to allow independent verification. Second, comparing crude rates with age-standardized rates is invalid. This is because melanoma rates have been increasing in older people but decreasing in younger people during a period in which more Australians are surviving to old age. This means that crude melanoma rates calculated for the year 1982 relate to an entirely different population structure versus those calculated for 2011. Without properly accounting for these changes, the differences between crude and standardized rates are uninterpretable, and no valid comparisons can be made.

We considered the issue of population dilution in our article, and we referenced Dr. Czarnecki's original article positing his hypothesis (Czarnecki, 2014). We also referenced the subsequent paper by Baade et al. (2015) that elegantly disproved it. In their article, Baade et al. modeled melanoma incidence in Australia under the full range of hypothetical scenarios that might explain Australia's population growth between 1982 and 2011—that is, from being 100% attributable to migration to 0% attributable to migration. Regardless of the assumed level of migration, the decline in age-standardized melanoma incidence in Australia was apparent across all scenarios, from which the authors concluded that there is "strong evidence against the hypothesis that the observed decrease in melanoma incidence among young Australians since the mid-1990s can be explained solely by the increasing overseas migration and any resultant lowering of the 'at risk' population in Australia." We agree with their conclusion.

In summary, we agree that population dilution is of interest and may explain some of the decline in the Australian melanoma incidence rates, but we disagree with the assertion that melanoma incidence is rising in young susceptible Australians. As argued by others (Baade et al., 2015), the timing of the changes in melanoma incidence, coupled with the divergent trends among younger and older Australians, are consistent with birth cohort and period effects that are best explained by primary prevention campaigns that commenced nationally in the 1980s.

ORCIDs

David C. Whiteman: http://orcid.org/0000-0003-2563-9559
Adèle C. Green: http://orcid.org/0000-0002-2753-4841

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***David C. Whiteman*[1,2,*], *Adèle C. Green*[1,2,3] *and Catherine M. Olsen*[1,2]**

[1]*QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, QLD 4006, Australia;* [2]*The University of Queensland, School of Public Health, Herston Road, Herston, QLD 4006, Australia; and* [3]*Cancer Research UK Manchester Institute and Institute of Inflammation and Repair, University of Manchester, Manchester, UK*
[*]*Corresponding author e-mail: david.whiteman@qimrberghofer.edu.au*

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Association of Melanocortin-1 Receptor Variants with Pigmentary Traits in Humans: A Pooled Analysis from the M-Skip Project

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TO THE EDITOR

Skin pigmentation is due to the accumulation of eumelanin, which is brown-black pigment and photoprotective, and pheomelanin, which is yellow-red pigment and may promote carcinogenesis (Valverde et al., 1995). The melanocortin-1 receptor (*MC1R*) gene regulates the amount and type of pigment production and is a major determinant of skin phototype (Garcia-Borron et al., 2005; Valverde et al., 1995). Binding of α-melanocyte stimulating hormone to MC1R stimulates the enzymatic activity of adenylate cyclase enzyme, thereby elevating intracellular cyclic adenosine monophosphate (cAMP) levels. *MC1R* is highly polymorphic, especially in Caucasians: more than 200 coding region variants have been described to date (Garcia-Borron et al., 2014; Gerstenblith et al., 2007; Perez Oliva et al., 2009). Six variants—D84E, R142H, R151C, I155T, R160W, and D294H—have been designated as "R" alleles because of their strong association with the "red hair color" phenotype characterized by red hair, fair skin, freckles, and sun sensitivity. The V60L, V92M, and R163Q variants are found to have a weaker association with the red hair color phenotype and have been designated as "r" alleles (Garcia-Borron et al., 2014; Raimondi et al., 2008).

Previous studies demonstrated that several alleles are associated with phenotypic characteristics and that *MC1R* variants are associated with both

Abbreviations: cAMP, cyclic adenosine monophosphate; MC1R, melanocortin-1 receptor; SOR, summary odds ratio; WT, wild-type

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Table 1. Summary odds ratios for the association between combined *MC1R* variants and phenotypic characteristics

Phenotypic characteristic	MC1R	Studies/control	SOR (95% CI)	I^2 (%)[3]	*P*-value[3]
Hair color—fair versus dark[1]	Wild-type	13/1,371	1.00 (reference)		
	Any variant	13/2,758	**1.91 (1.38–2.65)**	**59**	**<0.01**
	1 variant	13/1,991	**1.55 (1.12–2.15)**	39	0.07
	2+ variants	13/767	**3.32 (2.34–4.72)**	**62**	**<0.01**
Hair color—red versus others	Wild-type	7/705	1.00 (reference)		
	Any variant	7/1,474	**3.54 (1.91–6.55)**	0	0.80
	1 variant	7/1,016	1.18 (0.57–2.44)	0	0.83
	2+ variants	7/458	**10.17 (5.28–19.58)**	0	0.77
Eye color—fair versus dark[2]	Wild-type	14/1,530	1.00 (reference)		
	Any variant	14/2,832	1.12 (0.96–1.30)	12	0.33
	1 variant	14/2,079	1.11 (0.94–1.32)	10	0.35
	2+ variants	14/753	1.16 (0.93–1.45)	0	0.80
Skin type—I, II versus III, IV	Wild-type	14/1,540	1.00 (reference)		
	Any variant	14/3,046	**2.26 (1.81–2.83)**	**49**	**0.02**
	1 variant	14/2,211	**1.95 (1.51–2.53)**	41	0.06
	2+ variants	14/8,35	**3.58 (2.68–4.78)**	**42**	**0.05**
Freckles—yes versus no	Wild-type	9/1,067	1.00 (reference)		
	Any variant	9/2,257	**2.52 (1.99–3.20)**	33	0.16
	1 variant	9/1,528	**2.00 (1.52–2.64)**	36	0.13
	2+ variants	9/729	**4.47 (3.25–6.15)**	38	0.12

Significant ORs and *P*-values are in bold.

Abbreviations: CI, confidence intervals; MC1R, melanocortin-1 receptor; OR, odds ratio; SOR, summary odds ratio.

[1]Fair hair colors were red, blond, dark blonde, light brown. Dark hair colors were brown, black, dark brown.

[2]Fair eye colors were blue, green, gray, hazel. Dark eye colors were brown, black.

[3]I^2 and Q test *P*-value are measures of between-study heterogeneity (see Supplementary Methods online).

melanoma and nonmelanoma skin cancer (Han et al., 2006; Pasquali et al., 2015; Scherer et al., 2008; Tagliabue et al., 2015) with a stronger role for darker-pigmented populations, suggesting that nonpigmentary pathways link *MC1R* with skin cancer development. Because the role and strength of each *MC1R* variant in determining specific phenotypic characteristics and the red hair color phenotype remains unclear, we performed a pooled analysis of individual-level data from the M-SKIP project, described in full elsewhere (Raimondi et al., 2012). We selected from the M-SKIP database all 5,366 cancer-free controls with *MC1R* gene sequenced and information on at least one of the following phenotypic characteristics: hair color, eye color, skin type, and freckles, thus including 16 independent studies from 18 publications (Supplementary Table S1 online).

We found greater summary odds ratios (SORs) for carriers of two *MC1R* variants compared with carriers of only one variant allele (Table 1). Furthermore carriage of any *MC1R* variant, one variant and two or more variants, compared with not having such variants (i.e., wild-type [WT] subjects), was significantly associated with fair hair color, skin type I/II, and presence of freckles. Red hair color was significantly associated with carrying any *MC1R* variant (SOR; 95% confidence interval: 3.54; 1.91–6.55) and with carrying two or more variants (SOR; 95% confidence interval: 10.17; 5.28–19.58), but not with carrying one *MC1R* variant (SOR; 95% confidence interval: 1.18; 0.57–2.44). No significant association was observed for light eye color and *MC1R*. Sensitivity analyses indicated that the observed between-study heterogeneity may be attributable to single studies: when we excluded the studies that were outliers, we obtained similar pooled odds ratios as the original ones, but no longer with evidence of heterogeneity (results not shown). No evidence of publication bias was found by Egger's test. All the investigated *MC1R* variants compared with WT subjects were positively associated with skin type I/II and freckles (Supplementary Table S2 online). The three variants that seemed to play the most important role in skin type determination and the presence of freckles were D84E, R151C, and D294H. Red hair color was significantly associated with all *MC1R* variants except for V92M and R163Q.

We visualized the associations between hair color, eye color, skin type, freckles, and the three main studied geographical areas by multiple correspondence analysis (Supplementary Figure S1a or b online). A two-dimensional multiple correspondence analysis solution, with dimension 1 on the horizontal axis and dimension 2 on the vertical axis, was considered the most adequate because the first and second dimension presented Benzecri-adjusted inertias of 85.31% and 11.31%, respectively (Supplementary Table S3 online), accounting for 96.62% of the total association. The extreme red hair color phenotype (red hair, skin type I, and freckles) was associated either with carrying at least two *MC1R* variants (Supplementary Figure S1a) or with the presence of major penetrant ("R") alleles (Supplementary Figure S1b). We suggest that dimension 1 can be interpreted as a "pigmentation score" because it differentiates well between dark and fair phenotypic characteristics. The median pigmentation score increased with increasing number of *MC1R* variants, and for single *MC1R* variants it was higher ($P < 0.0001$) compared with WT subjects (Supplementary Figure S2 online).

Seven of the nine *MC1R* variants analyzed in this study, V60L, D84E, R142H, R151C, I155T, R160W, and D294H, are clearly hypomorphic with significant reduction in cAMP signaling potential (Beaumont et al., 2007; Herraiz et al., 2012; Kadekaro et al., 2010; Scott et al., 2002). Within this group of variants, the lowest SOR for red hair, skin type I/II, or freckles corresponds to V60L. Interestingly, this variant was also the one with the smallest functional impairment in terms of coupling to the cAMP pathway, when the seven variants analyzed here were compared under identical experimental conditions (Herraiz et al., 2012).

Results also showed that V92M and R163Q behave as "r" alleles, with a weak albeit significant association with cutaneous phenotypic traits. In heterologous systems, V92M has been reported to display either a slight functional impairment (Herraiz et al., 2012) or normal coupling to the cAMP pathway (Beaumont et al., 2007), whereas R163Q apparently signals as efficiently as WT. Therefore, it appears that the ability of V92M or R163Q to activate the cAMP pathway is similar, if not identical to WT. This suggests that other mechanisms account for their association with cutaneous phenotypic characteristics, for example, V92M or R163Q might impair functional coupling to signaling module(s) different from the cAMP cascade. MC1R promiscuously binds to a variety of intracellular partners with signaling potential and this ability might depend on WT conformation. However, little is known as to the effects of other variants on MC1R binding to its various protein partners, and the phenotypic consequences of such molecular interactions also remain largely unknown. Further research is needed to understand the scaffolding properties of MC1R, the functional consequences of the formation of signaling complexes orchestrated by the receptor, and the effects on these processes of the myriad of natural variants in the *MC1R* gene.

ORCID

Leah Ferrucci: http://orcid.org/0000-0001-9488-7586

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The M-SKIP study group consists of the following members: Principal Investigator (PI): Sara Raimondi (European Institute of Oncology, Milan, Italy); Advisory Committee members: Philippe Autier (International Prevention Research Institute, Lyon, France), Maria Concetta Fargnoli (University of L'Aquila, Italy), José C. García-Borrón (University of Murcia, Spain), Jiali Han (Brigham and Women's Hospital and Harvard Medical School, Boston, MA), Peter A. Kanetsky (Department of Cancer Epidemiology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), Maria Teresa Landi (National Cancer Institute, NIH, Bethesda, MD), Julian Little (University of Ottawa, Canada), Julia Newton-Bishop (University of Leeds, UK), Francesco Sera (UCL Institute of Child Health, London, UK); Consultants: Saverio Caini (ISPO, Florence, Italy), Sara Gandini and Patrick Maisonneuve (European Institute of Oncology, Milan, Italy); Participant Investigators: Albert Hofman, Manfred Kayser, Fan Liu, Tamar Nijsten, and Andre G. Uitterlinden (Erasmus MC University Medical Center, Rotterdam, The Netherlands), Rajiv Kumar and Dominique Scherer (German Cancer Research Center, Heidelberg, Germany), Tim Bishop, Julia Newton-Bishop, and Faye Elliott (University of Leeds, UK), Eduardo Nagore (Instituto Valenciano de Oncologia, Valencia, Spain), DeAnn Lazovich (Division of Epidemiology and Community Health, University of Minnesota, MN), David Polsky (New York University School of Medicine, New York, NY), Johan Hansson and Veronica Hoiom (Karolinska Institutet, Stockholm, Sweden), Paola Ghiorzo and Lorenza Pastorino (University of Genoa, Italy), Nelleke A. Gruis and Jan Nico Bouwes Bavinck (Leiden University Medical Center, The Netherlands), Paula Aguilera, Celia Badenas, Cristina Carrera, Pol Gimenez-Xavier, Josep Malvehy, Miriam Potrony, Susana Puig, Joan Anton Puig-Butille, Gemma Tell-Marti (Hospital Clinic, IDIBAPS and CIBERER, Barcelona, Spain), Terence Dwyer (Murdoch Childrens Research Institute, Victoria, Australia), Leigh Blizzard and Jennifer Cochrane (Menzies Institute for Medical Research, Hobart, Australia), Ricardo Fernandez-de-Misa (Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Spain), Wojciech Branicki (Institute of Forensic Research, Krakow, Poland), Tadeusz Debniak (Pomeranian Medical University, Polabska, Poland), Niels Morling and Peter Johansen (University of Copenhagen, Denmark), Susan Mayne, Allen Bale, Brenda Cartmel and Leah Ferrucci (Yale School of Public Health and Medicine, New Haven, CT), Ruth Pfeiffer (National Cancer Institute, NIH, Bethesda, MD), Giuseppe Palmieri (Istituto di Chimica Biomolecolare, CNR, Sassari, Italy), Gloria Ribas (Fundación Investigación Clínico de Valencia Instituto de Investigación Sanitaria- INCLIVA, Spain), Chiara Menin (Veneto Institute of Oncology, IOV-IRCCS, Padua, Italy), Alexander Stratigos and Katerina Kypreou (University of Athens, Andreas Sygros Hospital, Athens, Greece), Anne Bowcock, Lynn Cornelius, and M. 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Study Centers: The University of Sydney and The Cancer Council New South Wales, Sydney (Australia): Bruce Armstrong (PI), Anne Kricker (co-PI), Melisa Litchfield (Study Coordinator). Menzies Institute for Medical Research, University of Tasmania, Hobart (Australia): Terence Dwyer (PI), Paul Tucker (Dermatopathologist), Nicola Stephens (Study Coordinator). British Columbia Cancer Agency, Vancouver (Canada): Richard Gallagher (PI), Teresa Switzer (Coordinator). Cancer Care Ontario, Toronto (Canada): Loraine Marrett (PI), Beth Theis (Co-Investigator), Lynn From (Dermatopathologist), Noori Chowdhury (Coordinator), Louise Vanasse (Coordinator), Mark Purdue (Research Officer). David Northrup (Manager for CATI). Centro per la Prevenzione Oncologia Torino, Piemonte (Italy): Roberto Zanetti (PI), Stefano Rosso (Data Manager), Carlotta Sacerdote (Coordinator). University of California, Irvine, CA: Hoda Anton-Culver (PI), Nancy Leighton (Coordinator), Maureen Gildea (Data Manager). University of Michigan, Ann Arbor, MI: Stephen Gruber (PI), Joe Bonner (Data Manager), Joanne Jeter (Coordinator). New Jersey Department of Health and Senior Services, Trenton, NJ: Judith Klotz (PI), Homer Wilcox (Co-PI), Helen Weiss (Coordinator). University of North Carolina, Chapel Hill, NC: Robert Millikan (PI), Nancy Thomas (Co-Investigator), Dianne Mattingly (Coordinator), Jon Player (Laboratory Technician), Chiu-Kit Tse (Data Analyst). University of Pennsylvania, Philadelphia, PA: Timothy Rebbeck (PI), Peter Kanetsky (Co-Investigator), Amy Walker (Laboratory Technician), Saarene Panossian (Laboratory Technician). Consultants: Harvey Mohrenweiser, University of California, Irvine, Irvine, CA; Richard Setlow, Brookhaven National Laboratory, Upton, NY.

Elena Tagliabue[1], Sara Gandini[1], José C. García-Borrón[2], Patrick Maisonneuve[1], Julia Newton-Bishop[3], David Polsky[4], DeAnn Lazovich[5], Rajiv Kumar[6], Paola Ghiorzo[7,8], Leah Ferrucci[9], Nelleke A. Gruis[10], Susana Puig[11], Peter A. Kanetsky[12], Tomonori Motokawa[13], Gloria Ribas[14], Maria Teresa Landi[15], Maria Concetta Fargnoli[16], Terence H. Wong[17], Alexander Stratigos[18], Per Helsing[19], Gabriella Guida[20], Philippe Autier[21], Jiali Han[22], Julian Little[23], Francesco Sera[24] and Sara Raimondi[1,*], for the M-SKIP Study group

[1]Division of Epidemiology and Biostatistics, European Institute of Oncology, Milan, Italy; [2]Department of Biochemistry, Molecular Biology and Immunology, University of Murcia and IMIB-Arrixaca, Murcia, Spain; [3]Section of Epidemiology and Biostatistics, Institute of Cancer and Pathology, University of Leeds, Leeds, UK; [4]The Ronald O. Perelman Department of Dermatology, New York

University School of Medicine, NYU Langone Medical Center, New York, New York, USA; [5]Division of Epidemiology and Community Health, University of Minnesota, Minnesota, USA; [6]Division of Molecular Genetic Epidemiology, German Cancer Research Center, Heidelberg, Germany; [7]Department of Internal Medicine and Medical Specialties, University of Genoa, Italy; [8]IRCCS AOU San Martino-IST, Genoa, Italy; [9]Department of Chronic Disease Epidemiology, Yale School of Public Health, Yale Cancer Center, New Haven, Connecticut, USA; [10]Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands; [11]Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona, University of Barcelona, CIBER de Enfermedades Raras, Spain; [12]Department of Cancer Epidemiology, H. Lee Moffitt Cancer Center and Research Institute2, Tampa, Florida, USA; [13]Skin Research Department, POLA Chemical Industries, Yokohama, Japan; [14]Department of medical oncology and hematology, Fundación Investigación Clínico de Valencia Instituto de Investigación Sanitaria- INCLIVA, Valencia, Spain; [15]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland, USA; [16]Department of Dermatology, University of L'Aquila, L'Aquila, Italy; [17]NHS Forth Valley, UK; [18]First Department of Dermatology, Andreas Sygros Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [19]Department of Pathology, Oslo University Hospital, Oslo, Norway; [20]Department of Basic Medical Sciences, Neuroscience and Sense Organs, University of Bari, Bari, Italy; [21]International Prevention Research Institute, Lyon, France; [22]Department of Epidemiology, Richard M. Fairbanks School of Public Health, Melvin & Bren Simon Cancer Center, Indiana University, Indianapolis, Indiana, USA; [23]School of Epidemiology, Public Health and Preventive Medicine, University of Ottawa, Ottawa, Canada; and [24]Department of Social and Environmental Health Research, London School of Hygiene & Tropical Medicine, London, UK

[*]Corresponding author e-mail: sara.raimondi@ieo.it

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.2016.05.099.

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Low Levels of Genetic Heterogeneity in Matched Lymph Node Metastases from Patients with Melanoma

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TO THE EDITOR

In our previous experience, a high consistency of *BRAF* and *NRAS* mutation patterns was observed between primary tumors and lymph node metastases in patients with advanced melanoma (Colombino et al., 2012). Conversely, increasing rates of discrepancies in *BRAF/NRAS* mutation patterns were found between primary melanomas and metastases in other sites (brain or, mostly, skin) (Colombino et al., 2012). When the distribution of *BRAF/NRAS* mutations was evaluated in a larger cohort, the high rate of consistency in sequence variations of these two genes was further confirmed between primary melanomas and lymph node metastases (142/156; 91%) (Colombino et al., 2013; unpublished data). However, intraindividual heterogeneity of *BRAF* mutations has been

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