

## Melanocytic Nevi, Nevus Genes, and Melanoma Risk in a Large Case-Control Study in the United Kingdom

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### Abstract

**Background:** Increased number of melanocytic nevi is a potent melanoma risk factor. We have carried out a large population-based case-control study to explore the environmental and genetic determinants of nevi and the relationship with melanoma risk.

**Methods:** We report nevus phenotype in relation to differing patterns of sun exposure, inherited variation at loci shown in recent genome-wide association studies to be nevus genes, and risk.

**Results:** Increased numbers of nevi were associated with holiday sun exposure, particularly on intermittently sun-exposed body sites (test for  $P_{\text{trend}} < 0.0001$ ). Large nevi were also associated with holiday sun exposure ( $P = 0.002$ ). Single nucleotide polymorphisms (SNP) on chromosomes 9 and 22 were associated with increased numbers of nevi ( $P = 0.04$  and  $P = 0.002$  respectively) and larger nevi ( $P = 0.03$  and  $P = 0.002$ ), whereas that on chromosome 6 was associated only with large nevi ( $P = 0.01$ ). Melanoma risk was associated with increased nevus count, large nevi, and atypical nevi for tumors in all body sites (including rare sites) irrespective of age. The risk persisted when adjusted for inheritance of nevus SNPs.

**Conclusions:** The at-risk nevus phenotype is associated with behaviors known to increase melanoma risk (holiday sun exposure). Although SNPs on chromosomes 6, 9, and 22 were shown to be nevus genes, they explained only a small proportion of melanoma risk and nevus phenotype; therefore, several nevus genes likely remain to be identified.

**Impact:** This article confirms the importance of nevi in melanoma pathogenesis and increases understanding of their genetic determinants. *Cancer Epidemiol Biomarkers Prev*; 19(8); 2043–54. ©2010 AACR.

### Introduction

Cutaneous melanoma is predominantly a cancer of pale-skinned peoples. Within those pale-skinned populations, individuals with skin that tends to burn in the sun are at increased risk (1). The genetic basis of this increased susceptibility is becoming increasingly understood as a result both of candidate gene approaches, which resulted in the identification of variation in the *melanocortin 1 receptor* (*MC1R*) as a susceptibility gene (2, 3), and of genome-wide association studies, which identified the tyrosinase gene

and the *ASIP* locus as common susceptibility genes (4–6). Sunburn and intermittent (holiday) sun exposure are the dominant environmental determinants of melanoma risk (rather than large cumulative exposures) in temperate climates, with stronger evidence for both intermittent sun exposure and cumulative exposures in hotter countries (7).

Although sun-sensitive characteristics are common at-risk phenotypes, the most potent phenotypic risk factor for melanoma is the presence of increased numbers of melanocytic nevi (8, 9). Melanocytic nevi are benign proliferations of melanocytes that are postulated to result from sun-induced mutations in oncogenes, typically *BRAF* (10) and less frequently *NRAS* (11). In the majority of such neoplasms, subsequent melanocyte senescence is induced by tumor suppressor proteins such as p16, and the nevus therefore ceases to grow and becomes stable or even involutes (12). In a proportion of individuals, however, a greater number of nevi develop, and commonly, the melanocytes continue to proliferate for longer before senescence is induced. Therefore, these individuals often have bigger nevi (5 mm or more in diameter), and those individuals with a particularly large number of nevi are said to have the dysplastic nevus or atypical mole syndrome. Twin studies have provided strong evidence that the number of nevi is predominantly genetically

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**Table 1.** Association between different patterns of sun exposure and total body nevus number, number of large normal nevi, and presence of atypical nevi adjusted for case-control status

Sun exposure	n (%)	Total body nevus number*			
		Controls median (Q1, Q3)	Cases median (Q1, Q3)	$\beta$ (95% CI)	$P_{\text{trend}}$
Sunburn under age 20 y					
No	902 (68)	15 (7, 29)	42 (21, 76)	—	—
Yes	423 (32)	18 (10, 37)	42 (22, 98)	0.03 (−0.08 to 0.15)	0.55
Sunburn at/over age 20 y					
No	802 (61)	14 (6, 29)	42 (21, 83)	—	—
Yes	504 (39)	19 (10, 36)	43 (20, 89)	0.10 (−0.01 to 0.21)	0.06
Average weekday exposure at cooler months (h/d)					
≤0.9	463 (34)	15 (7, 32)	42 (23, 79)	—	—
0.9-1.5	463 (34)	17 (8, 29)	43 (20, 85)	0.01 (−0.12 to 0.13)	—
>1.5	440 (32)	15 (6, 29)	42 (22, 89)	0.01 (−0.12 to 0.13)	0.91
Average weekday exposure at warmer months (h/d)					
≤1.4	489 (36)	15 (8, 28)	42 (23, 84)	—	—
1.4-2.1	415 (30)	17 (7, 32)	42 (21, 81)	0.03 (−0.10 to 0.15)	—
>2.1	466 (34)	15 (6, 29)	43 (20, 89)	−0.01 (−0.13 to 0.11)	0.87
Average weekend exposure at cooler months (h/d)					
≤2.5	498 (36)	14 (7, 30)	44 (23, 78)	—	—
2.5-3.5	466 (34)	17 (8, 34)	41 (20, 85)	0.04 (−0.08 to 0.16)	—
>3.5	416 (30)	15 (6, 28)	44 (20, 94)	−0.03 (−0.15 to 0.10)	0.71
Average weekend exposure at warmer months (h/d)					
≤4.0	538 (39)	14 (7, 27)	42 (21, 78)	—	—
4.0-5.0	424 (31)	15 (6, 31)	46 (23, 94)	0.03 (−0.09 to 0.15)	—
>5.0	417 (30)	18 (7, 36)	41 (19, 87)	−0.04 (−0.17 to 0.08)	0.52
Average daily exposure (h/d)					
≤1.9	492 (36)	15 (7, 29)	44 (24, 80)	—	—
1.9-2.5	419 (31)	17 (8, 34)	40 (21, 88)	−0.01 (−0.13 to 0.12)	—
>2.5	443 (33)	15 (6, 29)	43 (20, 89)	−0.06 (−0.18 to 0.07)	0.38
Average holiday exposure (h/y)					
≤46.5	493 (36)	14 (5, 27)	33 (19, 59)	—	—
46.5-71.5	411 (30)	15 (7, 31)	48 (25, 93)	0.23 (0.10-0.35)	—
>71.5	467 (34)	17 (10, 34)	51 (23, 97)	0.29 (0.17-0.41)	<0.0001
Average holiday exposure (10 am-2 pm; h/y)					
≤23.3	502 (37)	14 (5, 26)	34 (19, 63)	—	—
23.3-38.0	435 (32)	15 (7, 31)	50 (26, 96)	0.28 (0.16-0.40)	—
>38.0	429 (31)	17 (10, 36)	46 (21, 93)	0.28 (0.16-0.40)	<0.0001
Average holiday exposure below 45°N (h/y)					
≤6.5	456 (33)	11 (4, 23)	30 (14, 61)	—	—
6.5-26.7	446 (33)	15 (8, 34)	42 (23, 87)	0.19 (0.07-0.32)	—
>26.7	469 (34)	20 (10, 36)	56 (27, 101)	0.31 (0.19-0.43)	<0.0001
Average holiday exposure below 45°N (10 am-2 pm; h/y)					
≤3.3	471 (35)	12 (4, 23)	32 (17, 61)	—	—
3.3-13.0	434 (32)	15 (8, 32)	43 (23, 94)	0.20 (0.07-0.32)	—
>13.0	461 (34)	20 (10, 35)	52 (26, 96)	0.26 (0.13-0.38)	<0.0001

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determined (13-16), with a smaller effect of sun exposure (17). Genome-wide association studies have recently identified several loci that determine nevus number (6, 18).<sup>4</sup>

<sup>4</sup> Duffy et al. In press.

We report here a large case-control study of melanoma done in the north of United Kingdom, in which the risk associated with nevus phenotype was investigated in relation to patterns of sun exposure and the inheritance of three single nucleotide polymorphisms (SNP) on chromosomes 6, 9, and 22, in previously identified nevus genes (18).<sup>4</sup>

**Table 1.** Association between different patterns of sun exposure and total body nevus number, number of large normal nevi, and presence of atypical nevi adjusted for case-control status (Cont'd)

Sun exposure	No. of large nevi <sup>†</sup>		Presence of atypical nevi <sup>‡</sup>		
	Rate ratio (95% CI)	P <sub>trend</sub>	Risk ratio (95% CI)	P <sub>trend</sub>	P <sub>trend</sub> <sup>§</sup>
Sunburn under age 20 y					
No	—	—	—	—	—
Yes	1.09 (0.90-1.32)	0.37	1.12 (0.92-1.38)	0.25	0.20
Sunburn at/over age 20 y					
No	—	—	—	—	—
Yes	1.18 (0.98-1.41)	0.08	1.07 (0.87-1.30)	0.54	0.86
Average weekday exposure at cooler months (h/d)					
≤0.9	—	—	—	—	—
0.9-1.5	1.09 (0.88, 1.34)	—	0.83 (0.65, 1.04)	—	—
>1.5	1.15 (0.92-1.43)	0.22	0.96 (0.76-1.20)	0.68	0.84
Average weekday exposure at warmer months (h/d)					
≤1.4	—	—	—	—	—
1.4-2.1	0.97 (0.78-1.21)	—	0.78 (0.61-0.99)	—	—
>2.1	1.21 (0.98-1.49)	0.07	0.86 (0.69-1.07)	0.16	0.23
Average weekend exposure at cooler months (h/d)					
≤2.5	—	—	—	—	—
2.5-3.5	1.18 (0.96-1.45)	—	0.90 (0.71-1.12)	—	—
>3.5	1.01 (0.81-1.26)	0.84	0.94 (0.75-1.19)	0.58	0.82
Average weekend exposure at warmer months (h/d)					
≤4.0	—	—	—	—	—
4.0-5.0	1.07 (0.87-1.32)	—	1.15 (0.92-1.45)	—	—
>5.0	0.98 (0.80-1.22)	0.94	1.15 (0.91-1.45)	0.21	0.10
Average daily exposure (h/d)					
≤1.9	—	—	—	—	—
1.9-2.5	0.94 (0.76-1.17)	—	0.91 (0.72-1.15)	—	—
>2.5	1.16 (0.94-1.43)	0.18	0.91 (0.72-1.14)	0.39	0.68
Average holiday exposure (h/y)					
≤46.5	—	—	—	—	—
46.5-71.5	1.29 (1.03-1.60)	—	1.45 (1.14-1.84)	—	—
>71.5	1.38 (1.12-1.71)	0.002	1.24 (0.97-1.58)	0.08	0.38
Average holiday exposure (10 am-2 pm; h/y)					
≤23.3	—	—	—	—	—
23.3-38.0	1.15 (0.93-1.43)	—	1.15 (0.91-1.45)	—	—
>38.0	1.33 (1.07-1.64)	0.009	1.08 (0.85, 1.37)	0.48	0.85
Average holiday exposure below 45°N (h/y)					
≤6.5	—	—	—	—	—
6.5-26.7	1.03 (0.83-1.28)	—	1.58 (1.21-2.04)	—	—
>26.7	1.06 (0.85-1.31)	0.61	1.57 (1.21-2.02)	0.0006	0.01
Average holiday exposure below 45°N (10 am-2 pm; h/y)					
≤3.3	—	—	—	—	—
3.3-13.0	1.03 (0.83-1.28)	—	1.58 (1.22-2.03)	—	—
>13.0	0.98 (0.79-1.22)	0.88	1.51 (1.17-1.94)	0.001	0.02

\*Median total body nevus number (first quartile, third quartile) for controls and cases are presented separately but subsequent analyses are for the total sample adjusted for case-control status. Age-sex-corrected log-transformed body nevus number was used to test for association, and the linear regression coefficient ( $\beta$ ) and its 95% CIs are presented.

<sup>†</sup>Analyses were carried out using Negative Binomial regression models, and rate ratios are presented.

<sup>‡</sup>Modified Poisson regression models with robust error variance were used to analyze presence of atypical nevi and risk ratios are presented.

<sup>§</sup>Log-transformed body nevus number was further adjusted for in the trend test.

**Table 2.** Association between nevus genotype and total body nevus number, large normal nevus number, and presence of atypical nevi adjusted for case-control status

Nevus genotype	N (%)	Total body nevus number*			
		Controls median (Q1, Q3)	Cases median (Q1, Q3)	$\beta$ (95% CI)	$P_{\text{trend}}$
rs12203592_chr6					
CC	872 (62%)	16 (8, 29)	41 (21, 77)	—	—
CT	463 (33%)	15 (6, 30)	44 (21, 94)	0.04 (−0.07 to 0.15)	—
TT	64 (5%)	14 (8, 24)	33 (14, 62)	−0.27 (−0.51 to −0.03)	0.43
rs7023329_chr9					
GG	343 (26%)	17 (8, 32)	43 (21, 93)	—	—
GA	713 (53%)	15 (7, 29)	41 (20, 78)	−0.15 (−0.28 to −0.02)	—
AA	289 (21%)	15 (7, 24)	41 (20, 77)	−0.15 (−0.30 to −0.00)	0.04
rs2284063_chr22					
GG	611 (44%)	17 (8, 35)	45 (21, 89)	—	—
GA	620 (45%)	15 (7, 27)	40 (20, 77)	−0.12 (−0.23 to −0.02)	—
AA	152 (11%)	13 (6, 28)	39 (20, 77)	−0.24 (−0.41 to −0.07)	0.002

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## Materials and Methods

### Ascertainment of cases and controls

Studies were approved by the UK Multi-Centre Research Ethics Committee and the Patient Information Advisory Group. Population-ascertained incident melanoma cases were recruited to a case-control study in a geographically defined area of Yorkshire and the Northern region of the United Kingdom (67% participation rate) as has been described elsewhere (19). All patients gave written informed consent to participation. A total of 960 patients (ages between 18 and 76 y) were diagnosed in the period from September 2000 to December 2005 (19). The cases were identified through clinicians, pathology registers, and the Northern and Yorkshire Cancer Registry to ensure maximal ascertainment. During the study, a pragmatic approach was adopted to recruitment: during two time periods, patients with very thin tumors who were unlikely to contribute to information on the determinants of outcome were excluded given limited resources. During other time periods, all patients with invasive melanoma were eligible to sample the whole melanoma population for studies on etiology. Thus, between September 2000 and December 2001, and from July 2003 to December 2005, patients with a Breslow thickness of <0.75 mm were not invited to participate. Between January 2002 and June 2003, all patients with invasive melanoma were invited to participate.

The 513 population-ascertained controls were identified by the cases' family doctors as not having cancer, and were randomly invited from individuals with the same sex and within the same 5-year age group as a case (55% response rate). Descriptive statistics were obtained from the cancer registry of the characteristics of cases diagnosed in a similar time period to this recruitment

to show the comparability of the sample with the incident case population as described elsewhere.<sup>5</sup>

### Data collection

An initial questionnaire (including a residence calendar) was completed by the participants, at home, and mailed to the interviewer, and more detailed sun exposure data were subsequently collected by telephone based on the residence calendar as described by Armstrong et al. (20). Comprehensive calendar data on self-reported weekday and weekend sun exposure habits throughout life were collected, as were details about latitude of residence and sun exposure habits during vacations. Age, sex, natural hair color at age 18 years, propensity to burn, ability to tan, skin color of inside upper arm, and freckling as a child using Gallagher's freckle chart (21) were recorded, based on self-report. Self-reported freckling as a child showed good correlation with nurses' examined score,  $P < 0.0001$  for linear trend. The nurse examined mean scores were 10.0, 14.5, 24.2, and 40.4, respectively, for self-reported categories none, very few, few/some, and many, respectively.

Cases and controls were also examined by research nurses, who recorded eye color (blue/gray, green/hazel, or brown) and freckling scores for face, arms, and shoulders using Gallagher's chart (21). Three nurses were trained to count nevi by JNB. Nevi of  $\geq 2$  mm in diameter were counted on exposed skin: sites not examined were the genitalia and breasts in women. The counts were subdivided into 18 body sites; nevi of  $\geq 5$  mm in diameter and clinically atypical nevi were separately tabulated in

<sup>5</sup> Newton-Bishop et al. Submitted for publication.

**Table 2.** Association between nevus genotype and total body nevus number, large normal nevus number, and presence of atypical nevi adjusted for case-control status (Cont'd)

Nevus genotype	No. of large nevi <sup>†</sup>		Presence of atypical nevi <sup>‡</sup>	
	Rate ratio (95% CI)	<i>P</i> <sub>trend</sub>	Risk ratio (95% CI)	<i>P</i> <sub>trend</sub>
rs12203592_chr6				
CC	—	—	—	—
CT	0.88 (0.73-1.06)	—	0.86 (0.69-1.06)	—
TT	0.52 (0.33-0.83)	0.01	0.67 (0.38-1.18)	0.05
rs7023329_chr9				
GG	—	—	—	—
GA	0.79 (0.64-0.98)	—	0.82 (0.66-1.03)	—
AA	0.77 (0.59-0.99)	0.03	0.94 (0.71-1.23)	0.54
rs2284063_chr22				
GG	—	—	—	—
GA	0.75 (0.62-0.90)	—	0.99 (0.81-1.21)	—
AA	0.73 (0.54-0.98)	0.002	1.05 (0.76-1.46)	0.87

\*Median total body nevus number (first quartile, third quartile) for controls and cases are presented separately, but subsequent analyses are for the total sample adjusted for case-control status; age-sex-corrected log-transformed body nevus number was used to test for association, and linear regression coefficient ( $\beta$ ) and its 95% CIs are presented.

<sup>†</sup>Analyses were carried out using Negative Binomial regression models and rate ratios are presented.

<sup>‡</sup>Modified Poisson regression models with robust error variance were used to analyze presence of atypical nevi, and risk ratios are presented.

each body site. An atypical nevus was defined as a nevus of  $\geq 5$  mm in diameter, with variable pigmentation and an irregular or diffuse edge.

### Genotyping

DNA was extracted from blood from consenting participants to allow detection of SNPs (19). The SNPs rs12203592 (*IRF4*, chromosome 6), rs7023329 (*MTAP*, chromosome 9), and rs2284063 (*PLA2G6*, chromosome 22) were genotyped using the Taqman genotyping assays C\_31918199\_10, C\_29146385\_10, and C\_2458775\_1, respectively (Applied Biosystems). Two microliter PCRs were performed in 384-well plates using 10 ng of DNA (dried), using 0.05  $\mu$ L assay mix and 1  $\mu$ L Universal Master Mix (Applied Biosystems) according to the manufacturer's instructions. End point reading of the genotypes was done using an ABI 7900HT Real-time PCR system (Applied Biosystems).

The entire coding region of the *MC1R* gene was screened for variants by Sanger sequencing. The single *MC1R* exon was PCR amplified in three overlapping fragments. Each fragment was sequenced in both directions using the primers from the initial amplification. Sequencing reactions were done as previously described (22). The sequencing results were collated, and variants identified were assigned a status as "R" (strong association with red hair phenotype), "r" (weak association with red hair phenotype), or not associated with red hair, according to a previous widely used classification (23).

### Statistical methods

Several sun exposure measures were derived from the questionnaire data: average hours of weekday sun exposure, average hours of weekend sun exposure, average daily sun exposure, average holiday exposure, and average exposure on holidays in sunny climates (defined as at latitude below 45°). Aggregated sun exposure variables were classified into thirds based on the distribution in the control population. Data on self-reported significant sunburns (defined as causing pain for  $\geq 2$  d) were dichotomized as ever/never reporting sunburn, both under the age of 20 years and at or over the age of 20 years.

Distribution of whole body nevus number was fairly skewed, and log transformation was applied to attain normality (Supplementary Fig. S1). Because nevi develop in the earlier years of life and then involute, estimated age-sex adjusted log-transformed nevus numbers for all participants were calculated based on regression of the log body nevus number on age and sex in the control population. Residual log body nevus number was then calculated as the log-transformed total body nevus number with the estimated age-sex-adjusted value subtracted. We further classified body nevus number into quarters based on the residual log body nevus number distribution in the control population.

Linear regression models were used to estimate the effects (regression coefficients,  $\beta$ ) of sun exposure patterns and nevus genotype on residual log body nevus number. Negative binomial regression models were used to estimate effects of sun exposure and nevus genotype on

nevus number, and rate ratios and 95% confidence intervals (CI) were estimated. Modified Poisson regression with robust error variance models were used to analyze the presence of atypical nevi. Interactions between sun exposure and nevus genotype on nevus phenotype were tested by comparing a model with main effects and an interaction term with a reduced model with only main effects, using the likelihood ratio test. Unconditional logistic regression models were used to examine the effects of nevus phenotype and nevus genotype on melanoma risk, and odds ratios (OR) and 95% CI were estimated. Sums-of-squares  $R^2$  ( $R_{SS}^2$ ), defined as

$$R_{SS}^2 = 1 - \frac{SSE}{SST} = 1 - \frac{\sum_i (y_i - \hat{p}_i)^2}{\sum_i (y_i - \bar{p})^2},$$

in which  $y_i = 0$  for control and 1 for case,  $\hat{p}_i$  is the estimated probability of being affected, based on the fitted model, and  $\bar{p} = \sum_i y_i / N$  was used to assess the proportion of variation explained by covariates in the logistic regression models (24). These analyses were carried out using the GLM, GENMOD, and LOGISTIC procedures in SAS version 9.1 for PC (Copyright, SAS Institute, Inc.).

## Results

As nevus number is believed to be determined, at least in part, by sun exposure, we first looked at the relationship between counts of all nevi and clinically atypical nevi, and different patterns of sun exposure (Table 1). Correlations with sun exposure were determined for cases and controls separately and then for all cases and controls together. In Table 1, it can be seen that there was a strong positive correlation between total nevus number and holiday sun exposure. For example, cases in the highest tertile of holiday sun exposure below 45° had a median nevus count of 56 compared with 30 for those in the lowest tertile, and a similar increase was seen in controls. There was no convincing relationship between either average daily exposure or sunburn and nevus number.

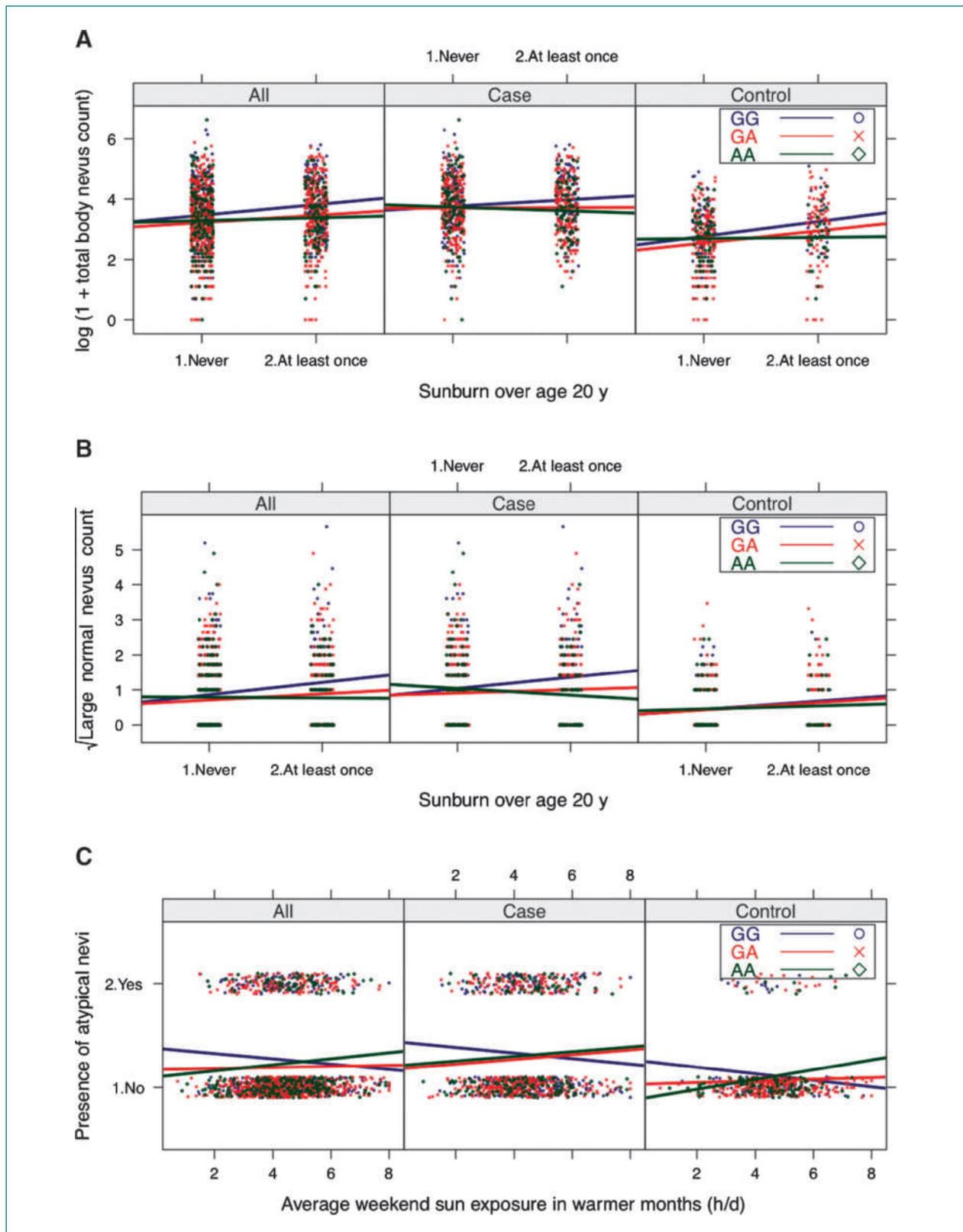
Holiday sun exposure was also associated with an increased number of atypical nevi, particularly for very sunny holidays at below 45° latitude (presence of at least one atypical nevus 58% more likely, respectively, for those in the middle and highest tertiles of exposure compared with the lowest). As total nevus number and number of atypical nevi have been shown in other studies to be highly correlated (25), we looked at the relationship between sun exposure and atypical nevi corrected for total nevus number. The effect of holiday sun exposure on atypical nevi persisted only for holidays at <45° latitude ( $P = 0.01$ ).

We examined the relationship between sun exposure and nevi of  $\geq 5$  mm in diameter, and the data support a relationship between holiday sun exposure and number of large nevi, although the effect was only seen for holidays at latitudes higher than 45°, individuals in the high-

est tertile of exposure having on average 45% more large nevi than those in the lowest tertile of holiday sun exposure above 45° (rate ratio = 1.45;  $P_{\text{trend}} = 0.0007$ ; data not shown). We investigated the relationship between sun exposure in continuously exposed body sites such as the arms and in intermittently exposed sites such as the trunk (Supplementary Table S1). The relationship between holiday exposure and nevus number was more marked for intermittently exposed sites [ $P < 0.0001$  for all categories of average holiday exposure, regression coefficient  $\beta = 0.32$  on log scale corresponding to a 38% increase in nevus number comparing highest to lowest tertile of exposure] than for continuously exposed sites [ $P = 0.0005$ ,  $\beta = 0.20$ , 22% increase]. There was weak evidence that higher average weekend sun exposure in warmer months was associated with fewer nevi in continuously exposed sites ( $P = 0.05$ ;  $\beta = -0.11$ , 10% decrease).

We then confirmed the relationship between inheritance of SNPs on chromosomes 9 (rs7023329), 6 (rs12203592), and 22 (rs2284063) and nevus phenotype (Table 2). The A allele at the SNP on chromosome 9 was associated with reduced total nevus number ( $P_{\text{trend}} = 0.04$ ; median nevus count of 41 in cases with the AA genotype compared with 43 in those with GG genotype and a similar reduction to 15 from 17 in controls) and reduced number of large nevi ( $\geq 5$  mm;  $P_{\text{trend}} = 0.03$ ; number of large nevi in those with AA genotype 77% of that for the GG genotype). There was no significant protective effect for clinically atypical nevi, but the regression coefficients were of a similar order of magnitude as for large nevi. The association between this SNP and fewer nevi was similar for 1 and for 2 A alleles, suggesting a dominant effect. The chromosome 9 SNP showed weak evidence of an interaction between sunburn and sun exposure and genotype (Supplementary Table S2) on nevus phenotype, represented graphically in Fig. 1. The effect of sunburn at or over the age of 20 years for heterozygotes was a 14% increase in total body nevus number ( $P = 0.09$ ) and a 33% increase in large nevi ( $P = 0.03$ ). The effect of sunburn for GG homozygotes was a 31% increase in total body nevus number ( $P = 0.01$ ) and a 40% increase in large nevi ( $P = 0.07$ ). There was no effect of sunburn on nevi in individuals with the AA genotype ( $P = 0.69$  and 0.41 for total body nevus and number of large normal nevi, respectively).

The T allele for the chromosome 6 *IRF4* SNP showed some evidence of association with fewer large nevi (rate ratio 0.52 for TT versus CC genotype;  $P_{\text{trend}} = 0.01$ ; Table 2) and less significantly with reduced risk of atypical nevi but not total nevus count. The A allele at the SNP on chromosome 22 was associated with both a reduced total number of nevi (median of 39 in cases with the AA genotype compared with 45 with the GG genotype and a less striking difference in controls; overall  $P_{\text{trend}} = 0.002$ ) and a reduced number of large nevi (rate ratio 0.73 for AA versus GG,  $P_{\text{trend}} = 0.002$ ), but there was no association with clinically atypical nevi. There was some evidence of an interaction between this SNP and sunburn under the



**Figure 1.** Interaction between sun exposure and rs7023329\_chr9 variant on (A) total body nevus number, (B) large normal nevi, and (C) presence of atypical nevi.

**Table 3.** Association between nevus phenotype and nevus genotype and risk of melanoma

Risk factor	Control	Case	Univariate OR (95% CI)	Multivariable OR1 (95% CI)*	Adjusted OR2 (95% CI)†
Residual log body nevus number quartile					
Q1	126 (25)	55 (6)	—	—	—
Q2	126 (25)	127 (14)	2.31 (1.55-3.45)	1.98 (1.29-3.03)	2.64 (1.71-4.08)
Q3	126 (25)	205 (22)	3.73 (2.53-5.49)	3.12 (2.07-4.70)	4.32 (2.84-6.59)
Q4	126 (25)	551 (59)	10.02 (6.91-14.52)	7.47 (5.01-11.14)	11.66 (7.78-17.48)
Atypical nevi					
0	462 (92)	657 (70)	—	—	—
1+	42 (8)	281 (30)	4.70 (3.33-6.64)	2.80 (1.91-4.10)	3.95 (2.75-5.66)
rs12203592_chr6					
CC	279 (57)	598 (65)	—	—	—
CT	185 (38)	279 (30)	0.70 (0.56-0.89)	0.66 (0.51-0.86)	0.67 (0.52-0.87)
TT	22 (5)	42 (5)	0.89 (0.52-1.52)	1.04 (0.57-1.91)	0.80 (0.44-1.44)
Linear trend			0.80 (0.66-0.96)	0.83 (0.67-1.03)	0.76 (0.61-0.94)
rs7023329_chr9					
GG	94 (20)	250 (29)	—	—	—
GA	274 (58)	441 (50)	0.61 (0.46-0.80)	0.65 (0.48-0.88)	0.58 (0.43-0.77)
AA	107 (23)	184 (21)	0.65 (0.46-0.90)	0.70 (0.48-1.01)	0.63 (0.45-0.90)
Linear trend			0.80 (0.68-0.95)	0.85 (0.70-1.02)	0.79 (0.67-0.94)
rs2284063_chr22					
GG	193 (40)	421 (47)	—	—	—
GA	226 (47)	396 (44)	0.80 (0.63-1.02)	0.86 (0.66-1.13)	0.82 (0.64-1.06)
AA	66 (14)	87 (10)	0.60 (0.42-0.87)	0.72 (0.48-1.09)	0.61 (0.42-0.89)
Linear trend			0.79 (0.67-0.93)	0.86 (0.71-1.04)	0.79 (0.67-0.94)
Total number of variants (T allele for rs12203592_chr6, and A allele for rs7023329_chr9 and rs2284063_chr22)					
0/1	122 (26)	303 (35)	—	—	—
2	154 (33)	306 (36)	0.80 (0.60-1.07)	0.81 (0.59-1.11)	0.75 (0.56-1.02)
3	133 (29)	186 (22)	0.56 (0.42-0.77)	0.67 (0.48-0.95)	0.55 (0.40-0.76)
4+	57 (12)	60 (7)	0.42 (0.28-0.64)	0.58 (0.36-0.92)	0.43 (0.28-0.67)
Linear trend			0.77 (0.69-0.86)	0.85 (0.75-0.95)	0.77 (0.69-0.86)

\*All risk factors listed in the table except for total number of variants were included in the multivariable model (multivariable OR1); age-sex-corrected log body nevus number and presence of atypical nevi were adjusted in OR1 for total number of variants.

†Adjusted for age, sex, hair color, freckling as child, general skin type without other nevus phenotype or genotype.

age of 20 years on the number of large and atypical nevi (Supplementary Table S2). SNPs were associated similarly with nevus number in continuously exposed and intermittently exposed sites (Supplementary Table S3).

The major interest in nevus genes is their effect on melanoma risk, and we therefore looked at the relationship between nevus phenotype and genotype (Table 3) and risk. In these analyses, we corrected for other variables in the table and also for age, sex, and sun sensitivity phenotypes such as hair color and freckling. The analysis confirmed the strong relationship between nevus number and melanoma risk, with a crude OR for melanoma of 10.02 (95% CI, 6.91-14.52) comparing the top quartile with the lowest quartile of nevus count. The relationship persisted when adjusted for the number of atypical nevi and genotype (OR, 7.47; 95% CI, 5.01-11.14) and sun sensitivity phenotypes (OR, 11.66; 95% CI, 7.78-17.48). Inheritance of the rarer allele at the SNPs on chromosomes 6, 9, and 22 was associated with a reduced risk of melanoma,

and these effects persisted when adjusted for other SNPs and the nevus phenotype [ORs of 0.83 (95% CI, 0.67-1.03), 0.85 (95% CI, 0.70-1.02), and 0.86 (95% CI, 0.71-1.04) per allele, respectively, for SNPs on chromosomes 6, 9, and 22, assuming an additive mode of inheritance].

As sun sensitivity is associated with an increased risk of melanoma, and inheritance of variants in the *MC1R* gene is the major determinant of sun sensitivity (23, 26), we investigated the relationship between the three nevus SNPs and melanoma risk stratified by the presence or absence of 1 or more *MC1R* variants, that is, variants known to be strongly associated with the sun-sensitive phenotype (Supplementary Table S4; ref. 27), but there was no evidence of a differential effect. In Table 4, we report the association between nevus number, number of atypical nevi, and inheritance of the nevus SNPs and melanoma risk at different body sites. It is seen that nevus number, number of atypical nevi, and inheritance of the chromosome 6 and 9 nevus SNPs were more strongly associated with risk of

**Table 4.** Association between nevus phenotype and genotype and melanoma risk by tumor site

Risk factor	Control	Trunk	OR (95% CI)	Limbs	OR (95% CI)	Head and neck	OR (95% CI)	Rare sites*	OR (95% CI) <sup>†</sup>	All sites	OR (95% CI)
Residual log body nevus number quartile <sup>‡</sup>											
Q1	126 (25)	12 (4)	—	23 (5)	—	18 (15)	—	2 (4)	—	55 (6)	—
Q2	126 (25)	41 (13)	3.47 (1.65-7.32)	51 (12)	2.59 (1.43-4.69)	25 (21)	1.72 (0.82-3.60)	10 (18)	—	127 (14)	2.64 (1.71-4.08)
Q3	126 (25)	68 (21)	5.48 (2.68-11.18)	92 (21)	4.90 (2.78-8.64)	31 (26)	2.14 (1.05-4.38)	14 (25)	3.31 (1.31-8.39)	205 (22)	4.32 (2.84-6.59)
Q4	126 (25)	202 (63)	17.58 (8.86-34.85)	274 (62)	13.32 (7.78-22.80)	45 (38)	3.61 (1.81-7.22)	30 (54)	6.42 (2.81-14.66)	551 (59)	11.66 (7.78-17.48)
Presence of atypical nevi <sup>‡</sup>											
0	462 (92)	198 (61)	—	316 (72)	—	100 (84)	—	43 (77)	—	657 (70)	—
1+	42 (8)	125 (39)	2.27 (1.44-3.59)	124 (28)	2.07 (1.31-3.27)	19 (16)	1.46 (0.73-2.94)	13 (23)	1.82 (0.73-4.55)	281 (30)	2.08 (1.41-3.06)
rs12203592_chr6 <sup>§</sup>											
CC	279 (57)	220 (70)	—	273 (63)	—	71 (60)	—	34 (63)	—	598 (65)	—
CT	185 (38)	78 (25)	0.54 (0.39-0.74)	141 (31)	0.78 (0.59-1.03)	41 (35)	0.87 (0.57-1.34)	19 (35)	0.84 (0.47-1.52)	279 (30)	0.70 (0.56-0.89)
TT	22 (5)	15 (5)	0.87 (0.44-1.71)	20 (5)	0.93 (0.50-1.74)	6 (5)	1.07 (0.42-2.74)	1 (2)	0.37 (0.05-2.86)	42 (5%)	0.89 (0.52-1.52)
Linear trend	—	—	0.68 (0.53-0.88)	—	0.85 (0.68-1.07)	—	0.94 (0.66-1.33)	—	0.77 (0.46-1.29)	—	0.80 (0.66-0.96)
rs7023329_chr9 <sup>§</sup>											
GG	94 (20)	90 (30)	—	115 (28)	—	29 (25)	—	16 (31)	—	250 (29)	—
GA	274 (58)	146 (49)	0.56 (0.39-0.79)	209 (51)	0.62 (0.45-0.86)	61 (54)	0.72 (0.44-1.19)	25 (49)	0.54 (0.27-1.05)	441 (50)	0.61 (0.46-0.80)
AA	107 (23)	61 (21)	0.60 (0.39-0.91)	89 (22)	0.68 (0.46-1.01)	24 (21)	0.73 (0.40-1.34)	10 (20)	0.55 (0.24-1.27)	184 (21)	0.65 (0.46-0.90)
Linear trend	—	—	0.76 (0.61-0.94)	—	0.82 (0.67-1.00)	—	0.85 (0.62-1.16)	—	0.71 (0.46-1.11)	—	0.80 (0.68-0.95)
rs2284063_chr22 <sup>§</sup>											
GG	193 (40)	148 (49)	—	194 (46)	—	56 (47)	—	23 (43)	—	421 (47)	—
GA	226 (47)	126 (41)	0.73 (0.54-0.99)	187 (44)	0.82 (0.62-1.09)	57 (48)	0.87 (0.57-1.32)	26 (48)	0.97 (0.53-1.75)	396 (44)	0.80 (0.63-1.02)
AA	66 (14)	31 (10)	0.61 (0.38-0.99)	45 (11)	0.68 (0.44-1.04)	6 (5)	0.31 (0.13-0.76)	5 (9)	0.64 (0.23-1.74)	87 (10)	0.60 (0.42-0.87)
Linear trend	—	—	0.76 (0.62-0.95)	—	0.82 (0.68-1.00)	—	0.69 (0.50-0.95)	—	0.85 (0.56-1.30)	—	0.79 (0.67-0.93)

\*Rare sites include acral, nodal with no known primary, anal, buttock, foot, subungual, and vulval.

<sup>†</sup>Quartile 1 and quartile 2 were grouped together in the comparison of residual log body nevus number for rare tumor sites.

<sup>‡</sup>OR adjusted for age, sex, hair color, freckling as child, general skin type, and (for atypical nevi only) residual log body nevus number.

<sup>§</sup>Crude ORs were reported.

**Table 5.** Association between nevus phenotype and genotype and melanoma risk by age subgroup

Risk factor	Age, <50 y				Age, ≥50 y			
	Control	Case	OR (95% CI)	<i>P</i> <sub>trend</sub>	Control	Case	OR (95% CI)	<i>P</i> <sub>trend</sub>
Residual log body nevus number quartile*								
Q1	45 (31%)	26 (7%)	—	—	81 (23%)	29 (5%)	—	—
Q2	30 (21%)	55 (14%)	3.08 (1.51-6.28)	—	96 (27%)	72 (13%)	2.59 (1.46-4.59)	—
Q3	34 (24%)	91 (23%)	4.48 (2.28-8.79)	—	92 (26%)	114 (21%)	4.44 (2.54-7.74)	—
Q4	35 (24%)	224 (57%)	10.27 (5.38-19.62)	<0.0001	91 (25%)	327 (60%)	13.34 (7.76-22.94)	<0.0001
Presence of atypical nevi*								
No	125 (87%)	250 (63%)	—	—	337 (94%)	407 (75%)	—	—
Yes	19 (13%)	146 (37%)	1.64 (0.90-3.01)	0.11	23 (6%)	135 (25%)	2.33 (1.39-3.91)	0.001
rs12203592_chr6 <sup>†</sup>								
CC	76 (55%)	250 (65%)	—	—	203 (58%)	348 (65%)	—	—
CT	55 (40%)	116 (30%)	0.64 (0.43-0.97)	—	130 (37%)	163 (30%)	0.73 (0.55-0.98)	—
TT	6 (4%)	18 (5%)	0.91 (0.35-2.38)	0.11	16 (5%)	24 (4%)	0.88 (0.45-1.69)	0.08
rs7023329_chr9 <sup>†</sup>								
GG	30 (22%)	105 (29%)	—	—	64 (19%)	145 (29%)	—	—
GA	73 (54%)	193 (52%)	0.76 (0.46-1.23)	—	201 (59%)	248 (49%)	0.55 (0.39-0.77)	—
AA	33 (24%)	70 (19%)	0.61 (0.34-1.08)	0.09	74 (22%)	114 (22%)	0.68 (0.45-1.03)	0.06
rs2284063_chr22 <sup>†</sup>								
GG	50 (36%)	182 (48%)	—	—	143 (41%)	239 (46%)	—	—
GA	64 (46%)	164 (43%)	0.70 (0.46-1.08)	—	162 (47%)	232 (44%)	0.86 (0.64-1.14)	—
AA	24 (17%)	35 (9%)	0.40 (0.22-0.74)	0.003	42 (12%)	52 (10%)	0.74 (0.47-1.17)	0.15

NOTE: There was no statistically significant difference in melanoma risk between age groups for the nevus genotypes ( $P = 0.76$ ,  $0.75$ , and  $0.12$  for SNPs on chromosomes 6, 9, and 22, respectively).

\*OR adjusted for age, sex, hair color, freckling as child, general skin type, and (for atypical nevi only) residual log body nevus number.

<sup>†</sup>Crude ORs were reported.

truncal melanoma than melanoma at other common sites. Nevus number was however also strongly associated with risk of melanoma on the limbs and, notably, also with melanoma at other sites, including rare sites (pooled acral lentiginous melanoma, genital melanoma, and melanoma arising in the ear nose and throat).

Finally, in Table 5, we report the effect of nevus number and inheritance of nevus SNPs on melanoma arising at different ages because nevus phenotype changes with age. The risk associated with increased nevus number was similar under the age of 50 years and in older individuals. The risk associated with atypical nevi seemed greater in cases with ages over 50 ( $P = 0.001$ ) than in those under the age of 50 years ( $P = 0.11$ ), but the difference was not statistically significant. Similarly, the risk associated with inheritance of the nevus SNP on chromosome 22 seemed to differ by age, so that the A allele was more strongly protective for those under the age of 50 years ( $P = 0.003$ ) than those with ages over 50 years ( $P = 0.15$ ), but the difference was not statistically significant. The risk associated with the chromosome 6 and 9 SNPs was similar in the two age groups.

We estimated the proportion of the variation in melanoma risk explained by nevus genotype, nevus phenotype, and pigmentation phenotype (Supplementary

Table S5). The nevus SNPs, considered independently, each explained <1% of the variation, and by combining all SNPs, only 2% was explained. The nevus phenotype however explained 19% and pigmentation phenotypes explained 4% of the variation. The highest proportion of risk explained was 23%, when the nevus SNPs, and nevus and pigmentation phenotypes were combined.

## Discussion

As melanoma continues to increase in incidence in many parts of the world, it remains crucial to identify risk factors for melanoma and to understand the effects of both environmental exposures and susceptibility genes on risk. We report here on the effect of nevus-related SNPs associated with melanoma risk in a large case-control sample recruited in the United Kingdom. In this area of the world, the number of melanoma cases has increased (28), so that it has become the commonest cancer in young British adults (Office of National Statistics Data).

Pooled data analyses of case-control studies have confirmed that the major environmental exposure associated with melanoma risk is holiday sun exposure (7), particularly in temperate climates, and that the strongest phenotypic risk factor is the presence of increased number of nevi

(9). Previous twin studies in adolescents have suggested that holiday sun exposure is predictive of an increased number of nevi (17), and several studies in healthy individuals have shown a relationship between sun exposure and nevus number (16, 29-31). In this study, we have provided further evidence that holiday sun exposure, rather than total (daily) sun exposure, is the major determinant of nevus number in adults, and that holiday sun exposure is also correlated with larger nevi (indicative of more proliferative melanocytes) and (to a lesser extent) clinically atypical nevi. The data reported here do support the view that behavior in the sun is associated with melanocyte proliferation, at least in those with a susceptible phenotype.

It is of interest that we found some weak evidence that nevus number on continuously sun-exposed sites such as the arms was lower ( $P = 0.05$ ) in those with higher levels of weekend sun exposure in warmer months in this temperate climate. This is consistent with our recent observation that higher levels of weekend sun exposure were protective for melanoma in the United Kingdom.<sup>5</sup> That nevus number seemed to be negatively correlated with greater weekend sun exposure in a temperate climate might suggest that although sunny holidays and sunburn increased nevus number, regular (moderate) sun exposure may also be in some way related to the inhibition of melanocyte proliferation. We suggest that this is consistent with the observation that larger, clinically atypical nevi are most numerous on intermittently exposed skin, such as the back (30), although the number of small nevi on the arms may be higher (31). Thus, the data support the view that the relationship between sun exposure and melanoma risk is complex.

In previous case-control (32) studies, increased nevus number and actinic keratoses (as a marker of chronic sun exposure in the fair skinned) were both reported to be predictive of melanoma risk, but the phenotypic markers were negatively correlated. That is, that increased number of nevi and actinic keratoses were both risk factors but they occurred in different people, indicating the probability of two different "routes" to melanoma. Other observations added to the suggestion that there was more than one route to melanoma (33), and this has been substantiated more recently by the identification of biological differences between tumors that have developed on intermittently sun-exposed body sites and those on continuously exposed sites (34). In this study, we have shown that increased nevus number is related to holiday sun exposure, and although nevus number was predictive of melanoma risk overall, the strongest relationship was with melanoma on the trunk (Table 4), which is the most intermittently sun-exposed body site. Our data are therefore consistent with this "two-route hypothesis," and indeed with other studies including a pooled data analysis of case-control study data in women, recently reported (35, 36), which was carried out to test the hypothesis. However, it is of note that in our study, increased nevus number was associated with melanoma risk

for tumors at all body sites, suggesting that patients with the atypical mole syndrome are at increased risk of melanoma even in rare sites such as acral lentiginous melanoma, and that the relationship between tumor site and different biological pathways to melanoma is likely complex.

We have confirmed that SNPs in *IRF4* on chromosome 6 and additional SNPs on chromosomes 9 and 22 influence the nevus phenotype and are associated with melanoma risk. Of these SNPs, those on chromosomes 9 and 22 were most strongly predictive of nevus number. The relationship between the *IRF4* SNP and nevus number is reported to be complex and dependent on age,<sup>4</sup> which may explain the lack of association with total nevus count in these data.

We showed that the proportion of melanoma risk explained by these SNP genotypes is small compared with what is explained by nevus phenotype. The evidence is strong from twin studies that nevus number is primarily under genetic control (13, 15, 16); the implication is therefore that there are significant numbers of nevus (hypothesized to be melanoma susceptibility) genes yet to be identified.

In summary, these data confirm that increased nevus number and bigger nevi (indicative of a more highly proliferative melanocyte population) are associated with increased melanoma risk. That association is particularly strong for melanoma on the trunk and limbs, but is detected for all body sites, even in non-sun-exposed skin. Thus, although our data support the "two-route hypothesis to melanoma," they also support the view that melanomas related to the inheritance of nevus genes occur all over the skin. The three SNPs on chromosomes 6, 9, and 22 are related both to nevus number and to melanoma risk, but a large proportion of the nevus phenotype remains unexplained. Further exploration of the genetic determinants of nevi using both genome-wide association and candidate gene studies will be needed to better understand the hereditary variation that determines melanocyte responses to sun exposure and therefore melanoma risk.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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<sup>4</sup> Duffy et al. In press.

<sup>5</sup> Newton-Bishop et al. Submitted for publication.

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