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Germline mutations in *CDKN2A* are frequently identified among melanoma kindreds and are associated with increased atypical nevus counts. However, a clear relationship between pathogenic *CDKN2A* mutation carriage and other nevus phenotypes including counts of common acquired nevi has not yet been established. Using data from GenoMEL, we investigated the relationships between *CDKN2A* mutation carriage and 2-mm, 5-mm, and atypical nevus counts among blood-related members of melanoma families. Compared with individuals without a pathogenic mutation, those who carried one had an overall higher prevalence of atypical (odds ratio = 1.64; 95% confidence interval = 1.18−2.28) nevi but not 2-mm nevi (odds ratio = 1.06; 95% confidence interval = 0.92−1.21) or 5-mm nevi (odds ratio = 1.26; 95% confidence interval = 0.94−1.70). Stratification by case status showed more pronounced positive associations among non-case family members, who were nearly three times (odds ratio = 2.91; 95% confidence interval = 1.75−4.82) as likely to exhibit nevus counts at or above the median in all three nevus categories simultaneously when harboring a pathogenic mutation (vs. not harboring one). Our results support the hypothesis that unidentified nevogenic genes are co-inherited with *CDKN2A* and may influence carcinogenesis.

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Abbreviations: CI, confidence interval; IQR, interquartile range; OR, odds ratio

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INTRODUCTION

Germline mutations in the *CDKN2A* gene are frequently identified in familial melanoma (Goldstein et al., 2006, 2007), with prevalence in families with three or more members diagnosed with melanoma ranging between 20% and 50% (Goldstein and Tucker, 2001; Harland et al., 2014; Kefford et al., 1999). In contrast, these mutations account for only 1−2% of population-based melanoma cases (Harland et al., 2014). Germline mutations in *CDKN2A* have also been associated with familial atypical multiple mole melanoma syndrome, an autosomally dominant condition exemplified by a family history of melanoma and high numbers of atypical nevi (Eckerle Mize et al., 2009; Goldstein et al., 2007). However, estimating the prevalence of familial atypical multiple mole melanoma has been difficult due to intra- and interfamily variability in the familial atypical multiple mole melanoma phenotype (Goldstein et al., 2000; Lynch et al., 2002; Rulyak et al., 2003), and a clear relationship between *CDKN2A* mutation classification and number of atypical nevi has not yet been established (Bishop et al., 2000; de Snoo et al., 2008; Nielsen et al., 2010).

Few studies have examined the relationship between germline *CDKN2A* mutational status and number of common melanocytic nevi among melanoma families, even though evidence from previous genome-wide association studies suggests that variation near the *CDKN2A* locus is associated with nevus count (Barrett et al., 2011; Falchi et al., 2009). Here, we evaluate associations between germline *CDKN2A* pathogenic mutation classification and nevus phenotype among participants in research performed by the GenoMEL consortium (www.genomel.org). A better understanding of *CDKN2A*'s influence on nevogenesis among blood-related cases and non-cases of melanoma may aid in the search of other risk-modifying nevogenic genes. In addition, robust phenotypic indicators of *CDKN2A* pathogenic mutation carriers, especially among non-case members (i.e., individuals who have not been diagnosed with melanoma) of melanoma families, could influence clinicians' surveillance and prevention strategies in this high-risk population.

RESULTS

CDKN2A genotype was available for at least one member of 896 (78%) families comprising 3,990 individuals, of whom 1,651 (41%) also submitted to nevus phenotyping (Table 1). All analyses were confined to this final analytic cohort of 1,651 participants. The median values of 2 mm, 5 mm, and atypical nevus counts were similar among those with and without a pathogenic *CDKN2A* mutation, although we observed a higher degree of variation among pathogenic mutation carriers compared with those without a pathogenic mutation (Figure 1). Total nevus count (i.e., the sum of 2-mm, 5-mm, and atypical nevus counts) was highly correlated ($r = 0.99$) with number of 2-mm nevi. Median 2-mm nevus counts for those with and without a pathogenic mutation were 54 (interquartile range [IQR] = 102) and 47 (IQR = 87), respectively. For 5-mm nevus counts, those with a pathogenic mutation had a median value of 2 (IQR = 5), whereas a median value of 1 (IQR = 5) was observed among individuals without a pathogenic mutation. Those with and without a pathogenic mutation had a median value of 0 for atypical nevus counts with an IQR of 2 for pathogenic mutation carriers and 1 for those without a pathogenic mutation.

Compared with individuals without a pathogenic *CDKN2A* mutation, pathogenic mutation carriers had an overall higher prevalence of atypical nevi (odds ratio [OR] = 1.64; 95% confidence interval [CI] = 1.18−2.28). Moreover, pathogenic mutation carriers were almost twice as likely as those without a pathogenic mutation (OR = 1.83; 95% CI = 1.25−2.67) to exhibit nevus counts at or above the center-specific medians in all three categories of nevi (mole gestalt scores of 3 vs. 0). Pathogenic *CDKN2A* mutation carriage was not associated with common acquired (2-mm, 5-mm) nevus counts (Table 2). Total nevus count was not associated with carriage of *CDKN2A* mutations and, as expected, point estimates were nearly identical to those observed for 2-mm nevus counts (data not tabulated).

Upon stratification by melanoma case status, we observed more pronounced positive associations between *CDKN2A* pathogenic mutation carriage and nevus counts among the non-case family members. Among non-case participants, those harboring a pathogenic mutation were nearly three times as likely to show the highest mole gestalt score (3 vs. 0) compared with those without a pathogenic mutation (OR = 2.91; 95% CI = 1.75−4.82) and exhibited approximately twice as many atypical nevi compared with non-case participants without a pathogenic mutation (OR = 1.98; 95% CI = 1.34−2.90). In contrast, carriage of a pathogenic mutation was inversely associated with mole gestalt score (3 vs. 0) (OR = 0.90; 95% CI = 0.53−1.53) and showed a modest, but statistically nonsignificant, positive association with number of atypical nevi (OR = 1.47; 95% CI = 0.92−2.33) compared with wild type carriage among case participants (Table 2).

We further explored associations stratified by GenoMEL study centers grouped according to proximity to the equator to assess the relative influence of increasing daylight hours and one stratified by anatomic site of first melanoma classified by relative duration of UVR exposure. Latitude did not show a statistically significant influence on the association between any *CDKN2A* mutation carriage and nevus phenotype (P-interaction > 0.05 for all nevus phenotype categories), nor could we discern any clear patterns of association according to relative UVR exposure of anatomic site of first verified melanoma (see Supplementary Tables S1 and S2 online).

DISCUSSION

Within melanoma families, we observed higher mole gestalt scores among pathogenic *CDKN2A* mutation carriers compared with those without a pathogenic mutation, indicating that carriers tended to have more nevus-laden phenotypes. Estimates within individual nevus phenotype categories (i.e., 2-mm, 5-mm, and atypical nevus counts) indicate that pathogenic mutation carriers exhibit greater numbers of atypical nevi compared with non-carriers.

To date, few studies have examined the influence of germline *CDKN2A* mutation carriage on common acquired nevus counts among melanoma-prone families. A longitudinal study of a large melanoma family from Utah reported

increasing nevus counts among carriers of the specific V126D mutation compared with the wild type over a 15-year interval (Florell et al., 2004). However, the impact of the mutation on atypical nevi is unclear, because total nevus count was reported. Twin studies identified a quantitative trait locus (microsatellite marker D9S942) for nevus density in a noncoding region of *CDKN2A* (Falchi et al., 2006; Zhu et al., 1999, 2007), which may suggest a broader role of *CDKN2A* in nevogenesis among most individuals who do not harbor a rare germline mutation. However, an adolescent twin study from the UK found no evidence for D9S942 as a quantitative trait locus influencing nevus density (Barrett et al., 2003), and a familial-based investigation of a potentially nevogenic variant (A148T) near D9S942 also found no association with common acquired nevus counts (Bertram et al., 2002). Germline mutations in *CDKN2A* are strongly associated with familial atypical multiple mole melanoma syndrome, and individual members of these families often have abundant numbers of atypical and common nevi (Gruis et al., 1995; Hussussian et al., 1994; Soura et al., 2016). However, not all individuals with *CDKN2A* mutations present with excessive or even higher nevus counts. Studies of Dutch and Swedish melanoma kindreds have reported low atypical and common nevus counts among *CDKN2A* mutation carriers (Ipenburg et al., 2016; Nielsen et al., 2010). Similar findings were reported among melanoma families from the UK (Newton Bishop et al., 1994). The range of atypical nevi (0–94) observed in GenoMEL family members with pathogenic *CDKN2A* mutations further highlights the influence of *CDKN2A* on phenotypic heterogeneity.

Evaluating individual nevus types among GenoMEL participants suggests that germline pathogenic mutations at *CDKN2A* are more predictive of number of atypical nevi compared with common acquired nevi (2-mm and 5-mm nevi), a result that is consistent with previous findings (Bishop et al., 2000). These results are also interesting in light of recent research that suggests that intermediate lesions, a classification that includes atypical/dysplastic nevi, are likely to exhibit hemizygous loss of *CDKN2A*, supporting a role for this locus in the development of histological atypia in nevi (Shain et al., 2015). The defining criteria of atypical nevi in this study were clinical and not pathologically based; it is possible that very subtle atypical nevi could have been misclassified as 5-mm nevi. Furthermore, although we took a conservative approach when assigning pathogenicity to *CDKN2A* variants/mutations, it is possible that our designation of some common variants as not pathogenic is not accurate. We based our assessment on evidence of a deleterious effect, and for some of the common variants there is no such evidence to date.

Our observation of distinct differences in associations according to case status is interesting. Non-case members of melanoma-prone families showed relatively strong associations of *CDKN2A* pathogenic mutation carriage with mole gestalt score and number of atypical nevi, whereas corresponding associations among case family members tended to be attenuated. These differences may be due, in part, to the higher proportion of pathogenic *CDKN2A* mutations among case (42%) compared with non-case (25%) individuals.

Table 1. *CDKN2A* status in melanoma families and family members participating in the GenoMEL Study by ascertainment center[1]

Center	Total Number of Families	Number of Families with ≥1 Member Who Is *CDKN2A* Genotyped	Number of Family Members with Known *CDKN2A* Genotype	Number of Family Members Phenotyped with Known *CDKN2A* Genotype
Barcelona, Spain	25	25	116	83
Bethesda, USA	49	48	782	468
Cesena, Italy	24	24	116	17
Copenhagen, Denmark	18	15	18	0
Genoa, Italy	14	14	45	31
Leeds, UK	76	74	282	216
Leiden, The Netherlands	61	59	600	240
Ljubljana, Slovenia	4	4	11	10
Lund, Sweden	8	8	97	74
Montevideo, Uruguay	4	4	23	23
Paris, France	181	181	588	161
Philadelphia, USA	36	36	104	47
Porto Alegre, Brazil	10	5	12	4
Queensland, Australia	230	22	172	11
Riga, Latvia	5	5	8	5
Salt Lake City, USA	1	1	3	3
Santiago, Chile	2	2	6	6
São Paulo, Brazil	12	7	28	25
Stockholm, Sweden	27	25	118	113
Sydney, Australia	319	311	820	85
Tel Aviv, Israel	28	21	25	25
Valencia, Spain	15	5	16	4
Total	1,149	896	3,990	1,651

[1]Melanoma families are defined by three or more members with a verified melanoma or two first degree relatives with verified melanomas. Married-in relatives not belonging to a melanoma family lineage are excluded.

Because pathogenic germline mutations in *CDKN2A* and number of nevi are both important risk factors for melanoma, if *CDKN2A* influences nevogenesis, we might expect to see diminished associations between pathogenic *CDKN2A* mutation carriage and nevus phenotype among case compared with non-case individuals. The higher nevus count distributions we observed among case compared with non-case individuals tends to support this hypothesis (see Supplementary Figure S1 online). It is also possible that case members are affected by yet-to-be-discovered nevogenic genes that co-

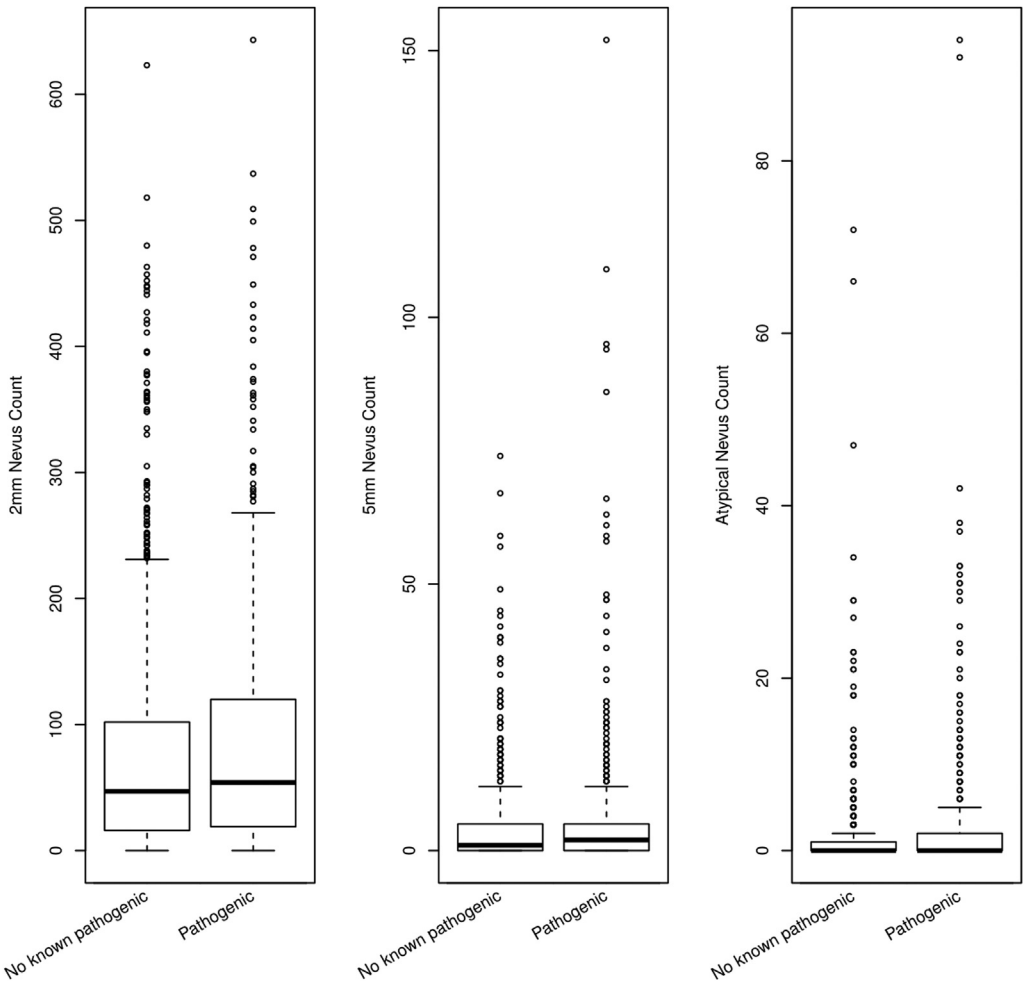

Figure 1. 2-mm, 5-mm, and atypical nevus count distributions among GenoMEL melanoma family members across all ascertainment centers according to *CDKN2A* mutational status. Crude nevus counts are plotted and are not representative of center-specific measures adopted for statistical modeling. Heavy horizonal lines indicate 50th percentile counts, boxes indicate 25th and 75th percentile counts, whiskers indicate 5th and 95th percentile counts, and circles represent values in the top 5% of counts.

segregate with *CDKN2A* and either modify *CDKN2A*'s nevogenic function or influence nevogenesis independently. Another possible explanation is that non-case family members may be more likely to inherit unidentified lower-penetrance genes that are important risk modifiers of nevus formation, potentially hinder melanoma initiation, and co-segregate with *CDKN2A*.

Zhu et al. (2007) have speculated that environmental factors affecting spontaneous somatic mutation rates (e.g., UVR exposure) in tumor suppressor genes may help explain nevus count variation among individuals and familial correlations in nevus counts (Zhu et al., 2007). However, our analyses by latitude of ascertainment center and anatomic site of melanoma—arguably two proxy measures of UVR exposure—did not show meaningful nevus phenotype differences across strata. This exploratory analysis did not take into consideration behaviors that influence UVR exposure (e.g., sunbathing/tanning, sunscreen usage, apparel).

In summary, our results are consistent with those of previous studies reporting that CDKN2A plays a role in nevogenesis (Bishop et al., 2000; Cannon-Albright et al., 1994; Florell et al., 2004; Shain et al., 2015; Zhu et al., 1999). In general, pathogenic mutation carriers are significantly more likely to exhibit higher-than-median nevus counts in all three categories of nevus phenotype simultaneously compared with those without pathogenic *CDKN2A* mutations, as evidenced by our mole gestalt score results. Acknowledging the potential nevus phenotype overlap between those with and without a pathogenic *CDKN2A* mutation (Bishop et al., 2000), we examined associations based on case status among melanoma family members. Associations between *CDKN2A* pathogenic mutational status and nevus phenotype according to case status contrasted sharply. These differences may be explained if *CDKN2A* possesses a degree of nevogenic function, because case family members exhibited higher nevus counts and were more likely to harbor a pathogenic *CDKN2A* mutation compared with non-case members, which could result in diminished associations among case members. Our findings are generally supportive of the hypothesis that unidentified nevogenic genes are co-inherited with *CDKN2A* (Florell et al., 2004).

MATERIALS AND METHODS

Over the past two decades, GenoMEL has aggregated data from individuals belonging to melanoma families from around the globe. We refer to participants with a melanoma diagnosis at the time of recruitment as *cases,* whereas family members who had not been diagnosed with melanoma at the time of recruitment are referred to

Table 2. Associations between nevus phenotypes and *CDKN2A* mutational status among members of melanoma families

Nevus Phenotype	Individual *CDKN2A* Mutational Status	Overall[1]		Case Members Only (n = 757)[2]		Non-case Members Only (n = 894)[2]		*P*-Value Interaction[6]
		OR (95% CI)	*P*-Value	OR (95% CI)	*P*-Value	OR (95% CI)	*P*-Value	
2-mm nevi	No known pathogenic	1.00	0.45	1.00	0.49	1.00	0.93	0.45
	Pathogenic	1.06 (0.92–1.21)		1.06 (0.90–1.26)		0.99 (0.83–1.19)		
5-mm nevi	No known pathogenic	1.00	0.18	1.00	0.31	1.00	0.27	0.95
	Pathogenic	1.26 (0.94–1.70)		1.21 (0.87–1.69)		1.31 (0.86–1.99)		
Atypical nevi	No known pathogenic	1.00	0.02	1.00	0.16	1.00	0.01	0.27
	Pathogenic	1.64 (1.18–2.28)		1.47 (0.92–2.33)		1.98 (1.34–2.90)		
Mole gestalt (3 vs. 0)[3]	No known pathogenic	1.00	0.004	1.00	0.69	1.00	0.0001	0.002
	Pathogenic	1.83 (1.25–2.67)		0.90 (0.53–1.53)		2.91 (1.75–4.82)		
Mole gestalt (2 vs. 0)[4]	No known pathogenic	1.00	0.05	1.00	0.35	1.00	0.004	0.02
	Pathogenic	1.38 (1.00–1.91)		0.79 (0.48–1.29)		1.96 (1.26–3.06)		
Mole gestalt (1 vs. 0)[5]	No known pathogenic	1.00	0.28	1.00	0.36	1.00	0.15	0.25
	Pathogenic	1.21 (0.86–1.71)		0.80 (0.50–1.29)		1.42 (0.89–2.25)		

Abbreviations: CI, confidence interval; IQR, interquartile range; OR, odds ratio.

[1]Adjusted for age at phenotyping, sex, age at phenotyping × sex, melanoma affected status, center, and familial clustering within study center. Married-in relatives not belonging to a melanoma family lineage are excluded. *P*-values correspond to overall score tests.

[2]Adjusted for age at phenotyping, sex, age at phenotyping × sex, center, and familial clustering within study center. Married-in relatives not belonging to a melanoma family lineage are excluded. *P*-values correspond to overall score tests.

[3]Mole gestalt is modeled in a generalized estimating equation model excluding individuals with values of 1 and 2 for mole gestalt to achieve the contrast estimates.

[4]Mole gestalt is modeled in a generalized estimating equation model excluding individuals with values of 1 and 3 for mole gestalt to achieve the contrast estimates.

[5]Mole gestalt is modeled in a generalized estimating equation model excluding individuals with values of 2 and 3 for mole gestalt to achieve the contrast estimates.

[6]*P*-value for the association between the interaction of *CDKN2A* mutation carriage with case status and nevus phenotype.

as *non-cases*. Currently, GenoMEL consists of 29 centers from Australia, Europe, the Middle East, and North and South America.

GenoMEL used a common protocol for data collection from prospectively enrolled participants, although family identification and recruitment procedures were allowed to differ among study centers. Additionally, centers had a degree of autonomy over the data collection process, which resulted in different contributions across various protocol components. Thus, not all centers completed all portions of the research protocol for each enrolled participant. Regulatory approval was obtained by the institutional review boards of each GenoMEL study center, and written informed consent was obtained for each participant. Individuals who signed informed consent were asked about their personal and familial melanoma histories and to submit to a full phenotypic examination by research staff, which included an evaluation of nevus counts by anatomic site. Training was carried out for all staff performing phenotyping on participants in the prospective study in the UK. Consolidation of that training was subsequently carried out in Italy. Several GenoMEL study centers had extant data previously collected from members of melanoma families under local regulatory approval, and where possible this information was harmonized with data arising from participants enrolled in the prospective GenoMEL study.

A *melanoma family* was defined by the presence of three or more cases of confirmed cutaneous melanoma in the same lineage, or two cases of confirmed cutaneous melanoma in first degree relatives. Melanoma case family members with a diagnosis of mucosal or ocular melanoma did not contribute to defining a melanoma family and were excluded from analysis. Confirmation of diagnosis was made by pathology report (75%), physician letter or clinical document verifying melanoma diagnosis (19%), death certificate (2%), or cancer registry data (4%). Individuals who are members of melanoma families by virtue of marriage and not ancestry, or for whom family relationship information was ambiguous or missing, were excluded from this study. Family members who reported a melanoma, but for whom verification of diagnosis was not available, were also excluded from analyses.

Nevi of 2 mm or greater but less than 5 mm in diameter (herein referred to as *2-mm nevi*) were counted on exposed skin, in addition to nevi of 5 mm or greater in diameter (herein referred to as *5-mm nevi*) and clinically atypical nevi; sites not examined were the genitalia and female breasts. An atypical nevus was defined as a nevus of 5 mm or greater in diameter and containing a flat component, with at least two of the following characteristics: variable pigmentation, asymmetrical shape, or diffuse border. We also derived a summary variable from 2-mm, 5-mm, and atypical nevus counts to describe an individual's overall nevus phenotypic landscape. Specifically, individuals were assigned a dichotomous score within each category of 2-mm, 5-mm, and atypical nevus count according to the study center-specific median. Individuals with at least the median nevus count were scored as 1, with those exhibiting fewer than the median nevus count scored as 0; each individual then received an aggregate "mole gestalt" summary score between 0 and 3 based on the sum of these three dichotomous scores.

Germline DNA of consenting participants was screened for mutations in *CDKN2A* (exons 1α, 1β, 2, and 3), as previously described (Harland et al., 2008). Mutation evaluation, predominantly by sequencing or denaturing high performance liquid chromatography followed by sequencing, was conducted at each study center.

Previous evaluation has confirmed consistent mutation detection across the consortium (Harland et al., 2008). Sequencing results were collated, and mutational status was assigned according to pathogenicity as outlined in Supplementary Table S3 online. Briefly, pathogenic variants were adjudicated based on demonstrated (i.e., published) impact on the biological function of *CDKN2A* or bioinformatically inferred deleterious impact on *CDKN2A* function and evidence of co-segregation within melanoma families. Variants not meeting any of these criteria were classified as benign (Taylor et al., 2016). Individual participants were classified based on presence of a pathogenic mutation; benign variant carriers and wild-type individuals were combined for analyses and classified as having "no known pathogenic" mutations at *CDKN2A*. Individuals who carried both a pathogenic mutation and a benign mutation were classified as pathogenic.

We used the generalized estimating equation method implemented in SAS, version 9.4 (SAS Institute, Cary, NC) to calculate ORs and 95% CIs for associations between nevus phenotypes and *CDKN2A* mutational status. For our nevus count outcomes we used Poisson regression (2-mm nevi) or negative binomial regression when nevus counts were right-skewed (5-mm and atypical nevi), whereas a multinomial model was used to evaluate the "mole gestalt" variable. Designating a type I error rate of $\alpha = 0.05$, we performed score tests of the null hypothesis that no differences exist between nevus counts within strata of mutational status. Analyses were adjusted for age at phenotyping, sex, the interaction between age and sex, melanoma status, and study center; we accounted for the non-independence of observations arising from familial clustering within study center using the repeated subject statement of the GENMOD SAS procedure.

We examined associations by latitude by grouping GenoMEL ascertainment centers according to equatorial proximity. Among family members with a diagnosis of melanoma, we also examined associations between *CDKN2A* mutational status and nevus phenotype by anatomic location of an individual's first verified melanoma. Anatomic sites were classified as those usually exposed (head, neck, lower arms and scalp—male), intermittently exposed (trunk, back, upper arms, lower legs, and scalp—female), and usually unexposed (buttock, upper legs) to UVR.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.07.829.

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