and considered as wild type in statistical analyses.
Type 2 variants have been shown to result in diminished *MC1R* function, and/or to be associated with fair pigmentation characteristics (RHC),[29] and/or to be strongly associated with the risk of melanoma in previous studies.
*Fisher's exact test.
†*MC1R* variants unable to stimulate cAMP production as strongly as the wild type receptor in response to alpha melanocyte stimulating hormone (α-MSH).[2–26]
‡*MC1R* variants showing a decreased α-MSH binding affinity.[25]
§*MC1R* variants previously shown to be associated with melanoma risk.[8 13 30]
¶*MC1R* variants previously shown to be associated with the RHC phenotype.[29]
††*MC1R* variants predicted to be damaging (deducted from the Polyphen program).

risk, although it should be noted that for Asp294His statistical results were nearly significant (p = 0.058).

MC1R variants allelic frequency observed in incident cases (genetic investigation within one year after MM diagnosis) was not statistically different from that observed in prevalent cases (36.8% *v* 45%; p = 0.25, Fisher's exact test).

The PAR% of MM associated with the main *MC1R* variants is indicated in table 4. Val60Leu has the highest PAR, followed by Arg151Cys and Arg160Trp.

Effect of *MC1R* on the age of onset of melanoma

No effect of *MC1R* genotype on the age of onset of melanoma was observed: median age at diagnosis of MM patient carriers of *MC1R* variants was 46.17 *v* 39.88 years for the MM group without variant (p = 0.087; standard deviation 17.11 *v* 17.17 using the ANOVA test). Median age at diagnosis was lower for patients carrying *CDKN2A* mutations (35.5 years, range 23–64), but this was not statistically significant (p = 0.23, ANOVA test), presumably because of the small number of *CDKN2A* mutated patients studied.

Distribution of *MC1R* variants in the different MM subgroups

Table 5 shows the distribution of *MC1R* variants among the five MM subgroups. The highest *MC1R* allelic frequencies were observed in FAM and MPM subgroups (50%), whereas the lowest were observed in the NPIM group (28.5%). However, there was no statistical difference in *MC1R* allelic frequencies between the different subgroups (p = 0.37), or between the simple MM group and the group with more stringent genetic criteria (FAM, MPM, NPIM, MM <25 years of age, MM associated with another cancer) (p = 0.35).

Table 3 *MC1R* genotype in patients with melanoma and in control subjects; association of *MC1R* variants with the risk (odds ratios) of MM

MC1R genotype	Patients (n = 108)	Controls (n = 105)	p	Odds ratios
Wt/Wt	31.5 (34)	68.6 (72)	_	Reference
Wt/V	52.8 (57)	26.7 (28)	<.0001	**4.3** (2.34–7.93)
V/V	14.8 (16)	4.7 (5)	<.0002	**6.78** (2.29–20.03)

n,number.
Wt represents the wild type allele; V represents variant alleles.
Odds ratios (indicated with 95% confidence intervals) compare Wt/V (heterozygotes) and V/V (two functional variants) with Wt/Wt.

Table 4 Percentage population attributable risk associated with the most frequent *MC1R* variants

Variant	*PAR (%)	CI (95%)
Val60Leu	10.18	(3.93; 16.03)
Arg151Cys	6.55	(2.31; 10.61)
Arg160Trp	5.62	(1.35; 9.72)
Asp294His	2.31	(0.75; 5.27)

*The percentage population attributable risk (PAR%) associated with the four frequent *MC1R* variants was calculated as previously described,[48] and is indicated with 95% confidence intervals (CI).

Table 5 *MC1R* variants allelic frequencies among the different subgroups

Subgroup	*MC1R* variants		Allelic frequency
1	Present*	Absent	
1(n = 18)	15	3	50%
2 (n = 17)	14	3	50%
3 (n = 20)	12	8	32%
4 (n = 11)	8	3	41%
5 (n = 14)	8	6	28.5%
6 (n = 28)	20	8	46%
Total (n = 108)	77	31	41%

n, number.
1, familial melanoma; 2, multiple primary melanoma; 3, melanoma before the age of 25 years; 4, melanoma associated with another cancer; 5, non-photo induced melanoma; 6, simple melanoma.
*, number of patients carrying at least one *MC1R* variant described in table 2.

Effect of *MC1R* on pigmentation characteristics

In order to study the effects of *MC1R* on pigmentation characteristics and naevus count, cases and controls were grouped together. Statistical analysis confirmed previous observations, in that *MC1R* variants were significantly associated with light hair colour (red, blond, light brown, $p<0.0001$); skin type I–II ($p = 0.0015$); solar lentigines ($p = 0.0019$); and either light or medium skin colour (Mantel Haenszel trend test, $p = 0.047$). In addition, *MC1R* variants were also associated with light eye colour ($p = 0.027$). Conversely, *MC1R* variants were not associated with naevus count ($p = 0.19$) (data not shown).

Persistence of MM risk after stratification on pigmentation characteristics and UV exposure

Clinical and epidemiological data indicated further investigation of the association between *MC1R* functional variants and MM risk. Table 6 shows the significant persistence of MM risk according to *MC1R* variants after stratification for the different melanoma associated clinical risk factors (skin types I or II, medium or light skin, red or light hair, light eye colour). These results strongly suggest that *MC1R* variants are an independent risk factor for the development of MM.

In addition, *MC1R* MM risk also persisted after stratification on classical UV exposure risk factors (severe sunburns and high intermittent UV exposure, both before and after the age of 15 years), suggesting that *MC1R* confers an MM risk independently of UV exposure (table 6). Interestingly, in multiple logistic regression analyses, which take into account all these potential confounders, the presence of *MC1R* variants was the second most important MM risk factor risk (odds ratio 4.52, 95% confidence interval 2.15–9.52), after a high naevus count (odds ratio 12.66, 95% confidence interval 4.81–33.33, table 7).

DISCUSSION

Numerous studies have shown that *MC1R* is highly polymorphic in the Caucasian population in the British Isles, Holland, and Australia,[12 18 28 42–44] and that some *MC1R* variants are associated with the risk of MM and non-melanoma skin cancer in populations of Celtic and/or Germanic origin (Australia, Scotland, Ireland, and the Netherlands).[8 13 33 45]

In this study, we have shown a strong association between functional *MC1R* variants and MM in the French population, which is of a different geographical origin. This confirms and highlights the role of *MC1R* in genetic predisposition to MM.

MC1R variants were previously found to be associated with RHC[18 19 27 28] and sun sensitivity.[46] In our study, *MC1R* functional alleles were also associated with medium or light skin colour, skin types I and II, fair hair colour (red, blond, and light brown), and presence of solar lentigines, further demonstrating that *MC1R* plays a crucial role in human pigmentation and response to UV radiation in a French population. However, it should be noted that the individual allelic frequencies of the main *MC1R* functional variants in our control population (table 2) were much lower than those reported in previous studies.[8 13 45] This difference in allele frequencies might due to the particular characteristics of the French population, which has a darker complexion and a different genetic ancestry from the other populations studied (that were mainly of Celtic origin). In addition, we observed a very low *MC1R* variant frequency in control subjects with skin type I/II (23%) (table 6). As it has been shown that pigmentation is a complex process under the control of at least 50 different loci in mice,[49] it seems reasonable to assume that other polymorphisms (localised in pigmentation genes different from *MC1R*) also play an important role in the genetic control of human pigmentation and could explain our result.

Two large studies reported that *MC1R* variants increase MM risk, with a mean odds ratio varying between 2 and 4, depending on whether one or two variants were present.[8 31] It is difficult to compare our data with the previous studies, as the coding sequence of *MC1R* was completely examined in only one of them.[13] However, we confirmed the effect of *MC1R* variants on MM risk, increasing the risk by a factor of four when one variant was present, and by seven when two variants were present (table 3). The *MC1R* related MM risks were nearly the double those previously reported,[8 13] a result most likely due to an increased frequency of the *MC1R* consensus genotype in our control population.

Separate analyses of individual *MC1R* variant alleles showed that three variant alleles (all of which are common) were significantly associated with an increased risk of MM (table 2). Arg151Cys and Arg160Trp showed the highest risk, as in previous studies.[8 31] However, Val60Leu, which was not or was weakly associated with MM risk in Australia and the Netherlands,[8 31] appeared here to be the most frequent variant and to be associated with an important MM risk (odds ratio 3.99, 95% confidence interval 2.03–7.82). Two

Table 6 Role of *MC1R* on MM risk after stratification on clinical risk factors and UV exposure parameters

Risk factor		Allele	*Cases	*Controls	p	OR	95%CI
Skin type	I/II	Total	49	43	–	–	–
		Wt/Wt	22 (11)	67 (29)	–	Reference	–
		Wt/V	51 (25)	23 (10)	0.0001	6.59	2.40–18.09
		V/V	27 (13)	9 (4)	0.0006	8.57	2.29–32.01
	III/IV	Total	52	62	–	–	–
		Wt/Wt	42 (22)	69 (43)	–	Reference	–
		Wt/V	52 (27)	29 (18)	0.007	2.93	1.33–6.43
		V/V	6 (3)	2 (1)	0.13$	5.86	0.43–314.79
Skin colour	Light/medium	Total	93	76	–	–	–
		Wt/Wt	30 (28)	71 (54)	–	Reference	–
		Wt/V	53 (49)	24 (18)	<0.0001	5.25	2.59–10.65
		V/V	17 (16)	5 (4)	0.0002	7.71	2.35–25.28
Eye colour	Light	Total	52	34	–	–	–
		Wt/Wt	37 (19)	79 (27)	–	Reference	–
		Wt/V	42 (22)	12 (4)	0.0004	7.81	2.32–26.38
		V/V	21 (11)	9 (3)	0.015	5.21	1.28–21.24
	Dark	Total	45	71	–	–	–
		Wt/Wt	31 (14)	63 (45)	–	Reference	–
		Wt/V	58 (26)	34 (24)	0.002	3.48	1.54–7.88
		V/V	11 (5)	3 (2)	0.018$	8.04	1.12–89.57
Hair colour	Red/blond/light brown	Total	47	29	–	–	–
		Wt/Wt	28 (13)	66 (19)	–	Reference	–
		Wt/V	45 (21)	21 (6)	0.004	5.12	1.62–16.14
		V/V	28 (13)	14 (4)	0.017	4.75	1.26–17.86
	Dark brown/black	Total	50	76	–	–	–
		Wt/Wt	40 (20)	70 (53)	–	Reference	–
		Wt/V	54 (27)	29 (22)	0.002	3.25	1.52–6.97
		V/V	6 (3)	1 (1)	0.077$	7.95	0.58–424.97
Naevus count	⩽50	Total	53	98	–	–	–
		Wt/Wt	30 (16)	68 (67)	–	Reference	–
		Wt/V	53 (28)	28 (27)	<0.0001	4.34	2.03–9.28
		V/V	17 (9)	4 (4)	0.0005$	9.42	2.21–45.91
Severe sunburns at <15 years of age	No	Total	49	68	–	–	–
		Wt/Wt	35 (17)	72 (41)	–	Reference	–
		Wt/V	55 (27)	27 (18)	0.0018	3.62	1.59–8.23
		V/V	10 (5)	1 (1)	0.016$	12.06	1.17–583.78
	Yes	Total	49	37	–	–	–
		Wt/Wt	31 (15)	62 (23)	–	Reference	–
		Wt/V	47 (23)	27 (10)	0.011	3.53	1.31–9.46
		V/V	22 (11)	11 (4)	0.026	4.22	1.13–15.72
Severe sunburns at at >15 years of age	No	Total	41	41	–	–	–
		Wt/Wt	34 (14)	71 (41)	–	Reference	–
		Wt/V	56 (23)	26 (15)	0.0007	4.49	1.85–10.93
		V/V	10 (4)	3 (2)	0.06$	5.86	0.72–68.94
	Yes	Total	57	47	–	–	–
		Wt/Wt	32 (18)	66 (31)	–	Reference	–
		Wt/V	47 (27)	28 (13)	0.0004	3.58	1.48–8.63
		V/V	21 (12)	6 (3)	0.0036	6.89	1.71–27.72
Holidays exposure at at <15 years of age	High	Total	86	84	–	–	–
		Wt/Wt	36 (31)	68 (57)	–	Reference	–
		Wt/V	48 (41)	26 (22)	0.0003	3.43	1.74–6.75
		V/V	16 (14)	6 (5)	0.002	5.15	1.70–15.64
Holidays exposure at at >15 years of age	High	Total	86	83	–	–	–
		Wt/Wt	35 (30)	71 (59)	–	Reference	–
		Wt/V	50 (43)	25 (21)	<0.0001	4.03	2.04–7.97
		V/V	15 (13)	4 (3)	0.0004	8.52	2.25–32.23

Wt, wild type allele; V, variant alleles.
*Results are indicated both by percentage of patients, and by number of patients (in parentheses).
Odds ratios (OR) compare Wt/V (heterozygotes) and V/V (two functional variants) with Wt/Wt.
CI, 95% confidence interval.

variants, Asp84Glu and Asp294His alleles, that were previously associated with an important MM risk,[8 30 31] were not here significantly associated with MM. This could probably be related to the size of our effectives, as their low frequency could have masked their effects on MM risk. We found no association with MM risk for the frequent variant Val92Met, in agreement with a previous report.[31]

There is a discrepancy concerning the effect of this substitution on *MC1R* function. One study[40] suggested that the Val92Met substitution reduced the binding affinity of *MC1R* for α-MSH, whereas another found that melanocytes homozygous for Val92Met responded dose dependently to α-MSH with stimulation of cAMP formation, tyrosinase activity, and proliferation, suggesting that this polymorphism did not represent a loss of function of *MC1R*.[47]

Table 7 *Statistical multiple analysis of MM risk factors

Risk factor	p	OR	95% CI
Naevus count>50	<0.0001	12.66	4.81–33.33
MC1R variant	<0.0001	4.52	2.15–9.52
Light eye colour	0.007	3.11	1.49–6.49
Solar lentigines	0.019	2.33	1.14–4.78

*Logistic regression multiple analysis includes all clinical risk factors and *MC1R* variants, and was performed using a stepwise procedure.
OR, odds ratio; CI, 95% confidence interval.

The PAR% of MM associated with the main *MC1R* variants (table 4) was much higher than that associated with *CDKN2A* mutations, which are very rare in most series of unselected melanoma.[50]

The allelic frequency of *MC1R* variants was the highest in MM patients with the most stringent genetic criteria (multiple and familial MM subgroups, table 5). However, our groups were too small to draw definitive conclusions, and we observed no statistical difference between the different MM subgroups; these results need to be assessed with a higher number of patients.

There is a debate as to whether the *MC1R* MM risk is independent or not of other melanoma clinical risk factors (skin type, mole count, and eye, hair, and skin colours). Initially, *MC1R* variants were shown to increase MM risk only in individuals whose darker complexions would normally be considered protective, the association between MM and *MC1R* variants disappearing in persons with light skins.[8] More recently, the role of *MC1R* was also demonstrated in individuals with skin types I and II and light hair colour in the Netherlands population.[31] We further investigated whether the association between *MC1R* variants and MM is independent of clinical risk factors in our French population, and showed the persistent effect of *MC1R* variants on MM risk after stratification for the different clinical risk factors (table 6). This shows that *MC1R* variants and pigmentary characteristics are independent MM risk factors, and confirms and extends the results of the Dutch study.[31] Furthermore, the effect of *MC1R* variants also persists after stratification on UV exposure parameters, particularly in the absence of severe sunburns (table 6) that are classically considered to be important risk factors for melanoma formation.[51]

The exact mechanisms underlying the increased risk to individuals carrying *MC1R* variants of developing MM are not known. First, recent investigations suggest that the quality of melanin, rather than its quantity, determines skin cancer risk.[52] In fact, increase of red yellow phaeomelanin in *MC1R* variant melanocytes could contribute to mutagenesis and confer susceptibility to MM and non-melanoma skin cancer by generating free radicals following UV exposure. In addition, melanocytes with non-functional *MC1R* exhibit a pronounced increase of sensitivity to the cytotoxic effect of UV radiation compared with melanocytes expressing functional *MC1R*.[47]

Secondly, *MC1R* variants could affect some UV induced cellular protective effects. The cell cycle inhibitor $p16^{INK4a}$, that plays a major role in genetic predisposition to familial melanoma and sporadic MM formation, appears to have an important role in a cell cycle checkpoint in skin after UV exposure.[53 54] As it has been shown that this UV epidermal induction of $p16^{INK4A}$ is induced by α MSH via *MC1R*,[55] the presence of *MC1R* variants could alter this effect, thereby reducing its tumour suppressor function and providing a physiopathological basis for the formation of melanoma cells. An additional hypothesis is that UV irradiation, in the presence of *MC1R* variants, induces an important oxidative stress with formation of reactive oxygen species that have been shown to lead to a decrease of interaction between $p16^{INK4A}$ and *Cdk4* proteins, thus promoting carcinogenesis.[56]

Thirdly, in our study, *MC1R* variants were also present in 57% of patients with non-photo induced melanoma, further suggesting that *MC1R* could act on MM risk via non-pigmentary mechanisms. One of these mechanisms could be an autocrine effect of α-MSH on melanoma cells. It has recently been demonstrated that α-MSH significantly reduces the growth and progression (via decreasing fibronectin binding) of wild type *MC1R* melanoma cells, but has no effect on melanoma cells with *MC1R* variants.[57]

Finally, we showed that in multiple logistic regression analysis taking into account all MM clinical and genetic risk factors, *MC1R* variants were identified as the second MM risk factor following a high naevus count (table 7). This result inclines us to consider the *MC1R* genotype to be an important MM marker risk, at least equal to eye colour, hair colour, or skin type in MM risk evaluation.

In conclusion, we have shown that carrying particular *MC1R* variants increases MM risk in France. The association between MM and *MC1R* variants persists once skin, hair, and eye colours, skin type, mole count, and AMS phenotypes are taken into account. In addition, *MC1R* variants were identified as the second MM risk factor following a high naevus count. If our data could be confirmed in larger series, this would suggest that assessing *MC1R* status, in association with clinical risk factors, might be useful in identifying high risk groups to be targeted for prevention.

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