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Common genetic variants associated with melanoma risk or naevus count in patients with wildtype *MC1R* melanoma

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Summary

Background Hypomorphic MC1R variants are the most prevalent genetic determinants of melanoma risk in the white population. However, the genetic background of patients with wildtype (WT) MC1R melanoma is poorly studied.
Objectives To analyse the role of candidate common genetic variants on the melanoma risk and naevus count in Spanish patients with WT MC1R melanoma.
Methods We examined 753 individuals with WT MC1R from Spain (497 patients and 256 controls). We used OpenArray reverse-transcriptase polymerase chain reaction to genotype a panel of 221 common genetic variants involved in melanoma, naevogenesis, hormonal pathways and proinflammatory pathways. Genetic models were tested using multivariate logistic regression models. Nonparametric multifactor dimensionality reduction (MDR) was used to detect gene–gene interactions within each biological subgroup of variants.
Results We found that variant rs12913832 in the HERC2 gene, which is associated with blue eye colour, increased melanoma risk in individuals with WT MC1R [odds ratio (OR) 1·97, 95% confidence interval (CI) 1·48–2·63; adjusted $P < 0·001$; corrected $P < 0·001$]. We also observed a trend between the rs3798577 variant in the oestrogen receptor alpha gene (ESR1) and a lower naevus count, which was restricted to female patients with WT MC1R (OR 0·51, 95% CI 0·33–0·79; adjusted $P = 0·002$; corrected $P = 0·11$). This sex-dependent association was statistically significant in a larger cohort of patients with melanoma regardless of their MC1R status ($n = 1497$; OR 0·71, 95% CI 0·57–0·88; adjusted $P = 0·002$), reinforcing the hypothesis of an association between hormonal pathways and susceptibility to melanocytic proliferation. Last, the MDR analysis revealed four genetic combinations associated with melanoma risk or naevus count in patients with WT MC1R.
Conclusions Our data suggest that epistatic interaction among common variants related to melanocyte biology or proinflammatory pathways might influence melanocytic proliferation in individuals with WT MC1R.

What is already known about this topic?

- Genetic variants in the MC1R gene are the most prevalent melanoma genetic risk factor in the white population. Still, 20–40% of cases of melanoma occur in individuals with wildtype MC1R.
- Multiple genetic variants have a pleiotropic effect in melanoma and naevogenesis. Additional variants in unexplored pathways might also have a role in melanocytic proliferation in these patients.

- Epidemiological evidence suggests an association of melanocytic proliferation with hormonal pathways and proinflammatory pathways.

What does this study add?

- Variant rs12913832 in the *HERC2* gene, which is associated with blue eye colour, increases the melanoma risk in individuals with wildtype MC1R.
- Variant rs3798577 in the oestrogen receptor gene is associated with naevus count regardless of the MC1R status in female patients with melanoma.
- We report epistatic interactions among common genetic variants with a role in modulating the risk of melanoma or the number of naevi in individuals with wildtype MC1R.

What is the translational message?

- We report a potential role of hormonal signalling pathways in melanocytic proliferation, providing a basis for better understanding of sex-based differences observed at the epidemiological level.
- We show that gene–gene interactions among common genetic variants might be responsible for an increased risk for melanoma development in individuals with a low-risk phenotype, such as darkly pigmented hair and skin.

Cutaneous melanoma is a complex and heterogeneous disease caused by the interaction of environmental, phenotypic and genetic risk factors.[1] Genetic risk factors involved in sporadic cutaneous melanoma include moderate-risk and low-risk common genetic variants.[2] The melanocortin 1 receptor gene (*MC1R*), which is a key regulator of human pigmentation,[3] is the main moderate-risk gene for melanoma.[4] *MC1R* variants are classified according to their association with the red hair colour phenotype, which is characterized by fair skin, red hair, freckles and sun sensitivity.[4] Individuals carrying hypomorphic variants in MC1R have increased melanoma risk compared with individuals who have wildtype (WT) MC1R, regardless of their phenotypic characteristics.[5]

Patients with WT MC1R melanoma account for a remarkable fraction of cases of melanoma, especially in Mediterranean and Latin American populations (20–40% of cases).[6–10] However, the genetic background associated with melanoma susceptibility in these individuals has been poorly studied. Melanoma low-risk common genetic variants are located in genes associated with naevogenesis and pigmentation traits, telomere maintenance, metabolism, and the immune system.[11–13] Interaction among these variants might be responsible for increased melanoma risk in individuals with WT MC1R.

Presence of a high number of naevi is one of the strongest phenotypic predictors of melanoma risk.[14] Naevogenesis is a polygenic process with a strong heritable component.[15–17] The role of MC1R in naevogenesis is still unclear,[18,19] but different genetic variants have been associated with the number, clinical characteristics and dermoscopic patterns of naevi.[20–24] Most of these variants have a pleiotropic effect in naevogenesis and melanoma.[25] Genetic variants involved in unexplored biological pathways might also be associated with an increased risk of developing melanocytic lesions.

Epidemiological data suggest a potential role of hormonal pathways in naevogenesis and melanoma development. The total naevus count (TNC) typically peaks during puberty, which is characterized by an increase in circulating sex hormones levels, and children with higher TNC have a higher frequency of family history of breast cancer.[26] Higher TNC is also associated with higher breast cancer risk in both premenopausal[27] and postmenopausal women.[28] Furthermore, there is a bidirectional association between breast cancer and melanoma occurrence,[29] and there are sex-related differences in melanoma incidence.[30]

The immune pathogenic background of autoimmune diseases might also be involved in melanocytic proliferation. Patients with immune-mediated skin diseases, such as atopic dermatitis[31] and psoriasis,[32] have a lower TNC than unaffected individuals. Also, immunosuppressive therapies in patients with inflammatory autoimmune diseases, including Crohn disease, have been associated with melanoma development[33,34] and eruptive naevi.[35,36]

Here, we aimed to investigate the role of candidate common genetic variants on the risk of developing melanoma in individuals with WT MC1R and to evaluate their association with the TNC in the subgroup of patients with melanoma. Genetic variants analysed included variants involved in melanomagenesis and naevogenesis, hormonal pathways and/or proinflammatory pathways.

Patients and methods

Study population

Three different series of individuals were included in the study: (i) 1738 patients with melanoma from the Melanoma

Unit of the Hospital Clínic de Barcelona (HCB) (Spain); (ii) 169 individuals with no history of melanoma with phenotypic and/or naevus data available, recruited for this study or undergoing continuous dermatological screening at HCB, and (iii) 500 healthy controls representative of the Spanish population provided by the Spanish National DNA Bank-Carlos III (BNADN) (Spain). Genomic DNA was obtained in all cases (Appendix S1; see Supporting Information).

Clinical, phenotypic and naevus data from individuals recruited at HCB were prospectively obtained by direct examination or retrospectively recovered from the patients' digital dermoscopy images by trained dermatologists. The number of naevi was categorized into low or high TNC (≤ 50 vs. > 50 naevi larger than 2 mm, respectively). Phenotypic and naevus data were not available for control individuals recruited at BNADN. The age reported refers to the moment of diagnosis in patients with melanoma, the moment of phenotyping in individuals without melanoma from HCB, and the moment of DNA sampling in controls from BNADN.

The study was approved by the clinical research ethics committee of HCB (HCB/2015/0820). Each participant signed written informed consent according to the Declaration of Helsinki.

MC1R molecular screening

The whole coding region of *MC1R* was sequenced as previously reported (Appendix S1).[37] In terms of *MC1R* status, patients carrying no *MC1R* variant (consensus sequence) or only synonymous *MC1R* variants were considered to have the WT.

Selection and genotyping of candidate genetic variants

We selected 256 candidate genetic variants based on previous results of the group and/or information available in public databases such as PubMed and GWAS Central (Table S1; see Supporting Information). Variant genotyping was performed using OpenArray® technology (Thermo Fisher Scientific, Waltham, MA, USA). Quality filtering of genotyping data was performed using PLINK v1.07,[38] and only data from individuals with WT *MC1R* (793 of 2407). In short, we excluded four sex-related variants, 25 variants with low genotyping rates (< 95%), one variant that failed the Hardy–Weinberg equilibrium test in the control cohort ($P \leq 0{\cdot}001$), one variant with a minor allele frequency (MAF) < 0·05, and four variants with linkage disequilibrium ($r^2 > 0{\cdot}8$; from each of four pairs the variant with the most missing data was removed). Next, we excluded 40 individuals with ≥ 5% of missing variants. Eventually, 221 variants met the quality criteria in the 753 remaining individuals (average genotyping rate of 99·7%) (Figure 1). Details on genetic variant selection and quality filtering are provided in Appendix S1.

Statistical analysis

Statistical analyses were performed using PLINK v1.07[38] and SPSS Statistics 23.0 (IBM, Armonk, NY, USA). Demographic characteristics were compared using Student *t*-tests for continuous variables, and the Pearson χ^2-test or Fisher exact test for categorical variables, as appropriate. To estimate the association of each variant with melanoma susceptibility or TNC, we calculated odds ratios (ORs) and their 95% confidence intervals (CIs) using multivariable logistic regression analysis under additive models and adjusting for age and sex. For specific variants, we performed sex-stratified analyses. We applied Benjamini–Hochberg false discovery rate (FDR-BH) as a multiple comparison test, considering all variants analysed or only those variants involved in a specific biological set. All tests were two sided and were considered statistically significant at $P < 0{\cdot}05$.

Multifactor dimensionality reduction

We used a multifactor dimensionality reduction (MDR) approach (www.epistasis.org/software) (scikit-mdr-0.4.4, mdr_3.0.2, scikit-learn-0.24.2) to identify the genetic variant combinations that provided the best discrimination of the status of the patients (i.e. melanoma status or TNC). For each outcome of interest, we performed separate MDR analyses including only those variants from a specific biological set that showed statistical significance in the corresponding multivariable logistic regression analysis prior to the FDR-BH correction. For each comparison, we explored all possible locus combinations in 10-fold cross-validation and selected the classifier with the highest testing balanced accuracy. Statistical significance was evaluated using a 100-fold permutation test ($P < 0{\cdot}05$). The character of the interactions was estimated through an entropy-based approach, which enables the estimation of the information gain (IG) associated with each attribute or pair of attributes.[39] Further details are provided in Appendix S1.

Results

In 1738 patients with melanoma and 669 healthy individuals, we genotyped 256 candidate genetic variants associated with melanoma, naevogenesis, pigmentation traits, hormonal pathways and/or proinflammatory pathways (Table S1). *MC1R* molecular characterization was performed in 96·7% (2328 of 2407) of these individuals. Among them, 65·9% (1535 of 2328) harboured at least one nonsynonymous *MC1R* variant, and 34·1% (793 of 2328) were carrying synonymous variants or had a WT *MC1R* gene (all considered as having WT *MC1R*). Further analyses were performed within 753 individuals with WT *MC1R* with high genotyping completion rates (≥ 95%) for the 221 candidate variants that met the quality criteria, including 497 patients with melanoma (mean age 54·8 years, SD 17·2; 50·5% male) and 256 control individuals (mean age 47·6 years, SD 9·06; 50·8% male) (Figure 1).

Among controls, phenotypic data were available only for those individuals recruited at HCB (n = 37). Table 1 summarizes the demographic and clinical characteristics of the individuals from HCB included in the analysis. We found no significant differences regarding the phototype or hair colour between individuals with and without a personal history of

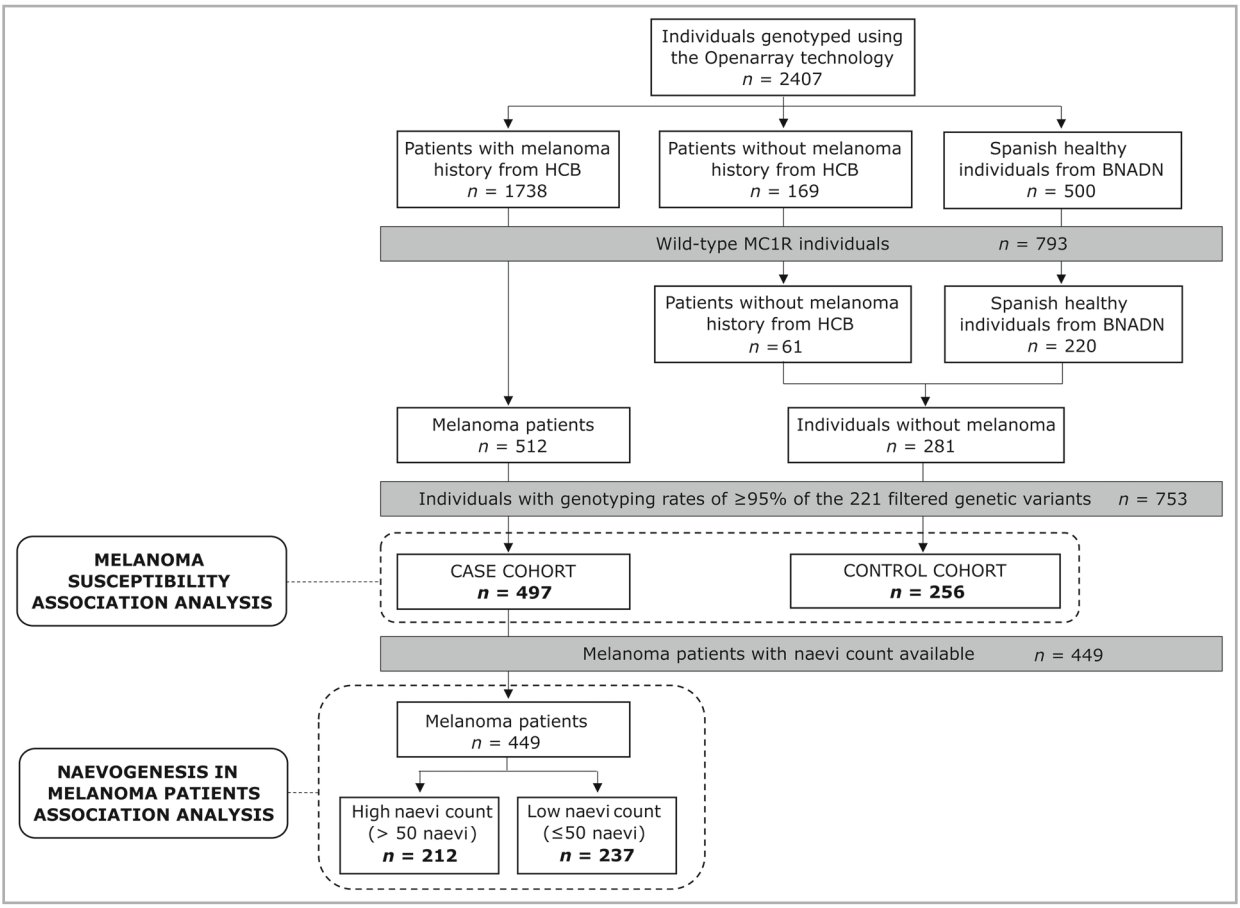

Figure 1 Flowchart of the study cohorts. BNADN, Banco Nacional de ADN Carlos III (Spain); HCB, Hospital Clínic de Barcelona (Spain).

melanoma, highlighting the role of MC1R in these phenotypic features. In contrast, patients with melanoma presented with light eyes (i.e. blue, grey, green) more frequently than controls (42·4% vs. 17%, respectively; P = 0·022), indicating the role of other genes beyond MC1R in eye colour determination.[40] Interestingly, three individuals with WT MC1R (two cases and one control) presented with the red hair colour phenotype. Lastly, 48·1% (234 of 486 with available data) of all individuals from HCB presented with a high TNC (> 50 naevi). This may be explained by the fact that most individuals without melanoma were under dermatological surveillance precisely for having a high TNC and/or atypical naevi.

For subsequent analyses, the filtered variants were grouped into three different sets based on the biological processes in which they are involved: 78 variants associated with melanoma and/or naevogenesis (set M), 40 variants associated with breast cancer and/or hormonal signalling pathways (set B), and 106 variants associated with autoimmune diseases or related to the immune system (set I) (Table S1).

Genetic variants associated with melanoma risk in individuals with wildtype *MC1R*

We first assessed the association of the candidate variants with melanoma risk in 497 patients with melanoma and 256 control individuals (Figure 1). The multivariable logistic regression analysis adjusted by age and sex revealed 17 variants significantly associated with melanoma risk in individuals with WT MC1R. Variants rs12913832 (*HERC2*), rs751173 (near *MTAP*), rs1800682 (*ACTA2*), rs1385736 (near *VEGFC*), rs4902642 (near *ZFP36L1*), rs2228570 (*VDR*), rs4934436 (near *FAS*) and rs10036538 (*PPARGC1B*) were associated with an increased melanoma risk (Table 2). In contrast, variants rs2218220 (near *MTAP*), rs1847472 (*BACH2*), rs35414 (*SLC45A2*), rs11805303 (*IL23R*), rs1799801 (*ERCC4*), rs13073817 (3p24.3 intergenic region), rs9469220 (near *HLA-DQB1*), rs3181100 (*CD28*) and rs9858542 (*BSN*) were associated with decreased melanoma risk in these individuals (Table 2). Only the rs12913832 *HERC2* variant, which is associated with blue eye colour,[41,42] reached significance after FDR-BH correction (OR 1·97, 95% CI 1·48–2·63; adjusted P < 0·001; corrected P < 0·001) (Table 2).

Genetic variants associated with naevus count in patients with wildtype *MC1R* melanoma

Next, we examined whether the same candidate genetic variants were associated with TNC in the subgroup of patients with WT MC1R melanoma with naevus data available (237 with a low TNC vs. 212 with a high TNC) (Figure 1). The

Table 1 Demographic and phenotypic characteristics of the individuals with wildtype MC1R with or without a personal history of melanoma recruited at the Hospital Clínic de Barcelona

	Patients with melanoma history n = 497	Patients without melanoma history n = 37	Total n = 534	P-value
Age (years),[a] mean (SD)	54·8 (17·2)	40·6 (18.9)	53·8 (17·4)	**< 0·001**
Sex				0·61
Male	251 (50·5)	17 (45.9)	268 (50·2)	
Female	246 (49·5)	20 (54.1)	266 (49·8)	
Phototype				0·87
Light (I–II)	265 (55·8)	20 (54.1)	285 (55·7)	
Dark (III–IV)	210 (44·2)	17 (45.9)	227 (44·3)	
Missing	22	0	22	
Eye colour				**0·022**
Blue/grey	102 (21·7)	2 (5.6)	104 (20·6)	
Green	97 (20·7)	4 (11.1)	101 (20·0)	
Brown	258 (55·0)	29 (80.6)	287 (56·8)	
Black	12 (2·6)	1 (2.8)	13 (2·6)	
Missing	28	1	29	
Hair colour				0·16
Red	2 (0·4)	1 (2.8)	3 (0·6)	
Blonde	101 (21·6)	5 (13.9)	106 (21·1)	
Brown	318 (68·1)	28 (77.8)	346 (68·8)	
Black	46 (9·9)	2 (5.6)	48 (9·5)	
Missing	30	1	31	
Total naevus count				0·15
Low (≤ 50)	237 (52·8)	15 (40.5)	252 (51·9)	
High (> 50)	212 (47·2)	22 (59.5)	234 (48·1)	
Missing	48	0	48	

The data are presented as n (%) unless otherwise indicated. Bold text indicates a statistically significant association with a P-value <0·05. [a]Age refers to the moment of diagnosis in patients with melanoma and the moment of phenotyping in individuals without a personal history of melanoma.

multivariable logistic regression analysis adjusted by age and sex showed 16 variants significantly associated with TNC in these patients (Table 3). We identified the same significant associations when the sun exposure amount per year was included as a confounding factor in the model (data not shown). Variants associated with a higher TNC were rs4495224 and rs10512734 (both in the 5p13.1 intergenic region), rs10995271 (near *ZNF365*), rs16944 (near *IL1B*), rs12203592 (*IRF4*) and rs4871611 (8q24.13 intergenic region). Variants that showed a protective effect for a high TNC were rs3798577 (*ESR1*); rs7517810 and rs12035082 (both near *TNFSF18*); rs1408799 (near *TYRP1*); rs17042407 (near *IL1A*); rs7975128 (*VDR*); rs1982151 (*RMI1*); rs7872878 (*TNFSF8*); rs1799964 (near *LTA* and *TNF*); and rs1738074 (*TAGAP*). None of these associations remained statistically significant after FDR-BH correction. However, when statistical correction was performed independently within each biological set of variants, we identified a trend between the rs3798577 *ESR1* variant and lower TNC (OR 0·62, 95% CI 0·47–0·83; adjusted P = 0·002; corrected P = 0·061) (Table 3).

The *ESR1* gene encodes an oestrogen receptor that is clinically relevant in breast cancer and several gynaecological malignancies.[43,44] As hormonal pathways might be associated with TNC, we performed an additional analysis stratifying by sex. Interestingly, the tendency observed in the prior analysis was maintained only among women (OR 0·51, 95% CI 0·33–0·79; adjusted P = 0·003; corrected P = 0·11) and not among men (adjusted P = 0·13; corrected P = 0·69).

To provide more evidence of this association, we further explored this variant in the entire cohort of patients with melanoma regardless of their *MC1R* status (782 with a low TNC vs. 715 with a high TNC), despite this not being the main objective of the study. In this case, the rs3798577 *ESR1* variant was significantly associated with lower TNC (OR 0·79, 95% CI 0·67–0·92; adjusted P = 0·003). Once again, the association was maintained only among women (OR 0·71, 95% CI 0·57–0·88; adjusted P = 0·002) and not among men (adjusted P = 0·28).

Gene–gene interaction analyses

As the genetic variants that proved statistically significant in the association analyses are involved in specific biological pathways, we performed independent MDR analysis within each biological set of variants to investigate epistatic interactions among them (Table 4).

In terms of predicting the melanoma status in individuals with WT *MC1R*, the best classifier among variants from set M was the combination of variants rs3181100 (*CD28*),

Table 2 Genetic variants associated with melanoma risk in individuals with wildtype MC1R determined by a logistic regression model based on additive effects of allele dosage

BS of variants[a]	Genetic variant ID	Gene or locus	Chr.	Position[b]	MA	MAF cases	MAF controls	N	OR (95% CI)	Adjusted P-value[c]	Corrected P-value[d]	Corrected P-value[e]
M	rs3181100	*CD28*	2	204572006	G	0·36	0·42	750	0·78 (0·61–0·99)	**0·042**	0·55	0·36
M	rs35414	*SLC45A2*	5	33969628	T	0·37	0·44	753	0·76 (0·61–0·96)	**0·021**	0·54	0·29
M	rs2218220	Near *MTAP*	9	21756089	T	0·45	0·52	752	0·71 (0·57–0·90)	**0·004**	0·47	0·17
M	rs751173	Near *MTAP*	9	21707372	C	0·49	0·43	753	1·37 (1·08–1·72)	**0·009**	0·47	0·21
M	rs1800682	*ACTA2*	10	90749963	G	0·50	0·43	753	1·34 (1·07–1·67)	**0·011**	0·47	0·21
M	rs4934436	Near *FAS*	10	90783320	C	0·46	0·41	751	1·29 (1·02–1·62)	**0·031**	0·55	0·34
M	rs2228570	*VDR*	12	48272895	A	0·34	0·29	752	1·44 (1·05–1·97)	**0·022**	0·54	0·29
M	rs12913832	*HERC2*	15	28365618	G	0·44	0·31	752	1·97 (1·48–2·63)	**< 0·001**	**< 0·001**	**< 0·001**
M	rs1799801	*ERCC4*	16	14041958	C	0·31	0·34	742	0·76 (0·59–0·98)	**0·036**	0·55	0·36
B	rs10036538	*PPARGC1B*	5	149155588	G	0·34	0·30	751	1·36 (1·01–1·83)	**0·042**	0·55	0·78
I	rs11805303	*IL23R*	1	67675516	T	0·25	0·29	753	0·70 (0·51–0·97)	**0·030**	0·55	0·63
I	rs13073817	3p24.3	3	18706858	A	0·29	0·34	752	0·75 (0·57–0·99)	**0·040**	0·55	0·63
I	rs9858542	*BSN*	3	49701983	A	0·29	0·36	743	0·76 (0·58–0·99)	**0·042**	0·55	0·63
I	rs1385736	Near *VEGFC*	4	177602165	A	0·31	0·28	752	1·48 (1·08–2·03)	**0·015**	0·50	0·56
I	rs1847472	*BACH2*	6	90973159	A	0·36	0·42	753	0·74 (0·59–0·93)	**0·009**	0·47	0·56
I	rs9469220	Near *HLA-DQB1*	6	32658310	G	0·42	0·47	749	0·79 (0·63–0·99)	**0·040**	0·55	0·63
I	rs4902642	Near *ZFP36L1*	14	69210199	A	0·39	0·36	750	1·38 (1·06–1·80)	**0·016**	0·50	0·56

BS, biological set; Chr., chromosome; CI, confidence interval; MA, minor allele based on the whole sample (tested allele); MAF, minor allele frequency; OR, odds ratio. Bold text indicates a statistically significant association with a P-value < 0·05. [a]Genetic variants are grouped into the following biological sets: M, variants associated with melanoma and/or naevogenesis; B, variants associated with breast cancer and/or hormonal signalling pathways; I, variants associated with autoimmune diseases or related to the immune system. [b]Position is reported for GRCh37/hg19. [c]P-value adjusted for age and sex. [d]P-value corrected by the Benjamini–Hochberg false discovery rate method for multiple comparisons, including all variants. [e]P-value corrected by the Benjamini–Hochberg false discovery rate method for multiple comparisons, including only variants from the corresponding biological set of variants (M, B or I).

rs2228570 (*VDR*), and rs751173 and rs2218220 (both near *MTAP*) (testing balanced accuracy of 58%; P = 0·010). Variants rs2228570 and rs2218220 had the largest main effect in the model (IG = 0·75%). The entropy-based interaction graph showed a synergistic effect between the rs2228570 *VDR* variant and both *MTAP* variants: rs751173 (IG = 0·23%) and rs2218220 (IG = 0·13%) (Figure 2a). Within variants from set I, the best predictor was the combination of variants rs11805303 (*IL23R*), rs9858542 (*BSN*), rs1385736 (near *VEGFC*) and rs13073817 (3p24.3 intergenic region) (testing balanced accuracy of 57%; P = 0·020). The nature of epistasis among the four loci was of high redundancy effect, which can be interpreted as an additive or correlation effect among them. The intergenic variant rs1385736 showed the largest main effect in this model (IG = 0·96%) (Figure 2b).

The *MDR* analysis revealed that the best classifier for predicting the TNC in patients with WT *MC1R* melanoma using variants from set M was the combination of variants rs7975128 (*VDR*), rs7872878 (*TNFSF8*) and rs17042407 (near *IL1A*) (testing balanced accuracy of 60%; P = 0·010). The entropybased interaction graph shows that variant rs17042407 has the higher individual effect in this model, as the variant alone explains 1·25% of the entropy. Another important factor in this model is the interaction between variants rs7975128 and rs7872878, which has a synergistic effect that explains the most entropy of this model (IG = 1·54%) (Figure 2c). Within variants from set I, the best predictor was the combination of variants rs10995271 (near *ZNF365*), rs12035082 (near *TNFSF18*), and rs10512734 and rs4495224 (both in 5p13.1) (testing balanced accuracy of 63%; P = 0·010), with high redundancy effect among them. The highest individual IG values were observed for both variants in 5p13.1 (1·18% and 2·46%) (Figure 2d).

These findings were confirmed when we analysed the specific genotypic associations for each combination of genetic variants (Figures S1–S4; see Supporting Information).

Discussion

The specific genetic background of patients with WT *MC1R* melanoma is poorly studied. Here, we have evaluated the role of 221 common genetic variants in individuals from Spain with WT *MC1R*, with the intention of identifying genetic variants with an effect on melanoma susceptibility or naevogenesis, which would otherwise be masked by the effect of *MC1R* variants. Using classical logistic regression models, we identified several variants associated with melanoma risk in this subgroup of individuals. However, only variant rs12913832 in

Table 3 Genetic variants associated with total naevus count (TNC) in patients with wildtype MC1R melanoma determined by a logistic regression model based on additive effects of allele dosage

BS of variants[a]	Genetic variant ID	Gene or locus	Chr.	Position[b]	MA	MAF high TNC	MAF low TNC	N	OR (95% CI)	Adjusted P-value[c]	Corrected P-value[d]	Corrected P-value[e]
M	rs17042407	Near *IL1A*	2	113558914	C	0·22	0·28	448	0·60 (0·38–0·95)	**0·027**	0·63	0·47
M	rs16944	Near *IL1B*	2	113594867	A	0·38	0·32	447	1·41 (1·02–1·94)	**0·037**	0·63	0·47
M	rs12203592	*IRF4*	6	396321	T	0·18	0·15	448	2·36 (1·03–5·41)	**0·042**	0·63	0·47
M	rs1408799	Near *TYRP1*	9	12672097	T	0·34	0·39	449	0·70 (0·51–0·96)	**0·026**	0·63	0·47
M	rs1982151	*RMI1*	9	86617265	A	0·24	0·29	449	0·64 (0·42–0·97)	**0·034**	0·63	0·47
M	rs7872878	*TNFSF8*	9	117682077	C	0·43	0·49	448	0·73 (0·55–0·98)	**0·039**	0·63	0·47
M	rs7975128	*VDR*	12	48245828	A	0·33	0·40	448	0·70 (0·52–0·96)	**0·028**	0·63	0·47
B	rs3798577	*ESR1*	6	152421130	C	0·39	0·51	449	0·62 (0·46–0·83)	**0·002**	0·34	0·061
I	rs12035082	Near *TNFSF18*	1	172898377	C	0·40	0·48	447	0·69 (0·51–0·92)	**0·011**	0·63	0·38
I	rs7517810	Near *TNFSF18*	1	172853460	T	0·22	0·28	448	0·61 (0·40–0·92)	**0·018**	0·63	0·38
I	rs4495224	5p13.1	5	40477515	C	0·38	0·26	447	1·68 (1·17–2·42)	**0·006**	0·61	0·38
I	rs10512734	5p13.1	5	40393605	G	0·34	0·26	444	1·63 (1·09–2·42)	**0·017**	0·63	0·38
I	rs1799964	Near *LTA, TNF*	6	31542308	C	0·23	0·28	448	0·65 (0·43–0·98)	**0·039**	0·63	0·61
I	rs1738074	*TAGAP*	6	159465977	T	0·41	0·47	448	0·74 (0·55–0·99)	**0·045**	0·63	0·61
I	rs4871611	8q24.13	8	126537570	G	0·49	0·41	448	1·36 (1·01–1·83)	**0·046**	0·63	0·61
I	rs10995271	Near *ZNF365*	10	64438486	C	0·39	0·31	448	1·48 (1·07–2·06)	**0·018**	0·63	0·38

BS, biological set; Chr., chromosome; CI, confidence interval; MA, minor allele based on the whole sample (tested allele); MAF, minor allele frequency; OR, odds ratio. Bold text indicates a statistically significant association with a P-value < 0·05. [a]Genetic variants are grouped into the following biological sets: M, variants associated with melanoma and/or naevogenesis; B, variants associated with breast cancer and/or hormonal signalling pathways; I, variants associated with autoimmune diseases or related to the immune system. [b]Position is reported for GRCh37/hg19. [c]P-value adjusted for age and sex. [d]P-value corrected by the Benjamini–Hochberg false discovery rate method for multiple comparisons, including all variants. [e]P-value corrected by the Benjamini–Hochberg false discovery rate method for multiple comparisons, including only variants from the corresponding biological set of variants (M, B or I).

Table 4 Interaction of genetic variants by multifactor dimensionality reduction analysis

Trait classification	Biological set of variants[a]	Interaction model	Training BACC	Testing BACC	P-value
Melanoma vs. without melanoma	M	rs2218220 (near *MTAP*), rs751173 (near *MTAP*), rs2228570 (*VDR*) and rs3181100 (*CD28*)	0·63	0·58	0·0099
	I	rs11805303 (*IL23R*), rs9858542 (*BSN*), rs1385736 (near *VEGFC*) and rs13073817 (3p24.3)	0·62	0·57	0·020
High vs. low TNC in patients with melanoma	M	rs17042407 (near *IL1A*), rs7975128 (*VDR*) and rs7872878 (*TNFSF8*)	0·63	0·60	0·0099
	I	rs4495224 (5p13.1), rs10512734 (5p13.1), rs10995271 (near *ZNF365*) and rs12035082 (near *TNFSF18*)	0·66	0·63	0·0099

BACC, balanced accuracy; TNC, total naevus count. [a]Genetic variants are grouped into the following biological sets: M, variants associated with melanoma and/or naevogenesis; I, variants associated with autoimmune diseases or related to the immune system.

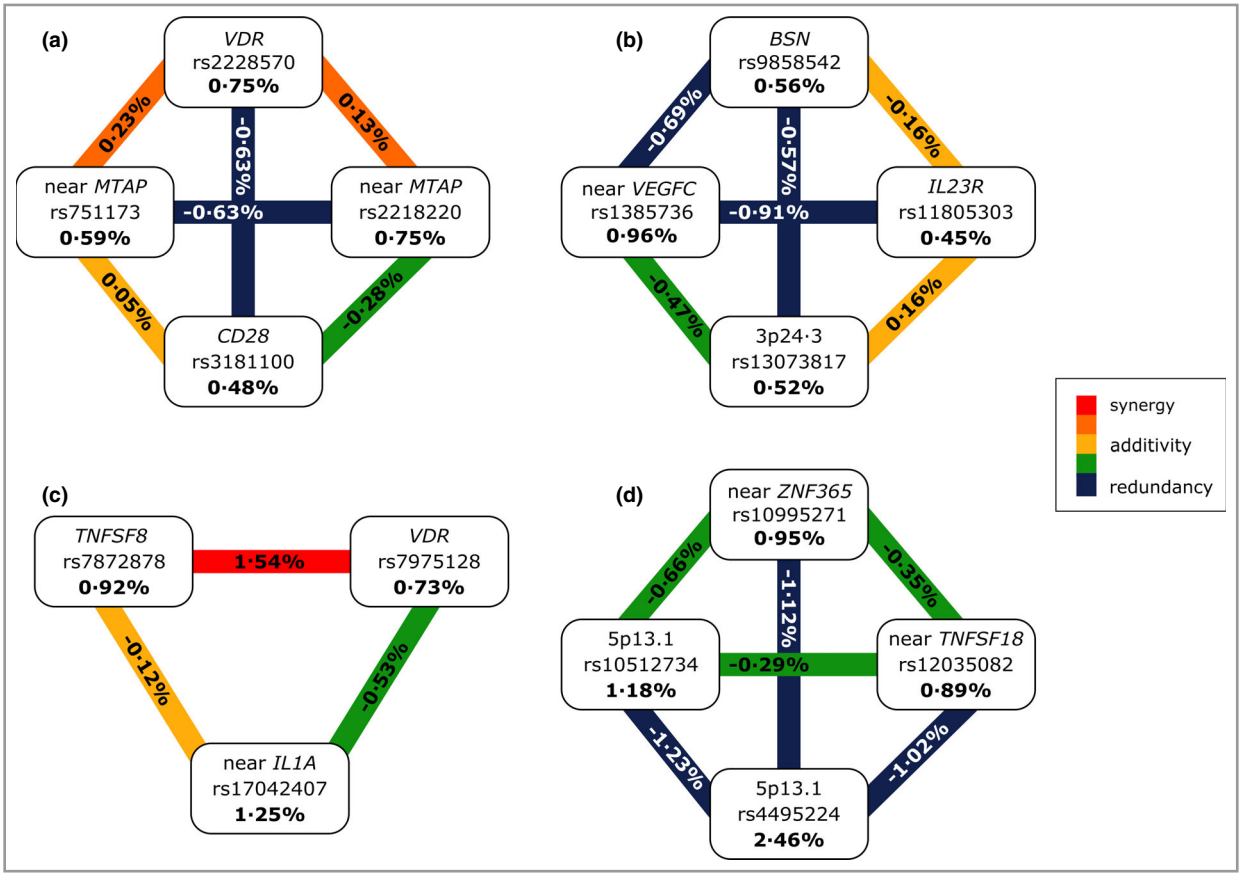

Figure 2 Multifactor dimensionality reduction (MDR) entropy-based interaction circle graphs for each classifier: (a) classifier with variants from set M (melanoma and/or naevogenesis) for melanoma risk prediction in individuals with wildtype (WT) MC1R; (b) classifier with variants from set I (autoimmune diseases or immune system) for melanoma risk prediction in individuals with WT MC1R; (c) classifier with variants from set M for naevus count prediction in patients with WT MC1R melanoma; (d) classifier with variants from set I for naevus count prediction in patients with WT MC1R melanoma. Entropy values in the cells of particular factors indicate the information gain of individual variants, whereas the entropy values indicated on the lines connecting two factors represent the entropy of interaction, meaning the individual gain of each pairwise combination. The colour of the lines represents the type of interaction: a red line represents a high degree of synergy, a yellow line represents independence or additivity, a green line represents a low degree of redundancy, and a blue line represents a high degree of redundancy.

HERC2 reached significance after correction for multiple comparison testing (OR 1·97; $P < 0·001$). This variant is the main determinant of blue eye colour in European populations,[41,42] which is, in turn, a risk factor for melanoma.[45]

A limitation of our study is that the comparison of phenotypic data, including eye colour, was restricted to individuals recruited at the same hospital. Despite this being a limited cohort, we were able to detect the expected different frequencies

between cases and controls, with a higher proportion of individuals with light-coloured eyes among patients with melanoma than in controls (42% vs. 17%, respectively). Thus, our findings support the previously reported role of the *HERC2* gene in melanoma susceptibility,[46] specifically in individuals with a low-risk phenotype such as darkly pigmented hair and skin.

We also found several genetic associations with the TNC in patients with WT *MC1R* melanoma, but none of them reached statistical significance after correction. However, we identified a trend between variant rs3798577 in *ESR1* and lower TNC, which was restricted to female patients with melanoma. Given the relevance of the finding, we assessed only this genetic variant in the entire cohort of patients with melanoma regardless of their *MC1R* status in order to provide more evidence of this association. Also in this case, the effect of variant rs3798577 was restricted to female patients (OR 0·71; P = 0·002).

The *ESR1* gene encodes oestrogen receptor alpha, which has been associated with cancer susceptibility. In particular, variant rs3798577 has been associated with breast cancer susceptibility,[47,48] and other variants in the same gene have been associated with melanoma susceptibility.[49,50] There is previous evidence of an association between TNC and breast cancer susceptibility,[26–28] but to our knowledge this is the first study to report a genetic factor linking them. The fact that hormones affect people of both sexes differently might explain why the observed correlation between the *ESR1* variant and TNC is sex dependent. Taken together, our findings reinforce the hypothesis of an association between hormonal pathways and susceptibility to melanocyte proliferation.

Beyond the effect of single genes, complex genetic interactions play an important role in determining susceptibility to complex human diseases or phenotypes.[51,52] Conventional statistical methods have limited power for evaluating large numbers of genetic variants due to conservative statistical correction, as observed in this study. Alternative methods, such as MDR, are specifically designed for detecting epistasis in large genetic studies.[53–55] Indeed, MDR has proved epistasis in several diseases and phenotypes, including skin photosensitivity,[56] human pigmentation[57] and melanoma susceptibility.[58]

It is important to differentiate the goal of MDR from that of polygenic risk scores. Polygenic risk scores provide an estimate of genetic liability to a trait at the individual level and, in combination with traditional risk factors, may help identify patients with a higher risk of developing a complex disease or trait.[59] Instead, the primary goal of MDR is hypothesis generation for further studies, which should be validated in additional cohorts.[53] Here, we have identified four genetic combinations within specific biological categories, which incorporate epistatic interactions with an effect on melanoma proliferation in individuals with WT *MC1R*.

On the one hand, we have identified two epistatic models associated with melanoma susceptibility in these individuals. The first model includes four genetic variants associated with melanoma, naevus and/or pigmentation traits: rs3181100 in *CD28*, rs2228570 in *VDR*, and rs2218220 and rs751173 located at chromosome 9p21. Both the CD28 and VDR (vitamin D receptor) proteins have a role in immune response modulation,[60,61] and genetic variation in the entire 9p21 locus has been associated with melanoma development.[62,63] Interestingly, the combination of the *VDR* variant with both 9p21 variants had the greatest effect, emphasizing the potential role of this locus in melanoma susceptibility in individuals with WT *MC1R*. The second prediction model includes the effect of four genetic variants related to the immune system and/or autoimmune diseases: rs1385736 near *VEGFC*, rs11805303 in *IL23R*, rs9858542 in *BSN*, and rs13073817 located in chromosome 3p24.3. Except for the first one, these variants have shown a strong association with susceptibility to Crohn disease,[64–66] suggesting that genetic variants related to proinflammatory pathways underlying autoimmune diseases might have a role in melanoma risk in individuals with WT *MC1R*.

On the other hand, we have also identified two prediction models of the TNC in patients with WT *MC1R* melanoma. The first one includes the epistatic effect of four intergenic variants related to proinflammatory pathways: rs10995271 near *ZNF365*, rs12035082 near *TNFSF18*, and rs4495224 and rs10512734 located in chromosome 5p13.1. Specifically, all these variants have shown a strong association with susceptibility to Crohn disease.[67–70] The other model includes three variants associated with melanoma, naevus and/or pigmentation traits: rs7975128 in *VDR*, rs7872878 in *TNFSF18*, and rs17042407 located near *IL1A*. The proteins encoded by these genes also play a role in the inflammatory process that underlies the pathology of autoimmune diseases.[71–73] Thus, our data support the idea that the immune system plays a role in naevogenesis and provide evidence for an association between autoimmune diseases and TNC in patients with WT *MC1R* melanoma.

Our study has several limitations that should be acknowledged. Firstly, phenotypic data and other relevant information, such as sun exposure habits, were available only for a small number of individuals without melanoma, as these data were not available for control individuals recruited at BNADN. This implies that well-established phenotypic or environmental risk factors have not been included in the logistic regression models. On the other hand, we have evaluated only a selection of candidate genetic variants, which were in turn biologically classified based on the literature or previous analyses. Studies involving a larger number of genes are necessary to better elucidate the genetic background of individuals with WT *MC1R*. Also, MDR requires high computational efficiency when applied to high-order interactions and/or genome-wide data.[74] For this reason, MDR analyses were restricted to those variants within each biological subset that showed a statistically significant association in the logistic regression analysis. The inclusion of a larger number of variants in MDR analyses, independently of the biological subset, might have uncovered additional interactions with a potential role in melanoma susceptibility or TNC in individuals with WT *MC1R*. Another limitation is that the null associations we observed in the present analysis may have been due to limited statistical power, especially in the case of genetic variants with a minor allele

frequency < 15%. Lastly, the observed genetic associations should be replicated in an independent and larger cohort of patients with WT *MC1R* melanoma.

In conclusion, the evaluation of candidate common genetic variants in individuals with WT *MC1R* showed that the rs12913832 *HERC2* variant increases the melanoma risk in these individuals. Also, we found that the rs3798577 *ESR1* variant might be associated with TNC in female patients with melanoma regardless of their *MC1R* status. Finally, our data suggest that epistasis among genetic variants related to melanocyte biology or proinflammatory pathways might play a role in melanoma susceptibility or TNC determination in individuals with WT *MC1R*.

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Conflicts of interest

The authors declare they have no conflicts of interest.

Ethics statement

The study was approved by the clinical research ethics committee of the Hospital Clínic de Barcelona (HCB/2015/0820). Each participant signed written informed consent according to the Declaration of Helsinki.

Data availability

The datasets generated during this study are available from the authors upon request.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1 Supporting methods.

Figure S1 Cross-tables showing genotype combinations among genetic variants from set M for melanoma risk prediction in individuals with wildtype *MC1R*.

Figure S2 Cross-tables showing genotype combinations among genetic variants from set I for melanoma risk prediction in individuals with wildtype *MC1R*.

Figure S3 Cross-tables showing genotype combinations among genetic variants from set M for naevus count prediction in patients with wildtype *MC1R* melanoma.

Figure S4 Cross-tables showing genotype combinations among genetic variants from set I for naevus count prediction in patients with wildtype *MC1R* melanoma.

Table S1 A complete list of the 256 selected common genetic variants.

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