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Characterization of patients at high risk of melanoma in Austria*

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Conflicts of interest

None declared.

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Summary

Background Risk of melanoma is determined by genetic and exogenous factors. Only a few studies have included both characteristics in a comprehensive multivariable analysis.

Objectives To find determinants of patients at high risk of melanoma in Austria, including phenotype, genotype and lifestyle characteristics in comprehensive analyses.

Methods In total, 1668 patients with melanoma from the M3 case–control study were studied. Overall, 567 participants were sequenced for *CDKN2A*, 232 for *CDK4*, 123 for *MITF* encoding the variant E318K and 964 for *MC1R*.

Results Patients with melanoma with a positive family history (n = 190, 11·6%), multiple primary melanomas (n = 261, 15·7%) and younger age (< 50 years, n = 675, 40·5%) were defined as being at high risk. All other patients with melanoma were defined as the reference group. We found significant differences between those two groups and between the high-risk subgroups (positive family history, multiple primary melanomas and younger age). Pigmentation phenotype was associated with the high-risk group in general (childhood freckling, odds ratio 1·46, P = 0·007; blond/reddish hair colour, odds ratio 1·43, P = 0·011). Patients with a positive family history and patients with early-onset disease were similar regarding both their phenotypic characteristics and external factors. Established high-risk mutations in *CDKN2A* were found in cases with a positive family history (n = 12) or multiple melanomas (n = 2). Moreover, we found three patients carrying the MITF p.E318K variant, two with a *CDK4* variant and seven with nonsynonymous *MC1R* variants with undescribed biological significance, of which four were predicted as damaging.

Conclusions Austrian patients could represent a reservoir for novel genetic variants. Further investigation of populations in Central and Eastern Europe might reveal more novel and disease-relevant variants.

What's already known about this topic?

- Melanoma risk is determined by environmental and genetic risk factors.
- The frequency and type of disease-causing gene mutations vary between different countries.

What does this study add?

- This is the first comprehensive description of Austrian patients at high risk of melanoma.
- The heterogeneity of the different subgroups suggests diverse pathways.
- We present functional prediction of *MC1R* and *CDK4* variants with unknown biological significance in high-risk patients, and three novel cases with *MITF* variants in high-risk patients.

What is the translational message?

- Identification of high-risk individuals helps to reduce mortality.
- The risk of melanoma is comprised of a complex interplay between genetic and environmental factors; however, genetic mutations associated with melanoma might differ from country to country.
- Knowledge of these mutations and adjustment of criteria for testing could be required for adequate risk assessments.

Melanoma is still one of the most life-threatening types of cancer, with an increasing number of incident cases worldwide.[1] Exact knowledge of its cause is the basis for disease prevention, particularly for those at high risk. The risk is determined by a complex interplay of external and genetic factors. In specialized centres, genetic testing is suggested for patients who are regarded as being at high risk.[2] *CDKN2A* is the best-described high-risk melanoma gene, followed by *CDK4*, which has been described in only a few families worldwide. Additionally, numerous low-risk variants in *MC1R* modulate the risk, in addition to high-risk mutations. Furthermore, variations in other genes such as *MITF* and *POT1* have been described as being associated with familial melanoma.

In spite of numerous studies describing phenotypic features or genetic factors separately, there are only a few studies where risk factors were analysed comprehensively.[3,4] Here we present for the first time a comprehensive study of established genetic variants and phenotypic and lifestyle characteristics associated with melanoma in a central European population, to improve the knowledge of determinants of patients at high risk of melanoma and the different subgroups.

Patients and methods

Patients

All 1668 histologically confirmed patients with melanoma who were recruited from 2008 to 2015 as part of the M3 case–control study were included.[5,6] The participants originated mainly from Vienna, the province of Lower Austria, and Burgenland (Table S1; see Supporting Information). To address a potential selection bias, patients were included from different Austrian hospitals. The data were collected by blood draw and a questionnaire, as described elsewhere.[5] Those with an unavailable pathology report or occult melanoma were not included in this study.

We categorized cases as high risk when they fulfilled at least one of the following criteria: (i) a positive family history (n = 190); (ii) multiple primary melanomas (n = 261); and (iii) early onset of melanoma (n = 675) (Table 1). All three criteria used for the categorization of high-risk patients were based on the signs for high-risk melanoma genes according to the Genomel Consortium.[7] Other patients with one primary melanoma (n = 764), without a positive family history and aged > 50 years at diagnosis were used as the reference group. All of the comparative analyses were performed using the reference group.

The following patient characteristics were analysed: age at diagnosis, sex, skin reaction to sun exposure, hair and eye colour, childhood freckling, outdoor occupation, solarium use,

Table 1 High-risk subgroups

	Positive family history (n = 190)	Multiple primary melanomas (n = 261)	Early-onset melanoma (n = 675)
Positive family history	–	44 (16·9)	95 (14·1)
Multiple primary melanomas	44 (23·2)	–	103 (15·3)
Early-onset melanoma (< 50 years)	95 (50·0)	103 (39·5)	–

Values are n (%).

suncream use, weeks of holiday in the lifetime, number of sunburns from birth to age 10 years, time spent outdoors at leisure, localization of the primaries, tumour thickness according to Breslow, sentinel lymph node status, distant metastasis and *MC1R* gene status. In case of multiple primaries, melanoma-specific data always refers to the first primary melanoma.

Written informed consent was obtained from each participant. This study was approved by the ethics committee of the Medical University of Vienna.

Statistics

In the univariable analysis nominal variables were analysed using a χ^2-test. The continuous variables, represented by mean values and their SDs, were analysed with an independent-sample *t*-test. High-risk patients and all subgroups were compared with patients with sporadic melanoma. All variables had been adjusted for sex and age in a multivariable analysis, except for the patients with early-onset melanoma. For this subgroup all significant variables of the univariable analysis were included in the multivariable model. All statistical tests were two sided with a significance level of $\alpha = 0{\cdot}05$ and a 95% confidence interval (CI), and were performed using SPSS version 21 (IBM, Armonk, NY, U.S.A.).

Genotyping

Sequencing data were available for *CDKN2A* from 474 participants, for exon 2 of *CDK4* from 109 patients and for *MC1R* from 875 participants, as described previously.[6,8] Additionally, 93 new cases were sequenced for *CDKN2A*, 123 for *CDK4* and *MITF*, and 89 for *MC1R*. *MC1R* was analysed in all cases of melanoma (964 in total) as part of an association study.[6] Among those 964 patients genotyped for *MC1R*, 452 high-risk patients and 115 cases of sporadic melanoma were sequenced for *CDKN2A*. *CDK4* and *MITF* were sequenced in high-risk patients only.

MC1R variants were classified as variants strongly associated with red hair colour (R), including p.D84E, p.R142H, p.R151C, p.R160W and p.D294H, and variants weakly associated (r), including p.V60L, p.V92M and p.R163Q. The specific sequencing protocols and primers are described in the Supplementary Methods and Table S2 (see Supporting Information).

Prediction analysis

The nonsynonymous *MC1R* variants and the two *CDK4* variants have been predicted with different prediction tools, as described before:[8] MutationTaster2,[9] PolyPhen-2 (Polymorphism Phenotyping-v2, HumDiv and HumVar),[10] PROVEAN (Protein Variation Effect Analyzer),[11] SIFT (sorts intolerant from tolerant substitutions),[12] SNAP2 (screening for non-acceptable polymorphisms-2),[13] Panther,[14] GERP++,[15] phyloP[16] and CADD (Combined Annotation Dependent Depletion).[17] For *MC1R* the Ensembl transcript ID ENST00000555147 was used, and for *CDK4* ENST00000257904 was used.

Results

Firstly, all characteristics of high-risk patients with melanoma (n = 904) were compared against the reference group (n = 764), as shown in Tables 2 and 3. After adjusting for sex and age, childhood freckling [odds ratio (OR) 1·46, 95% confidence interval (CI) 1·11–1·93, P = 0·007] blond/reddish hair colour (OR 1·43, 95% CI 1·09–1·88, P = 0·011) and male sex (OR 1·40, 95% CI 1·07–1·82, P = 0·013) were associated with high-risk status (Table 2). Melanomas in the high-risk group were less likely to be localized on the head and neck compared with the reference group (P = 0·005, Table 2). The tumour thickness was lower in high-risk patients (mean 1·2 vs. 1·8 mm, P = 0·001); however, this failed to remain significant in the multivariable model (OR 0·94, 95% CI 0·87–1·02, P = 0·12). In total, 11·4% (n = 190) of all patients with melanoma had at least one reported melanoma diagnosis in the family, 15·7% (n = 261) had multiple primary melanomas and 40·5% (n = 719) were diagnosed before the age of 50 years. All of the results of the univariable analysis are shown in Table 3.

We then studied the phenotypic differences between the subgroups of high-risk patients (Table 4). In the multivariable model, blond/reddish hair colour (OR 2·05, 95% CI 1·39–3·04, P < 0·001), childhood freckling (OR 1·62, 95% CI 1·10–2·38, P = 0·015) and sunburns aged 0–10 years (OR 1·01, 95% CI 1·00–1·03, P = 0·044) were significantly associated with positive family history. Male sex (OR 1·75, 95% 95% CI 1·26–2·41, P = 0·001) was significantly associated with multiple primary melanomas. Suncream use (OR 2·52, 95% CI 1·79–3·53, P < 0·001), solarium use (OR 2·11, 95% CI 1·47–3·02, P < 0·001), childhood freckling (OR 1·73, 95% CI 1·28–2·33, P < 0·001), Breslow index (OR 0·89, 95% CI 0·81–0·98, P = 0·013), outdoor occupation (OR 0·64, 95% CI 0·47–0·87, P = 0·005) and localization (head and neck as the reference, P = 0·006 for extremities and P = 0·019 for trunk) were significantly associated with early-onset melanoma (Table 4).

Regarding genetic mutations, 14 patients (2·5%) carried a disease-causing mutation in *CDKN2A*. This included 12 of 155 cases (7·7%) with a positive family history who were tested for *CDKN2A*. The following established high-risk mutations were found in this study group: p.R24H (11 carriers; two carriers were shared in two families each and cosegregated with melanoma), p.N71T (one carrier), p.G101W (one carrier) and p.V126D (one carrier). Among the *CDKN2A* high-risk mutation carriers, 12 had another member in the pedigree with melanoma: nine cases were from a family with two cases, two carriers had three further cases, and one carrier had four cases in the pedigree (Table S3; see Supporting Information). In addition to the previously reported cases,[8] we found four more participants with the p.A148T mutation, one with the c.150+ 37G>GC variant and one more patient

Table 2 Patients' characteristics (univariable and multivariable analysis)

Characteristic	Sporadic (n = 764)	High risk (n = 904)	P-value[a]	OR	95% CI	P-value[b]
Diagnosis age (years), mean ± SD	64 ± 8·8	44 ± 14·2	< 0·001	0·87	0·86–0·89	< 0·001
Sex						
Male	441 (57·7)	449 (49·7)	0·001	1·40	1·07–1·82	0·013
Female	323 (42·3)	455 (50·3)				
Skin reaction to sun exposure						
Rather red	497 (66·6)	653 (74·2)	0·001	1·22	0·91–1·62	0·19
Rather brown	249 (33·4)	227 (25·8)				
Unknown	18	24				
Hair colour						
Blond/reddish	237 (31·1)	324 (35·9)	0·038	1·43	1·09–1·88	0·011
Black/brown	525 (68·9)	578 (64·1)				
Unknown	2	2				
Eye colour						
Fair	524 (68·6)	616 (68·1)	0·85	1·10	0·84–1·45	0·49
Dark	240 (31·4)	288 (31·9)				
Childhood freckling						
Yes	211 (29·7)	376 (43·4)	< 0·001	1·46	1·11–1·93	0·007
No	499 (70·3)	490 (56·6)				
Unknown	54	38				
Outdoor occupation						
Yes	224 (40·6)	212 (32·9)	0·006	0·90	0·65–1·23	0·50
No	328 (59·4)	432 (67·1)				
Unknown	212	260				
Solarium use						
Yes	105 (14)	245 (27·4)	< 0·001	1·02	0·73–1·43	0·89
No	645 (86)	649 (72·6)				
Unknown	14	10				
Time spent outdoors at leisure						
> 3 h	559 (74·9)	621 (69·9)	0·022	0·90	0·67–1·22	0·50
< 3 h	187 (25·1)	268 (30·1)				
Unknown	18	15				
Holiday weeks						
Mean ± SD	14 ± 11·4	13·4 ± 11·1	0·34	1·00	0·99–1·01	0·70
Unknown (n)	71	70				
Sunburns 0–10 years						
Mean ± SD	6 ± 12·1	7·5 ± 11·3	0·011	1·01	0·99–1·02	0·34
Unknown (n)	24	16				
Suncream use						
Yes	457 (61·1)	700 (78·2)	< 0·001	0·94	0·70–1·26	0·67
No	291 (38·9)	195 (21·8)				
Unknown	16	9				
Multiple primary melanoma						
Yes	0	261 (28·9)				
No	764 (100)	643 (71·1)				
Localization						
Head/neck	111 (14·5)	85 (9·4)	0·005	Ref		
Extremities	273 (35·7)	348 (38·5)		0·68	0·44–1·05	0·083
Trunk	380 (49·7)	471 (52·1)		0·71	0·47–1·08	0·11
Breslow index (mm), mean ± SD	1·8 ± 4·3	1·2 ± 1·5	0·001	0·94	0·87–1·02	0·12
Sentinel node						
Positive	56 (18·1)	63 (20·6)	0·44	1·15	0·66–2·02	0·63
Negative	253 (81·9)	243 (79·4)				
Unknown	455	598				
Distant metastasis						
Yes	43 (5·6)	61 (6·7)	0·35	1·42	0·85–2·37	0·19
No	721 (94·4)	843 (93·3)				
MC1R						
R or r	278 (74·7)	369 (81·3)	0·023	1·42	0·89–2·27	0·14
Wild-type	94 (25·3)	85 (18·7)				

Values are n (%) unless stated otherwise. OR, odds ratio; CI, confidence interval; Ref, reference value. [a]χ^2-test for nominal variables and t-test for continuous variables. [b]Multivariable analysis, adjusted for sex and age. P-values > 0·05 are significant. The total missing numbers were 42 for skin reaction to sun exposure, four for hair colour, 92 for childhood freckling, 472 for outdoor occupation, 24 for solarium use, 33 for time spent outdoors at leisure, 141 for holiday weeks and 40 for sunburns aged 0–10 years.

Table 3 Univariable subgroup analysis

Characteristic	Sporadic (n = 764)	Positive family history (n = 190)	P-value[a]	Multiple primary melanoma (n = 261)	P-value[a]	Early-onset melanoma (n = 675)	P-value[a]
Diagnosis age (years), mean ± SD	63·9 ± 8·8	48·8 ± 15·1	< 0·001	54 ± 15·6	< 0·001	38 ± 8·3	–
Sex							
Male	441 (57·7)	101 (53·2)	0·26	162 (62·1)	0·22	288 (42·7)	< 0·001
Female	323 (42·3)	89 (46·8)		99 (37·9)		387 (57·3)	
Skin reaction to sun exposure							
Rather red	497 (66·6)	141 (75·8)	0·016	189 (73·3)	0·048	485 (74·2)	0·002
Rather brown	249 (33·4)	45 (24·2)		69 (26·7)		169 (25·8)	
Unknown	18	4		3		21	
Hair colour							
Blond/reddish	237 (31·1)	74 (39·2)	0·035	94 (36·0)	0·14	236 (35·0)	0·12
Black/brown	525 (68·9)	115 (60·8)		167 (64·0)		438 (65·0)	
Unknown	2	1		0		1	
Eye colour							
Fair	524 (68·6)	141 (74·2)	0·13	187 (71·6)	0·35	449 (66·5)	0·40
Dark	240 (31·4)	49 (25·8)		74 (28·4)		226 (33·5)	
Childhood freckling							
Yes	211 (29·7)	81 (44)	< 0·001	92 (37·2)	0·028	290 (44·7)	< 0·001
No	499 (70·3)	103 (56)		155 (62·8)		359 (55·3)	
Unknown	54	6		14		26	
Outdoor occupation							
Yes	224 (40·6)	53 (43·8)	0·51	75 (37·9)	0·51	134 (28·1)	< 0·001
No	328 (59·4)	68 (56·2)		123 (62·1)		343 (71·9)	
Unknown	212	69		63		198	
Solarium use							
Yes	105 (14)	51 (27·1)	< 0·001	42 (16·3)	0·37	213 (32·0)	< 0·001
No	645 (86)	137 (72·9)		216 (83·7)		452 (68·0)	
Unknown	14	2		3		10	
Time spent outdoors at leisure							
> 3 h	559 (74·9)	53 (28·6)	0·2	194 (75·2)	0·93	449 (67·7)	0·003
< 3 h	187 (25·1)	132 (71·4)		64 (24·8)		214 (32·3)	
Unknown	18	5		3		12	
Holiday weeks							
Mean ± SD	13·9 ± 11·4	14 ± 11·3	0·88	14·5 ± 12·3	0·55	13·1 ± 10·2	0·17
Unknown (n)	71	17		12		58	
Sunburns 0–10 years							
Mean ± SD	6 ± 12·1	8·6 ± 13·3	0·013	7·5 ± 10·8	0·099	7·5 ± 10·8	0·016
Unknown (n)	24	3		4		11	
Suncream use							
Yes	457 (61·1)	146 (78·1)	< 0·001	178 (69·0)	0·023	561 (84·1)	< 0·001
No	291 (38·9)	41 (21·9)		80 (31·0)		106 (15·9)	
Unknown	16	3		3		8	
Localization							
Head/neck	111 (14·5)	24 (12·6)	0·22	35 (13·4)	0·37	44 (6·5)	< 0·001
Extremities	273 (35·7)	58 (30·5)		83 (31·8)		284 (42·1)	
Trunk	380 (49·7)	108 (56·8)		143 (54·8)		347 (51·4)	
Breslow index (mm), mean ± SD	1·8 ± 4·3	1 ± 1·25	0·018	1·2 ± 1·3	0·02	1·2 ± 1·6	0·002
Sentinel node							
Positive	56 (18·1)	12 (21)	0·56	13 (20)	0·68	48 (20·7)	0·45
Negative	253 (81·9)	44 (79)		51 (80)		184 (79·3)	
Unknown	455	134		197		443	
Distant metastasis							
Yes	43 (5·6)	7 (3·7)	0·28	24 (9·2)	0·044	41 (6·1)	0·72
No	721 (94·4)	183 (96·3)		237 (90·8)		634 (93·9)	

(continued)

Table 3 (continued)

Characteristic	Sporadic (n = 764)	Positive family history (n = 190)	P-value[a]	Multiple primary melanoma (n = 261)	P-value[a]	Early-onset melanoma (n = 675)	P-value[a]
MC1R							
R or r	278 (74·7)	75 (87)	0·013	103 (78·6)	0·37	275 (79·9)	0·097
Wild-type	94 (25·3)	11 (13)		28 (21·4)		69 (20·1)	

Values are n (%) unless stated otherwise. [a]χ^2-test for nominal variables and t-test for continuous variables. P-values < 0·05 are significant.

Table 4 Multivariable subgroup analysis

	Positive family history (n = 190)			Multiple primary melanomas (n = 261)			Early-onset melanoma (n = 675)		
Characteristic	OR	95% CI	P-value[a]	OR	95% CI	P-value[a]	OR	95% CI	P-value[b]
Diagnosis age	0·88	0·86–0·90	< 0·001	0·92	0·91–0·93	< 0·001			
Sex: male	1·20	0·83–1·75	0·34	1·75	1·26–2·41	0·001	0·99	0·73–1·35	0·95
Skin reaction to sun exposure: mostly red	1·35	0·88–2·09	0·17	1·19	0·85–1·68	0·31	1·00	0·72–1·39	0·99
Hair colour: blond/reddish	2·05	1·39–3·04	< 0·001	1·34	0·97–1·86	0·078			
Eye colour: fair	1·20	0·80–1·81	0·38	1·20	0·86–1·68	0·30			
Childhood freckling: yes	1·62	1·10–2·38	0·015	1·35	0·97–1·89	0·074	1·73	1·28–2·33	< 0·001
Outdoor occupation: yes	1·03	0·65–1·63	0·91	0·89	0·62–1·29	0·55	0·64	0·47–0·87	0·005
Solarium use: yes	1·27	0·80–2·04	0·32	0·88	0·57–1·36	0·57	2·11	1·47–3·02	< 0·001
Time spent outdoors at leisure: > 3 h	1	0·65–1·53	1·00	0·97	0·68–1·40	0·88	0·74	0·54–1·02	0·067
Holiday weeks	1·00	0·99–1·02	0·72	1·00	0·99–1·02	0·83			
Sunburns at 0–10 years	1·01	1·00–1·03	0·044	1·00	0·99–1·02	0·62	1·01	0·99–1·02	0·51
Suncream use: yes	1·30	0·85–2·01	0·23	0·90	0·64–1·26	0·54	2·52	1·79–3·53	< 0·001
Localization									
Head/neck	Ref			Ref			Ref		
Extremities	0·47	0·25–0·88	0·019	0·76	0·46–1·25	0·28	2·09	1·23–3·54	0·006
Trunk	0·64	0·36–1·14	0·128	0·83	0·52–1·32	0·43	1·85	1·11–3·10	0·019
Breslow index	0·88	0·77–1·01	0·068	0·91	0·82–1·00	0·051	0·89	0·81–0·98	0·013
Sentinel lymph node: positive	1·06	0·48–2·35	0·89	1·17	0·57–2·40	0·66			
Distant metastasis: yes	0·79	0·38–1·64	0·52	1·84	1·05–3·23	0·032			
MC1R: R or r	2·13	0·99–4·60	0·054	1·08	0·63–1·84	0·78			

OR, odds ratio; CI, confidence interval; Ref, reference value. [a]Multivariable analysis, adjusted for sex and age. [b]Multivariable analysis, adjusted for all significant variables of the univariable analysis. P-values < 0·05 are significant.

each carrying the p.R24P, p.A34V and p.P81R mutations. We also found a patient with a novel synonymous variant p.T93T. No high-risk mutations were found in the reference group.

Sequencing of *CDK4* revealed two variants that had not been described in association with melanoma before. One variant (c.−60C>CG) was located in the 5′-untranslated region. The carrier was a 20-year-old male patient diagnosed with a spitzoid melanoma with a tumour thickness of 1·1 mm. The other variant, p.V154L, was located in exon 4, a protein-coding region. This female patient had a positive family history (father) and a melanoma with 0·8-mm Breslow thickness, excised at the age of 42 years. No further *CDK4* mutations, including the known high-risk variants, were found. The p.E318K-causing variant of the *MITF* gene was detected in three cases, all from the high-risk group (2·4% of 123 tested participants). High-risk patients more frequently carried red hair *MC1R* variants (R or r) compared with the reference group (81·3% vs. 74·7%, P = 0·023). When the subgroups were analysed separately, patients with a positive family history had the highest number of *MC1R* carriers (86·2% vs. 74·7%, P = 0·022). The most frequent red hair variants were p.R160W for R and p.V60L for r.

High-risk patients carrying a disease-causing *CDKN2A* mutation were significantly younger at first melanoma diagnosis (mean 37 years vs. 47 years, P = 0·019) and were more likely to be female (71% vs. 44·1%, P = 0·044) than high-risk wild-type patients in a univariable analysis (Table 5). They had more multiple primary melanomas (mean 2·6 vs. 1·7, P = 0·005) and were more likely to have another family member with melanoma (mean 1·1 vs. 0·4, P < 0·001), which remained significant after adjustment for age and sex: multiple primary melanoma count, OR 1·64 (95% CI 1·23–2·20, P = 0·001) and mean family members with melanoma, OR 3·81 (95% CI 1·98–7·32, P < 0·001).

Table 5 Characteristics of carriers of *CDKN2A* disease-causing variants compared with wild-type patients (only high-risk patients included)

Characteristic	*CDKN2A* wild-type (n = 401)	*CDKN2A* high risk (n = 14)	P-value[a]	Odds ratio	95% CI	P-value[b]
Diagnosis age (years), mean ± SD	47 ± 15·9	37 ± 11·9	0·019	0·96	0·92–1·00	0·052
Sex						
Male	224 (55·9)	4 (29)	0·044	0·41	0·12–1·36	0·14
Female	177 (44·1)	10 (71)				
Skin reaction to sun exposure						
Rather red	284 (72·6)	11 (79)	0·62	1·27	0·34–4·73	0·72
Rather brown	107 (27·4)	3 (21)				
Unknown	10	0				
Hair colour						
Blond/reddish	149 (37·2)	6 (43)	0·67	1·08	0·36–3·26	0·89
Black/brown	251 (62·7)	8 (57)				
Unknown	1	0				
Eye colour						
Fair	277 (69·1)	13 (93)	0·057	6·01	0·77–46·80	0·087
Dark	124 (30·9)	1 (7)				
Childhood freckling						
Yes	159 (41·3)	7 (54)	0·37	1·39	0·45–4·31	0·57
No	226 (58·7)	6 (46)				
Unknown	16	1				
Outdoor occupation						
Yes	118 (37·1)	5 (38)	0·92	1·68	0·49–5·76	0·41
No	200 (62·9)	8 (62)				
Unknown	83	1				
Solarium use						
Yes	108 (27·1)	5 (38)	0·365	0·89	0·27–2·93	0·85
No	291 (72·9)	8 (62)				
Unknown	2	1				
Time spent outdoors at leisure						
> 3 h	289 (73·5)	8 (57)	0·18	0·57	0·19–1·72	0·32
< 3 h	104 (26·5)	6 (43)				
Unknown	8	0				
Holiday weeks						
Mean ± SD	13·6 ± 11·4	7·7 ± 7·2	0·065	0·91	0·83–1·00	0·044
Unknown (n)	22	1				
Sunburns 0–10 years						
Mean ± SD	6·8 ± 10	7 ± 9·8	0·92	1·00	0·95–1·05	0·95
Unknown (n)	5	0				
Suncream use						
Yes	306 (76·9)	12 (86)	0·44	0·85	0·17–4·25	0·84
No	92 (23·1)	2 (14)				
Unknown	3	0				
Multiple primary melanoma						
Yes	184 (45·9)	9 (64)	0·18	4·03	1·23–13·18	0·021
No	217 (54·1)	5 (36)				
Multiple primary melanoma count, mean ± SD	1·7 ± 1·1	2·6 ± 1·7	0·005	1·64	1·23–2·20	0·001
Positive family history						
Yes	131 (32·7)	12 (86)	< 0·001	15·48	3·33–71·99	< 0·001
No	270 (67·3)	2 (14)				
Family members with melanoma, mean ± SD	0·4 ± 0·6	1·1 ± 0·8	< 0·001	3·81	1·98–7·32	< 0·001
Localization						
Head/neck	45 (11·2)	2 (14)	0·51	Ref		
Extremities	147 (36·7)	3 (21)		0·23	0·035–1·54	0·13
Trunk	209 (52·1)	9 (64)		0·74	0·15–3·74	0·71
Breslow index, mean ± SD	1·2 ± 1·7	0·8 ± 0·6	0·309	0·71	0·35–1·43	0·33
Sentinel node						
Positive	28 (22·4)	0	0·29	0	NA	1·00
Negative	97 (77·6)	4 (100)				

(continued)

Table 5 (continued)

Characteristic	*CDKN2A* wild-type (n = 401)	*CDKN2A* high risk (n = 14)	P-value[a]	Odds ratio	95% CI	P-value[b]
Unknown	276	10				
Distant metastasis						
Yes	19 (4·7)	3 (21)	0·006	12·12	2·54–57·77	0·002
No	382 (95·3)	11 (79)				
MC1R						
R or r	175 (79·2)	10 (91)	0·35	3·15	0·38–26·02	0·29
Wild-type	46 (20·8)	1 (9)				

Values are n (%) unless stated otherwise. CI, confidence interval; Ref, reference value. [a]χ^2-test for nominal variables and t-test for continuous variables. [b]Multivariable analysis, adjusted for sex and age. P-values < 0·05 are significant.

In total, 46 different *MC1R* variants were found in all participants (Table S4; see Supporting Information). We found seven nonsynonymous variants in *MC1R* and two variants of *CDK4*, which, to the best of our knowledge, have not been tested with prediction tools before.[18–20] For those variants with unknown biological significance, 10 different prediction tools were applied to estimate their effect. For *MC1R*, the variant p.Y143C was found in three unrelated patients with melanoma, while the others occurred in only one person each. The prediction scores are listed in Table 6. Four variants (p.C35F, p.Y143C, p.A240T and p.I287N) were predicted to be damaging in > 75% of the used prediction tools. All carriers were classified as high risk, except for one patient carrying p.Y143C (for all phenotype data, see Table S5 in Supporting Information). The *CDK4* variant p.V154L was classified as being deleterious by five prediction tools. The other, c.−60 C>CG, was located in a noncoding region and could therefore be predicted with only four tools; one predicted it to be disease-causing.

Discussion

Individuals at high risk of melanoma should be recommended a more stringent skin examination for early recognition and risk-avoiding behaviour.[21] As there are different characteristics that define someone as high risk, a more precise description of each subgroup might lead to preventive measures that are better adjusted to individual needs. In this study, we could reveal distinct differences not only between the high-risk and the reference groups, but also between each subgroup. For example, patients with a positive family history were younger on average than the entire study population (49 years, vs. 53 years for the high-risk and reference groups taken together), confirming increased inherited risk. However, positive family history was also associated with childhood freckling and red hair phenotype in a multivariable analysis. These findings support the hypothesis that high-risk individuals were at increased risk due to genetic susceptibility, as both onset of the disease and pigmentation phenotype are genetically determined. Interestingly, melanomas in this group were thinner than in the reference group, which might indicate that knowledge of disease in the family leads to increased awareness and consequently to earlier consultation of a physician.

There are no indications for a slower growth of melanomas in patients with a positive family history, either in the literature or in our own study.[22] Patients with multiple melanomas were slightly older than patients with a positive family history. After adjustment for age and sex only male sex remained significant as a risk factor compared with the reference group. For patients with early-onset melanoma, similar to the subgroup with a positive family history, both childhood freckling and external factors (solarium use and outdoor occupation) remained significant in a multivariable analysis. The lower numbers of melanomas in the head and neck region in the early-onset subgroup suggest that sun protection during vacation might reduce melanoma risk in addition to general awareness and avoidance of unnecessary ultraviolet exposure.[23,24]

Besides these clinical and phenotypic differences, we noticed genetic differences between the subgroups as well. For example, disease-related *CDKN2A* mutations (and functional *MC1R* variants) were found mostly in association with a positive family history (12 of 14 carriers, 86%). Therefore, genetic testing seems to be particularly effective in this group. Carriers of high-risk mutations in *CDKN2A* were more likely to have multiple primary melanomas and were younger. Of the 46 *MC1R* variants in this population seven have not been analysed with prediction tools before. Four of them were classified as deleterious by ≥ 75% of the prediction tools. All of those variants predicted to be deleterious were found in the high-risk group. As shown above, both *CDK4* variants were predicted to be damaging. For the MITF p.E318K variant, we added three new carriers to the cases described previously in the literature, all in the high-risk group. The frequency of the MITF variant in our samples (2·4%) was similar to that in previously reported studies, with a frequency of 1·7–1·8%.[25,26] Although this variant is known to be associated with renal cell carcinoma, no such case was reported in either our patients or their families.

The study was limited by the anamnestic assessment of the family history. Therefore the actual number of cases of familial melanoma might be underestimated.

Table 6 Prediction of the *MC1R* and *CDK4* variants

	MC1R							*CDK4*	
Variant	c.104G>GT	c.322G>GA	c.428A>AG	c.466G>GA	c.623T>TC	c.718G>GA	c.860T>TA	c.11A>AG	c.104G>GT
Amino acid change	p.C35F	p.A108T	p.Y143C	p.V156M	p.V208A	p.A240T	p.I287N	p.V154L	
Polyphen2									
HumDiv	Probably damaging	Benign	Probably damaging	Benign	Benign	Possibly damaging	Probably damaging	Benign	–
Score	0·97	0	1	0·35	0	0·65	1	0·16	–
Provean									
HumVar	Possibly damaging	Benign	Probably damaging	Benign	Benign	Possibly damaging	Probably damaging	Benign	–
Score	0·68	0·01	0·99	0·32	0·01	0·58	0·99	0·092	–
Prediction	Deleterious	Neutral	Deleterious	Neutral	Deleterious	Deleterious	Deleterious	Deleterious	–
MutationTaster									
Score	−5·399	−0·183	−8·626	1·617	−3·096	−3·704	−5·957		–
Prediction	Disease causing	Polymorphism	Disease causing	Disease causing	Polymorphism	Disease causing	Disease causing	Disease causing	Disease causing
SIFT									
Effect	Damaging	Tolerated	Damaging	Tolerated	Damaging	Damaging	Damaging	Tolerated	–
Score	0	1	0	0·37	0	0	0	0·95	–
SNAP									
Prediction	Neutral	Neutral	Neutral	Neutral	Neutral	Effect	Effect	Neutral	–
Score	−5	−81	−12	−76	−27	11	62	−95	–
Expected accuracy	53%	93%	57%	87%	61%	59%	80%	97%	–
Panther									
Pdeleterious	0·698	0·161	0·980	0·156	0·294	0·404	0·798	0·131	–
CADD									
PHRED 12 score	16·72	0·095	25·7	8·295	8·081	24·9	31	18·95	14·98
GERP++									
Score	4·56	−8·69	4·81	1·77	2·32	4·82	5·27	4·66	−3·79
PhyloP									
Score	2·255	−1·7151	1·77998	0·07125	0·14892	2·16832	1·93532	2·55665	−0·4895
Summary: deleterious (% of total)	9 (90)	0	9 (90)	3 (30)	4 (40)	9 (90)	10 (100)	5 (50)	1 (25)
High-risk patient	Yes	Yes	Yes/yes/no	Yes	No	Yes	Yes	Yes	Yes

To the best of our knowledge, for the first time all established familial melanoma risk genes were assessed combined with exogenous factors in the same patients. In our study, risk in early-onset patients appeared to be determined by environmental factors. The others cases seem to be determined mainly by genetic or other unassessed factors. Sun protection at an early age, particularly during recreational activities, might have prevented melanomas in the early-onset subgroup.

For the genetic analysis, the majority of disease-related *CDKN2A* mutation carriers would have been missed in our study if we had tested families with a minimum of three cases. Therefore, our study supports the current recommendation to offer testing to families with two or more affected members.[7] We also revealed new nonsynonymous variants in *CDK4* and *MC1R* associated with high-risk patients. Further studies in central and eastern European countries might reveal novel disease-relevant genetic variants.

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Supporting Information

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

Table S1. Origin of the participants and subgroups in total numbers and percentages.

Table S2. Sequencing primers.

Table S3. *CDKN2A*, *MITF* and *CDK4* variant carriers.

Table S4. All *MC1R* variants.

Table S5. Phenotypes of the predicted *MC1R* and *CDK4* variants.