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TESI DI DOTTORATO DI RICERCA

MELANOCORTIN-1 RECEPTOR VARIANTS IN SKIN CARCINOGENESIS: A POOLED-ANALYSIS

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Dottoranda: **Sara Raimondi**

Tutor: Dr. **Claudia Specchia**

Coordinatore del Dottorato: Ch.mo Prof. **Adriano Decarli**

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Chapter 1

Melanoma and *MC1R* gene

Starting from the ó60s, a sharp increase in the incidence rate of cutaneous malignant melanoma has been observed in Caucasian people worldwide, both in men and women and in all age groups. Despite a recent flattening of the trend, cutaneous malignant melanoma has come to be one of the most frequent cancers in fair-skinned populations: as an example, it ranks fourth and third respectively in men and women in Australia [Lens et al. 2004], where the incidence rate values are among the highest in the world. During the same period, the trend in mortality rates followed, albeit in a less dramatic fashion, that of incidence rates: an initial increase in mortality rates involving, although unevenly, both sexes and most age groups, has been followed since the mid '90s by a slowdown or even a reversal of the trend [Lens et al. 2004; Karim-Kos et al. 2008]. The early detection owing to screening may be a reason, but does not appear as the only explanation for this fact [de Vries et al. 2003]. Worldwide, most elevated mortality rates are observed in Australia, Northern Europe and South Africa, and, although there is a wide geographic variability, both incidence and mortality rates for cutaneous malignant melanoma are everywhere higher in males than in females [Lens et al. 2004]. Among all the skin cancers, melanoma certainly represents the most lethal form and metastatic melanoma has a five-year survival rate of only 11% [Thompson et al. 2005].

As for all cancers, the occurrence of cutaneous malignant melanoma is the result of the interaction between host and environmental factors. Known risk factors include environmental UV radiation exposure, fair skin, family history of melanoma, high number of melanocytic naevi, light eye and hair pigmentation [Gandini S et al. 2005a; Gandini S et al. 2005b; Gandini S et al. 2005c].

Melanocortin-1-receptor gene (*MC1R*, MIM#155555) is responsible for constitutive pigment variation in humans and has been shown to be a risk factor for melanoma. It is located on chromosome 16q24.3 and encodes for a seven pass transmembrane G-protein coupled-receptor of 317 amino acids, which has a high affinity for Ŭ-melanocyte-stimulating hormone (Ŭ-MSH) and adrenocorticotropin [Mountjoy et al. 1992; Busca et al. 2000]. The binding of Ŭ-MSH to the functional MC1R on melanocytes stimulates the synthesis of eumelanin [Burchill et al. 1993], which determines black/brown pigment. *MC1R* therefore contributes to determine pigmentation by regulating the relative proportion of eumelanin and phaeomelanin (red/yellow pigment). Eumelanin has been shown to reduce the accumulation of DNA photoproducts, while phaeomelanin may contribute to cancer risk because it generates free radicals following UV exposure [Ranadive et al. 1986; Kollias et al. 1991; Rouzaud et al. 2005]. *MC1R* is highly polymorphic in the Caucasian population: more than 80 variants have been recently described [Gerstenblith et al. 2007]. Some of these variants result in partial loss of the receptorôs signaling ability, since they are unable to stimulate cyclic adenosine monophosphate (cAMP) production as strongly as the wild-type receptor in response to Ŭ-MSH stimulation [Schioth et al. 1999; Beaumont et al. 2007]. It results in a quantitative shift of melanine synthesis from eumelanin to phaeomelanin, which is associated with the ñred hair colorò (RHC) phenotype, characterized by fair pigmentation (fair skin, red hair, and freckles), and by sun sensitivity (poor tanning response and solar lentigines).

Genetic association studies have found that the *MC1R* variants D84E, R151C, R160W, and D294H, defined as ó*R*ô alleles [Gerstenblith et al. 2007; Beaumont et al. 2007], were strongly associated with the RHC phenotype [Box et al. 1997; Smith et al. 1998; Palmer et al. 2000; Flanagan et al. 2000; Bastiaens et al. 2001a; Kennedy et al. 2001; Branicki et al. 2007]. Other two less frequent variants (R142H and I155T) have also been classified as *R* alleles [Beaumont et al. 2007] basing on findings of strong familial association with RHC phenotype [Flanagan et al. 2000; Duffy et al. 2004]. The V60L, V92M, and R163Q variants seem to have a relatively weak association with RHC phenotype

and are designated as ór ô alleles [Duffy et al. 2004; Beaumont et al. 2007]. Several studies in different populations have reported that the risk of melanoma is higher among individuals who carry *MC1R* variant alleles than among individuals who are wild type for *MC1R* [Valverde et al. 1996; Ichii-Jones et al. 1998; Palmer et al. 2000; Kennedy et al. 2001; van der Velden et al. 2001; Matichard et al. 2004; Landi et al. 2005; Stratigos et al. 2006; Fernandez et al. 2007]. Melanoma risk attributable to *MC1R* may arise through the determination of the tanning response of skin to UV light, which can then either ameliorate or exacerbate the genotoxic effects of sunlight [Bliss et al. 1995]. However, the relationship between some *MC1R* variants and melanoma also in darkly-pigmented Caucasian populations suggests that *MC1R* signaling pathway may have an additional role in skin carcinogenesis beyond the UV-filtering differences between dark and fair skin [Gerstenblith et al. 2007].

1.1. Previous meta-analysis on *MC1R* variants and melanoma

We previously investigated the association of the nine main variants in *MC1R* gene (V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, and D294H) with melanoma and RHC phenotype by a meta-analysis of 20 published studies [Raimondi S et al. 2008]. Eleven studies on *MC1R* and melanoma, and 9 on *MC1R* and phenotype were included in the analysis. The 7 variants D84E, R142H, R151C, I155T, R160W, R163Q, and D294H were significantly associated with melanoma development, with Odds Ratios (OR) (95% Confidence Intervals-CI-) ranging from 1.42 (1.09-1.85) for R163Q to 2.45 (1.32-4.55) for I155T. The *MC1R* variants R160W and D294H were associated both with red hair and fair skin, while D84E, R142H, and R151C were strongly associated with red hair only- ORs (95%CI) ranged from 2.99 (1.51-5.91) for D84E to 8.10 (5.82-11.28) for R151C. The association of D84E, R142H, R151C, R160W, and D294H with melanoma could be at least partly explained by pigmentary pathways: red hair and fair skin individuals are

unable to increase melanin levels in the skin in response to high exposure to UV light and therefore increase the levels of phaeomelanin, which is mutagenic and cytotoxic [Rouzaud et al. 2005]. The two variants I155T and R163Q were found to be positively associated with melanoma risk but not with red hair nor with fair skin. These results suggest that, for these variants, melanoma risk could be mainly increased via non-pigmentary pathways. It is well documented that Ŭ-MSH has immunomodulatory and anti-inflammatory functions [Luger et al. 2003; Rogers et al. 2006], therefore the association between *MC1R* and skin cancer could be a result of inflammatory or immune mechanisms influencing tumorigenesis. Modulation of melanocyte growth, development and differentiation, and increased DNA damage possibly associated with production of reactive oxygen species are other hypothesized mechanisms contributing to *MC1R* carcinogenesis [Suzuki et al. 1996; Rouzaud et al. 20057. No association with melanoma or phenotype was found for V60L and V92M variants.

Although the results of our previous meta-analysis were informative, firm conclusions on the specific contribution of each *MC1R* variant in melanoma development due to pigmentary and non-pigmentary pathways could not be reached using only published data. The main limitations were the lack of genotyping information in some allele-based studies that were therefore excluded from the analysis; and the lack of presentation of the number of individuals who carried no *MC1R* variant, which did not allow us to compare subjects carrying each *MC1R* variant with subjects carrying no variant. Furthermore, using only published data, we could not take into account possible confounding factors and interaction of *MC1R* with other genes and environmental exposures, and we could not study the independent effect of *MC1R* variants on melanoma stratifying by RHC phenotype.

1.2. Pooled-analysis of genetic epidemiology data

In order to overcome the problems above described, I decided to perform a pooled-analysis of individual data on *MC1R* gene, SKin cancer and Phenotypic characteristics (M-SKIP project).

Pooled-analyses has several advantages over other study types, especially in the genetic epidemiology field. Since millions of Single Nucleotide Polymorphisms (SNPs) were identified by the SNP Consortium [Sachidanandam et al. 2001], a growing number of studies have reported the association of SNPs in candidate genes with several diseases. However individual studies of typical size usually have low statistical power to find true associations given the polygenic nature of most common diseases, leaving alone the various forms of potential interactions between genetic, phenotypic and environmental factors. The advent of genome-wide association studies allowed genotyping of hundreds of thousands of SNPs across the genome on a usually large number of subjects, but information on a wide spectrum of epidemiological and lifestyle factors were seldom collected, although the role of these factors in complex diseases is undoubtedly crucial.

Meta-analysis of genetic epidemiological studies has been adopted to increase the power of smaller candidate gene studies by summarizing results from multiple studies. However the lack of access to individual data precludes in-depth investigations, including analyses of gene-gene and gene-environment interaction, and appropriate stratified analyses. This may potentially lead to false-positive or false-negative results, or biased magnitudes of associations, as previously pointed out [Chatzinasiou et al. 2011].

Pooled-analysis of the primary data has been shown to have critical methodological advantages over meta-analysis [Ioannidis et al. 2002; Seminara et al. 2007] and has been applied successfully in the genetic epidemiology field [Ioannidis et al. 2001; Ioannidis et al. 2002; Raimondi et al. 2006; Paracchini et al. 2007; Klug et al. 2009; Bracci et al. 2010; Truong et al. 2010; Lurie et al. 2011]. Pooled-analysis uses standardized definitions of cases, outcomes and covariates, as well as the same

analytical methods, thus limiting potential sources of heterogeneity across different studies. It also allows investigators to better control for confounding factors, evaluate alternative genetic models and estimate the joint effect of multiple genes. Finally, population-specific effect and gene-gene and gene-environment interactions may be better assessed using pooled-analysis [Boffetta et al. 2007]. The pooling of data from observational studies has become more common recently, and different approaches of data analysis have been applied [Smith-Warner et al. 2006]. Methodological guidelines to correctly design and conduct pooled-analyses are needed to facilitate application of such methods, thus providing a better summary of the actual findings on specific fields. Moreover, the awareness of the potential problems connected with the establishment of international collaborations and data pooling might help investigators to avoid or overcome them. For these reasons, before presenting the results from the M-SKIP project, I will describe the project itself, by explaining the procedures that were used to identify studies, contact investigators, collect and standardize data.

Chapter 2

The M-SKIP Project

The M-SKIP (Melanocortin-1 receptor gene, SKin cancer and Phenotypic characteristics) project is an international pooled-analysis, which aims to investigate the role of *MC1R* variants in skin carcinogenesis, by pooling individual patient data on sporadic skin cancer cases and controls with genetic information on *MC1R* variants. The project was founded by the Italian Association of Cancer Research (AIRC) in 2011 as a My First AIRC Grant (MFAG 11831). The specific aims of the pooled-analysis are:

1) to assess the association between *MC1R* variants and skin cancer overall, and by skin cancer type;

2) to assess the association between *MC1R* variants and phenotypic characteristics, including hair and eye color, skin color, skin type, common and atypical naevi, and freckles.

3) to evaluate the complex interplay between *MC1R* variants and phenotypic characteristics in skin cancer development.

Beyond these investigations, other studies on the M-SKIP data could be proposed by each member of the M-SKIP study group, following the project guidelines (Appendix 1). The database will therefore be the start point for future investigation on the genetic and molecular epidemiology of melanoma and non-melanoma skin cancer (NMSC) and will represent a reference and guide for all investigators working in this research field. Collaboration within investigators will significantly improve the knowledge and accelerate our understanding of the association between *MC1R* variants, skin cancer and phenotype.

The M-SKIP study will have an important impact on the genetic melanoma research. First of all, this pooled-analysis will help elucidating the dual pathway hypothesis by investigation into a molecular distinction between those individuals whose melanomas arise on chronic sun-exposed skin from those in whom tumors will develop on sun-protected skin or from melanocytic nevi. If a dual pathway for melanoma is supported, public-health messages can be tailored to the population at risk. Moreover, the identification of *MC1R* variants associated with melanoma in darkly-pigmented subjects would be a start point for further molecular studies investigating the non-pigmentary mechanisms leading to cancer development, in order to identify and block them. Finally, if specific *MC1R* variants are associated with a higher melanoma risk in certain sub-populations, genetic-based innovative techniques for early detection may be developed and applied to these populations.

2.1. Innovation of the project

Melanoma is a complex and heterogeneous disease with genetic, phenotypic and environmental factors contributing to its development. As the incidence of melanoma continues to increase, there is a great deal of interest in determining DNA variants at ñmelanoma gene(s)ò to identify at-risk patients before disease develops and also to solicit targets for potential therapies. Until now, the genetic studies have typically focused on families, and identified two high-penetrance melanoma susceptibility genes: *CDKN2A* and *CDK4*. However, only approximately 5-12% of melanoma cases occur in a familial setting, while the vast majority of melanoma cases are sporadic; therefore there is a need to identify also low-risk, low-penetrance alleles quite prevalent in the general population, which may be responsible for sporadic melanoma development.

Very well-structured collaborative groups studying the genetics of melanoma already exist. The most important were GenoMEL, coordinated by Julia Newton-Bishop, who investigates the

genetics of familial melanoma, and GEM, coordinated by Marianne Berwich, who explores the genetic and environmental epidemiology of melanoma by comparing single- with multiple-primary melanoma cases. Both these consortia selected patients with higher risk of melanoma compared to the general population. The innovation of M-SKIP project is to create a new collaborative group with the aim of studying the still not deeply investigated area of the genetics of sporadic melanoma, which represent up to 95% of melanoma cases. Although reducing the probability to found rare genetic variants, our data are particularly suitable to find associations between low-penetrance alleles and skin cancer development at a population level. *MC1R* is up to now the main important gene found to play a role in sporadic melanoma, and a large amount of data on *MC1R* variants has been already collected by investigators all over the world. The still ongoing collaboration with GenoMEL and GEM will let integrate information, data and results among the three groups, and give a complete picture of the genetics of melanoma.

With respect to Genome Wide Association Studies (GWAS), that have been recently conducted to find gene loci predisposing to melanoma development, the M-SKIP project integrates also epidemiological information and takes into account relationships and interactions between lifestyle, epidemiological and genetic factors.

2.2. The identification of data sets and data collection

An Advisory Committee of 10 international investigators with great expertise in skin cancer and genetic research was established. Members of the Advisory Committee are listed in the project guidelines (Appendix 1). The invitation letters for the identified investigators, the datasheet including all the variables to be collect and the guidelines, rules, and general conditions applicable to the M-SKIP project had been prepared and firstly approved by the Advisory Committee members.

Published epidemiological studies on *MC1R* variants, melanoma, non-melanoma skin cancer (NMSC) and phenotypic characteristics associated with melanoma [Gandini et al. 2005a; Gandini et al. 2005c] were searched until April 2010 in the following databases: PubMed, ISI Web of Science (Science Citation Index Expanded) and Embase, using the keywords ñMC1Rò and ñmelanocortin 1 receptorò alone and in combination with the terms ñmelanomaò, ñbasaliomaò, ñbasal cell carcinomaò, ñsquamous cell carcinomaò, ñskin cancerò, ñhair colorò, ñskin colorò, ñskin typeò, ñeye colorò, ñneviò, ñfrecklesò, and ñsolar lentiginesò, with no search restriction. The computer search was supplemented by consulting the bibliographies of the articles and reviews. We also tried to identify unpublished datasets by personal communication with participant investigators, members of the Advisory Committee, and with attendees of scientific meetings.

We selected papers according to the following inclusion criteria: 1) observational studies on single-primary sporadic skin cancer cases with information on any *MC1R* variant or 2) control series with information on any *MC1R* variant and at least one phenotypic characteristic under study. Permanent exclusion criteria were: 1) populations selected for *MC1R* status or for other genetic factors, 2) studies including only familial and/or multiple-primary melanoma cases, because we wanted to study *MC1R*-melanoma association at a population level, therefore excluding cases for whom the role of genetics is probably stronger. In the first step of the project, we also excluded GWAS, because their different study design and genotyping methodology would significantly increase the heterogeneity of our data; however GWAS with epidemiological data would be included in a next step of the project and their results would be compared with those of classical genetic epidemiological studies.

The original search provided 748 papers, among them 111 were considered potentially interesting and full-text articles were retrieved and evaluated. We excluded 49 articles for the following reasons: duplicate populations (N=20), no data on outcome (case/control status or any of the

studied phenotypic characteristics) or on *MC1R* variants (N=12), case reports, commentaries or reviews (N=6), GWAS (N=6), populations selected for genetic factors (N=4) and multiple primary melanoma cases only (N=1). The remaining 62 independent studies were considered eligible for inclusion in the pooled analysis.

For each independent study, we identified the corresponding investigator and retrieved his/her contact information. Each investigator was invited to join the M-SKIP project: this required them to sign a participation form and a document attesting to approval of the study guidelines (Appendix 1), and then to provide their data in electronic form without restrictions on format. A detailed list of variables relevant for skin cancer was provided and, for each available variable in the list, the authors were required to compile a form with a clear and complete description on how it was collected and coded. Investigators did not send any personal identifier with data, but only identification codes. Finally, investigators were asked to send a signed statement declaring that the original study was approved by an Ethic Committee and/or that study subjects provided a written consent to participate in the original study.

Data collection started in May 2009 and was closed in December 2010. During this period, 43 investigators were contacted and invited to share data. Thirty-one (72%) agreed to participate and provided data on 28,998 subjects, including 13,511 skin cancer cases (10,182 melanomas) and 15,477 controls from 37 independent published [Bastiaens et al. 2001a; Bastiaens et al. 2001b; Kennedy et al. 2001; Fargnoli et al. 2003; Dwyer et al. 2004; Kanetsky et al. 2004; Pastorino et al. 2004; Landi et al. 2005; Debniak et al 2006; Fargnoli et al. 2006a; Fargnoli et al. 2006b; Han et al. 2006; Kanetsky et al 2006; Stratigos et al. 2006; Anno et al. 2007; Branicki et al. 2007; Fernandez et al. 2007; Motokawa et al. 2007; Stefanaki et al. 2007; Anno et al. 2008; Fargnoli et al. 2008; Fernandez-de-Misa et al. 2008a; Fernandez-de-Misa et al. 2008b; Mengel-From et al. 2008; Motokawa et al. 2008; Nan et al. 2008; Pastorino et al. 2008; Scherer et al. 2008; Branicki et al.

2009; Brudnik et al. 2009; Casula et al. 2009; Council et al. 2009; Dimisianos et al. 2009; Gapska et al. 2009; Ghiorzo et al. 2009; Höiom et al. 2009; Liu et al. 2009; Nan et al. 2009; Scherer et al. 2009; Kanetsky et al. 2010; Wong et al. 2010] and 2 unpublished studies. Both the unpublished datasets came from investigators who were originally contacted for their published data and who had further data of (still) unpublished studies. Among the 12 non-participant investigators, seven did not reply to our invitation letter, three were not able to retrieve the original dataset and two were not interested in the project. The total number of skin cancer cases and controls from the 25 independent studies [Valverde et al. 1995; Valverde et al. 1996; Box et al. 1997; Ichii-Jones et al. 1998; Smith et al. 1998; Clairmont et al. 1999; Jones et al. 1999; Rana et al. 1999; Strange et al. 1999; Flanagan et al. 2000; Healy et al. 2000; Palmer et al. 2000; Akey et al. 2001; Box et al. 2001; Jiménez-Cervantes et al. 2001; Ramachandran et al. 2001; Voisey et al. 2001; Shriver et al. 2003; Duffy et al. 2004; Matichard et al. 2004; Naysmith et al. 2004; Jannot et al. 2005; Liboutet et al. 2006; Beaumont et al. 2007; Mossner et al 2007; Guedj et al. 2008; Shekar et al. 2008; Binkley et al. 2009; Galore-Haskel et al. 2009; Latreille et al. 2009; Duffy et al. 2010; Elfakir et al. 2010; Hacker et al. 2010] of non-participant investigators was 5,135 and 8,262, respectively. The study design of non-participant studies was caseï control for 13 studies, control-only for 11 studies, and case-only for one study.

2.3. Quality control, data coding and creation of the standardized dataset

We inspected the data for completeness and resolved inconsistencies with the investigator of each study. Unpublished data submitted to the M-SKIP project were evaluated by an internal peer-review process: the principal investigator and one Advisory Committee member randomly selected evaluated the sent information and data, assessed their quality, and decided whether the unpublished data could be included in the M-SKIP data base or not.

Among all the collected datasets, a number of subjects were excluded due to the following reasons: multiple-primary melanoma cases (N=1596), missing data on *MC1R* variants (N=1081), non-skin melanoma cases (N=150), subjects with atypical mole syndrome and no skin cancer (N=58), non first-primary melanoma cases (N=24), familial melanoma cases, defined as subjects with two first-degree relatives or three or more any-degree relatives with melanoma (N=25), other reasons including: unknown case/control status, duplicate subjects, or inappropriate controls (N=232).

The following study-related variables were recoded uniformly: study country, study design, source of controls, application of caseï control matching, methods to define phenotypic characteristics, genotyping methodology, whether genotyping was done in the same center for cases and controls and was blinded for case/control status, and DNA source. In addition, the variables listed in Table 2.1 were retrieved from each study if available, checked for quality, recoded in a standardized manner and entered in the main database. Finally, data on *MC1R* variants were entered for each subject. Quality controls and data coding were performed independently by two investigators, and inconsistencies were solved via consensus. A complete and clear description of all the rules we applied to code data was compiled.

Each final, recoded dataset was sent to the owner investigator in order to be checked and approved. When a final agreement on a code could not be made with the corresponding author of each study, the data was considered missing. No missing imputation was performed for any of the considered variables.

Table 2.1. List of the main variables, number of original studies and related subjects per variable

Variable	Studies (%) N=39	Melanoma cases (%) n=7806	NMSC cases (%) n=3151	Controls (%) n=14875
Age	37 (95%)	7761 (99%)	3150 (100%)	14550 (98%)
Gender	39 (100%)	7801 (100%)	3151 (100%)	14853 (100%)
Ethnicity	38 (97%)	6770 (87%)	3142 (100%)	13833 (93%)
Body mass index	8 (21%)	557 (7%)	1380 (44%)	2226 (15%)
Smoking status	6 (15%)	2266 (29%)	419 (13%)	2286 (15%)
Intermittent sun exposure	21 (54%)	4493 (58%)	1266 (40%)	2286 (15%)
Continuous sun exposure	21 (54%)	4909 (62%)	741 (24%)	1938 (13%)
Sunburns	25 (64%)	4210 (54%)	1288 (41%)	2968 (20%)
Artificial UV exposure	16 (41%)	3842 (49%)	298 (9%)	1058 (7%)
Family history of skin cancer	27 (69%)	6660 (85%)	1289 (41%)	3318 (22%)
Family history of cancer other than skin	19 (49%)	4445 (57%)	371 (12%)	1630 (11%)
Melanoma body site	24 (62%)	6271 (80%)	NA	NA
Melanoma histology	19 (49%)	4868 (62%)	NA	NA
Breslow thickness	24 (62%)	5907 (76%)	NA	NA
Hair color	34 (87%)	6841 (88%)	2590 (82%)	11889 (80%)
Eye color	31 (79%)	5990 (77%)	2456 (78%)	10720 (72%)
Skin color	23 (59%)	3517 (45%)	826 (26%)	2963 (20%)
Skin type	31 (79%)	6590 (84%)	1992 (63%)	4540 (31%)
Common nevi	19 (49%)	3817 (49%)	442 (14%)	1181 (8%)
Atypical nevi	11 (28%)	2681 (34%)	642 (20%)	1447 (10%)
Freckles	21 (54%)	4028 (52%)	737 (23%)	2333 (16%)
Solar lentigines	6 (15%)	1419 (18%)	442 (14%)	1088 (7%)

NA=not applicable; NMSC=non melanoma skin cancer

2.4. Brief description of the collected data

The final dataset was created in June 2011 and included data on 7,806 melanoma cases, 3,151 NMSC cases (2,211 BCC, 788 SCC and 152 with both), and 14,875 controls.

Distribution of data according to study country in which the study was performed is presented in Table 2.2. The majority of data came from Europe, especially from southern European populations. There was no significant difference in participation rate according to study area (Fisher exact test p-value: 0.25).

The main characteristics of the studies included in the M-SKIP database are described in Table 2.3. The majority are caseï control studies (54%) with population or healthy controls and caseï control matching. Phenotypic characteristics were frequently assessed by self-administered questionnaire (41%) or examination by a dermatologist or research nurse (36%). The majority of studies sequenced the entire coding region of the *MC1R* (67%) and used blood as DNA source (62%).

Table 2.2. Summary of data included in the M-SKIP project by geographical location

Study area	Invited investigators	Participant investigators (studies)	Melanoma cases	NMSC cases	Controls
Africa	1	0 (0)	0	0	0
Asia	3	2 (2)	0	0	345
Australia	4	2 (3)	744	298	290
Northern Europe[a]	8	6 (6)	858	1629	8095
Central Europe[b]	6	3 (4)	977	639	2398
Southern Europe[c]	9	8 (12)	2,747	0	2263
North America	13	11 (12)	2,480	585	1484
TOTAL	**43**[d]	**31**[d] **(39)**	**7808**	**3151**	**14875**

NMSC=non melanoma skin cancer
[a] includes Denmark, Norway, Sweden, The Netherlands, UK
[b] includes France, Germany, Poland
[c] includes Greece, Italy, Spain
[d] one investigator collected data for two different areas (North America and Asia)

Table 2.3. Main characteristics of the included studies

	Studies (%) N=39	Melanoma cases (%) n=7806	NMSC cases (%) n=3151	Controls (%) n=14875
Study design				
Case-control	21 (54%)	5092 (65%)	2052 (65%)	6852 (46%)
Case only	11 (28%)	2646 (34%)	0	0
Control only	6 (15%)	0	0	1464 (10%)
Cohort	1 (3%)	68 (1%)	1099 (35%)	6559 (44%)
Source of controls				
Hospital	6 (21%)	509 (10%)	1169 (37%)	1847 (12%)
Population or healthy[a]	21 (75%)	4651 (90%)	1982 (63%)	12872 (87%)
Mixed	1 (4%)	0	0	156 (1%)
Case-control matching[b]				
No	10 (45%)	3151 (61%)	1739 (55%)	9578 (71%)
Yes	12 (55%)	2009 (39%)	1412 (45%)	3833 (29%)
Phenotype assessment				
Self-administered questionnaire	16 (41%)	2768 (35%)	672 (21%)	1875 (13%)
Examination by an expert	14 (36%)	3970 (51%)	1380 (44%)	4392 (30%)
Instrumental measure	2 (5%)	0	0	222 (1%)
Mixed	5 (13%)	297 (4%)	1099 (35%)	7247 (49%)
No measure	2 (5%)	771 (10%)	0	1139 (8%)
Genotype assessment				
Sequencing analysis	26 (67%)	5942 (76%)	1059 (34%)	4813 (32%)
Others[c]	13 (33%)	1864 (24%)	2092 (66%)	10062 (68%)
DNA source				
Blood	24 (62%)	4645 (60%)	2743 (87%)	13304 (89%)
Buccal cells	14 (36%)	3161 (40%)	408 (13%)	1326 (9%)
Tissue	1 (3%)	0	0	245 (2%)

NMSC=non melanoma skin cancer

[a] healthy subjects are blood donors, friends or relatives of cases

[b] individual or frequency

[c] includes RFLP, SNaPshot, allele discrimination assay

2.4.1. Statistical power

We calculated that the minimum required sample size to find a statistically significant association between a *MC1R* variant and melanoma assuming a similar association to that observed in our previous meta-analysis [Raimondi et al. 2008] (Odds Ratio (OR)ر=1.5) is around 7,500 cases and 7,500 controls for rare variants (1-2% allele frequency in controls), and 1,400 cases and 1,400 controls for common variants (8-10% allele frequency in controls), with 90% statistical power. Sample size for gene-environment interaction analysis was also calculated with the program POWER, version 3.0 [García-Closas et al. 1999]. Considering the study of a simple two-way interaction between an environmental factor and a rare *MC1R* variant, around 5,000 cases and 5,000 controls would be needed to observe a multiplicative interaction effect of 2.0, arising to 16,000 cases and 16,000 controls to observe a smaller multiplicative effect of 1.5, both with 90% statistical power. For common *MC1R* variants, the same gene-environment interaction effects of 2.0 and 1.5 could be observed with around 1,200 cases and 1,200 controls, and with around 3,500 cases and 3,500 controls, respectively. Our sample size therefore is appropriate for the purpose of the analysis, and large enough to allow stratified and interaction analyses, especially to find even small interaction effects with the most frequent variants, and larger interaction effects for less common variants.

2.5. Possible difficulties of the study and proposed solutions

While conducting a pooled-analysis of genetic epidemiology data, several problems and difficulties may occur. I listed here some problems we faced in the M-SKIP project and advise on possible strategies to avoid or reduce their impact on the pooled analysis.

2.5.1. Participation bias

Some identified investigators may decide not to join the project. In the M-SKIP project we tried to prevent this situation by involving the Advisory Committee in all the decisional aspects of the project, as well as in establishing personal contacts with the investigators who were undecided about participation. After the first letter of invitation, new letters were sent to the potential participants who had not answered yet, in order to remind on the project. Advantages of being part of this project were highlighted: data are available for all participants, who can decide to analyze them for their specific interests, upon approval of the Advisory Committee; each investigator is co-author of publications arising from the project.

Although the previous strategy let to obtain a high participation rate, not all the identified investigators agreed to join the project. In order to investigate any possible participation bias, I compared the main characteristics of the studies included in the pooled-analysis with the published characteristics of the studies not included in the pooled-analysis, as study design, source of controls, study country, distribution of age and gender, *MC1R* genotyping methodology.

2.5.2. Data quality

The quality of collected data, and especially of unpublished data, may be poor. I tried to solve this problem with different strategies within the M-SKIP project. First, unpublished data submitted to the M-SKIP project were evaluated by an internal peer-review process, as described in section 2.3. Second, if there were discordances among the collected data or between sent data and published ones, or if I had any doubt on the quality of data, I contacted the participant investigator to asked clarification, additional material, and further information. After that, I decided whether the data could be included in the main data base or not.

2.5.3. Data standardization

Collected data may be not completely comparable: different studies may indeed collect data in different ways and may provide different variables categories. This may especially occur for phenotypic characteristics, sun exposure, sunburns, and naevi count. In order to standardize these variables, we wrote a complete and clear list of rules. I report here as an example the rules we used to standardize sun exposure variables, in order to provide suggestions on how to recode variables with highly heterogeneous assessment among studies.

Intermittent and continuous sun exposure was coded as hours of exposure per day if the information was structured in this way. If not, we converted it to hours/day on a scale of 0 as no exposure and 6 as the maximum hours of exposure per day. For example, for datasets with four classes of exposure (never, seldom, often, always), we recoded the classes as 0, 2, 4, 6 hours/day. If individual sun exposure was collected over different time periods, we calculated the average exposure weighting for years of exposure in each time period. Other continuous variables (i.e. days of exposure per year, average hours of exposure per year) were converted to hours/day using the following algorithm:

1) calculate the variable mean on all the study subjects as:

$$\mu = \sum_{i=1}^{n} x_i / n \qquad (2.1)$$

where x_i is the measure of the continuous variable on subject *i*, and *n* is the study sample size;

2) calculate the average hours of exposure/day (ν) over all the datasets with the variable coded (or recoded) in this way as in 1);

3) recode each observation basing on the proportion $x_i : \mu = \tilde{x}_i : \nu$ as:

$$\tilde{x}_i = \nu x_i / \mu \qquad (2.2)$$

4) set as 6 (maximum hours of exposure per day) the value of all calculated values greater than 6.

The assumption underlying this coding was that the average sun exposure pattern for study subjects was similar for different studies (and countries). Since we will use this variable only for confounding adjustment and/or effect modifier analyses, the purpose was to regroup subjects with a similar pattern of sun exposure, although the precise individual amount of sun exposure could not be estimated.

As a general rule, when a variable (i.e. common nevi count) was collected into classes, we recoded each class by using its median. The maximum numbers for open categories were chosen according to the available M-SKIP data.

2.5.4. Different outcomes and confounders

It is possible that not all the datasets collected the same confounding variables and investigated the same outcomes. For this latter problem, before starting the statistical analysis, I pooled together all the studies with the same outcomes and calculate the corresponding statistical power.

In order to deal with the problem of different sets of variables collected by different studies, I applied a two stage approach and a method previously proposed [Jackson et al. 2009] and better discussed in the next Chapter.

2.5.5. *MC1R* genotyping

It is possible that not all the investigators sequenced the full gene. When the *MC1R* gene was sequenced, all the variants of the gene could be identified and patients with no *MC1R* variants (wild-type) could be clearly defined. However, some studies searched just for the main variants using genotyping methodologies like Restriction Fragment Length Polymorphism (RFLP) or SNP-shot. This way, only some *MC1R* variants could be identified and ñwild-typeò subjects in these

studies were those with none of the searched *MC1R* variants (they could carry, however, other not searched *MC1R* variants). I created different variables to code *MC1R* gene. First, I coded each of the nine main variants (V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D284H), so that it was 0 if the subject had no that variant, 1 if they had 1 allele of that variant, 2 if they had 2 alleles of that variant,....Second, I coded *MC1R* just for the studies which sequenced the entire gene, as 0 if no variant was found (ñwild-typeò subjects), 1 if just one variant was found, 2 if two variants were found,.... The analyses on the most studied variants were carried out on the largest number of datasets, while the analyses of variants combined were carried out just on the subgroup of studies which sequenced the gene. I evaluated the impact of genotyping methodology on the summary estimate of the studied association by meta-regression analysis.

Chapter 3

Association between *MC1R* variants and melanoma

The first aim of the M-SKIP project is to investigate the association between *MC1R* variants and skin cancer, with particular focus on melanoma. For this latter investigation, I selected from the M-SKIP database 17 independent melanoma case-control studies, that overall included data on 5,160 melanoma cases and 12,119 controls. According with the participant investigator in the M-SKIP project, the 17 studies were: Gruis [Bastiaens et al. 2001a; Bastiaens et al. 2001b; Kennedy et al. 2001], Dwyer [Dwyer et al. 2004], Ghiorzo [Pastorino et al. 2004; Pastorino et al. 2008; Ghiorzo et al. 2009], Landi [Landi et al. 2005], Debniak [Debniak et al 2006; Gapska et al. 2009], Fargnoli [Fargnoli et al. 2003, Fargnoli et al. 2006a; Fargnoli et al. 2006b; Fargnoli et al. 2008], Han [Han et al. 2006; Nan et al. 2008; Nan et al. 2009], Stratigos [Stratigos et al. 2006; Stefanaki et al. 2007; Dimisianos et al. 2009], Ribas [Fernandez et al. 2007], Branicki [Branicki et al. 2007; Branicki et al. 2009; Brudnik et al. 2009], Cornelius [Council et al. 2009], Hansson [Höiom et al. 2009], Kayser [Liu et al. 2009], Kumar [Scherer et al. 2009], Nagore [Scherer et al. 2009], Palmieri [Casula et al. 2009], Kanetsky [Kanetsky et al. 2004; Kanetsky et al. 2010]. A description of the main characteristics of the studies is reported on Table 3.1. The majority of studies come from Europe (N=13, 76%), particularly from South Europe (N=7, 54%). Thirteen studies (76%) included healthy controls (population controls, blood donors, friends or relatives of cases), while the remaining four (24%) recruited hospital controls. Overall, the average age of controls was higher than that of cases (62 versus 53 years), while the proportion of males was the same for both cases and controls (44%). All the studies collected information on the following potential confounders: age, gender and family history of melanoma. The assessment of further confounders varied among different studies.

Table 3.1. Description of the main characteristics of the 17 case-control studies included in the pooled-analysis

First author, year	Country	Controls type	N Cases/ controls	Age (SD)		% of males		Possible confounders assessed beyond age, gender and family history of melanoma
				Cases	Controls	Cases	Controls	
Gruis, 2001	The Netherlands	Hospital	115/378	49 (12)	58 (11)	37	42	Sun exposure (chronic, intermittent), sunburns (lifetime), naevi (common, atypical)
Dwyer, 2004	Australia	Healthy	159/290	44 (10)	44 (10)	41	46	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood)
Ghiorzo, 2004	Italy	Healthy	254/507	52 (16)	51 (17)	50	47	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (common, atypical)
Landi, 2005	Italy	Healthy	165/171	49 (15)	46 (13)	49	49	Sun exposure (chronic, intermittent), sunburns (lifetime)
Debniak, 2006	Poland	Healthy	349/313	53 (14)	53 (13)	32	24	sunburns (lifetime, childhood)
Fargnoli, 2006	Italy	Hospital	155/163	49 (14)	49 (14)	40	50	Sun exposure (chronic, intermittent), sunburns (childhood), naevi (common, atypical)
Han, 2006	USA	Healthy	219/241	64 (8)	58 (7)	0	0	Sunburns (lifetime)
Stratigos, 2006	Greece	Hospital	123/155	52 (16)	44 (15)	51	54	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (common, atypical)
Ribas, 2007	Spain	Healthy	108/188	51 (15)	53 (14)	46	39	Sunburns (childhood), naevi (common)
Branicki, 2009	Poland	Hospital	116/489	62 (14)	43 (19)	35	40	-
Cornelius, 2009	USA	Healthy	83/166	51 (15)	77 (7)	45	50	-
Hansson, 2009	Sweden	Healthy	675/477	53 (19)	42 (12)	47	64	-
Kayser, 2009	The Netherlands	Healthy	68/6559	70 (8)	72 (9)	47	41	-
Kumar, 2009	Germany	Healthy	512/1064	58 (15)	54 (12)	56	56	-
Nagore, 2009	Spain	Healthy	1031/558	52 (16)	37 (12)	46	62	-
Palmieri, 2009	Italy	Healthy	259/75	49 (14)	61 (15)	48	27	-
Kanetsky, 2010	USA	Healthy	769/325	49 (14)	48 (13)	49	43	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (atypical)
Total			**5,160/12,119**	**53 (16)**	**62 (16)**	**44**	**44**	

The main studied *MC1R* variants, that I considered in my analyses, were nine: V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D294H.

3.1. Preliminary analyses

3.1.1. Hardy-Weinberg equilibrium

The Hardy-Weinberg (HW) equilibrium is the simplest model of equilibrium in population genetics. It implies that: 1) given a locus with two alleles *A* and *a* and frequencies *p* and *q* respectively, the expected genotype frequencies for *AA*, *Aa* and *aa* are p^2, $2pq$ and q^2 and 2) that these allele and genotypes frequencies do not change in time. Deviation from HW equilibrium is taken as an indication that the alleles are not segregating independently; there are ógeneticô reasons for this, including non-random mating (which encompasses admixture), or that the alleles reflect recent mutations that have not reached equilibrium, as well as methodological reasons, e.g. that selection of subjects from the population is biased, that there is genotyping error, or that population stratification exists.

I assessed the departure of frequencies of each *MC1R* variant from expectation under HW equilibrium by Chi Square test in controls for each study. All the studies were in HW equilibrium for the following variants: V60L, D84E, V92M, I155T, and R163Q. For the remaining four variants departure from HW equilibrium was observed at least for one study, and in particular: one study (Kayser) for R142H variant, four studies (Dwyer, Fargnoli, Hannson, Stratigos) for R151C variant, two studies (Branicki, Kayser) for R160W variant, and one study (Branicki) for D294H variant. I took into account the deviation of these studies from HW equilibrium, and the consequent possible impact on the studied associations, by meta-regression analysis. The results are presented in the following section 3.4.

3.1.2. Choice of the inheritance model

Different inheritance models are possible and should be evaluated in order to choose the one that better fits the data. The main possible inheritance models are recessive or dominant. In the autosomal dominant mode of inheritance, heterozygous individuals are affected as well as variant homozygous individuals. Therefore, given a gene with the two possible alleles *A* and *a* (wild-type allele), with OR_{Aa} and OR_{AA} indicating the risk to develop the study disease for subjects with *Aa* and *aa* genotypes, respectively, the dominant model of inheritance may be expressed as follows: $OR_{AA}=OR_{Aa}>1$ and $OR_{aa}=1$. In the autosomal recessive model of inheritance, variant homozygous individuals are affected, while heterozygous individuals are normal. This model may be expressed as follows: $OR_{AA}>1$ and $OR_{Aa}=OR_{aa}=1$. Another inheritance model often observed is the multiplicative model, that implies a "dose" effect because it assumes a linear increase in OR with increasing the number of variant alleles. Therefore, according with this latter model of inheritance, $OR_{AA}>OR_{Aa}>1$ and $OR_{aa}=1$.

I tested these different possible inheritance models in each of the 17 studies included in the analysis and found that the dominant model was the one with the lowest Akaike's Information Criterion (AIC) for almost all the studies and variants, therefore I assumed this model of inheritance in all the following analyses.

AIC is a criterion for selecting an optimum model in a class of nested and non-nested models or models fitted on different samples. It takes into account both the binomial deviance and the degrees of freedom of each model and was defined as:

$$AIC(m) = -2L(m) + 2k(m) \qquad (3.1)$$

where *L(m)* is the maximum log-likelihood for the *m*-th model and *k(m)* is the number of predictors for the *m*-th model. Better models have smaller AIC.

3.1.3. Combining data into a single dataset with random effects models

A first analysis using data combined into one dataset, is to fit them with logistic regression models with random slope. Considering a dominant model, let ´$_{ik}$/X be the probability of melanoma for subject i (i=1,é ,nk) of study k (k=1,é ,K) conditional on the presence of a certain *MC1R* variant (X). I accounted for the fixed *MC1R* effect and the random selection of studies, assuming a model that relates *MC1R* and study effects linearly to the logit of the probabilities:

$$\text{logit}(\pi_{ik} / X) = \alpha + \beta X_{ik} + b_k X_{ik} \qquad (3.2)$$

In this model the transformed regression coefficient exp(**b**) is the odds of melanoma for a subject with the *MC1R* variant compared with a subject without the *MC1R* variant, and the b_k are the study-specific coefficients accounting for the random selection of studies, with $b_k \sim N(0, \sigma^2_b)$, where σ^2_b represents the between study variance of the *MC1R* effect.

The logistic regression model above described could be applied to different inheritance models and could include covariates, in order to adjust the studied associations by possible confounding factors. In order to include the available information from all the studies, missing values could be estimated in the model with multiple imputation and/or the creation of a missing-data indicator variable. However, when the majority of missing data are the results of non-availability of certain variables in some studies, as for the M-SKIP project, the use of both multiple imputation and the missing-data indicator would be likely to introduce a bias in comparison with the complete case method [Miettinen et al. 1985; Huberman et al. 1999] and a two-stage approach would be preferred.

I performed a preliminary analysis by combining data into a single dataset to estimate the crude effect of each *MC1R* variant on melanoma development, therefore I did not consider at this step all the possible confounders collected by each study. Otherwise, confounders were taken into account in the following analyses described in the next sections 3.2 and 3.3.

The results of this preliminary analysis are reported in Table 3.2. We found significant associations with melanoma for all variants except V92M and R163Q, with ORs ranging from 1.11 (95%CI: 1.01-1.22) for V60L to 2.46 (95%CI: 1.69-3.60) for D84E.

Table 3.2. Crude association between *MC1R* variants and melanoma

MC1R variant	N cases/N controls	OR (95%CI)
V60L	5048/5523	1.11 (1.01-1.22)
D84E	4261/4735	2.46 (1.69-3.60)
V92M	4577/4948	1.11 (0.98-1.26)
R142H	4365/4712	1.76 (1.31-2.38)
R151C	5081/5523	1.72 (1.52-1.95)
I1551T	4575/4947	1.43 (1.06-1.93)
R160W	5078/5538	1.87 (1.65-2.12)
R163Q	4925/5255	1.09 (0.93-1.28)
D294H	4609/5079	1.91 (1.53-2.38)

OR=Odds Ratio; CI=Confidence Intervals

3.2. Two-stage analysis

The two-stage analysis method [Stukel et al. 2001] allows to overcome the problem of the availability of different study covariates. The pooled-estimates of the association of *MC1R* variants with melanoma were calculated as follows.

First, study-specific ORs were calculated by applying logistic regression to the data from each study to estimate the odds of melanoma conditional on the presence of a *MC1R* variant (*X*), controlling for confounders Z_k. For study k ($k=1,\dots,K$), assuming just one confounder, the model is written as:

$$\text{logit}(\pi_{ik} / X) = \alpha_k + \beta_k X_{ik} + \gamma_k Z_{ik} \qquad (3.3)$$

where π_{ik} is the conditional probability of melanoma for subject i ($i=1,\dots,n_k$) of study k. Although *MC1R* variants were uniformly defined across studies, the confounders Z_k were specific to a particular study. The exposure log-odds ratio for study k is denoted β_k, the confounding log-odds

ratio is denoted γ_k, and the α_k are the study-specific intercepts. The β_k are assumed to vary across studies according to the second-stage model:

$$\beta_k = \beta + b_k + e_k \tag{3.4}$$

where β is the pooled-exposure log-odds ratio, b_k are random effects with $b_k \sim N(0, \tau^2_b)$, where τ^2_b represents the variability of the study-specific exposure effects β_k about the population mean β, and e_k are independent errors with $e_k \sim N(0, \tau^2_k)$, where τ^2_k describes the within-study variation of the β_k. In the first stage $\hat{\beta}_k$ and its variance $\hat{\sigma}^2_k$ are estimated from equation 3.3, separately for each study.

The two-stage estimator of the pooled exposure effect β is a weighted average of the $\hat{\beta}_k$, weighted by the inverse marginal variances of the $\hat{\beta}_k$, denoted $w_k = (\hat{\sigma}^2_k + \sigma_b{}^2)^{-1}$. Thus:

$$\hat{\beta} = (\sum_k w_k \hat{\beta}_k) / \sum_k w_k \tag{3.5}$$

$$\text{var}(\hat{\beta}) = (\sum_k w_k)^{-1} \tag{3.6}$$

Two methods [Stukel et al. 2001] are frequently used to estimate the random effects variance τ^2_b in equations 3.5 and 3.6. These methods are pseudo-maximum likelihood and moment estimation.

3.2.1. Results

I pooled adjusted study-specific estimates with the two-stage approach previously described. Missing data were imputed with multiple imputation models for variables with less than 20% of missing data, by using the iterative Markov chain Monte Carlo method [Horton et al. 2007]. Forest plots for each of the nine main *MC1R* variants are presented in Figure 3.1-3.9. A significant association with melanoma was observed for the six *MC1R* variants D84E (OR; 95%CI: 2.13; 1.44-3.17), V92M (1.15; 1.00-1.31), R142H (1.77; 1.14-2.75), R151C (1.62; 1.34-1.96), R160W (1.74; 1.43-2.13), and D294H (1.78; 1.40-2.28). No association was observed between melanoma and the

three *MC1R* variants V60L (OR; 95%CI: 1.14; 0.99-1.31), I155T (1.36; 0.97-1.90), and R163Q (1.10; 0.94-1.30).

Figure 3.1. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* V60L variant and melanoma

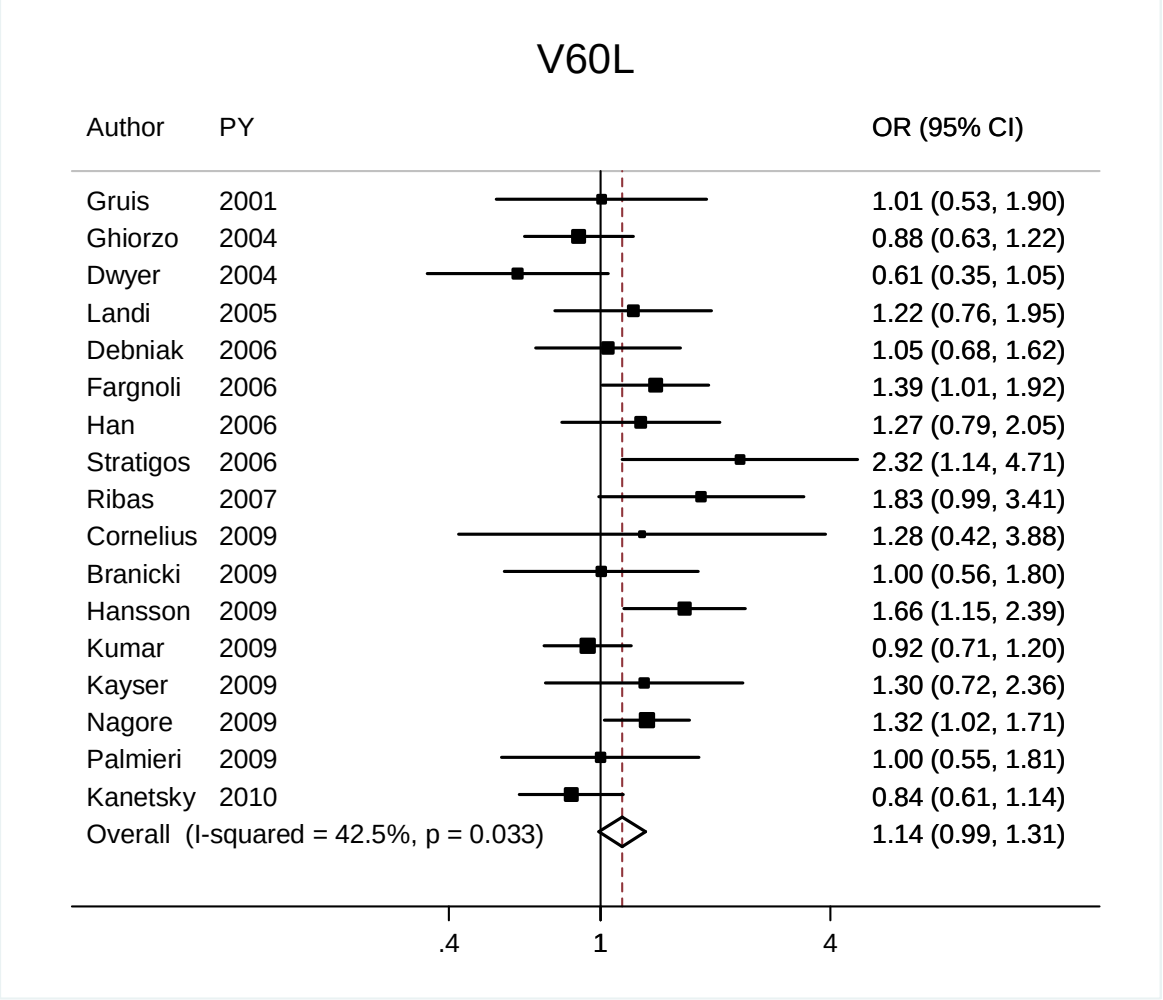


Figure 3.2. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* D84E variant and melanoma

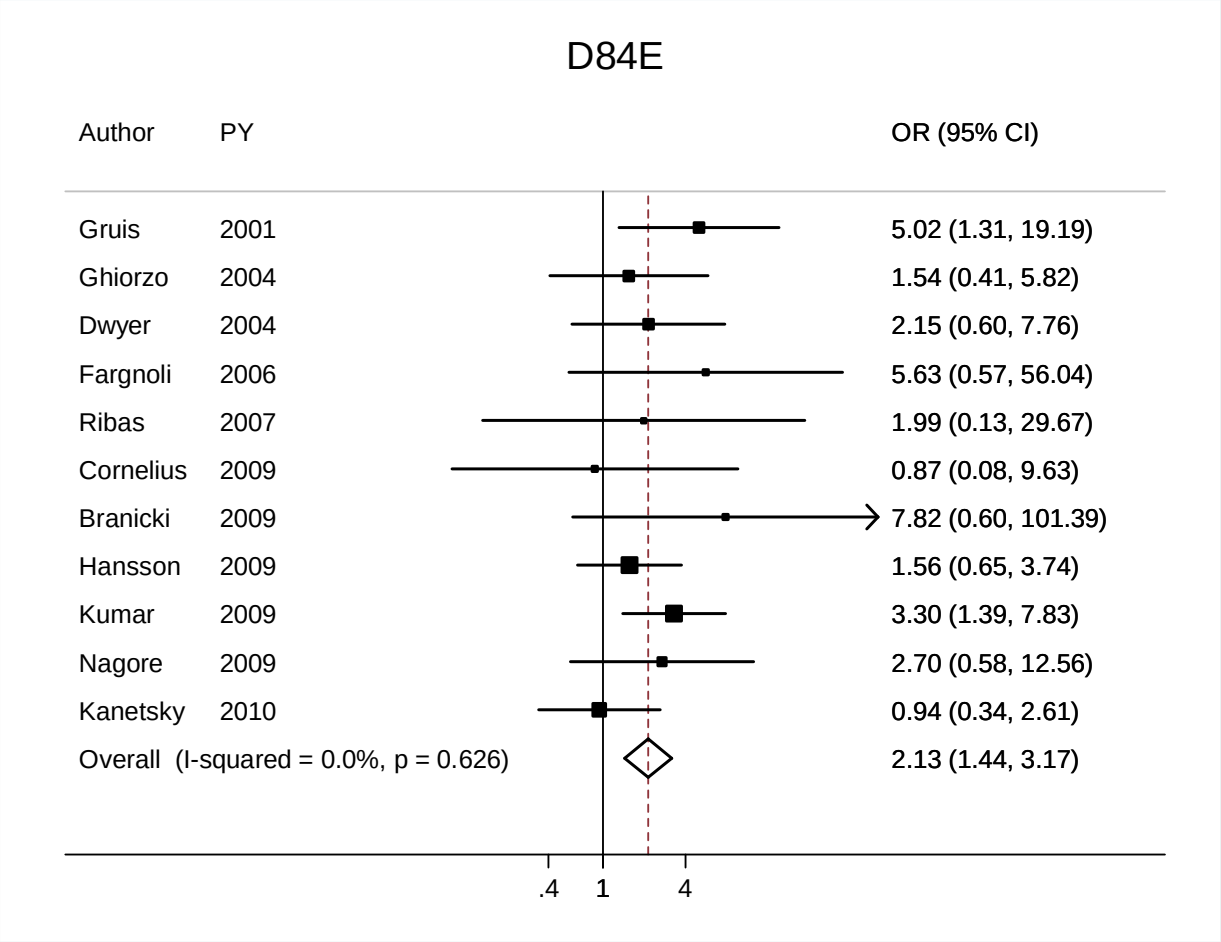


Figure 3.3. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* V92M variant and melanoma

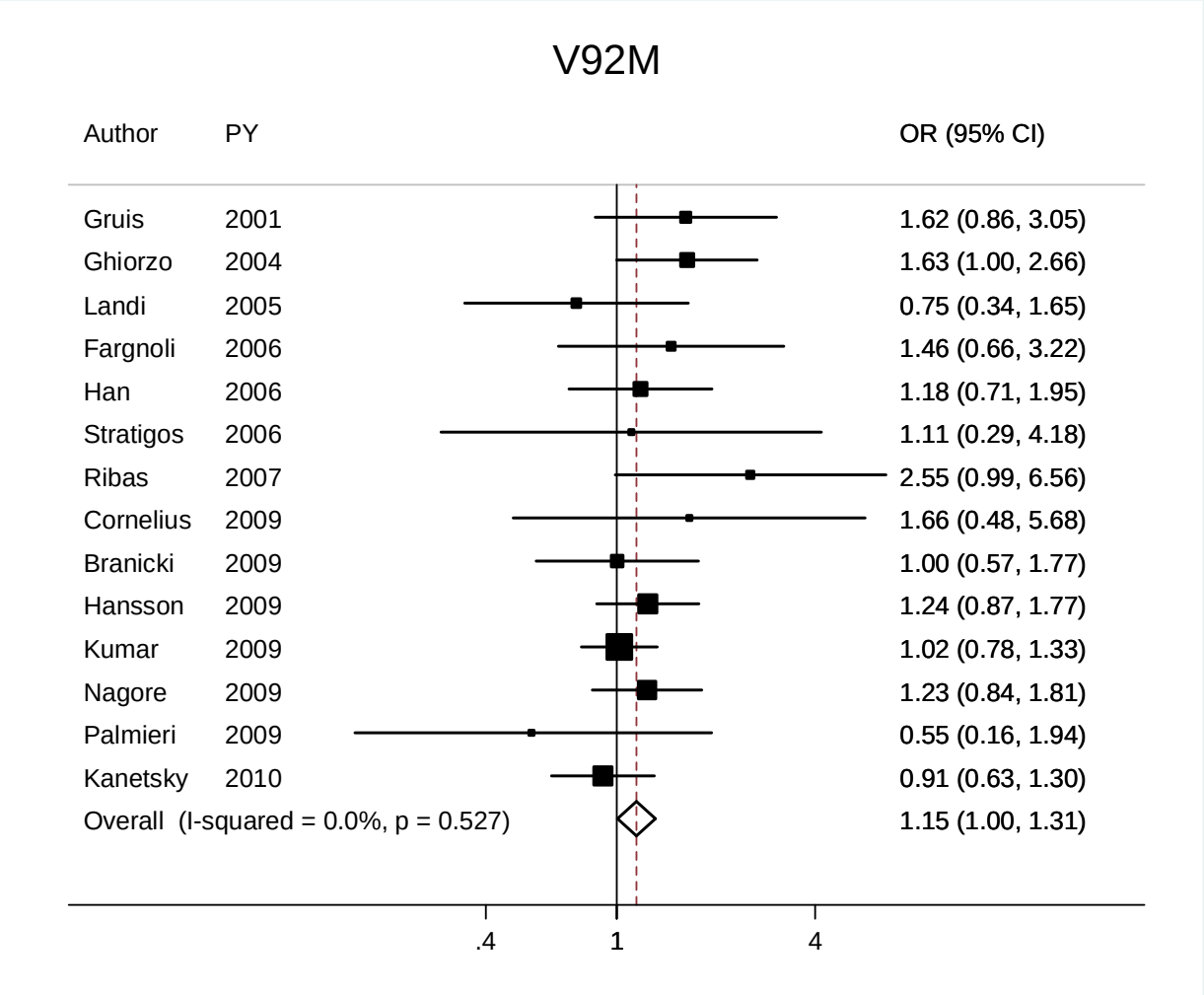


Figure 3.4. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* R142H variant and melanoma

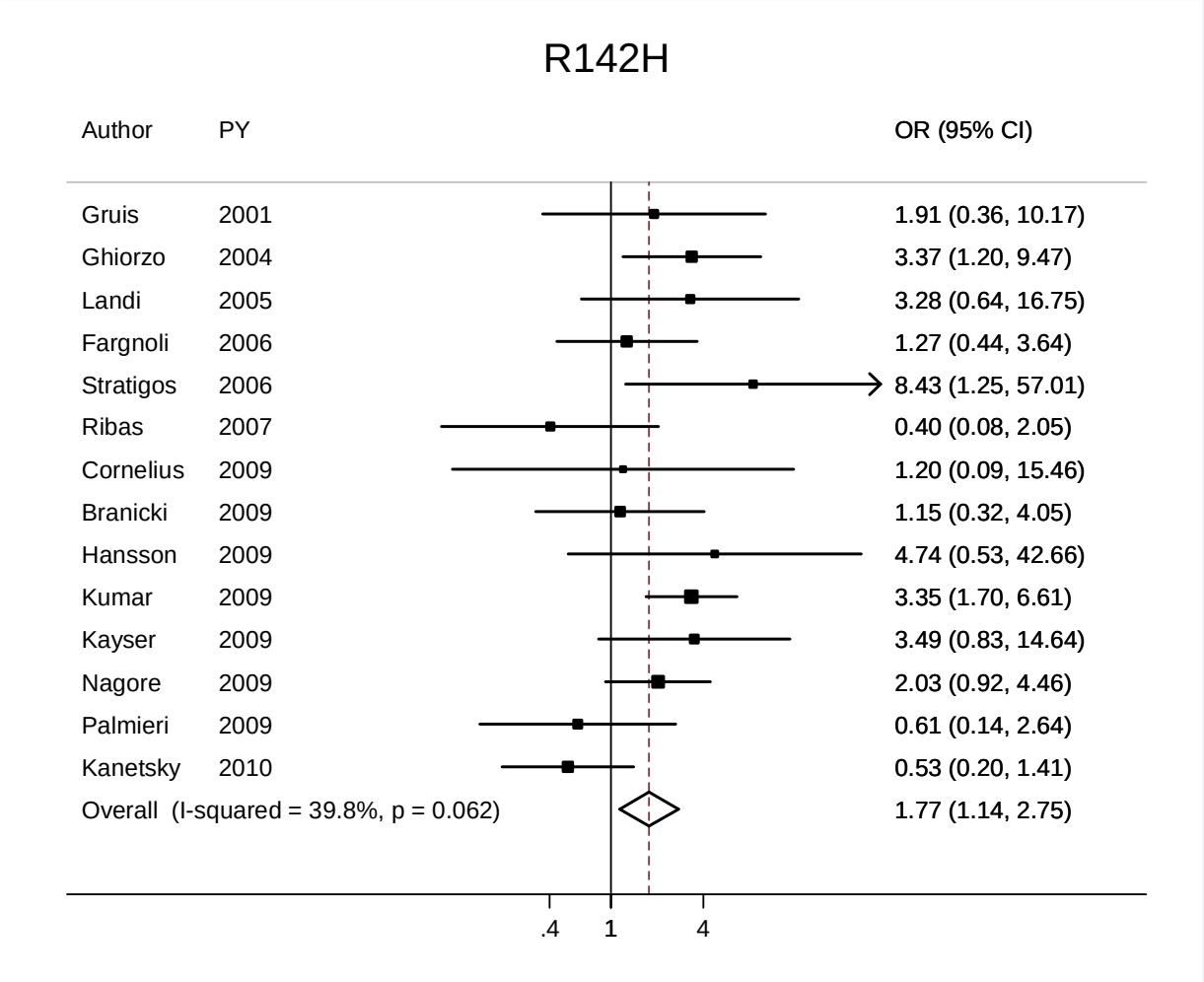


Figure 3.5. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* R151C variant and melanoma

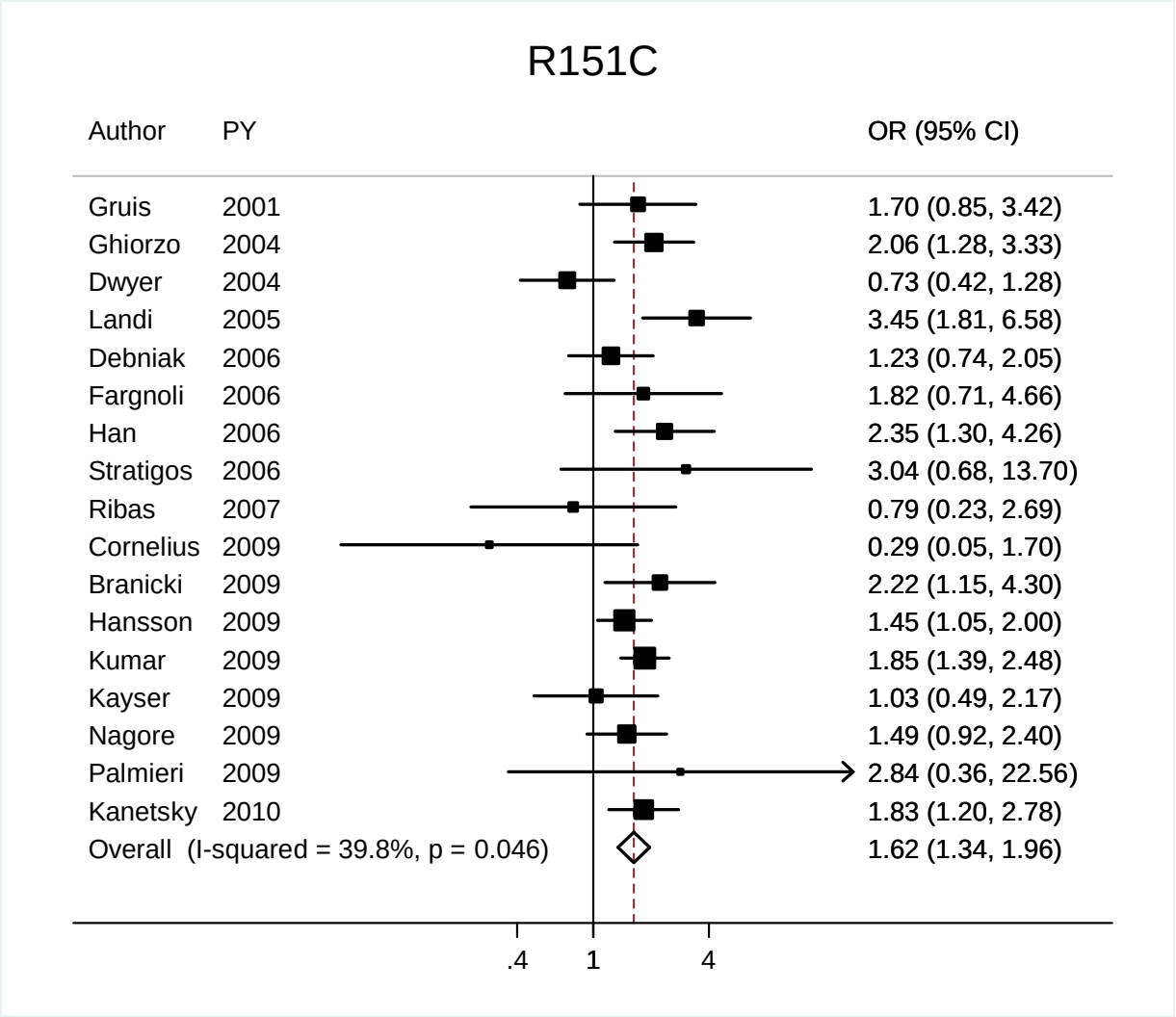


Figure 3.6. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* I155T variant and melanoma

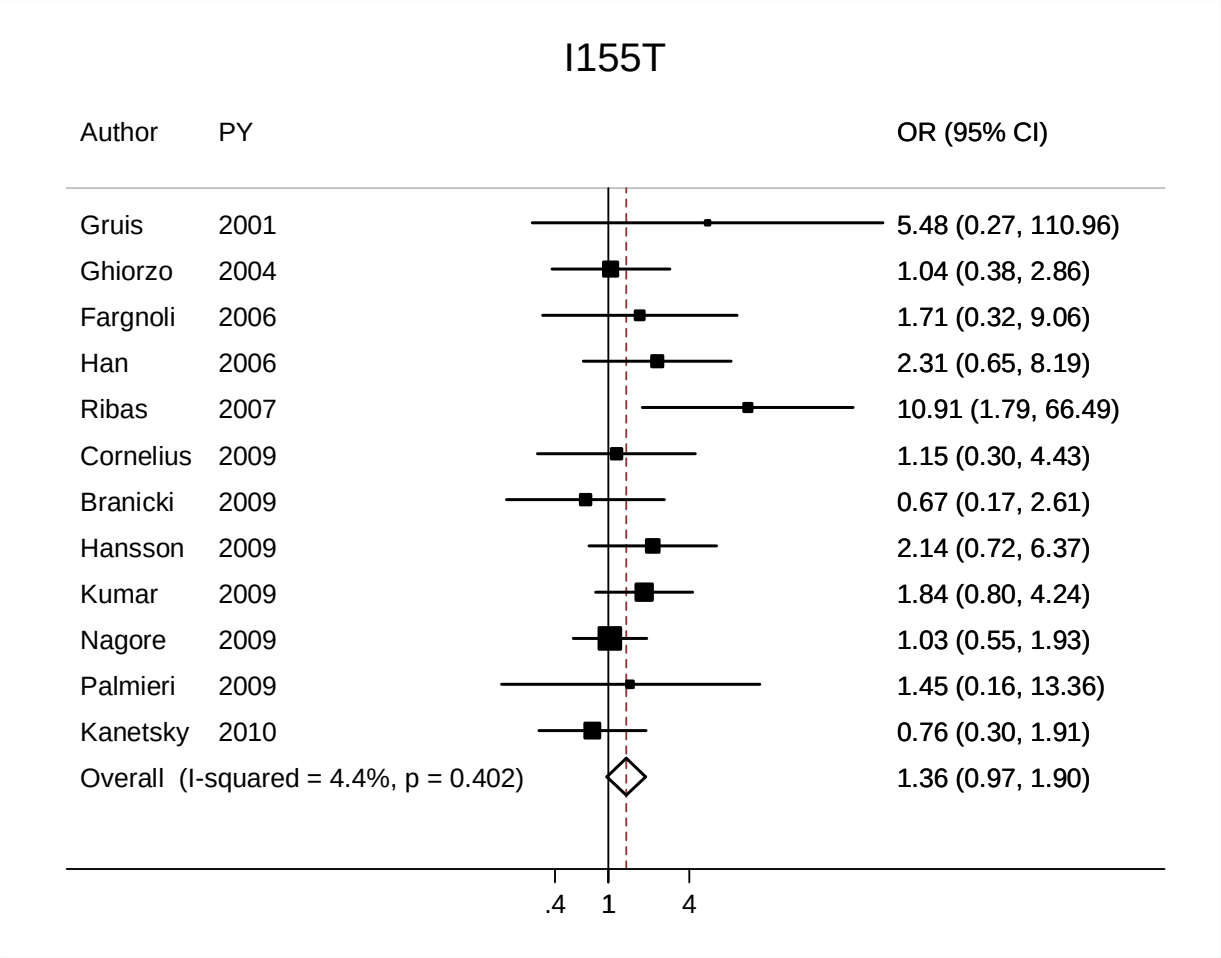


Figure 3.7. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* R160W variant and melanoma

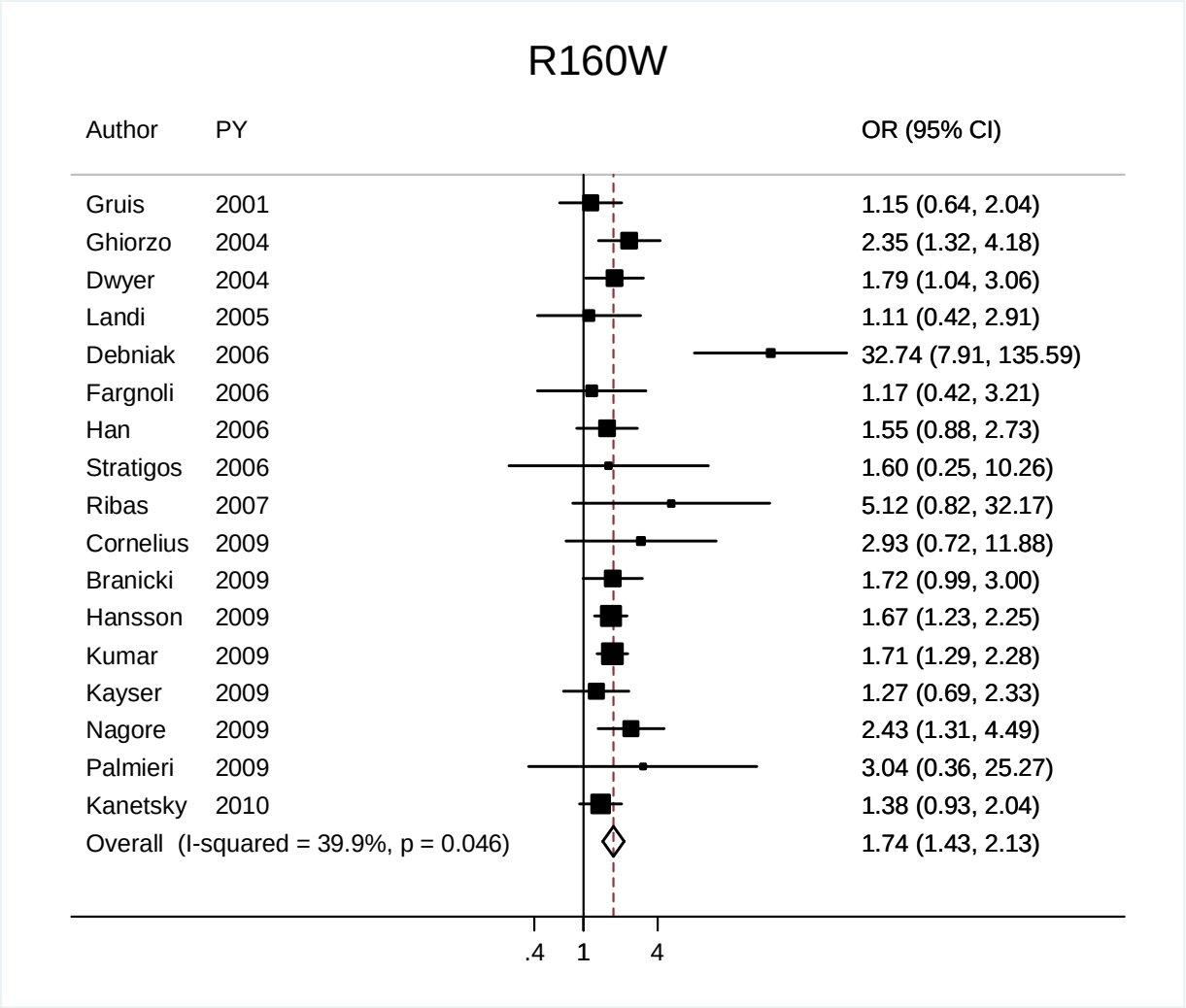


Figure 3.8. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* R163Q variant and melanoma

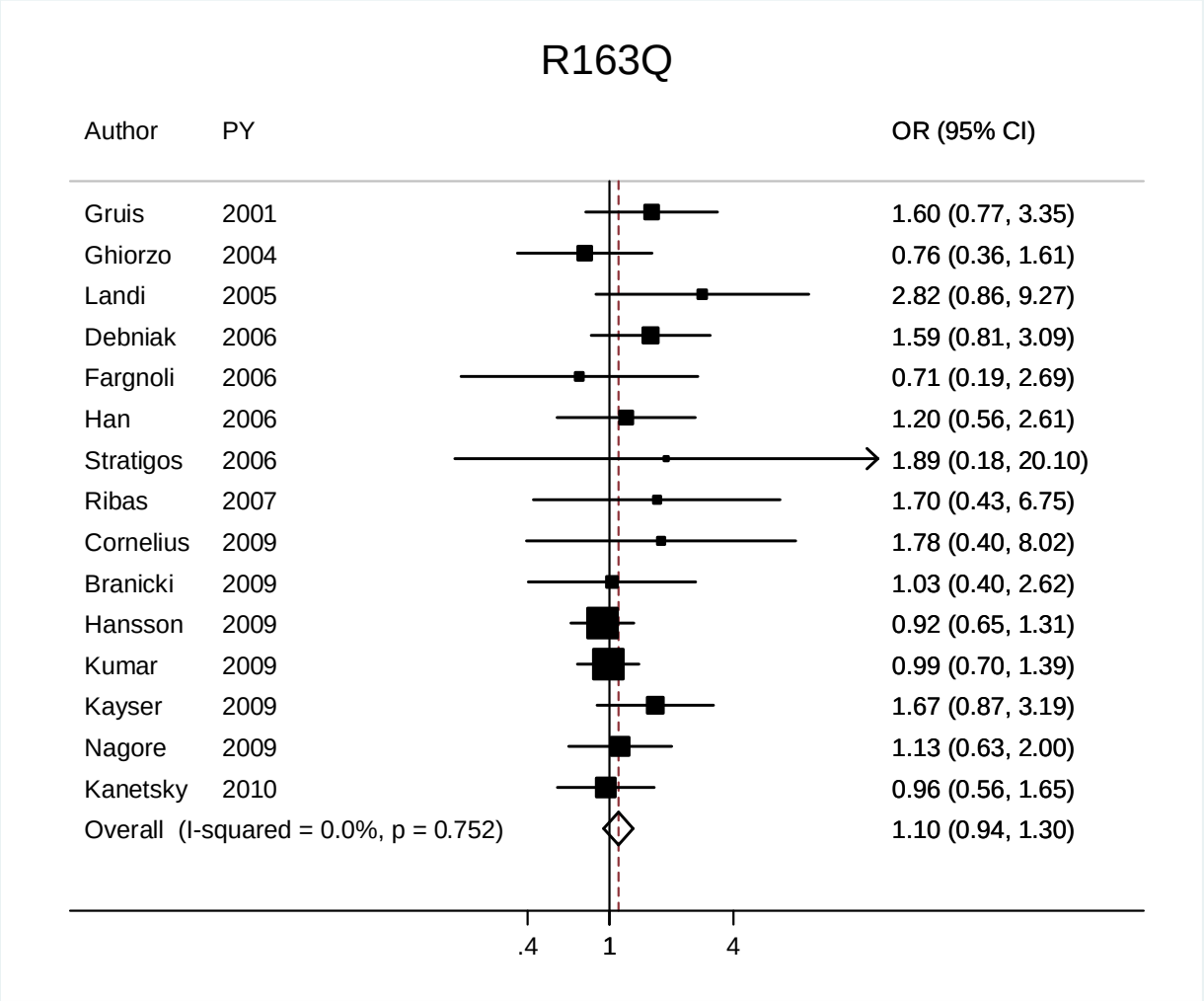
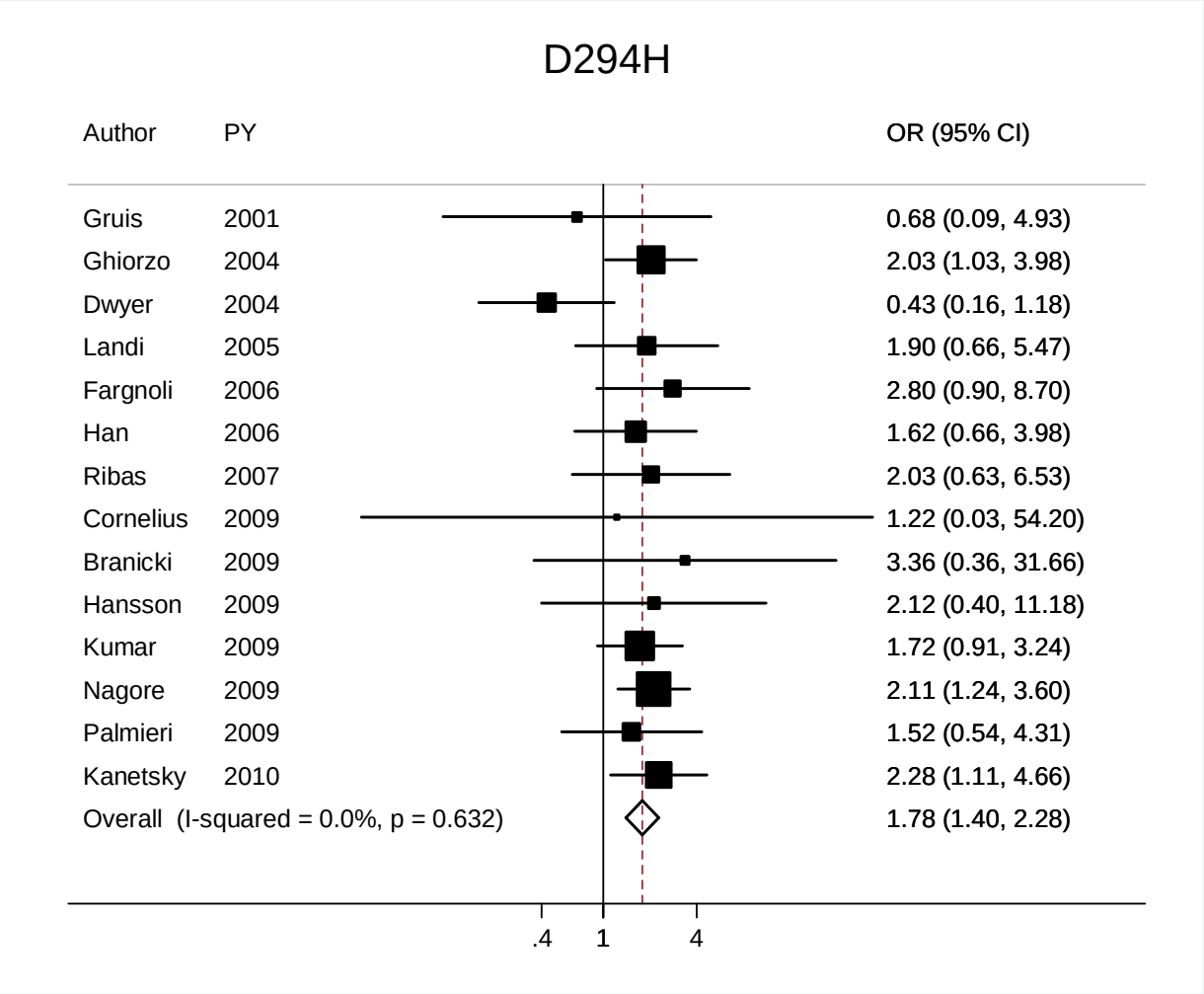


Figure 3.9. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between *MC1R* D294H variant and melanoma



3.3. Heterogeneous assessment of confounders

While performing pooled- and meta-analyses, one of the most common problems is to deal with heterogeneity among studies. The availability of individual data in pooled-analyses gives the opportunity to study in details the variability of the study estimates according with several possible stratification variables, and to take into account confounders of the studied association. However, the availability of confounders generally vary between studies, so that each study provides a different set of variables that may be used to obtain adjusted risk estimates. This way the risk

estimates obtained by different studies may not be comparable. In order to overcome this problem, I studied, validated and applied a method recently proposed [Jackson et al. 2009] to use information from all the available studies while adjusting for all the potential confounders. The method is based on a bivariate random-effects meta-analysis model previously described [van Houwelingen et al. 2002; Riley et al. 2007a; Riley et al. 2007b].

The present sub-chapter is set out as follows. In section 3.3.1 I will present the rationale of the proposed method, I will introduce the basics of a standard bivariate random effects meta-analysis, and I will describe the Jacksonôs method along with the procedure used to estimate the within-study correlations. In section 3.3.2 I will apply the proposed method to a simplified M-SKIP dataset. In section 3.3.3 I will compare the results obtained in section 3.3.2 with those obtained with the two-stage analysis above described and with two alternative and commonly used approaches: the application of a univariate meta-analysis to (1) a subset of studies with full confounder information and (2) all the studies but excluding some main confounders. In section 3.3.4 I will extend the application of the proposed method to the more complex case of the original M-SKIP data. Finally, I will discuss in section 3.3.5 strengths and possible pitfalls of the presented method, and I will comment the most important results for the M-SKIP Project.

3.3.1. Bivariate meta-analysis approach to overcome the problem of missing confounders in pooled-analysis of observational studies

Results from observational studies are susceptible to the distorting influence of confounding variables that, unless they are properly adjusted for, can result in biasing the study association of interest. Although pooled-analyses of individual data avoids many typical difficulties of meta-analyses, one problem generally remains: it is unlikely that all studies provide information on the same set of potential confounders. In such situations, two simple approaches are commonly used:

either include in the analysis only those studies that provide full details of a set of potential confounders, or use all the available studies but omit some potentially important confounders. The first approach discards information and results in an inevitable loss of precision, while the second one may omit important confounders and therefore be misleading.

In 2009, Jackson et al. [Jackson et al. 2009] proposed a method to use the relationship between fully and partially adjusted estimates to make inferences about the fully adjusted effect, by including all the available studies in the analysis. Studies that provided full details of all potential confounders were used to obtain both fully and partially adjusted estimates, and hence were used to ascertain the nature of the association between the two, while those that provided only a subset of confounders were used to provide partially adjusted estimates alone. The authors proposed to perform a joint model for the fully and partially adjusted estimates by using a standard bivariate random effects model for meta-analysis [van Houwelingen et al. 2002; Riley et al. 2007a; Riley et al. 2007b].

Bivariate random effects approach for meta-analyses

A bivariate meta-analitic approach may be used when the parameter of interest is bivariate, for instance when there are two outcome variables. In this situation it is common to perform two separate univariate meta-analyses, one for each outcome. However, when there are two outcomes likely to be correlated (i.e. disease-free and overall survival), performing a separate meta-analysis for each outcome ignores such correlation. In contrast a bivariate meta-analysis model utilizes the correlation and jointly synthesizes the outcomes to estimate the two pooled effects simultaneously. It was previously demonstrated and discussed that a bivariate random effects meta-analysis is preferable to two normal univariate random effects meta-analyses, especially when some outcome data are missing at random [Riley et al. 2007a; Riley et al. 2007b]. A simple bivariate model for any

observed pair of outcome measures $\omega_i = (\hat{\theta}_{A,i}, \hat{\theta}_{B,i})$ with standard errors ($S_{A,i}$, $S_{B,i}$) and covariance $S_{AB,i}$ in study *i* is:

$$\begin{pmatrix} \hat{\theta}_{A,i} \\ \hat{\theta}_{B,i} \end{pmatrix} \sim N\left(\begin{pmatrix} \omega_{A,i} \\ \omega_{B,i} \end{pmatrix}, \begin{pmatrix} S_{A,i}^2 & S_{AB,i} \\ S_{AB,i} & S_{B,i}^2 \end{pmatrix} \right) \quad (i=1,...,N) \tag{3.7}$$

where $\omega_i = (\omega_{A,i}, \omega_{B,i})$ is the pair of the true outcomes for study *i*. The mixed model approach assumes the pair $(\omega_{A,i}, \omega_{B,i})$ to follow a bivariate normal distribution, where the true outcome measures in the studies are normally distributed around some common mean study outcomes with a between-study covariance matrix Σ:

$$\begin{pmatrix} \omega_{A,i} \\ \omega_{B,i} \end{pmatrix} \sim N\left(\begin{pmatrix} \omega_A \\ \omega_B \end{pmatrix}, \begin{pmatrix} \Sigma_{AA} & \Sigma_{AB} \\ \Sigma_{AB} & \Sigma_{BB} \end{pmatrix} \right) \tag{3.8}$$

Σ_{AA} and Σ_{BB} describe the variability among studies in the true outcome estimates, while Σ_{AB} is the covariance between the two true outcome estimates.

The resulting marginal model is:

$$\begin{pmatrix} \hat{\theta}_{A,i} \\ \hat{\theta}_{B,i} \end{pmatrix} \sim N\left(\begin{pmatrix} \omega_A \\ \omega_B \end{pmatrix}, \Sigma + C_i \right) \tag{3.9}$$

with *Ci* the covariance matrix.

Maximum likelihood estimation for this model can be then carried out, for example by using the SAS procedure Proc Mixed [van Houwelingen et al. 2002].

Bivariate model for missing confounders in pooled-analysis of observational studies

A model was developed for the simple scenario where all the studies included in a pooled-analysis provided the same set of confounders and only some studies provided all of the confounders. In the

study by Jackson et al. [Jackson et al. 2009], the assumed response was the time until event, and the proportional hazard model was used. I hereby extend the description of the method to a binary response variable with the use of the logistic regression model.

In a particular study, let X_1 denote the vector of covariates that are observed by all studies (including the covariate of particular interest) and let X_2 denote the column vector of covariates that are only observed by some studies. For each study where X_2 is observed the full logistic regression model for a binary outcome Y may be expressed as follows:

$$logit(Y = 1 | X_1, X_2) = \beta_0 + \boldsymbol{\beta}_1^f X_1 + \boldsymbol{\beta}_2 X_2 \tag{3.10}$$

For the partial model, i.e. without the covariates X_2, the model may be written as:

$$logit(Y = 1 | X_1) = \beta_0 + \boldsymbol{\beta}_1^p X_1 \tag{3.11}$$

In the full model, the superscript f of the vector $\boldsymbol{\beta}_1$ indicates that the coefficients $\boldsymbol{\beta}_1$ are calculated by taking into account all the covariates X_1 and X_2, while in the partial model the superscript p denotes quantities that are only partially adjusted, as they do not take into account the covariates X_2. Bold font is used for row vectors of parameters in these models. It is possible to obtain estimates $\hat{\boldsymbol{\beta}}_1^f, \hat{\boldsymbol{\beta}}_2$ (from the full model (3.10)) and $\hat{\boldsymbol{\beta}}_1^p$ (from the partial model (3.11)) for each study that provides details of X_2. For those studies that do not provide details of X_2, it is only possible to obtain the corresponding estimate from model (3.11).

Let the first entry in X_1 denote the covariate of particular interest. We are therefore interested only in inference regarding the first parameter in the vectors $\hat{\boldsymbol{\beta}}_1^f$ and $\hat{\boldsymbol{\beta}}_1^p$, denoted as $\hat{\beta}_1^f$ and $\hat{\beta}_1^p$, while the others represent potential confounders.

For any given study it is assumed that:

$$\begin{pmatrix} \hat{\beta}_1^f \\ \hat{\beta}_1^p \end{pmatrix} \sim N\left(\begin{pmatrix} \beta_1^f \\ \beta_1^p \end{pmatrix}, \begin{pmatrix} \hat{\sigma}_1^2 & \rho \hat{\sigma}_1 \hat{\sigma}_2 \\ \rho \hat{\sigma}_1 \hat{\sigma}_2 & \hat{\sigma}_2^2 \end{pmatrix} \right) \tag{3.12}$$

where $\hat{\sigma}_1^2$, $\hat{\sigma}_2^2$ and ρ are assumed to be fixed and known, a conventional assumption when using bivariate models in meta-analysis and a generalization of assuming that the within-study variances are fixed and known in more usual univariate analyses. In practice, while the within-study variances are easily estimable, the difficulty lies in estimating ρ. The underlying β_1^f and β_1^p may vary from study to study. This variation can be modeled as:

$$\begin{pmatrix} \beta_1^f \\ \beta_1^p \end{pmatrix} \sim N\left(\begin{pmatrix} \beta_f \\ \beta_p \end{pmatrix}, \begin{pmatrix} \tilde{\sigma}_1^2 & k\tilde{\sigma}_1\tau_2 \\ k\tilde{\sigma}_1\tau_2 & \tilde{\sigma}_2^2 \end{pmatrix} \right) \tag{3.13}$$

providing the marginal bivariate normal distribution for the study in question as:

$$\begin{pmatrix} \hat{\beta}_1^f \\ \hat{\beta}_1^p \end{pmatrix} \sim N\left(\begin{pmatrix} \beta_f \\ \beta_p \end{pmatrix}, \begin{pmatrix} \hat{\sigma}_1^2 + \tilde{\sigma}_1^2 & \rho \hat{\sigma}_1 \hat{\sigma}_2 + k\tilde{\sigma}_1\tau_2 \\ \rho \hat{\sigma}_1 \hat{\sigma}_2 + k\tilde{\sigma}_1\tau_2 & \hat{\sigma}_2^2 + \tilde{\sigma}_2^2 \end{pmatrix} \right) \tag{3.14}$$

Equation (3.14) is simply the standard bivariate model for meta-analysis, where the two outcomes are the partially and fully adjusted effects. This is an innovative use of the standard model, as more usually the outcomes are not defined so similarly.

Although the studies that fail to report X_2 do not provide direct evidence relating to β_f, they provide indirect information via their partially adjusted estimates and their assumed association with the fully adjusted estimates. The bivariate random-effects model therefore allows inferences concerning the fully adjusted effect to borrow strength from studies where fully adjusted estimates are unavailable. Missing estimates are not imputed in this procedure, but the relationship between the fully and partially adjusted estimates, for the studies where both estimates are available, is assumed to apply to those where only partially adjusted estimates can be obtained.

Once the fully and partially adjusted estimates have been obtained, the methodology becomes a fairly standard application of the bivariate random-effects model for meta-analysis, but with one difficulty: the within-study correlations are assumed known but need to be estimated. A nonparametric bootstrap estimate seems the best choice for situations where it is computationally feasible [Jackson et al. 2009] and it is here described.

A nonparametric bootstrap estimate of ψ

Nonparametric bootstrapping [Efron et al. 1994] is probably the simplest, but slowest, procedure for obtaining an estimate of ψ. For each study that provides details of X_2, participants can be sampled with replacement providing a bootstrap sample, where for each sampled individual all the collected variables are recorded. For each bootstrap sample, the ordered pair of estimates $\hat{\beta}_1^{f*}$ and $\hat{\beta}_1^{p*}$ provide the required bootstrap replication. The estimate ψ is obtained as their sample correlation.

The algorithm used to obtain ψ may be summarized in three simple steps as follows:

1) for each study, create a number (i.e. 500) of bootstrap samples with replacement, with the same sample size of the original study;

2) for each bootstrap sample *k*, obtain the fully adjusted coefficient $\hat{\beta}_{1,k}^{f}$ and the partially adjusted coefficient $\hat{\beta}_{1,k}^{p}$ for the covariate of interest, by fitting two different logistic regression models including the full and the partial set of confounders, respectively;

3) merge the two vectors $\hat{\beta}_1^{f}$ and $\hat{\beta}_1^{p}$ in a single dataset and calculate their Pearson correlation coefficient ψ .

Two analytic methods to estimate ψ where proposed and described elsewhere [Jackson et al. 2009].

3.3.2. Simple analysis of the M-SKIP data

The Jacksonôs method described above [Jackson et al. 2009] was proposed and applied to a pooled-analysis of cohort studies with time-to-event as response variable, and Cox proportional hazard models were therefore used in the analysis. In order to validate the proposed methodology to my pooled-analysis with a binary response variable and logistic regression models, I first applied an adaptation of the proposed method to a simplified dataset, including only age as possible confounding variable. The role of other possible confounders will be assessed and taken into account in section 3.3.4.

I assumed I was interested in age-adjusted estimate of the association between each of the nine *MC1R* variants with melanoma: this represented the ñfully adjusted estimateò in the model described in section 3.3.2. Using the same notation as above, here X_1 was the *MC1R* variant of interest and X_2 was the variable age. Since all the 17 studies reported information on age, it was possible to calculate for each *MC1R* variant the ñtrueò age-adjusted estimate, which represented the ñgold standardò (GS). I calculated it by using a two-stage analysis as described in the sub-chapter 3.2 [Stukel et al. 2001]: first, I calculated study-specific age-adjusted estimates, and then I pooled them by using a standard univariate random effects meta-analysis approach with maximum likelihood estimate [van Houwelingen et al. 2002]. Study-specific estimates and their corresponding standard errors were calculated by unconditional logistic regression models. Starting values for the estimates of the between-study variance were searched into the range 0.01 to 2.00, by 0.01, as previously suggested [van Houwelingen et al. 2002].

In order to validate the proposed Jacksonôs method, I then assumed, for each *MC1R* variant, that some randomly selected studies did not include information on age. Therefore, for each *MC1R* variant, I had *m* studies for which only crude estimates could be calculated, and *N-m* studies for which both age-adjusted and crude estimates could be obtained, where *N* is the overall number of

studies included in the analysis for each *MC1R* variant, with N_{max}=17. The pooled age-adjusted estimates were calculated by using the Jacksonôs bivariate random-effects meta-analysis above described, and were then compared with the GS estimates previously calculated. The within-study correlations between age-adjusted and crude estimates were calculated with the nonparametric bootstrap estimate described in section 3.3.1, with 500 bootstrap samples. For computationally reasons, the range of starting values for the estimate of the between-study variances and covariance was restricted from 0.01 to 0.06, by 0.01. This range was chosen in order to be consistent with the between-study variances estimated in the corresponding univariate model, and assuming a covariance *k*=1.00, as suggested [Riley et al. 2007b, Jackson et al. 2009] .

For the analysis of *MC1R* variants I assumed a dominant model, as previously discussed (section 3.1.2). Therefore, for each *MC1R* variant, subjects with at least one variant allele were compared to subjects with no variant alleles.

Results

The allele frequency calculated on the all the controls in the M-SKIP database was: 10.6% for V60L, 0.4% for D84E, 7.6% for V92M, 0.6% for R142H, 5.6% for R151C, 0.9% for I155T, 6.8% for R160W, 4.8% for R163Q, and 1.4% for D294H.

Table 3.3 reports the study-specific age-adjusted coefficients with their corresponding within-study standard errors for the association between each *MC1R* variant and melanoma development, and the pooled age-adjusted estimates, which represents the GS for the following analyses. Moreover, for each variant, the calculated pooled age-adjusted OR with its 95%CI is reported in Table 3.4. All the calculated ORs were higher than 1.00; a significant increase in melanoma risk was observed for carriers of the four *MC1R* variants D84E (OR; 95%CI: 2.21; 1.40-3.51), R151C (OR; 95%CI: 1.72; 1.44-2.04), R160W (OR; 95%CI: 1.71; 1.48-1.98), and D294H (OR; 95%CI: 1.90; 1.46-2.48).

Pooled estimates with the lowest standard errors were those obtained for V92M (ů=0.066), R160W (ů=0.068), V60L (ů=0.074), R151C (ů=0.082), and R163Q (ů=0.082), while higher standard errors was observed for the four rare variants (allele frequency<2%) R142H (ů=0.296), D84E (ů=0.207), I155T (ů=0.164), and D294H (ů=0.122).

Table 3.3. Estimated study-specific age-adjusted coefficients $ɓ_i$ for the association between each *MC1R* variant and melanoma development, with their corresponding standard errors $ů_i$, and pooled age-adjusted estimates. This analysis represents the Gold Standard because pooled estimates were calculated by using all the available study-specific age-adjusted estimates

Author	MC1R variants																	
	V60L		D84E		V92M		R142H		R151C		I155T		R160W		R163Q		D294H	
	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů	ɓ	ů
Gruis	0.235	0.290	2.028	0.646	0.353	0.300	0.356	0.865	0.354	0.335	0.928	1.466	0.324	0.265	0.498	0.350	-0.051	0.934
Dwyer	-0.457	0.275	0.714	0.641	-	-	-	-	-0.304	0.283	-	-	0.535	0.268	-	-	-0.846	0.508
Ghiorzo	-0.110	0.169	0.401	0.676	0.375	0.245	1.549	0.597	0.714	0.246	0.105	0.523	0.801	0.293	-0.366	0.381	0.770	0.353
Landi	0.218	0.238	-	-	-0.339	0.395	1.095	0.827	1.165	0.320	-	-	0.125	0.489	0.979	0.604	0.705	0.523
Debniak	0.128	0.224	-	-	-	-	-	-	0.328	0.255	-	-	4.118	1.012	0.431	0.332	-	-
Fargnoli	-0.357	0.232	1.149	1.160	0.253	0.369	0.171	0.500	1.057	0.436	0.331	0.773	0.388	0.480	-0.309	0.597	0.970	0.545
Han	0.236	0.239	-	-	0.201	0.251	-	-	0.907	0.298	1.072	0.640	0.492	0.282	0.264	0.390	0.525	0.447
Stratigos	0.955	0.293	-	-	0.149	0.482	1.542	0.838	1.050	0.579	-	-	1.012	0.781	0.183	1.051	-	-
Ribas	0.621	0.263	1.251	1.233	0.804	0.415	-0.407	0.690	0.114	0.546	1.978	0.803	1.655	0.830	0.792	0.619	1.430	0.511
Branicki	0.024	0.298	2.088	1.324	0.005	0.291	0.092	0.653	0.813	0.335	-0.404	0.691	0.547	0.281	0.038	0.474	1.102	1.171
Cornelius	0.197	0.550	-0.042	1.194	0.234	0.598	0.581	1.086	-0.561	0.773	-0.743	1.170	0.770	0.673	0.676	0.715	1.136	1.338
Hansson	0.403	0.185	0.431	0.436	0.288	0.181	1.524	1.113	0.438	0.164	0.575	0.556	0.513	0.152	-0.055	0.177	0.860	0.840
Kayser	0.263	0.304	-	-	0.217	0.194	1.250	0.731	0.031	0.379	-	-	0.031	0.379	0.510	0.332	-	-
Kumar	-0.080	0.135	1.173	0.441	-0.003	0.136	1.186	0.347	0.636	0.149	0.591	0.425	0.550	0.147	-0.037	0.176	0.523	0.323
Nagore	0.287	0.129	1.044	0.779	-	-	0.779	0.398	0.405	0.239	0.031	0.323	0.833	0.310	0.105	0.285	0.710	0.267
Palmieri	0.006	0.294	-	-	-0.561	0.635	-0.302	0.726	1.187	1.059	0.282	1.118	1.452	1.068	-	-	0.484	0.529
Kanetsky	-0.102	0.150	0.002	0.493	-0.071	0.172	-0.673	0.455	0.669	0.202	-0.178	0.438	0.424	0.190	0.054	0.257	0.850	0.351
POOLED	**0.122**	**0.074**	**0.795**	**0.207**	**0.129**	**0.066**	**0.366**	**0.296**	**0.540**	**0.082**	**0.281**	**0.164**	**0.539**	**0.068**	**0.112**	**0.082**	**0.643**	**0.122**

Table 3.4. Pooled age-adjusted Odds Ratio (OR) with 95% Confidence Intervals (CI) for the studied *MC1R* variants. This analysis represents the Gold Standard because pooled ORs were calculated by using all the available study-specific age-adjusted estimates

***MC1R* variant**	**N studies**	**OR (95%CI)**
V60L	17	1.13 (0.97-1.32)
D84E	**11**	**2.21 (1.40-3.51)**
V92M	14	1.14 (0.99-1.31)
R142H	14	1.44 (0.76-2.73)
R151C	**17**	**1.72 (1.44-2.04)**
I155T	12	1.32 (0.92-1.90)
R160W	**17**	**1.71 (1.48-1.98)**
R163Q	15	1.12 (0.94-1.34)
D294H	**14**	**1.90 (1.46-2.48)**

Note: significant results are in bold.

Tables 3.5 to 3.13 respectively report for each *MC1R* variant: the study-specific age-adjusted coefficients with their corresponding within-study standard errors for a number of *N-m* studies which I assumed to provide information on age, the study-specific crude coefficients with their corresponding within-study standard errors for all the *N* studies, and the bootstrap correlation coefficient ρ for the correlation between age-adjusted and crude estimates for the *N-m* studies where both estimates could be calculated. Moreover the same tables present the pooled age-adjusted and crude estimates obtained by applying the Jacksonôs approach previously described in section 3.3.1. For each variant, the calculated pooled age-adjusted OR with its 95%CI is reported in Table 13. For an immediate comparison, Table 3.14 also contains again the results obtained with the GS analysis. As for the GS analysis, all the calculated ORs were higher than 1.00; a significant increase in melanoma risk was again observed for carriers of the four *MC1R* variants D84E (OR; 95%CI: 2.20; 1.24-3.90), R151C (OR; 95%CI: 1.66; 1.38-1.99), R160W (OR; 95%CI: 1.73; 1.49-1.99), and D294H (OR; 95%CI: 1.69; 1.22-2.36), with similar ORs than the GS analysis, but wider 95%CI. Moreover, a significant increase in melanoma risk was also observed for the two *MC1R* variants I155T (OR; 95%CI: 1.58; 1.09-2.28) and R163Q (OR; 95%CI: 1.19; 1.00-1.42), which on the contrary were not found to be significantly associated with melanoma in the GS analysis. Pooled

estimates with the lowest standard errors were those obtained for V92M (ů=0.071), R160W (ů=0.074), R163Q (ů=0.089), and R151C (ů=0.092), while higher standard errors was observed for the rare variants R142H (ů=0.394), D84E (ů=0.291), I155T (ů=0.188), and D294H (ů=0.169), but also for the common V60L variant (ů=0.161). All the standard errors obtained with the Jacksonôs method were larger than the corresponding ones obtained with the GS analysis.

Table 3.5. Estimated study-specific age-adjusted and crude coefficients $б_{i(adj)}$ and $б_{i(crude)}$ for the association between V60L variant and melanoma development, with their corresponding standard errors $ů_{i(adj)}$ and $ů_{i(crude)}$, estimated bootstrap correlation coefficients $Ɉ_i$, and pooled estimates calculated with the Jacksonôs method

Author	$б_{i(adj)}$	$ů_{i(adj)}$	$б_{i(crude)}$	$ů_{i(crude)}$	$Ɉ_i$
Gruis	0.235	0.290	0.262	0.272	0.935
Dwyer	-0.457	0.275	-0.457	0.275	0.999
Ghiorzo	-0.110	0.169	-0.112	0.166	0.983
Landi			0.219	0.236	
Debniak	0.128	0.224	0.082	0.217	0.964
Fargnoli	-0.357	0.232	-0.346	0.232	0.997
Han	0.236	0.239	0.239	0.222	0.930
Stratigos			0.836	0.275	
Ribas			0.568	0.261	
Branicki	0.024	0.298	0.247	0.273	0.926
Cornelius	0.197	0.550	0.307	0.288	0.489
Hansson	0.403	0.185	0.445	0.174	0.938
Kayser			0.266	0.304	
Kumar			-0.069	0.133	
Nagore			0.184	0.116	
Palmieri			-0.006	0.278	
Kanetsky	-0.102	0.150	-0.098	0.149	0.999
POOLED	**0.148**	**0.161**	**0.149**	**0.159**	

Table 3.6. Estimated study-specific age-adjusted and crude coefficients $b_{i(adj)}$ and $b_{i(crude)}$ for the association between D84E variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients r_i, and pooled estimates calculated with the Jacksonôs method

Author	$b_{i(adj)}$	$s_{i(adj)}$	$b_{i(crude)}$	$s_{i(crude)}$	r_i
Gruis	2.028	0.646	1.576	0.596	0.989
Dwyer	0.714	0.641	0.712	0.641	1.000
Ghiorzo	0.401	0.676	0.474	0.675	1.000
Fargnoli	1.149	1.160	1.157	1.160	1.000
Ribas			1.260	1.231	
Branicki	2.088	1.324	2.147	1.229	0.999
Cornelius	-0.042	1.194	0.718	0.720	0.887
Hansson			0.582	0.423	
Kumar			1.113	0.437	
Nagore			1.267	0.761	
Kanetsky			-0.014	0.493	
POOLED	**0.789**	**0.291**	**0.801**	**0.182**	

Table 3.7. Estimated study-specific age-adjusted and crude coefficients $b_{i(adj)}$ and $b_{i(crude)}$ for the association between V92M variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients r_i, and pooled estimates calculated with the Jacksonôs method

Author	$b_{i(adj)}$	$s_{i(adj)}$	$b_{i(crude)}$	$s_{i(crude)}$	r_i
Gruis	0.353	0.300	0.394	0.280	0.932
Ghiorzo	0.375	0.245	0.427	0.243	0.991
Landi			-0.342	0.394	
Fargnoli	0.253	0.369	0.259	0.369	0.997
Han	0.201	0.251	0.082	0.232	0.936
Stratigos			0.053	0.446	
Ribas			0.781	0.414	
Branicki	0.005	0.291	0.007	0.268	0.927
Cornelius	0.234	0.598	-0.349	0.397	0.661
Hansson	0.288	0.181	0.232	0.169	0.936
Kayser			0.109	0.175	
Kumar			0.025	0.135	
Palmieri			-0.239	0.599	
Kanetsky	-0.071	0.172	-0.070	0.172	0.999
POOLED	**0.105**	**0.071**	**0.107**	**0.071**	

Table 3.8. Estimated study-specific age-adjusted and crude coefficients $b_{i(adj)}$ and $b_{i(crude)}$ for the association between R142H variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients r_i, and pooled estimates calculated with the Jacksonôs method

Author	$b_{i(adj)}$	$s_{i(adj)}$	$b_{i(crude)}$	$s_{i(crude)}$	r_i
Gruis	0.356	0.865	0.093	0.824	0.999
Ghiorzo	1.549	0.597	1.230	0.522	0.998
Landi			1.160	0.824	
Fargnoli	0.171	0.500	0.178	0.499	0.999
Stratigos			1.530	0.811	
Ribas			-0.442	0.688	
Branicki	0.092	0.653	0.268	0.581	0.988
Cornelius	0.581	1.086	0.294	0.923	0.982
Hansson	1.524	1.113	1.451	1.082	0.999
Kayser			1.227	0.730	
Kumar	1.186	0.347	1.092	0.343	0.990
Nagore			0.875	0.374	
Palmieri			-0.406	0.703	
Kanetsky	-0.673	0.455	-0.675	0.454	0.999
POOLED	**0.097**	**0.394**	**0.571**	**0.241**	

Table 3.9. Estimated study-specific age-adjusted and crude coefficients $b_{i(adj)}$ and $b_{i(crude)}$ for the association between R151C variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients r_i, and pooled estimates calculated with the Jacksonôs method

Author	$b_{i(adj)}$	$s_{i(adj)}$	$b_{i(crude)}$	$s_{i(crude)}$	r_i
Gruis	0.354	0.335	0.500	0.316	0.939
Dwyer	-0.304	0.283	-0.304	0.283	0.998
Ghiorzo	0.714	0.246	0.720	0.241	0.939
Landi			1.164	0.318	
Debniak	0.328	0.255	0.334	0.250	0.978
Fargnoli	1.057	0.436	1.054	0.435	0.996
Han	0.907	0.298	0.974	0.278	0.934
Stratigos			1.081	0.554	
Ribas			0.321	0.519	
Branicki	0.813	0.335	0.411	0.302	0.911
Cornelius	-0.561	0.773	-0.326	0.383	0.946
Hansson	0.438	0.164	0.527	0.154	0.927
Kayser			0.031	0.379	
Kumar			0.561	0.146	
Nagore			0.532	0.217	
Palmieri			1.584	1.039	
Kanetsky	0.669	0.202	0.655	0.201	0.997
POOLED	**0.506**	**0.092**	**0.503**	**0.092**	

Table 3.10. Estimated study-specific age-adjusted and crude coefficients $\beta_{i(adj)}$ and $\beta_{i(crude)}$ for the association between I155T variant and melanoma development, with their corresponding standard errors $\sigma_{i(adj)}$ and $\sigma_{i(crude)}$, estimated bootstrap correlation coefficients ρ_i, and pooled estimates calculated with the Jacksonôs method

Author	$\beta_{i(adj)}$	$\sigma_{i(adj)}$	$\beta_{i(crude)}$	$\sigma_{i(crude)}$	ρ_i
Gruis	0.928	1.466	1.196	1.418	1.000
Ghiorzo	0.105	0.523	0.087	0.513	1.000
Fargnoli	0.331	0.773	0.340	0.772	1.000
Han	1.072	0.640	0.830	0.620	0.998
Ribas			2.250	0.785	
Branicki	-0.404	0.691	-0.176	0.641	0.996
Cornelius	-0.743	1.170	0.536	0.621	0.992
Hansson	0.575	0.556	0.692	0.524	0.990
Kumar			0.651	0.421	
Nagore			0.109	0.285	
Palmieri			0.376	1.103	
Kanetsky			-0.172	0.438	
POOLED	**0.456**	**0.188**	**0.556**	**0.192**	

Table 3.11. Estimated study-specific age-adjusted and crude coefficients $\beta_{i(adj)}$ and $\beta_{i(crude)}$ for the association between R160W variant and melanoma development, with their corresponding standard errors $\sigma_{i(adj)}$ and $\sigma_{i(crude)}$, estimated bootstrap correlation coefficients ρ_i, and pooled estimates calculated with the Jacksonôs method

Author	$\beta_{i(adj)}$	$\sigma_{i(adj)}$	$\beta_{i(crude)}$	$\sigma_{i(crude)}$	ρ_i
Gruis	0.324	0.265	0.355	0.249	0.931
Dwyer	0.535	0.268	0.533	0.268	0.998
Ghiorzo	0.801	0.293	0.830	0.289	0.937
Landi			0.038	0.485	
Debniak	4.117	1.012	3.495	0.723	0.508
Fargnoli	0.387	0.480	0.392	0.479	0.996
Stratigos			0.764	0.741	
Ribas			1.699	0.826	
Branicki	0.546	0.281	0.451	0.255	0.898
Han	0.492	0.282	0.508	0.260	0.925
Cornelius	0.770	0.673	1.024	1.170	0.841
Hansson	0.513	0.152	0.485	0.142	0.939
Kayser			0.031	0.379	
Kumar			0.556	0.145	
Nagore			0.791	0.285	
Palmieri			1.278	1.049	
Kanetsky	0.424	0.190	0.423	0.190	0.999
POOLED	**0.546**	**0.074**	**0.544**	**0.074**	

Table 3.12. Estimated study-specific age-adjusted and crude coefficients $\beta_{i(adj)}$ and $\beta_{i(crude)}$ for the association between R163Q variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients ρ_i, and pooled estimates calculated with the Jacksonôs method

Author	$\beta_{i(adj)}$	$s_{i(adj)}$	$\beta_{i(crude)}$	$s_{i(crude)}$	ρ_i
Gruis	0.498	0.350	0.354	0.328	0.932
Ghiorzo	-0.366	0.381	-0.317	0.378	0.934
Landi			0.990	0.602	
Debniak	0.431	0.332	0.510	0.332	0.998
Fargnoli	-0.309	0.597	-0.303	0.597	1.000
Han	0.264	0.390	0.537	0.359	0.932
Stratigos			0.235	1.007	
Ribas			0.931	0.599	
Branicki	0.038	0.474	0.056	0.436	0.935
Cornelius	0.676	0.715	0.431	0.422	0.872
Hansson	-0.055	0.177	-0.165	0.166	0.937
Kayser			0.492	0.332	
Kumar			-0.032	0.175	
Nagore			-0.032	0.260	
Kanetsky	0.054	0.257	0.050	0.256	0.999
POOLED	**0.175**	**0.089**	**0.201**	**0.115**	

Table 3.13. Estimated study-specific age-adjusted and crude coefficients $\beta_{i(adj)}$ and $\beta_{i(crude)}$ for the association between D294H variant and melanoma development, with their corresponding standard errors $s_{i(adj)}$ and $s_{i(crude)}$, estimated bootstrap correlation coefficients ρ_i, and pooled estimates calculated with the Jacksonôs method

Author	$\beta_{i(adj)}$	$s_{i(adj)}$	$\beta_{i(crude)}$	$s_{i(crude)}$	ρ_i
Gruis	-0.051	0.934	0.278	0.844	0.995
Dwyer	-0.846	0.508	-0.484	0.508	1.000
Ghiorzo	0.770	0.353	0.729	0.343	0.996
Landi			0.675	0.520	
Fargnoli	0.970	0.545	0.975	0.545	1.000
Han	0.525	0.447	0.588	0.415	0.931
Ribas			1.423	0.510	
Branicki	1.102	1.171	1.045	0.919	0.988
Cornelius	1.136	1.338	1.855	0.828	1.000
Hansson	0.860	0.840	1.047	0.793	0.998
Kumar			0.502	0.319	
Nagore			0.685	0.242	
Palmieri			0.529	0.504	
Kanetsky			0.847	0.351	
POOLED	**0.527**	**0.169**	**0.771**	**0.139**	

Table 3.14. Pooled age-adjusted Odds Ratio (OR) with 95% Confidence Intervals (CI) for the studied *MC1R* variants calculated with the Jacksonôs method, and corresponding results for the GS analysis

***MC1R* variant**	**N studies**	**Jacksonôs OR (95%CI)**	**GS OR (95%CI)**
V60L	17	1.16 (0.85-1.59)	1.13 (0.97-1.32)
D84E	**11**	**2.20 (1.24-3.90)**	**2.21 (1.40-3.51)**
V92M	14	1.11 (0.97-1.28)	1.14 (0.99-1.31)
R142H	14	1.10 (0.51-2.39)	1.44 (0.76-2.73)
R151C	**17**	**1.66 (1.38-1.99)**	**1.72 (1.44-2.04)**
I155T*	**12**	**1.58 (1.09-2.28)**	1.32 (0.92-1.90)
R160W	**17**	**1.73 (1.49-1.99)**	**1.71 (1.48-1.98)**
R163Q*	**15**	**1.19 (1.00-1.42)**	1.12 (0.94-1.34)
D294H	**14**	**1.69 (1.22-2.36)**	**1.90 (1.46-2.48)**

Note: significant results are in bold.
* The significance of results for this variant is different according with the two methods.

3.3.3. Standard univariate meta-analyses on the M-SKIP simplified dataset

As previously pointed out, two possible and commonly used alternatives of the Jacksonôs method are to perform univariate random effects meta-analysis either to a subset of studies with information on the full set of confounders or to all the available studies but including only a subset of confounders. In the case of the simplified dataset previously described in section 3.3.2, this means that the two considered alternatives were:

1) to perform a univariate random effects meta-analysis on the subset of *N-m* studies with information on age;

2) to perform a univariate random-effects meta-analysis on the all the *N* studies by using only the crude estimates.

For both the analyses, study-specific estimates were calculated by unconditional logistic regression models and then pooled together, following the two-stage analysis approach [Stukel et al. 2001]. As for the GS analysis, starting values for the estimates of the between-study variance were searched in the range 0.01 to 2.00, by 0.01.

Results

Table 3.15 presents the pooled coefficients with their corresponding within-study standard errors for the association between each *MC1R* variant and melanoma development. Table 3.16 and Figure 3.1 presents, for each variant, the pooled age-adjusted OR with its 95%CI calculated on a subset of *N-m* studies, and the pooled crude OR with its 95%CI calculated on the *N* studies. For an immediate comparison, Table 3.16 and Figure 3.10 also contains again the results obtained with the GS analysis and with the Jacksonôs analysis. Moreover, Table 3.16 also includes results from a standard two-stage analysis as described in sub-chapter 3.2. This analysis pooled *N* crude study-specific estimates and *N-m* age-adjusted estimates.

Table 3.15. Estimated pooled age-adjusted and crude coefficients $\beta_{i(adj)}$ and $\beta_{i(crude)}$ for the association between the nine *MC1R* variants and melanoma development, with their corresponding standard errors $\sigma_{i(adj)}$ and $\sigma_{i(crude)}$, by using two separate univariate random-effects meta-analysis. The age-adjusted estimate was obtained for the subset of studies with information on age, the crude estimate was obtained by discarding information on age from all the studies

Variant	N (N-m) studies	$\beta_{i(adj)}$	$\sigma_{i(adj)}$	$\beta_{i(crude)}$	$\sigma_{i(crude)}$
V60L	17 (10)	0.008	0.084	0.127	0.067
D84E	11 (6)	1.051	0.334	0.815	0.189
V92M	14 (8)	0.171	0.088	0.104	0.063
R142H	14 (8)	0.111	0.510	0.531	0.201
R151C	17 (10)	0.499	0.110	0.524	0.081
I155T	12 (7)	0.312	0.264	0.355	0.153
R160W	17 (10)	0.528	0.083	0.535	0.066
R163Q	15 (9)	0.079	0.107	0.107	0.083
D294H	14 (8)	0.460	0.254	0.686	0.117

N= number of studies included in the calculation of crude estimate.
N-m= number of studies included in the calculation of age-adjusted estimate.

Table 3.16. Pooled age-adjusted and crude Odds Ratio (OR) with 95% Confidence Intervals (CI) for the studied *MC1R* variants calculated by three separate univariate random-effects meta-analysis, and corresponding results for the Jacksonôs method and for the GS analysis

MC1R variant	Univariate age-adjusted OR (95%CI)	Univariate crude OR (95%CI)	Standard two-stage OR (95%CI)	Jacksonôs OR (95%CI)	GS OR (95%CI)
V60L	1.01 (0.83-1.22)	1.14 (0.98-1.31)	1.11 (0.96-1.28)	1.16 (0.85-1.59)	1.13 (0.97-1.32)
D84E	**2.86 (1.21-6.75)**	**2.26 (1.48-3.44)**	**2.27 (1.46-3.51)**	**2.20 (1.24-3.90)**	**2.21 (1.40-3.51)**
V92M	1.19 (0.96-1.46)	1.11 (0.97-1.27)	1.13 (0.98-1.30)	1.11 (0.97-1.28)	1.14 (0.99-1.31)
R142H^	1.12 (0.33-3.73)	**1.70 (1.10-2.63)**	1.44 (0.76-2.76)	1.10 (0.51-2.39)	1.44 (0.76-2.73)
R151C	**1.65 (1.29-2.11)**	**1.69 (1.42-2.01)**	**1.72 (1.46-2.01)**	**1.66 (1.38-1.99)**	**1.72 (1.44-2.04)**
I155T*^À	1.37 (0.72-2.61)	**1.43 (1.02-2.00)**	**1.45 (1.04-2.00)**	**1.58 (1.09-2.28)**	1.32 (0.92-1.90)
R160W	**1.70 (1.40-2.05)**	**1.71 (1.49-1.96)**	**1.71 (1.48-1.97)**	**1.73 (1.49-1.99)**	**1.71 (1.48-1.98)**
R163Q*	1.08 (0.84-1.38)	1.11 (0.93-1.33)	1.11 (0.93-1.32)	**1.19 (1.00-1.42)**	1.12 (0.94-1.34)
D294H&	1.58 (0.87-1.89)	**1.99 (1.54-2.56)**	**1.89 (1.46-2.44)**	**1.69 (1.22-2.36)**	**1.90 (1.46-2.48)**

Significant results are in bold.

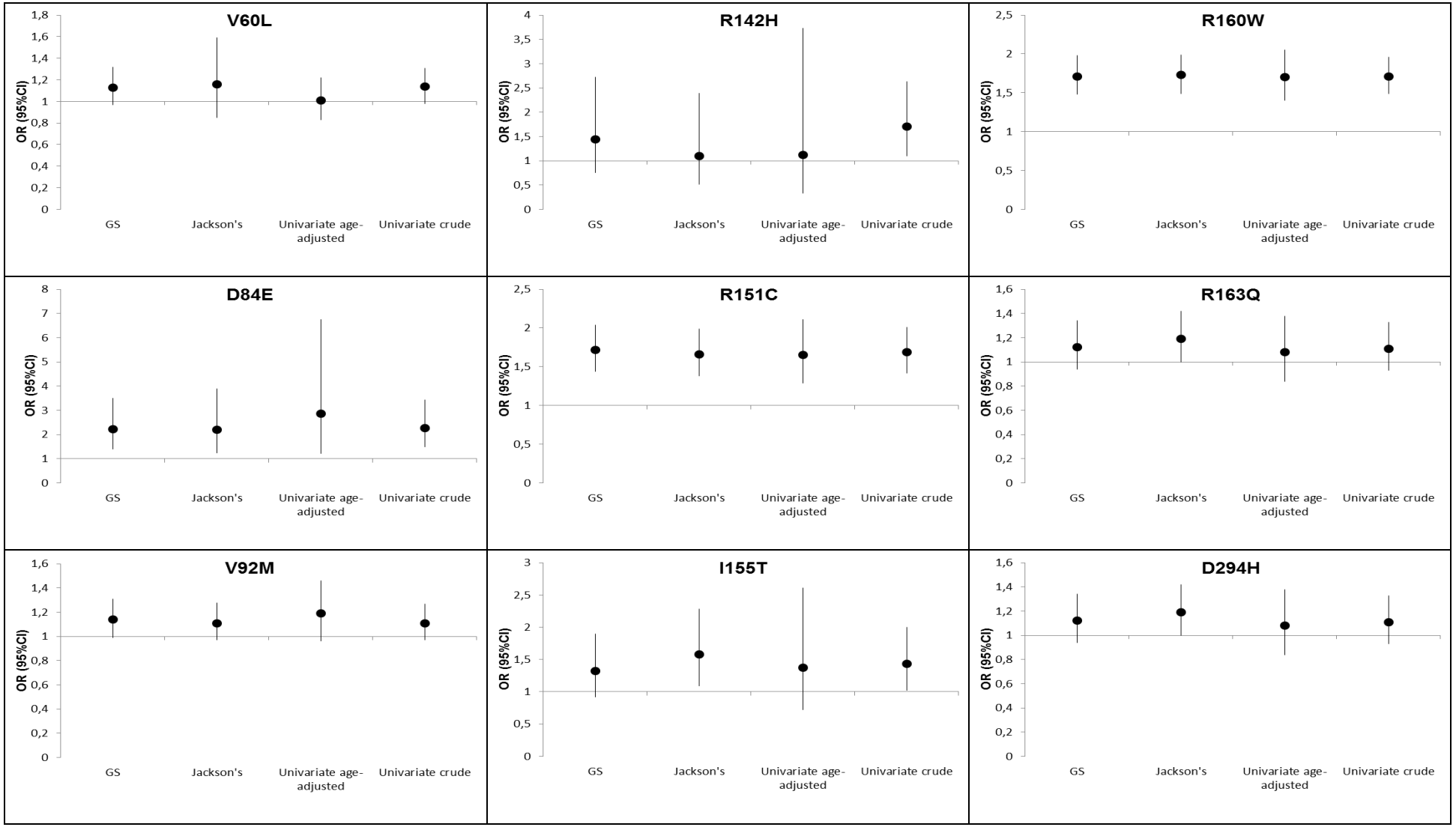
^The significance of results for this variant is different according with univariate crude and GS analysis.

*The significance of results for this variant is different according with Jacksonôs and GS analysis.

&The significance of results for this variant is different according with univariate age-adjusted and GS analysis.

ÀThe significance of results for this variant is different according with standard two-stage and GS analysis.

Figure 3.10. Pooled age-adjusted and crude Odds Ratio (OR) with 95% Confidence Intervals (CI) for the studied *MC1R* variants calculated by two separate univariate random-effects meta-analysis, and corresponding results for the Jacksonôs method and for the GS analysis



As for the GS analysis, all the calculated ORs were higher than 1.00.

Pooled age-adjusted OR indicated a significant increase in melanoma risk for carriers of the three *MC1R* variants D84E (OR; 95%CI: 2.86; 1.21-6.75), R151C (OR; 95%CI: 1.65; 1.29-2.11), R160W (OR; 95%CI: 1.70; 1.40-2.05), but not for the D294H variant, that was otherwise significantly associated with melanoma with the GS analysis.

Pooled crude OR indicated a significant increase in melanoma risk for carriers of the four *MC1R* variants D84E (OR; 95%CI: 2.26; 1.48-3.44), R151C (OR; 95%CI: 1.69; 1.42-2.01), R160W (OR; 95%CI: 1.71; 1.49-1.96), and D294H (OR; 95%CI: 1.99; 1.54-2.56). Moreover, a significant increase in melanoma risk was also observed for the two rare *MC1R* variants R142H (OR; 95%CI: 1.70, 1.10-2.63) and I155T (OR; 95%CI: 1.43; 1.02-2.00), which on the contrary were not found to be significantly associated with melanoma in the GS analysis.

Standard pooled age-adjusted estimates generally presented the wider 95%CI and therefore the highest standard errors, while standard pooled crude estimates presented similar standard errors to GS analysis. The Jacksonôs method generally produced intermediate 95%CI than standard age-adjusted and crude pooled estimate approach.

Looking at the point estimate, the standard crude estimate approach generally produced OR most similar to the GS ones, while the standard age-adjusted estimate approach often obtained the most different point estimates. As for the significance of the results, with the crude estimate and the Jacksonôs approaches, two rare variants were significantly associated with melanoma, despite they were not associated with melanoma with the GS approach; with the standard age-adjusted approach, the significant association of one variant with melanoma observed in the GS analysis was not reproduced.

Finally, the standard two-stage analysis seemed to produce the most closer results to GS analysis for both point and interval estimates in our dataset.

In conclusion, the Jacksonôs method seemed to have intermediate properties in the estimation of the ñtrueò OR, being generally more powerful than the standard age-adjusted estimate approach and more conservative that the standard crude estimate approach. The latter one generally had a good performance, probably due to the similarity between age-adjusted and crude estimates in our data.

3.3.4. Complete analysis of the M-SKIP data

Sections 33.2 presented an application of the Jacksonôs method to a simplified dataset obtained from the M-SKIP Project. In this section, I will extend the previous analyses to the complete dataset, by taking into account all the confounders available in each study. In the simple scenario described by Jackson et al. [Jackson et al. 2009], all the studies included in the pooled-analysis provided the same set of confounders and only some studies provided all of the confounders. This scenario is unlikely to happen in a real situation. More frequently, each study included in a pooled-analysis collected information on a different set of variables, providing more than two possible levels of adjustments.

In the complete M-SKIP dataset, all the studies collected information on age, gender and family history of melanoma. Some studies collected further information on other possible confounders like sun exposure (chronic and/or intermittent), sunburns (during lifetime and/or childhood) and naevi count (common and/or atypical). A summary of the available confounders is presented in Table 3.1. A strict application of a bivariate approach would force to consider as ñpartially adjusted estimatesò the 17 estimates adjusted by age, gender and family history, and as ñfully adjusted estimatesò the five estimates obtained by studies which included information on at least one confounder in each class of important confounders (sun exposure, sunburns, naevi count). With this choice, however, I would discharge information on additional confounders for five studies (Dwyer, Landi, Debniak,

Han, Ribas) which have data on more than the ñbasicò confounders, but no data on all the main classes of confounders.

In order to use all the possible available information, I decided to extend the application of the bivariate approach to a multivariate approach following described.

Statistical analysis on the complete dataset

I divided all the confounders in three classes of important confounders: sun exposure, sunburns and naevi count. I considered as ñlevel 1 adjusted estimatesò the ones adjusted by basic confounders (age, gender, family history), as ñlevel 2 adjusted estimatesò the ones adjusted by basic confounders + at least one confounder in the class of sunburns, as ñlevel 3 adjusted estimatesò the ones adjusted by basic confounders + at least one confounder in the class of sunburns and sun exposure, as ñlevel 4 adjusted estimatesò the ones adjusted by basic confounders + at least one confounder in all the three considered classes.

Using the same notation as in section 3.3.1, here X_1 is a vector including the *MC1R* variant of interest, age, gender and family history, X_2 is a vector including the variables related to sunburns, X_3 is a vector including the variables related to sun exposure, and X_4 is a vector including the variables related to naevi count.

For each study where X_1, X_2, X_3 and X_4 are observed, the full logistic regression model in (3.10) may be expressed as follows:

$$logit(Y=1|X_1,X_2,X_3,X_4) = \beta_0 + \beta_1^{L4} X_1 + \beta_2^{L4} X_2 + \beta_3^{L4} X_3 + \beta_4 X_4 \qquad (3.15)$$

where β_1^{L4}, β_2^{L4}, β_3^{L4} and β_4 are the vectors of coefficients for the fully adjusted model.

For each study where X_1, X_2, and X_3 are observed, the logistic regression model may be expressed as follows:

$$logit(Y=1|X_1,X_2,X_3)=\beta_0+\beta_1^{L3}X_1+\beta_2^{L3}X_2+\beta_3^{L3}X_3 \tag{3.16}$$

For each study where X_1 and X_2, are observed, the logistic regression model may be expressed as follows:

$$logit(Y=1|X_1,X_2)=\beta_0+\beta_1^{L2}X_1+\beta_2^{L2}X_2 \tag{3.17}$$

Finally, for each study where X_1 is observed, the logistic regression model may be expressed as follows:

$$logit(Y=1|X_1)=\beta_0+\beta_1^{L1}X_1 \tag{3.18}$$

Note that for the studies which provide information on all the possible confounders, I can estimate all the vectors $\beta_1^{Lj}, \beta_2^{Lj}, \beta_3^{Lj}$ and applying the previous models, while for studies including only basic confounders, only β_1^{L1} may be calculated.

We are interested in inferences regarding the first component of the vector β_1. For any given study with all the confounders assessed, it is assumed that:

$$\begin{pmatrix}\hat{\beta}_1^{L1}\\ \hat{\beta}_1^{L2}\\ \hat{\beta}_1^{L3}\\ \hat{\beta}_1^{L4}\end{pmatrix}\sim N\left(\begin{pmatrix}\beta_1^{L1}\\ \beta_1^{L2}\\ \beta_1^{L3}\\ \beta_1^{L4}\end{pmatrix},\Sigma=\begin{pmatrix}\hat{\sigma}_1^2 & \rho_{12}\hat{\sigma}_1\hat{\sigma}_2 & \rho_{13}\hat{\sigma}_1\hat{\sigma}_3 & \rho_{14}\hat{\sigma}_1\hat{\sigma}_4\\ \rho_{12}\hat{\sigma}_1\hat{\sigma}_2 & \hat{\sigma}_2^2 & \rho_{23}\hat{\sigma}_2\hat{\sigma}_3 & \rho_{24}\hat{\sigma}_2\hat{\sigma}_4\\ \rho_{13}\hat{\sigma}_1\hat{\sigma}_3 & \rho_{23}\hat{\sigma}_2\hat{\sigma}_3 & \hat{\sigma}_3^2 & \rho_{34}\hat{\sigma}_3\hat{\sigma}_4\\ \rho_{14}\hat{\sigma}_1\hat{\sigma}_4 & \rho_{24}\hat{\sigma}_2\hat{\sigma}_4 & \rho_{34}\hat{\sigma}_3\hat{\sigma}_4 & \hat{\sigma}_4^2\end{pmatrix}\right) \tag{3.19}$$

The underlying β_1^{L1}, β_1^{L2}, β_1^{L3} and β_1^{L4} may vary from study to study. This variation can be modelled as:

$$\begin{pmatrix} \beta_1^{L1} \\ \beta_1^{L2} \\ \beta_1^{L3} \\ \beta_1^{L4} \end{pmatrix}, \sim N\left(\begin{pmatrix} \beta_{L1} \\ \beta_{L2} \\ \beta_{L3} \\ \beta_{L4} \end{pmatrix}, C = \begin{pmatrix} \tau_1^2 & \kappa_{12}\tau_1\tau_2 & \kappa_{13}\tau_1\tau_3 & \kappa_{14}\tau_1\tau_4 \\ \kappa_{12}\tau_1\tau_2 & \tau_2^2 & \kappa_{23}\tau_2\tau_3 & \kappa_{24}\tau_2\tau_4 \\ \kappa_{13}\tau_1\tau_3 & \kappa_{23}\tau_2\tau_3 & \tau_3^2 & \kappa_{34}\tau_3\tau_4 \\ \kappa_{14}\tau_1\tau_4 & \kappa_{24}\tau_2\tau_4 & \kappa_{34}\tau_3\tau_4 & \tau_4^2 \end{pmatrix} \right) \tag{3.20}$$

providing the marginal mulitivariate normal distribution for the study in question as:

$$\begin{pmatrix} \breve{\beta}_1^{L1} \\ \breve{\beta}_1^{L2} \\ \breve{\beta}_1^{L3} \\ \breve{\beta}_1^{L4} \end{pmatrix} \sim N\left(\begin{pmatrix} \beta_{L1} \\ \beta_{L2} \\ \beta_{L3} \\ \beta_{L4} \end{pmatrix}, \Sigma + C \right) \tag{3.21}$$

Equation (3.21) represents a four-dimensional model for meta-analysis, where the four levels of possible adjustments are considered.

I calculated the pooled fully-adjusted estimate for each *MC1R* variant by using a two-stage analysis [Stukel et al. 2001] as described in sub-chapter 3.2. In the first step, each available study-specific estimate was calculated with unconditional logistic regression model including all the available covariates. Missing data were imputed with multiple imputation models for variables with less than 20% of missing data, by using the iterative Markov chain Monte Carlo method [Horton et al. 2007]. In the second step, the multivariate model above described was applied. The within-study correlations between each pair of adjustments levels were calculated with the nonparametric bootstrap estimate described in section 3.3.1, with 500 bootstrap samples. For computationally reasons, the starting values for the estimate of the between-study variances and covariance were set equal to the between-study variances estimated in the corresponding univariate model and assuming a covariance k_{ij}=1.00, as suggested [Riley et al. 2007b, Jackson et al. 2009] .

Results

The classification of studies according to their maximum level of adjustment is reported in Table 3.17. I can correctly classify all the studies but one (Ribas) which included information on X_1, X_2 and X_4, but not on X_3. In order to be conservative, I classified this study as ñlevel 2ò, however I calculated the estimate adjusted also by neavi count, in order to not discharge information on this potentially important confounder.

Table 3.17. Classification of studies into their maximum available level of adjustment

First author, year	Possible confounders assessed beyond age, gender and family history of melanoma	Maximum level of adjustment
Gruis, 2001	Sun exposure (chronic, intermittent), sunburns (lifetime), naevi (common, atypical)	LEVEL 4
Dwyer, 2004	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood)	LEVEL 3
Ghiorzo, 2004	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (common, atypical)	LEVEL 4
Landi, 2005	Sun exposure (chronic, intermittent), Sunburns (lifetime)	LEVEL 3
Debniak, 2006	Sunburns (lifetime, childhood)	LEVEL 2
Fargnoli, 2006	Sun exposure (chronic, intermittent), sunburns (childhood), naevi (common, atypical)	LEVEL 4
Han, 2006	Sunburns (lifetime)	LEVEL 2
Stratigos, 2006	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (common, atypical)	LEVEL 4
Ribas, 2007	Sunburns (childhood), naevi (common)	LEVEL 2
Branicki, 2009	-	LEVEL 1
Cornelius, 2009	-	LEVEL 1
Hansson, 2009	-	LEVEL 1
Kayser, 2009	-	LEVEL 1
Kumar, 2009	-	LEVEL 1
Nagore, 2009	-	LEVEL 1
Palmieri, 2009	-	LEVEL 1
Kanetsky, 2010	Sun exposure (chronic, intermittent), sunburns (lifetime, childhood), naevi (atypical)	LEVEL 4

Tables 3.18 to 3.26 respectively report for each *MC1R* variant: the study-specific coefficients adjusted for each of the four levels, where available, with their corresponding within-study standard errors, and the bootstrap correlation coefficient ρ for the correlation between each pair of available estimates. Moreover the same tables present the pooled estimates obtained for each of the four

levels of adjustment. For each variant, the calculated pooled fully adjusted OR with its 95%CI is reported in Table 3.27. All the calculated ORs but that for V60L were higher than 1.00; a significant increase in melanoma risk was observed for carriers of the six *MC1R* variants D84E (OR; 95%CI: 2.92; 1.69-5.04), R142H (OR; 95%CI: 2.24; 1.15-4.37), R151C (OR; 95%CI: 1.56; 1.27-1.92), I155T (OR; 95%CI: 2.84; 2.03-3.92), R160W (OR; 95%CI: 1.43; 1.19-1.72), and D294H (OR; 95%CI: 1.74; 1.34-2.27). Pooled fully adjusted estimates with the lowest standard errors were those obtained for V92M (ů=0.091), R160W (ů=0.093), R151C (ů=0.105), R163Q (ů=0.109), while higher standard errors was observed for the four rare variants R142H (ů=0.341), D84E (ů=0.279), I155T (ů=0.171) and D294H (ů=0.134), and for the common variant V60L (ů=0.165).

Table 3.18. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustment 4, 3, 2 and 1, respectively, to assess the association between V60L variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.005	0.324	0.228	0.294	0.930	0.233	0.294	0.912	0.995	0.234	0.290	0.898	0.987	0.992
Dwyer			-0.500	0.279		-0.462	0.276		0.989	-0.449	0.275		0.974	0.987
Ghiorzo	-0.173	0.247	-0.155	0.199	0.783	-0.132	0.168	0.695	0.856	-0.133	0.168	0.654	0.844	0.975
Landi			0.197	0.241		0.209	0.239		0.958	0.216	0.238		0.954	0.989
Debniak						0.045	0.223			0.059	0.222			0.831
Fargnoli	-0.733	0.266	-0.472	0.242	0.920	-0.380	0.236	0.854	0.932	-0.373	0.235	0.851	0.935	0.997
Han						0.242	0.243			0.275	0.241			0.986
Stratigos	0.840	0.362	1.010	0.309	0.768	0.928	0.301	0.739	0.953	0.892	0.288	0.598	0.793	0.838
Ribas						0.606	0.316			0.574	0.265			0.773
Branicki										0.004	0.298			
Cornelius										0.250	0.565			
Hansson										0.507	0.187			
Kayser										0.262	0.304			
Kumar										-0.079	0.134			
Nagore										0.279	0.131			
Palmieri										0.000	0.302			
Kanetsky	-0.177	0.160	-0.099	0.151	0.843	-0.098	0.151	0.843	0.997	-0.092	0.150	0.793	0.906	0.916
POOLED	**-0.039**	**0.165**	**0.138**	**0.108**		**0.121**	**0.085**			**0.109**	**0.075**			

Table 3.19. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between D84E variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	1.613	0.684	2.005	0.672	0.991	2.041	0.662	0.990	0.999	2.025	0.651	0.993	0.997	0.999
Dwyer			0.765	0.655		0.779	0.653		0.999	0.709	0.642		0.997	0.998
Ghiorzo	-0.578	1.016	0.360	0.801	0.823	0.434	0.678	0.622	0.755	0.434	0.676	0.620	0.752	1.000
Fargnoli	1.728	1.172	1.142	1.169	1.000	1.171	1.162	0.999	0.999	1.195	1.161	0.999	0.999	1.000
Ribas						0.687	1.379			1.244	1.238			0.999
Branicki										2.056	1.308			
Cornelius										-0.137	1.225			
Hansson										0.446	0.445			
Kumar										1.195	0.441			
Nagore										0.993	0.784			
Kanetsky	-0.060	0.521	-0.036	0.497	0.741	-0.050	0.496	0.738	1.000	0.008	0.495	0.602	0.801	0.801
POOLED	**1.072**	**0.279**	**0.656**	**0.253**		**0.662**	**0.246**			**0.804**	**0.204**			

Table 3.20. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between V92M variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.482	0.324	0.303	0.306	0.951	0.317	0.304	0.950	0.995	0.351	0.300	0.937	0.985	0.992
Ghiorzo	0.299	0.388	0.457	0.311	0.815	0.492	0.249	0.684	0.824	0.467	0.246	0.654	0.781	0.960
Landi			-0.283	0.399		-0.324	0.396		0.986	-0.335	0.396		0.975	0.991
Fargnoli	0.379	0.402	0.293	0.374	0.952	0.301	0.370	0.883	0.931	0.307	0.370	0.880	0.929	0.998
Han						0.165	0.256			0.169	0.254			0.975
Stratigos	0.101	0.673	0.207	0.513	0.637	0.300	0.503	0.609	0.973	0.229	0.472	0.540	0.804	0.811
Ribas						0.934	0.483			0.799	0.415			0.785
Branicki										0.003	0.290			
Cornelius										0.506	0.628			
Hansson										0.216	0.182			
Kumar										0.016	0.136			
Nagore										0.210	0.195			
Palmieri										-0.598	0.644			
Kanetsky	-0.098	0.184	-0.084	0.174	0.868	-0.092	0.173	0.866	0.993	-0.091	0.172	0.815	0.942	0.951
POOLED	**0.169**	**0.091**	**0.114**	**0.072**		**0.131**	**0.078**			**0.132**	**0.067**			

Table 3.21. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between R142H variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.648	0.853	0.333	0.878	0.999	0.364	0.858	0.999	1.000	0.368	0.864	0.999	0.999	1.000
Ghiorzo	3.102	1.122	2.707	1.072	0.999	1.214	0.528	0.246	0.246	1.231	0.525	0.242	0.242	0.998
Landi			1.188	0.832		1.127	0.831		1.000	1.086	0.829		0.999	1.000
Fargnoli	0.240	0.537	0.017	0.527	0.993	0.112	0.519	0.988	0.992	0.092	0.518	0.988	0.992	0.999
Stratigos	2.132	0.974	1.502	0.875	0.948	1.553	0.891	0.950	0.999	1.552	0.842	0.932	0.984	0.983
Ribas						-0.910	0.827			-0.376	0.691			0.616
Branicki										0.137	0.644			
Cornelius										0.183	1.304			
Hansson										1.557	1.121			
Kayser										1.251	0.731			
Kumar										1.209	0.347			
Nagore										0.706	0.402			
Palmieri										-0.494	0.752			
Kanetsky	-0.643	0.503	-0.598	0.460	0.477	-0.645	0.457	0.477	0.993	-0.632	0.456	0.410	0.938	0.947
POOLED	**0.806**	**0.341**	**0.497**	**0.262**		**0.496**	**0.225**			**0.489**	**0.203**			

Table 3.22. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between R151C variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.533	0.355	0.291	0.339	0.959	0.281	0.339	0.957	0.994	0.353	0.335	0.935	0.980	0.985
Dwyer			-0.315	0.286		-0.319	0.285		0.992	-0.301	0.284		0.980	0.988
Ghiorzo	0.310	0.343	0.649	0.286	0.818	0.725	0.245	0.692	0.878	0.725	0.243	0.663	0.850	0.976
Landi			1.238	0.330		1.140	0.322		0.951	1.165	0.320		0.944	0.994
Debniak						0.209	0.259			0.226	0.258			0.787
Fargnoli	0.597	0.480	1.011	0.451	0.931	0.988	0.444	0.895	0.943	0.994	0.443	0.894	0.940	0.998
Han						0.855	0.304			0.904	0.300			0.981
Stratigos	1.112	0.767	1.227	0.613	0.952	1.012	0.596	0.941	0.993	1.074	0.573	0.721	0.780	0.789
Ribas						-0.242	0.626			0.267	0.525			0.882
Branicki										0.798	0.337			
Cornelius										-1.250	0.909			
Hansson										0.371	0.165			
Kayser										0.030	0.379			
Kumar										0.616	0.148			
Nagore										0.399	0.244			
Palmieri										1.044	1.057			
Kanetsky	0.603	0.214	0.627	0.204	0.885	0.624	0.204	0.883	0.998	0.661	0.202	0.835	0.929	0.933
POOLED	**0.445**	**0.105**	**0.475**	**0.126**		**0.459**	**0.111**			**0.517**	**0.085**			

Table 3.23. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between I155T variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	1.701	1.535	1.115	1.512	1.000	0.999	1.489	1.000	1.000	0.904	1.468	0.999	1.000	1.000
Ghiorzo	-0.749	0.758	-0.105	0.656	0.710	0.038	0.517	0.524	0.710	0.045	0.515	0.511	0.695	0.992
Fargnoli	0.537	0.850	0.574	0.783	0.997	0.402	0.774	0.658	0.657	0.377	0.773	0.656	0.655	1.000
Han						0.836	0.646			0.917	0.655			0.996
Ribas						2.390	0.922			2.318	0.793			0.991
Branicki										-0.394	0.690			
Cornelius										0.136	0.691			
Hansson										0.759	0.557			
Kumar										0.610	0.426			
Nagore										0.025	0.322			
Palmieri										0.372	1.133			
Kanetsky	-0.276	0.470	-0.163	0.443	0.934	-0.136	0.443	0.925	0.996	-0.179	0.439	0.851	0.917	0.921
POOLED	**1.043**	**0.171**	**0.636**	**0.163**		**0.473**	**0.172**			**0.458**	**0.157**			

Table 3.24. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between R160W variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.136	0.295	0.230	0.275	0.930	0.283	0.272	0.919	0.983	0.349	0.268	0.908	0.965	0.985
Dwyer			0.580	0.275		0.551	0.273		0.989	0.533	0.269		0.957	0.965
Ghiorzo	0.940	0.537	1.028	0.383	0.717	0.855	0.293	0.544	0.779	0.850	0.293	0.548	0.760	0.973
Landi			0.103	0.492		0.140	0.489		0.948	0.123	0.489		0.940	0.997
Debniak						3.489	0.725			3.488	0.725			1.000
Fargnoli	0.155	0.516	0.403	0.487	0.955	0.410	0.483	0.924	0.964	0.432	0.481	0.919	0.959	0.996
Han						0.438	0.288			0.466	0.285			0.987
Stratigos	0.469	0.949	1.112	0.851	0.978	1.176	0.847	0.976	0.998	1.007	0.784	0.927	0.946	0.952
Ribas						1.634	0.937			1.534	0.853			0.993
Branicki										0.545	0.283			
Cornelius										1.075	0.714			
Hansson										0.511	0.153			
Kayser										0.238	0.310			
Kumar										0.539	0.146			
Nagore										0.887	0.314			
Palmieri										1.112	1.081			
Kanetsky	0.322	0.200	0.417	0.191	0.825	0.416	0.191	0.825	0.998	0.424	0.190	0.765	0.935	0.937
POOLED	**0.359**	**0.093**	**0.555**	**0.070**		**0.557**	**0.069**			**0.561**	**0.068**			

Table 3.25. Estimated coefficients $\beta_{i(L4)}$, $\beta_{i(L3)}$, $\beta_{i(L2)}$, and $\beta_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between R163Q variant and melanoma development, with their corresponding standard errors $\hat{u}_{i(L4)}$, $\hat{u}_{i(L3)}$, $\hat{u}_{i(L2)}$, and $\hat{u}_{i(L1)}$ and estimated bootstrap correlation coefficients $\rho_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$\beta_{i(L4)}$	$\hat{u}_{i(L4)}$	$\beta_{i(L3)}$	$\hat{u}_{i(L3)}$	$\rho_{i(L4L3)}$	$\beta_{i(L2)}$	$\hat{u}_{i(L2)}$	$\rho_{i(L4L2)}$	$\rho_{i(L3L2)}$	$\beta_{i(L1)}$	$\hat{u}_{i(L1)}$	$\rho_{i(L4L1)}$	$\rho_{i(L3L1)}$	$\rho_{i(L2L1)}$
Gruis	0.473	0.376	0.486	0.356	0.934	0.466	0.354	0.923	0.989	0.495	0.350	0.915	0.978	0.989
Ghiorzo	-0.150	0.603	-0.141	0.449	0.545	-0.278	0.384	0.456	0.876	-0.304	0.381	0.454	0.872	0.992
Landi			1.038	0.607		1.054	0.606		0.717	0.992	0.605		0.717	1.000
Debniak						0.461	0.340			0.462	0.339			0.612
Fargnoli	-0.338	0.676	-0.242	0.606	0.977	-0.259	0.599	0.521	0.573	-0.262	0.599	0.515	0.565	0.998
Han						0.186	0.394			0.173	0.392			0.986
Stratigos	0.635	1.207	0.781	1.054	0.992	0.564	1.052	0.993	0.999	0.185	1.044	0.993	0.999	0.999
Ribas						0.531	0.703			1.031	0.604			0.841
Branicki										0.027	0.477			
Cornelius										0.577	0.768			
Hansson										-0.079	0.179			
Kayser										0.511	0.332			
Kumar										-0.014	0.176			
Nagore										0.119	0.294			
Kanetsky	-0.042	0.275	0.045	0.259	0.874	0.057	0.258	0.868	0.998	0.045	0.257	0.831	0.950	0.954
POOLED	**0.203**	**0.109**	**0.201**	**0.085**		**0.169**	**0.083**			**0.092**	**0.082**			

Table 3.26. Estimated coefficients $b_{i(L4)}$, $b_{i(L3)}$, $b_{i(L2)}$, and $b_{i(L1)}$, for levels of adjustments 4, 3, 2 and 1, respectively, to assess the association between D294H variant and melanoma development, with their corresponding standard errors $u_{i(L4)}$, $u_{i(L3)}$, $u_{i(L2)}$, and $u_{i(L1)}$ and estimated bootstrap correlation coefficients $r_{i(LkLj)}$ for each pair of available estimates, and pooled estimates calculated for each level with a multivariate adaptation of the Jacksonôs method

Author	$b_{i(L4)}$	$u_{i(L4)}$	$b_{i(L3)}$	$u_{i(L3)}$	$r_{i(L4L3)}$	$b_{i(L2)}$	$u_{i(L2)}$	$r_{i(L4L2)}$	$r_{i(L3L2)}$	$b_{i(L1)}$	$u_{i(L1)}$	$r_{i(L4L1)}$	$r_{i(L3L1)}$	$r_{i(L2L1)}$
Gruis	-0.392	1.014	-0.287	0.993	0.998	-0.266	0.996	0.998	1.000	-0.045	0.932	0.997	0.999	0.999
Dwyer			-0.841	0.513		-0.803	0.510		0.999	-0.826	0.509		0.699	0.699
Ghiorzo	0.857	0.528	0.799	0.414	0.456	0.707	0.343	0.364	0.859	0.704	0.343	0.360	0.846	0.984
Landi			0.644	0.538		0.596	0.535		0.813	0.698	0.524		0.799	0.990
Fargnoli	1.029	0.579	1.038	0.551	0.982	1.031	0.547	0.965	0.981	1.028	0.547	0.963	0.979	0.999
Han						0.480	0.460			0.453	0.456			0.995
Ribas						0.710	0.595			1.426	0.511			0.410
Branicki										1.212	1.144			
Cornelius										0.202	1.934			
Hansson										0.750	0.849			
Kumar										0.543	0.323			
Nagore										0.747	0.272			
Palmieri										0.419	0.532			
Kanetsky	0.823	0.365	0.852	0.353	0.893	0.843	0.353	0.891	0.999	0.864	0.352	0.839	0.940	0.942
POOLED	**0.555**	**0.134**	**0.673**	**0.194**		**0.651**	**0.131**			**0.690**	**0.119**			

Table 3.27. Pooled fully adjusted Odds Ratio (OR) with 95% Confidence Intervals (CI) for the studied *MC1R* variants calculated with the multivariate adaptation of the Jacksonôs method

***MC1R* variant**	**N studies**	**OR (95%CI)**
V60L	17	0.96 (0.70-1.33)
D84E	**11**	**2.92 (1.69-5.04)**
V92M	14	1.18 (0.99-1.42)
R142H	**14**	**2.24 (1.15-4.37)**
R151C	**17**	**1.56 (1.27-1.92)**
I155T	**12**	**2.84 (2.03-3.97)**
R160W	**17**	**1.43 (1.19-1.72)**
R163Q	15	1.22 (0.99-1.52)
D294H	**14**	**1.74 (1.34-2.27)**

Note: significant results are in bold.

3.3.5. Discussion

Observational studies are likely to differ in terms of the information that they provide and are particular susceptible to the influence of confounders. Provided that fully and partially adjusted estimates are kept distinct and assuming that at least some studies provide enough information to produce fully adjusted estimates, the method proposed by Jackson et al. [Jackson et al. 2009] can be used to incorporate data from all the available studies.

I adapted the proposed model to a binary outcome with logistic regression model and case-control studies. Moreover, I extended the proposed approach to a multivariate (four-dimensional) rather than the bivariate analysis proposed by Jackson in the original paper.

From the validation study here performed, I found that the pooled age-adjusted estimates obtained with the Jacksonôs method were similar to the ñtrueò pooled age-adjusted estimates, but with two main differences: first, the 95%CI were always larger for Jacksonôs method estimates than for the GS estimates; second, with the Jacksonôs method two *MC1R* variants that were not significantly associated with melanoma in the GS analysis were found to have ORs significantly higher than 1.00. The wider 95%CI were due to larger standard errors for the estimates obtained with the bivariate Jacksonôs approach than for the univariate GS approach. The same difference between a bivariate and a univariate meta-analysis was previously observed in simulation studies [Riley et al.

2007b]. This may be explained with the fact that the between-study covariance $k\tau_1\tau_2$ is formulated so that k=1.00 and k is constrained to be no higher than 1.00; then to obtain the necessary solution for the between-study covariance, the maximum likelihood estimator can only increase the between-study variances $\hat{\tau}_1^2$ and $\hat{\tau}_2^2$, which do not have an upper bound constraint. Thus the between-study variance estimates are inflated to compensate for the constraint on k, finally providing larger variability estimates in bivariate rather than univariate meta-analyses. This may not be considered, however, a major concern as the maximum likelihood estimator for $\hat{\tau}_1^2$ and $\hat{\tau}_2^2$ is still asymptotically unbiased (the bias decreases as the number of studies increases) and the inflation is simply caused by the sensible and necessary constraint on k. Furthermore, the inflation is essentially conservative, leading to a larger standard error and mean-square error of the pooled estimates. The false significant results obtained for the two *MC1R* variants I155T and R163Q highlighted a possible problem in the Jacksonôs method to obtain an unbiased estimate of the association of interest. This could be attributable to the relatively low allele frequencies for these two variants, leading to a larger variability and possible bias in the final estimate. It may be therefore necessary to use caution when the study exposure is rare and the standard error of the pooled-estimate is relatively high.

By comparing the Jacksonôs estimates obtained in the validation study with those obtained with a univariate random-effect meta-analysis on a subset of studies with information on age, I observed that the point estimates were generally more similar to the ñtrueò ones for the Jacksonôs method, and also the 95%CI were more strict, giving a more precise estimate. This result therefore highlighted an advantage of using Jackson method than reducing the number of studies to be included in a univariate analysis.

Otherwise, when I compared the Jacksonôs estimates obtained in the validation study with those obtained with a univariate random-effect meta-analysis on all the crude estimates, and with those obtained from a standard two-stage approach as presented in section 3.2, I did not observe any

significant advantage of Jacksonôs method. On the contrary, pooled crude and standard two-stage estimates generally were more similar to the GS as point estimates and were more precise. This was probably due to the fact that in my study age was probably not a main confounder and crude and age-adjusted estimates were in fact very similar. For this reason, the univariate approach maintained some advantages on the bivariate approach, both for the lower standard errors obtained (as previously discussed) and for a more unbiased estimate. This latter point need a further consideration. The GS for a pooled fully adjusted estimates is indeed obtained by pooling fully adjusted estimates from all the studies, in a scenario where all the fully adjusted studies are available. With this scenario, a bivariate meta-analysis including both fully adjusted and partially adjusted estimates is not motivated, because we are only interested in the fully adjusted estimates and not in both estimates. Indeed, a bivariate meta-analysis is really motivated when we are interested in both outcomes and therefore we want an unbiased estimate for each outcome, which take into account the correlation between the two outcomes. In the application proposed by Jackson, the bivariate meta-analysis was used just to solve the problem of missing confounders in some studies, but has not a reason a priori, therefore, when partially and fully adjusted estimates were not significantly heterogeneous, a univariate meta-analysis may provide estimates which were less unbiased than a bivariate meta-analysis.

In conclusion I think that Jacksonôs method may be a very useful and good approach to deal with the problem of missing data in some observational studies when there is a significant difference between partially and fully adjusted estimates. In this situation, pooling together the two types of estimates is not recommendable because of the large heterogeneity, and pooling together just the partially adjusted estimates may lead to biased estimates when the confounders discharged are really influent in the estimate of the association of interest. Moreover, separate subgroup estimates are generally more biased and less precise than the ones obtained with the Jacksonôs method. Caution should be used in applying Jacksonôs method when the exposure of interest is rare and

when there is no heterogeneity between partially and fully adjusted estimates. In this case, a univariate meta-analytic approach may be preferable.

3.4. Heterogeneity estimate and meta-regression

Homogeneity among study-specific estimates was evaluated by Q statistic and I^2, and meta-regression analysis was performed to deeply investigate heterogeneity among study estimates. The Q statistic is a Chi Square test and thus have a limited power. For this reason I considered that statistically significant heterogeneity was present when the p-value was Ò0.10; furthermore I calculate the I^2 proposed by Higgins et al. [Higgins et al. 2002], which represents the percentage of total variation across studies that is attributable to heterogeneity rather than to chance. If a significant heterogeneity was found among study estimates, I performed meta-regression. This method extends a random-effects meta-analysis to estimate the extent to which one or more covariates, with values defined for each study in the analysis, explain heterogeneity in the study estimates. Meta-regression fits models with two additive components of variance, one representing the variance within units, the other the variance between units, and therefore is applicable to the pooled-analysis situation, where each unit is one study.

I first evaluated whether there was a statistically significant heterogeneity between study estimates according with different levels of adjustment. In this analysis I tested the difference between 1) estimates adjusted only for the base confounders (age, gender, family history of melanoma) versus all the other estimates, and 2) estimates adjusted by each of the four levels reported in Table 3.17. No significant heterogeneity was found for any of the nine studied variants and for each of the two analyses carried out. For this reason, I decided to use in all the following analyses the results obtained with the standard two-stage approach, that was found to provide more reliable pooled-estimates than the ones obtained with the Jackson method when the study-specific estimates were homogenous, as discussed in the section 3.3.5.

A significant heterogeneity among the study-specific estimates was found for the four *MC1R* variants V60L (I^2:42.5%, Q statistic p-value:0.033, Figure 3.1), R142H (I^2:39.8%, Q statistic p-value:0.062, Figure 3.4), R151C (I^2:39.8%, Q statistic p-value:0.046, Figure 3.5), and R160W (I^2:39.9%, Q statistic p-value:0.046, Figure 3.7). The pooled estimates for these variants but V60L revealed a statistically significant association with melanoma.

We evaluated by meta-regression the possible role in the observed heterogeneity of the following study-related variables: publication year, study area (Australia/North Europe, South Europe, North America), genotyping methodology (sequencing, others), deviation from HW equilibrium, source of controls, and source of DNA. None of these variable seemed to explain the observed heterogeneity for V60L, R151C and R160W variants. A significant p-value (p=0.04) was observed for the meta-regression model including DNA source for R142H variant. This was probably due to one (Kanetsky) of the two studies (Kanetsky, Branicki) that extracted DNA from buccal cells, which had a very low OR (0.53; 95%CI:0.20-1.41). By excluding this study from the analysis, the heterogeneity disappeared (I^2: 17.6%, Q statistic p-value: 0.27), and the pooled-OR (95%CI) increased to 2.08 (1.41-3.06).

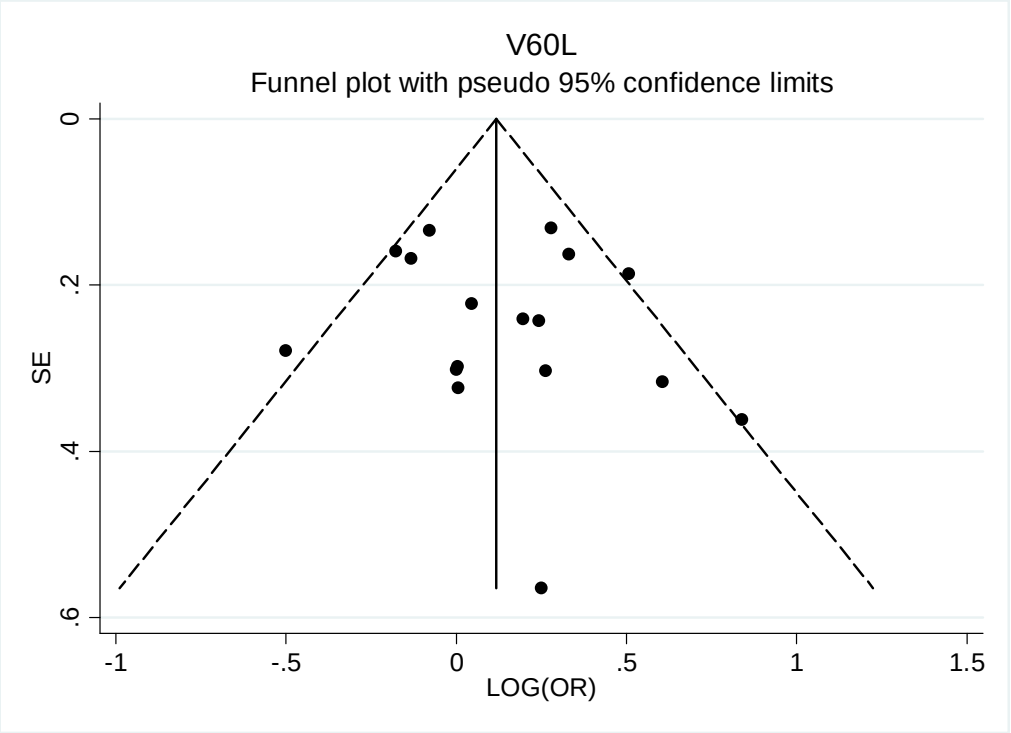
3.5. Participation bias

Funnel plots to evaluate participation bias were drawn and Egger's test was performed. A funnel plot shows the logarithm of the study-specific ORs against their Standard Errors (SEs); it assumes that studies with small sample size (large SE) should obtain an OR more distant from the real OR with respect to one study with a large sample size (small SE). When there is no participation/publication bias, the plot should be a symmetric funnel, viceversa it should appear asymmetric. The Egger test lets measuring the participation/publication bias in a formal way. The test performs a linear regression on the logarithm of ORs: the dependent variable is the Normal Standard Deviate (SND), defined as OR/SE, while the independent variable is the precision of the

estimate, defined as the inverse of SE, giving the following equation: SND=Ŭ+ɓ (1/SE). If there is no participation/publication bias, Ŭwill be equal to 0, while it will be significantly different from 0 when participation/publication bias will be present.

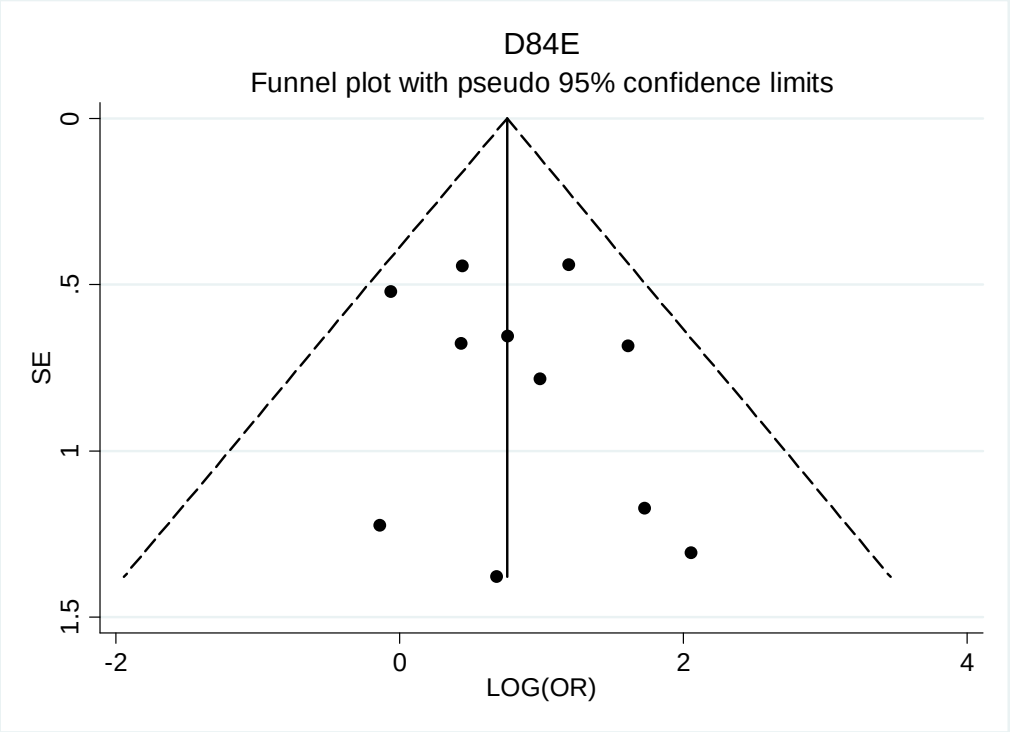
Funnel plots for each of the nine main *MC1R* variants are presented in Figure 3.11-3.19. I found evidence of publication bias for R163Q variant. Indeed the funnel plot in Figure 3.18 shows a blank area in the left-bottom part of the graph, which would include small studies with low OR.

Figure 3.11. Funnel plot for V60L variant



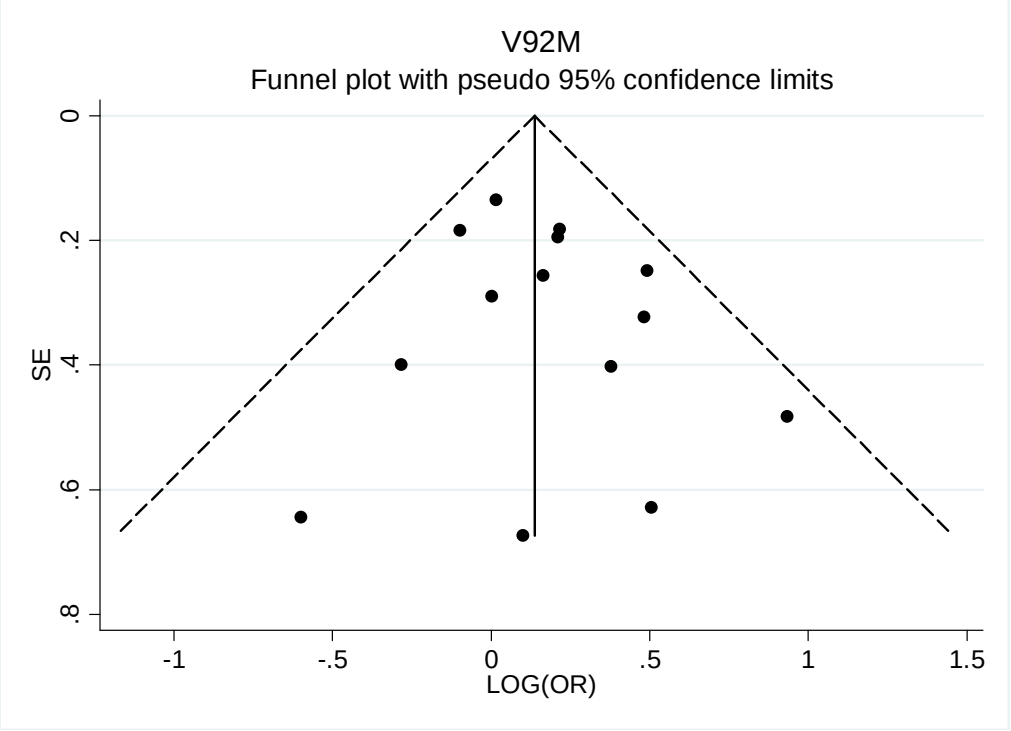
Eggerôs test : pvalue = 0.526

Figure 3.12. Funnel plot for D84E variant



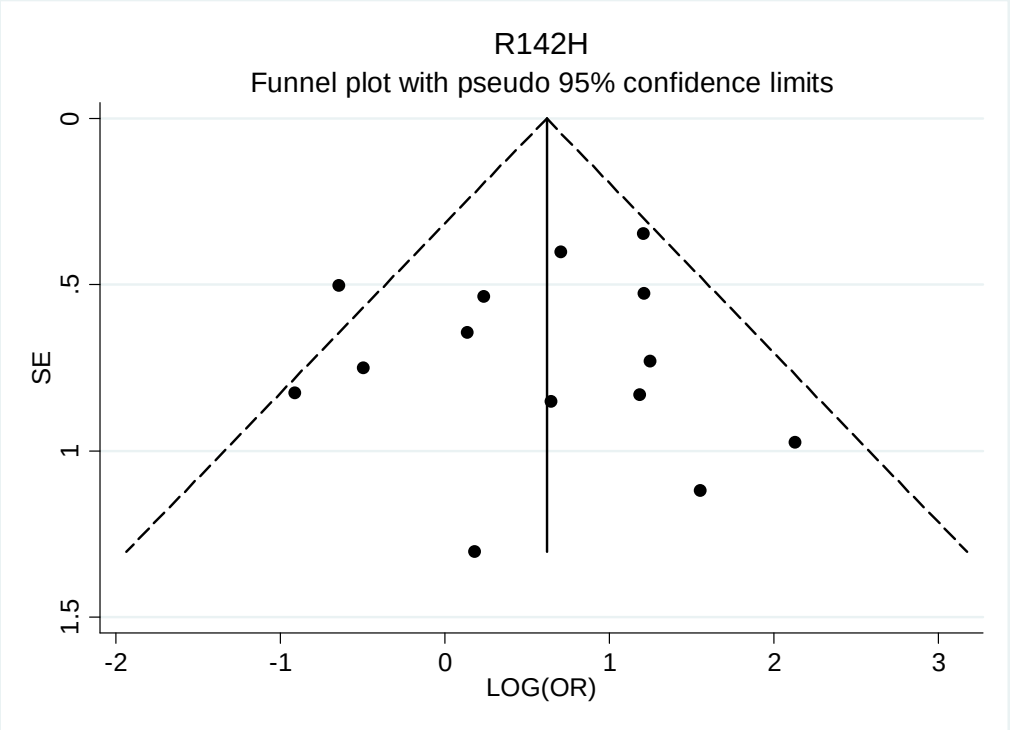
Eggerôs test : pvalue = 0.472

Figure 3.13. Funnel plot for V92M variant



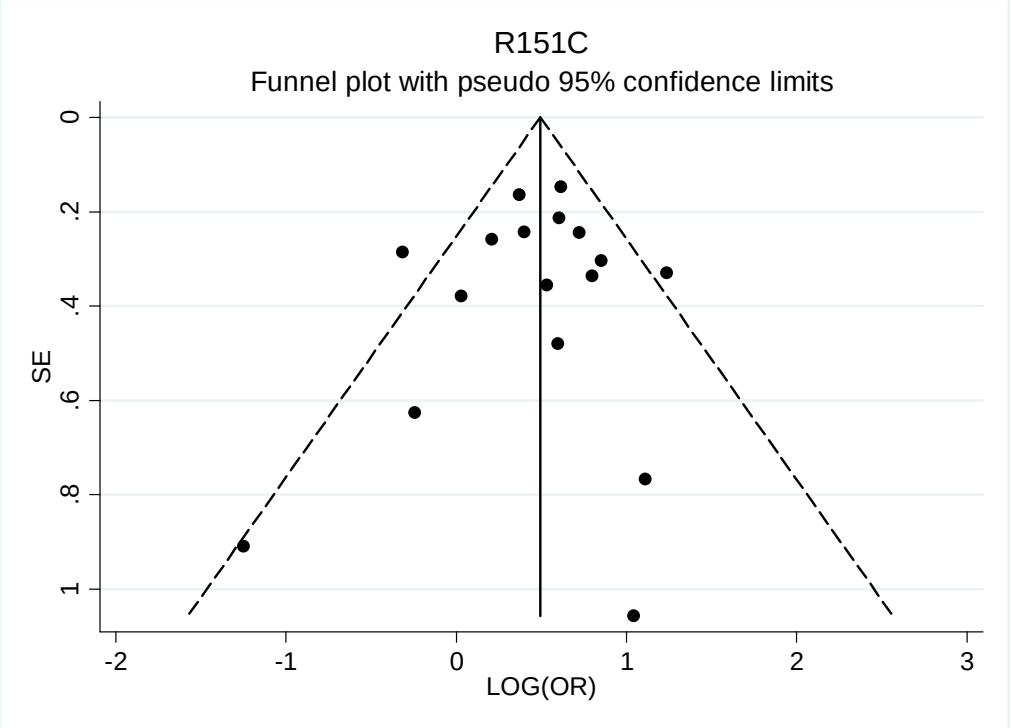
Eggerôs test : pvalue = 0.404

Figure 3.14. Funnel plot for R142H variant



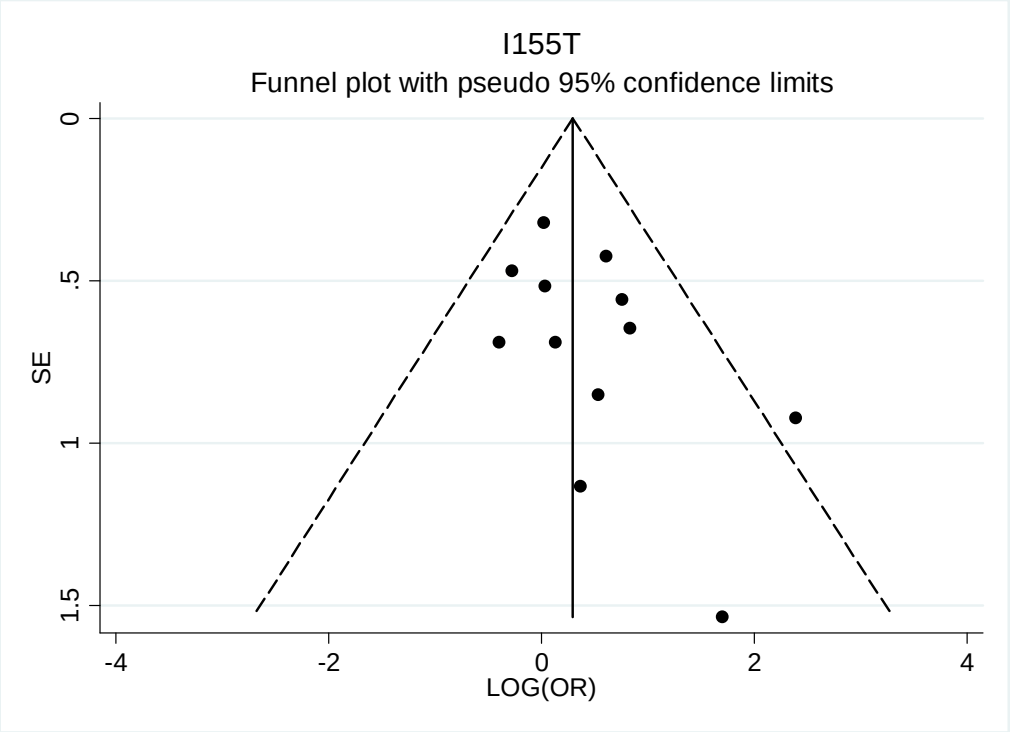
Eggerôs test : pvalue = 0.731

Figure 3.15. Funnel plot for R151C variant



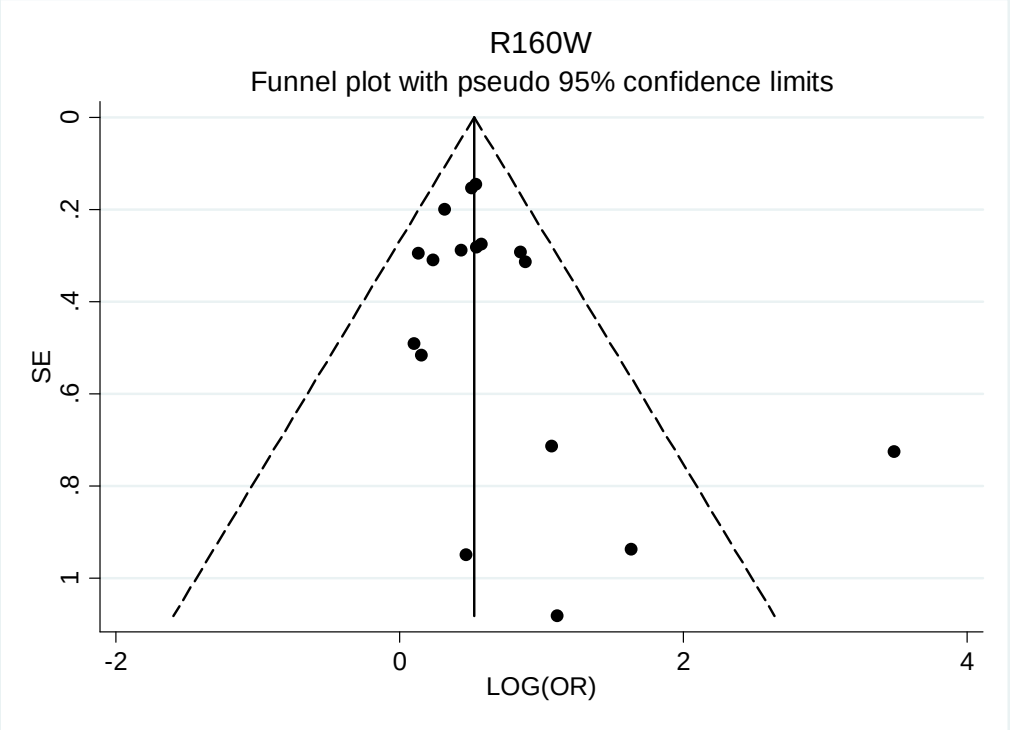
Eggerôs test : pvalue = 0.647

Figure 3.16. Funnel plot for I155T variant



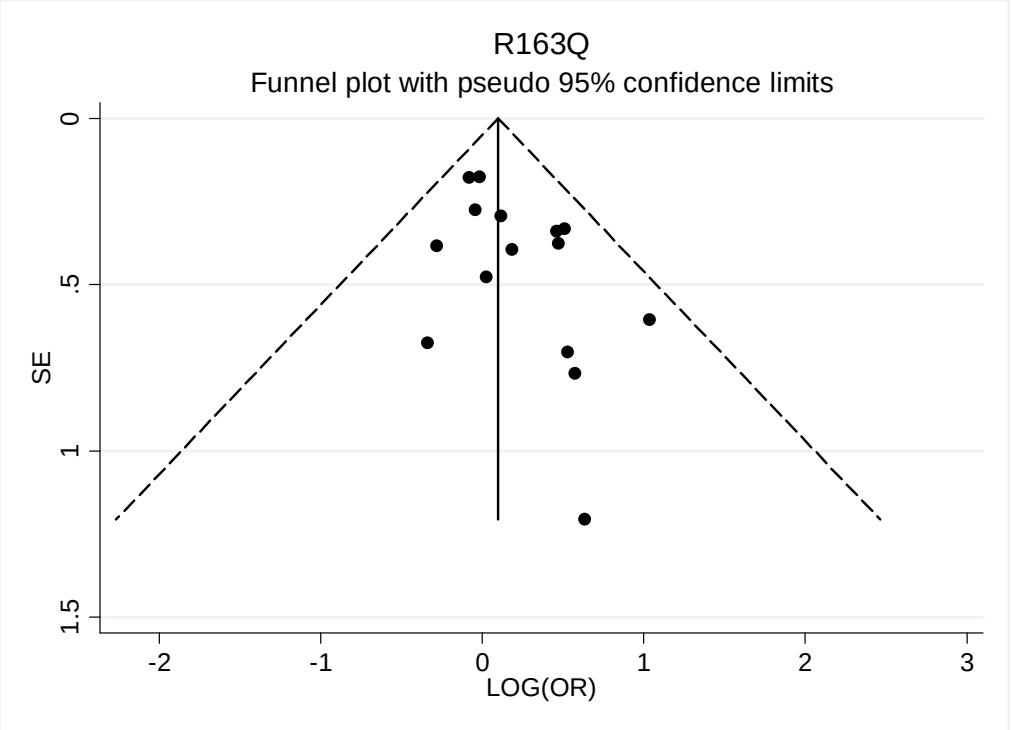
Eggerôs test : pvalue = 0.107

Figure 3.17. Funnel plot for R160W variant



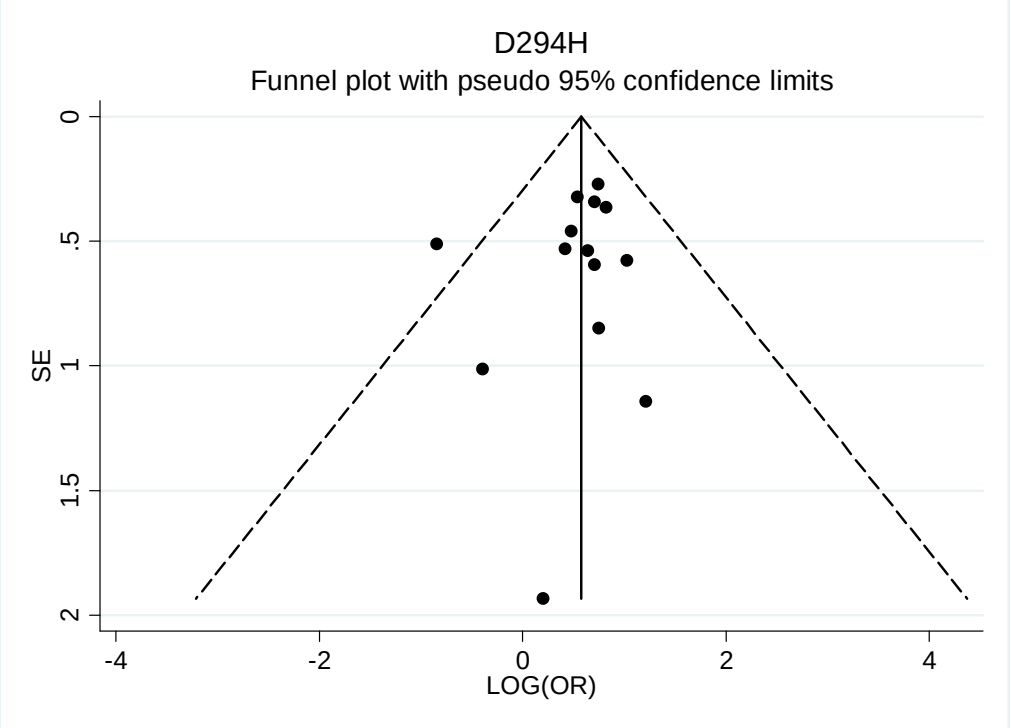
Eggerôs test : pvalue = 0.145

Figure 3.18. Funnel plot for R163Q variant



Eggerôs test : pvalue = 0.049

Figure 3.19. Funnel plot for D294H variant



Eggerôs test : pvalue = 0.484

3.5.1. Sensitivity analysis

For the three *MC1R* variants for which we observed a significant between-studies heterogeneity, I performed sensitivity analyses by excluding the studies that lies out of the funnel. For V60L, I excluded three studies [Dwyer, Stratigos, Hansoon] and recalculated the pooled-OR (95%CI) as 1.10 (0.98-1.23), which was similar to that previously obtained (1.14; 0.9-1.31, Figure 3.1), but with no more evidence of heterogeneity among studies (I^2:9.4%, Q statistic p-value:0.35). For R151C, I excluded two studies [Dwyer, Landi] and recalculated the pooled-OR (95%CI) as 1.67 (1.45-1.91), with no more evidence of between-studies heterogeneity (I^2:0.0%, Q statistic p-value:0.49). For R160W, I excluded one study [Debniak] and recalculated the pooled-OR (95%CI) as 1.65 (1.44-1.89), with no more evidence of between-studies heterogeneity (I^2:0.0%, Q statistic p-value:0.84).

Chapter 4

Gene-phenotype interaction

In the previous chapter I found that many *MC1R* variants were indeed associated with an increased risk of melanoma, independently from the main important confounders related with sun exposure. However, since *MC1R* is one of the most important genes which determine skin pigmentation, and since certain pigmentation characteristics were among the most important risk factors for melanoma, the study of the relationship between *MC1R* variants and melanoma risk could not avoid the investigation of gene-phenotype interaction. This is particularly important since melanoma is a complex and heterogeneous diseases, and it is therefore unlikely that it occurs as a result of a single disease SNP or a single phenotypic characteristic. It is probable that genetic, phenotypic and environmental factors interact and jointly contribute to its development. Thus it is crucial to investigate the role of gene-phenotype interactions and to identify combinations of genetic and phenotypic factors leading to an increased risk of melanoma.

If specific *MC1R* variants are associated with a higher melanoma risk in certain sub-populations, genetic-based innovative techniques for early detection may be developed and applied to these populations. Moreover, melanomas occurred to patients with certain combinations of *MC1R* variants and phenotypic characteristics may share similar mechanisms of development, and *ad hoc* therapies may be therefore applied to these categories of patients.

4.1. Analyses stratified by phenotypic characteristics

First, I want to investigate whether the observed association between *MC1R* variants and melanoma varied according with different phenotypic characteristics. I therefore performed stratified analyses for *MC1R* variants and melanoma according with the following phenotypic characteristics: skin color, skin type, hair color, eye color, and presence of freckles. The hypothesis of homogeneity of ORs among strata was tested by meta-regression models with random-effects and restricted maximum likelihood estimates, after the calculation of strata-specific OR in each study. The models took into account the two sources of variation (within and between studies). The correlation between the ORs calculated in the same studies was taken into account by using a bivariate approach previously described [van Houwelingen et al. 2002]. When more than two strata were considered in the analysis, I calculated both the overall p-value, evaluating whether there was any difference among strata-specific ORs, and the p-value for each strata compared with the reference strata category.

4.1.1. Skin color

Fair skin color is one of the identified risk factors for melanoma, according with a previous meta-analysis [Gandini et al. 2005c] and was also associated with certain *MC1R* variants [Beaumont et al. 2007; Raimondi et al. 2008]. For the present analysis, I classified skin color variable in each study with available information by using two categories: fair and medium/dark. Results of the stratified analysis for the association between each of the nine considered *MC1R* variants and melanoma according with skin color are reported in Table 4.1. No difference among ORs of fairer and darker subjects was observed for any of the studied *MC1R* variants. However, it is worthwhile to notice that ORs for darker pigmented subjects were higher than those of fairer pigmented subjects for almost all *MC1R* variants, with a borderline p-value observed for D294H variant (p-value:0.08).

Table 4.1. Stratified analysis for *MC1R* variants and melanoma association, according with skin color

Variant	Strata	OR (95%CI)	P-value
V60L	Fair	1.07 (0.67-1.71)	0.34
	Medium/Dark	1.33 (0.84-2.09)	
D84E*	Fair	1.56 (0.49-4.98)	0.54
	Medium/Dark	2.95 (0.56-15.58)	
V92M	Fair	1.20 (0.71-2.03)	0.66
	Medium/Dark	1.44 (0.78-2.67)	
R142H	Fair	2.68 (0.76-9.37)	0.55
	Medium/Dark	5.36 (0.64-45.03)	
R151C	Fair	1.20 (0.66-2.18)	0.27
	Medium/Dark	1.70 (0.93-3.11)	
I155T**	Fair	5.28 (0.25-113.36)	0.47
	Medium/Dark	2.02 (0.05-79.66)	
R160W	Fair	2.31 (0.81-6.57)	0.31
	Medium/Dark	3.48 (1.13-10.68)	
R163Q	Fair	0.78 (0.37-1.63)	0.10
	Medium/Dark	1.69 (0.97-2.94)	
D294H	Fair	1.06 (0.61-1.83)	0.08
	Medium/Dark	2.84 (1.11-7.26)	

OR= Odds Ratio; CI=Confidence Intervals
*Only one study with medium/dark category
**Only three studies with fair category and one with medium/dark category

4.1.2. Skin type

Skin types I and II were found to be associated with an increased risk of melanoma compared with skin types III and IV [Gandini et al. 2005c] and were also associated with certain *MC1R* variants [Beaumont et al. 2007]. For the present analysis, I maintained the classical categorization of skin type in four phototypes, as proposed by Fitzpatrick [Sachdeva 2009], and based on predisposition to sunburn and tanning ability. Specifically, the classification is as follows: skin type I ñburns easily, never tansò; skin type II ñburns easily, tans minimally with difficultyò; type III ñburns moderately, tans moderately and uniformlyò; type IV ñburns minimally, tans moderately and easilyò. Results of the stratified analysis for the association between each of the nine considered *MC1R* variants and melanoma according with skin type are reported in Table 4.2. A borderline difference among ORs of different skin types was observed for V60L variant (p-value: 0.06), with a significant p-value

(0.02) obtained for the difference between skin types IV and I. Again, it is interesting that ORs generally increased with increasing skin type for almost all *MC1R* variants.

Table 4.2. Stratified analysis for *MC1R* variants and melanoma association, according with skin type

Variant	Strata	OR (95%CI)	P-value (vs IV)	P-value (overall)
V60L	I	0.32 (0.13-0.81)	**0.02**	0.06
	II	0.89 (0.66-1.20)	0.46	
	III	1.08 (0.83-1.39)	0.90	
	IV	1.11 (0.64-1.94)		
D84E	I	3.80 (0.43-33.36)	0.60	0.53
	II	1.18 (0.44-3.12)	0.50	
	III	1.98 (0.55-7.13)	n.c.	
	IV	n.c.		
V92M	I	0.69 (0.22-2.16)	0.13	0.40
	II	1.07 (0.76-1.52)	0.17	
	III	1.24 (0.93-1.66)	0.27	
	IV	2.14 (0.85-5.40)		
R142H	I	1.70 (0.13-21.81)	0.67	0.40
	II	2.15 (0.82-5.63)	0.18	
	III	0.95 (0.41-2.23)	n.c	
	IV	n.c.		
R151C	I	1.57 (0.66-3.74)	0.52	0.85
	II	1.57 (1.13-2.18)	0.39	
	III	1.70 (1.27-2.28)	0.48	
	IV	2.36 (0.98-5.65)		
I155T*	I	0.97 (0.07-13.44)	0.84	0.93
	II	0.86 (0.39-1.89)	0.74	
	III	1.27 (0.47-3.43)	0.92	
	IV	1.50 (0.06-38.38)		
R160W	I	0.72 (0.29-1.82)	0.09	0.37
	II	1.59 (1.15-2.21)	0.56	
	III	1.53 (1.15-2.04)	0.49	
	IV	2.06 (0.92-4.61)		
R163Q	I	0.33 (0.03-4.28)	0.33	0.70
	II	1.24 (0.77-2.02)	0.94	
	III	1.01 (0.74-1.39)	0.65	
	IV	1.30 (0.46-3.67)		
D294H	I	1.26 (0.36-4.37)	0.82	0.62
	II	1.43 (0.81-2.51)	0.89	
	III	2.32 (1.35-4.01)	0.75	
	IV	1.65 (0.22-12.54)		

n.c.=not calculable; OR= Odds Ratio; CI=Confidence Intervals
Note: significant p-values are in bold
*Only one study with IV phototype

4.1.3. Hair color

Fair hair color, and red color in particular, is one of the identified risk factors for melanoma [Gandini et al. 2005c]. Red hair color is determined by *MC1R* gene and was therefore associated with certain *MC1R* variants [Beaumont et al. 2007; Raimondi et al. 2008]. For the present analysis, I classified hair color variable in each study with available information by using two different classifications: fair (blonde/red) versus dark (brown/black) and red versus all the other colors. Results of the stratified analysis for each *MC1R* variant and melanoma are reported in Tables 4.3 and 4.4 according with the two adopted classification, respectively. No difference among ORs of fairer and darker subjects was observed for any of the studied *MC1R* variants. As for the previous pigmentation characteristics, however, ORs for darker pigmented subjects were higher than those of fairer pigmented subjects for almost all *MC1R* variants. For R142H variant, a significantly lower OR was observed for red-haired subjects than subjects with other hair colors (p-value: 0.02). The same result was also found for V60L variant, but with a borderline p-value (0.06). Moreover. For almost all the *MC1R* variants, subjects with red hair had lower ORs than subjects with other colors.

Table 4.3. Stratified analysis for *MC1R* variants and melanoma association, according with hair color (fair ï blonde/red- versus dark ï brown/black- colors)

Variant	Strata	OR (95%CI)	P-value
V60L	Fair	0.90 (0.67-1.22)	0.12
	Dark	1.15 (0.90-1.47)	
D84E	Fair	1.30 (0.60-2.81)	0.18
	Dark	3.04 (1.05-8.75)	
V92M	Fair	1.41 (1.01-1.96)	0.50
	Dark	1.23 (0.96-1.56)	
R142H	Fair	1.42 (0.55-3.69)	0.68
	Dark	1.77 (0.78-4.04)	
R151C	Fair	1.45 (1.06-1.98)	0.41
	Dark	1.69 (1.24-2.29)	
I155T	Fair	0.80 (0.37-1.77)	0.40
	Dark	1.25 (0.63-2.49)	
R160W	Fair	1.40 (1.10-1.79)	0.35
	Dark	1.66 (1.28-2.15)	
R163Q	Fair	1.17 (0.80-1.71)	0.89
	Dark	1.22 (0.88-1.67)	
D294H	Fair	1.18 (0.62-2.23)	0.24
	Dark	1.82 (1.13-2.94)	

OR= Odds Ratio; CI=Confidence Intervals

Table 4.4. Stratified analysis for *MC1R* variants and melanoma association, according with hair color (red versus other colors)

Variant	Strata	OR (95%CI)	P-value
V60L	Red	0.37 (0.13-1.10)	0.06
	Other	1.06 (0.87-1.28)	
D84E	Red	1.01 (0.17-6.05)	0.40
	Other	2.23 (1.12-4.44)	
V92M	Red	0.46 (0.12-1.72)	0.14
	Other	1.26 (1.02-1.55)	
R142H	Red	0.29 (0.05-1.79)	**0.02**
	Other	2.24 (1.01-4.97)	
R151C	Red	1.13 (0.50-2.58)	0.37
	Other	1.65 (1.28-2.13)	
I155T*	Red	0.19 (0.02-2.24)	0.17
	Other	1.11 (0.65-1.92)	
R160W	Red	1.74 (0.80-3.77)	0.71
	Other	1.49 (1.24-1.79)	
R163Q	Red	n.c.	n.c.
	Other	1.17 (0.90-1.52)	
D294H	Red	2.78 (0.82-9.43)	0.40
	Other	1.60 (1.11-2.32)	

OR= Odds Ratio; CI=Confidence Intervals
Note: significant p-values are in bold
*Only one study with red hair

4.1.4. Eye color

Blue eye color was suggested to increase melanoma risk with respect to other eye colors [Gandini et al. 2005c]. *MC1R* does not directly determine eye color, but may be related also with this phenotypic characteristic via its association with RHC phenotype. For the present analysis, I classified eye color variable in each study with available information by using two categories: fair (blue, green, hazel, grey) and dark (brown, black). Results of the stratified analysis for this phenotypic characteristic are reported in Table 4.5. A significantly higher OR was found for subjects with dark eyes carrying the D294H variant than with fair eyes carrying the same variant (p-value: 0.02). For R151C variant I obtained a similar result, although the difference between the two OR did not reach the statistical significance (p=0.09). Moreover, the same trend was observed for almost all the other *MC1R* variants.

Table 4.5. Stratified analysis for *MC1R* variants and melanoma association, according with eye color (fair ï blue/green/grey/hazel versus dark ï brown/black-)

Variant	Strata	OR (95%CI)	P-value
V60L	Light Dark	1.07 (0.80-1.42) 0.99 (0.72-1.36)	0.65
D84E	Light Dark	2.39 (0.98-5.81) 1.53 (0.38-6.06)	0.57
V92M	Light Dark	1.08 (0.80-1.46) 1.39 (0.97-2.01)	0.26
R142H	Light Dark	1.90 (0.84-4.30) 1.40 (0.55-3.54)	0.55
R151C	Light Dark	1.40 (1.00-1.94) 2.02 (1.37-2.99)	0.09
I155T	Light Dark	0.60 (0.29-1.27) 1.43 (0.56-3.65)	0.16
R160W	Light Dark	1.63 (1.28-2.07) 1.46 (0.99-2.16)	0.65
R163Q	Light Dark	1.26 (0.91-1.75) 1.17 (0.72-1.92)	0.81
D294H	Light Dark	1.21 (0.75-1.95) 2.85 (1.64-4.94)	**0.02**

OR= Odds Ratio; CI=Confidence Intervals
Note: significant p-values are in bold

4.1.5. Freckles

The presence and the amount of freckles were known risk factors for melanoma development [Gandini et al. 2005c] and *MC1R* gene was strongly associated with this phenotypic characteristic [Beaumont et al. 2007]. Since very few studies in the M-SKIP project gave information on the amount of freckles for each subject, I classified freckles variable in each study as presence or absence of freckles. Results of the stratified analysis for the association between each of the nine considered *MC1R* variants and melanoma according with the presence of freckles are presented in Table 4.6. Carriers of V92M and of R160W with no freckles had significantly higher ORs than carriers of the same variants with freckles (p-values: 0.05 and 0.02, respectively). The same result was observed for V60L and D294H variants, although the p-values were borderline (0.09 and 0.07, respectively). The same trend was observed for almost all the remaining *MC1R* variants.

Table 4.6. Stratified analysis for *MC1R* variants and melanoma association, according with the presence of freckles

Variant	Strata	OR (95%CI)	P-value
V60L	Yes	0.81 (0.65-1.02)	0.09
	No	1.12 (0.84-1.50)	
D84E	Yes	1.16 (0.55-2.47)	0.20
	No	3.24 (0.83-12.65)	
V92M	Yes	0.80 (0.58-1.11)	**0.05**
	No	1.41 (0.89-2.25)	
R142H	Yes	1.77 (0.32-9.82)	0.75
	No	1.22 (0.16-9.52)	
R151C	Yes	1.37 (0.92-2.04)	0.39
	No	1.75 (1.03-2.95)	
I155T	Yes	0.59 (0.26-1.31)	0.34
	No	1.38 (0.28-6.72)	
R160W	Yes	1.28 (0.73-2.26)	**0.02**
	No	3.00 (1.57-5.74)	
R163Q	Yes	1.30 (0.73-2.33)	0.76
	No	1.46 (0.84-2.52)	
D294H	Yes	1.10 (0.60-2.04)	0.07
	No	3.67 (1.06-12.67)	

OR= Odds Ratio; CI=Confidence Intervals
Note: significant p-values are in bold

4.2. Explorative analysis of gene-phenotype interaction

The stratified analyses suggested that the role of some *MC1R* variants changed according with different phenotypic characteristics. Although this is an interesting result, it has small utility in clinical practice, because each subject is characterized by a combination of phenotypic characteristic, therefore it is difficult to understand which *MC1R* variant(s) may increase melanoma risk in each subject. For this reason, it would be valuable to identify combinations of phenotypic characteristics and *MC1R* variants, in order to better identify subgroup population characterized by a higher risk to develop melanoma.

In order to reach this goal, I decided to apply a method of statistical analysis recently proposed in the genetic field to select combinations of genes mostly associated with a disease: the logic regression [Kooperberg et al. 2001; Ruczinski et al. 2003; Kooperberg et al. 2005]. Within the M-SKIP project, I extended the application of this method to the study of both genetic and phenotypic factors, in order to find which combinations of *MC1R* variants and phenotypic characteristics were mostly associated with melanoma development. This approach is particularly useful for detecting subpopulations at high or low risk of disease, characterized by high-order interactions among covariates, and thus the methodology could be well applied to the study of complex diseases like cancer.

In section 4.2.1 I will introduce the basics of logic regression and explain how to find and select the best model. In section 4.2.2 I will describe the application of logic regression to a subset of data from the M-SKIP project. In section 4.2.3 I will compare the results presented in section 4.2.2 with those obtained with a classical approach based on logistic regression models. Finally I will discuss in section 4.2.4 strengths and possible pitfalls of the presented analysis.

4.2.1. Logic regression

Logic regression is a recently proposed tree-based statistical technique intended for situations where most predictors are binary, and the goal is to find Boolean combinations of these predictors that are associated with an outcome variable. Logic regression can be applied to any type of regression outcome as long as the proper scoring function is specified.

Let X_1, X_2,**é** , X_n be binary predictors, and let Y be a response variable. A logic regression model can be written as:

$$g(E[Y]) = \beta_0 + \sum_{j=1}^{k} \beta_j L_j, \tag{4.1}$$

where L_j is a Boolean expression of the predictors X_i, such as $L_j = X_4^C \wedge (X_5 \vee X_1 \vee X_3^C)$, with $\wedge$=AND, $\vee$=OR and ^{C}=NOT.

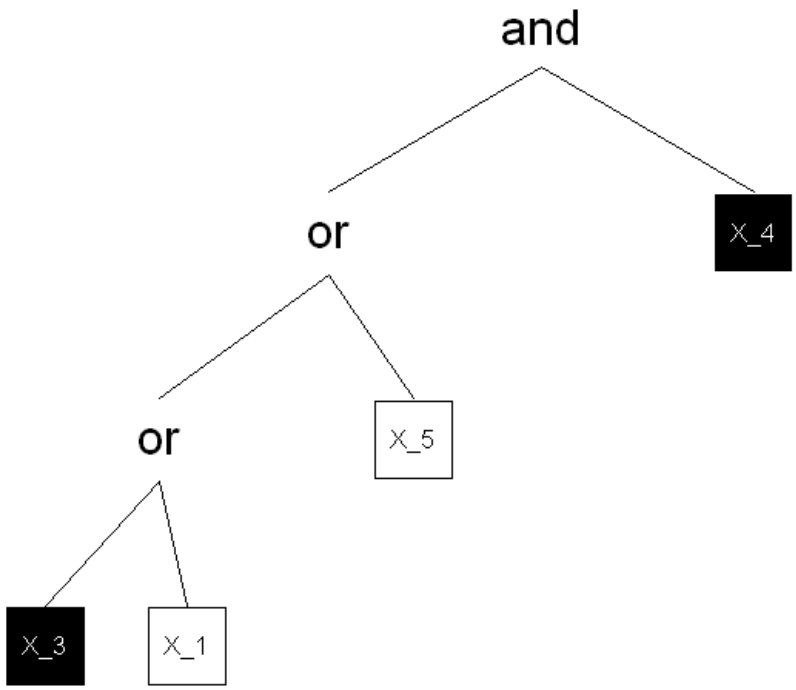
For a binary outcome the model (4.1) is a logistic regression model with Boolean expressions as covariates:

$$\log it(Y = 1 | X_1, X_2, ..., X_n) = \beta_0 + \sum_{j=1}^{k} \beta_j L_j \tag{4.2}$$

and the appropriate score function is the binomial deviance.

The goal is to find the Boolean expressions L_j that minimize the score function associated with a specific model type, estimating the parameters **b**$_j$ and the Boolean expressions L_j simultaneously. The output from logic regression is therefore represented as a series of trees, one for each Boolean predictor L_j, and the associated regression coefficient. The logic tree for the expression defined earlier is shown in Figure 4.5. Using this representation it is possible to start from a logic tree and obtain any other logic tree by a finite number of operations such as growing of branches, pruning of branches and changing of leaves.

Figure 4.5. Example of a logic tree representing the Boolean expression $(X_4)^C$ Ş $[X_5$ V X_1 V $(X_3)^C]$. White letters on black background denote the conjugate of the variable



Search for the best model

The searching of the optimum combination of variables which is mostly associated with the outcome may be obtained by a (stochastic) simulated annealing algorithm, which searches for Boolean combinations of predictors in the entire space of such combinations. The first step is to select the variable mostly associated with the outcome. Then, at each next step a new tree is selected at random among those that can be obtained by simple operations on the current tree. This new tree always replaces the current tree if it has a better score than the old tree, and it is accepted with a probability that depends on the difference between the scores of the old and the new tree and the stage of the algorithm, otherwise. For any pair of scores, the further ahead we are in the annealing scheme, the lower the acceptance probability, if the proposed new tree has a score worse than the score of the old state. This was done in order to overcome the problem of a possible local optimum: it is possible the search get ñstuckò if a better tree can be reached in more than one moves, but not in one move. This more probably happens in the first steps of the algorithm rather

than in the last steps. Since L_j and β_j are estimated simultaneously using the simulated annealing algorithm, for computational reasons the maximum number k of trees has to be preselected. In order to avoid too complex solutions, not clearly interpretable, it would be good to not select too many logic trees. The model presented in (4.1) may also include other continuous and/or binary predictors, as possible confounding factors to be included as covariates in the model. The previously described algorithm has a good chance to find a model that has the best or close to best possible score, but in the presence of noise in the data, typically overfits the data. In order to select the best model, it was therefore suggested to apply a combination of cross-validation, randomization tests and a Monte Carlo Markov Chain (MCMC)-based method.

Model selection

Let s be the model size of the best model obtained with the annealing simulating algorithm, and defined as the total number of leaves in the logic trees involved in the model. The goal of model selection methods is to assess how well the best model of size s performs in comparison to models of different sizes.

With cross-validation, the subjects of the dataset are split into m (usually m=10) equally sized groups. For each of the m groups of cases (say group k), the cases were removed from the data and the best scoring model of size s was found using only the data from the remaining m-1 groups, and the cases of group k were scored under this model. This yields score $\hat{U}_{sk}$. The cross-validated (test) score for the model size s is:

$$\varepsilon_s = \frac{1}{m}\sum_{k=1}^{m}\varepsilon_{sk} \tag{4.3}$$

The next step is to compare the cross-validated scores for models of various sizes and select the model with the lowest cross-validated score.

Another proposed method to select the best model is based on randomization. First, a test for identifying signal in the data should be carried out. Briefly, it tests the null hypothesis that there is

no association between the predictors X_1, X_2,**é** , X_n and the response *Y*. If that hypothesis was true, then the best model fit on the data with the response randomly permuted should yield about the same score as the best model fit on the original data. The procedure could be repeated *t* times, and the proportion of scores better than the score of the best model on the original data was used as an exact p-value. If there is evidence of signal in the data, then the second step is to select the optimal size *s* of the model that best describes the association between predictors and response. A sequence of randomization tests is carried out, with null hypothesis that the optimal model has size s_i and that the better scores obtained by models of larger sizes is due to noise. Randomization performed for each model of size s_i, with $s_i \in \{0,1,...,s\}$ is ñconditionedò on this model, assuming that the null hypothesis for model of size s_i is true. The response variable *Y* is permuted according with the null hypothesis and the number of scores better than the score of the optimum model with size *s* is computed. If the randomized scores are much worse, the true model size may be larger. The general rule is to chose the smallest model size where a fraction higher than *p* of the randomization scores have scores better than *s*.

Finally, a MCMC logic regression was recently proposed with the primary aim to identify all the interesting gene-gene interactions with a greater power than classic logic regression [Kooperberg et al. 2005]. I decided to apply this method, in combination with cross-validation and permutation tests described above, to select the model of the best size *s*. The method is clearly described elsewhere and its full application is not objective of the present study. I will briefly explain how it may be used for model selection. The goal of MCMC logic regression is to identify all models and combinations of covariates that are potentially associated with the outcome. Given a specific number of trees, the algorithm provides *n* logic regression models of different size, containing Boolean expression mostly associated with the outcome. The output gives the distribution of size of these visited models. If the highest percentage of visited models were of size *S*, it would be a suggestion that optimum model size is indeed *S*. Moreover the fraction p_i of models that contain a

particular covariate X_i is given and is a direct measure of the importance of this covariate for predicting the outcome. This information may be used to check whether the selected logic regression model indeed includes the most important covariates.

4.2.2. Application of logic regression to the M-SKIP dataset

For the present analysis of gene-phenotype interaction, I selected only the melanoma cases and controls with no missing data on the nine most studied *MC1R* variants and no missing data on the following phenotypic characteristics: hair and eye color, skin type, common and atypical nevi, freckles. I excluded from the analysis solar lentigines because the information was collected on a few number of subjects, and skin color because it partially overlaps with skin type, and it was less clearly defined than the latter one. I also excluded subjects with a not univocal definition of hair color (blonde/red, brown/black, other) and on eye color (brown/black, not blue, blue/green, other). I decided to include the variables related to naevi count because they represent an hereditary, not modifiable exposure, as the other considered phenotypic characteristics, although *MC1R* gene does not determine nor seems to be associated with naevi formation. The final dataset used for the analysis included data on 496 cases and 639 controls from three studies (Gruis, Ghiorzo, Debniak), which represents around 7% of the collected melanoma cases and controls.

In order to be included in logic regression models, the phenotypic characteristics were dichotomized by creation of dummy variables. Each of the nine *MC1R* variant was also dichotomized assuming a dominant model, as discussed in section 3.1.2, thus comparing carriers of no variant allele with carriers of 1 or 2 variant alleles. The variables considered in the analysis were 24: four hair colors (red, blonde, brown, black), three eye colors (blue, brown, green/grey/hazel), three skin types (I, II/III, IV), three classes of common naevi (Ò10, 11-45, >45), atypical naevi (any/none), freckles (any/none), and the nine *MC1R* variants V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D294H.

In order to find the combinations of *MC1R* variants and phenotypic characteristics mostly associated with melanoma, I applied logic regression and selected the optimum model as described in section 4.2.1. I allowed a maximum number of three logic trees, in order to not obtain a too complex model of difficult interpretation. For the selection procedure, the maximum number of leaves was set to 12, which is half of the included variables. This seemed a reasonable number in order to avoid too complex trees and too much time spent for randomization tests, otherwise allowing a large number of variables to be included in the final model.

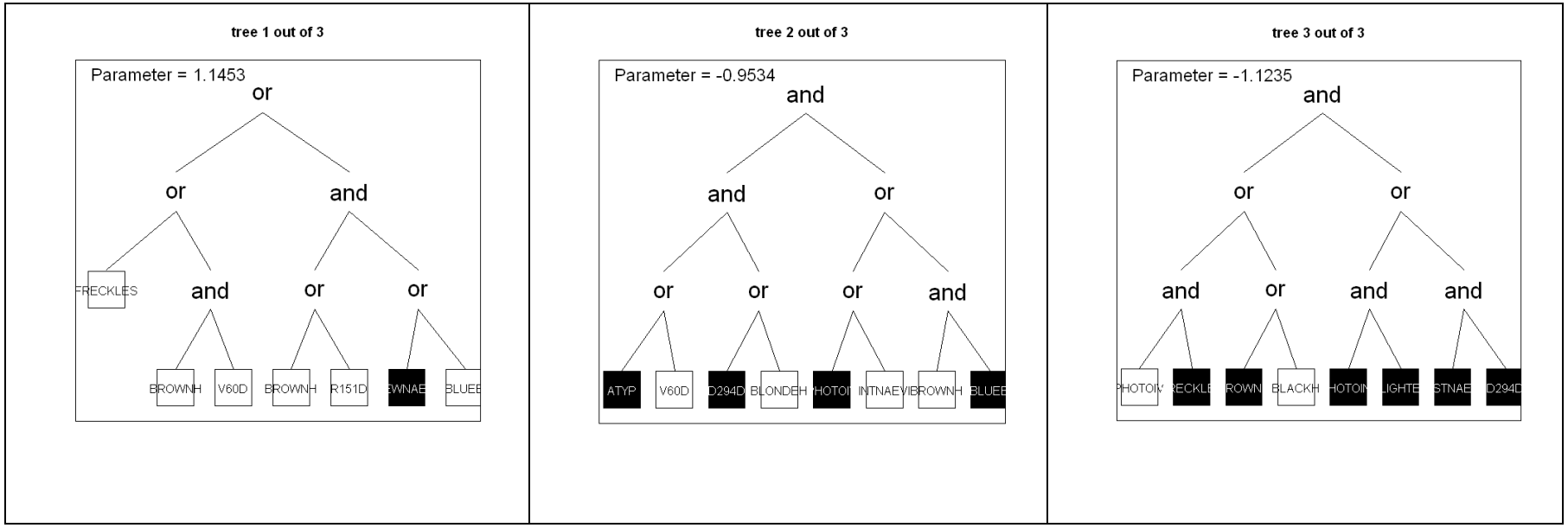
The parameter used for MCMC logic regression depended on the geometric prior on model size and was set to log2, as suggested [Kooperberg et al. 2011]. The number of iterations was set to 100,000.

The statistical analysis was performed using the package ñLogicRegò implemented in R software [Kooperberg et al. 2011].

Results

The best model with three trees included almost all the phenotypic characteristics and the three *MC1R* variants V60L, R151C and D294H (Figure 4.5).

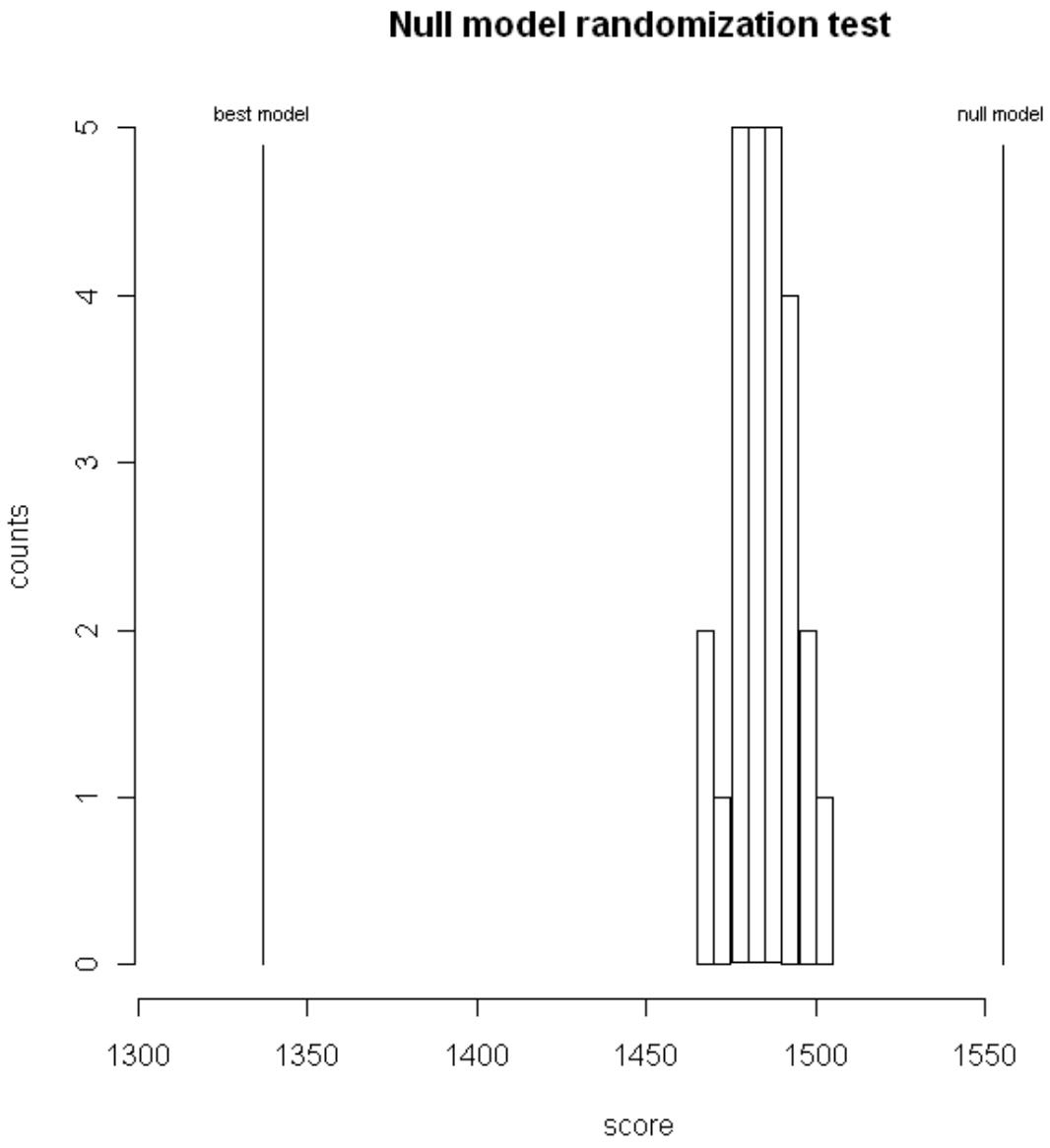
Figure 4.5. The logic trees of the optimum model obtained with the simulation annealing algorithm



ATYP=atypical naevi; BLACKH=black hair; BLONDEH=blonde hair; BLUEE=blue eyes; BROWNH=brown hair; FEWNAEVI=maximum of 10 common naevi; INTNAEVI=intermediate number of common naevi (11-45); LIGHTE=light eye color, other than blue (green, grey or hazel); MSTNAEVI=more than 45 common naevi; PHOTOINT=intermediate skin phototype (II or III); PHOTOIV=skin phototype IV

The randomization test assessing the existence of any association between the predictor variables and melanoma gave a p-value <0.0001 (0 randomized scores were better than the best score, Figure 4.6).

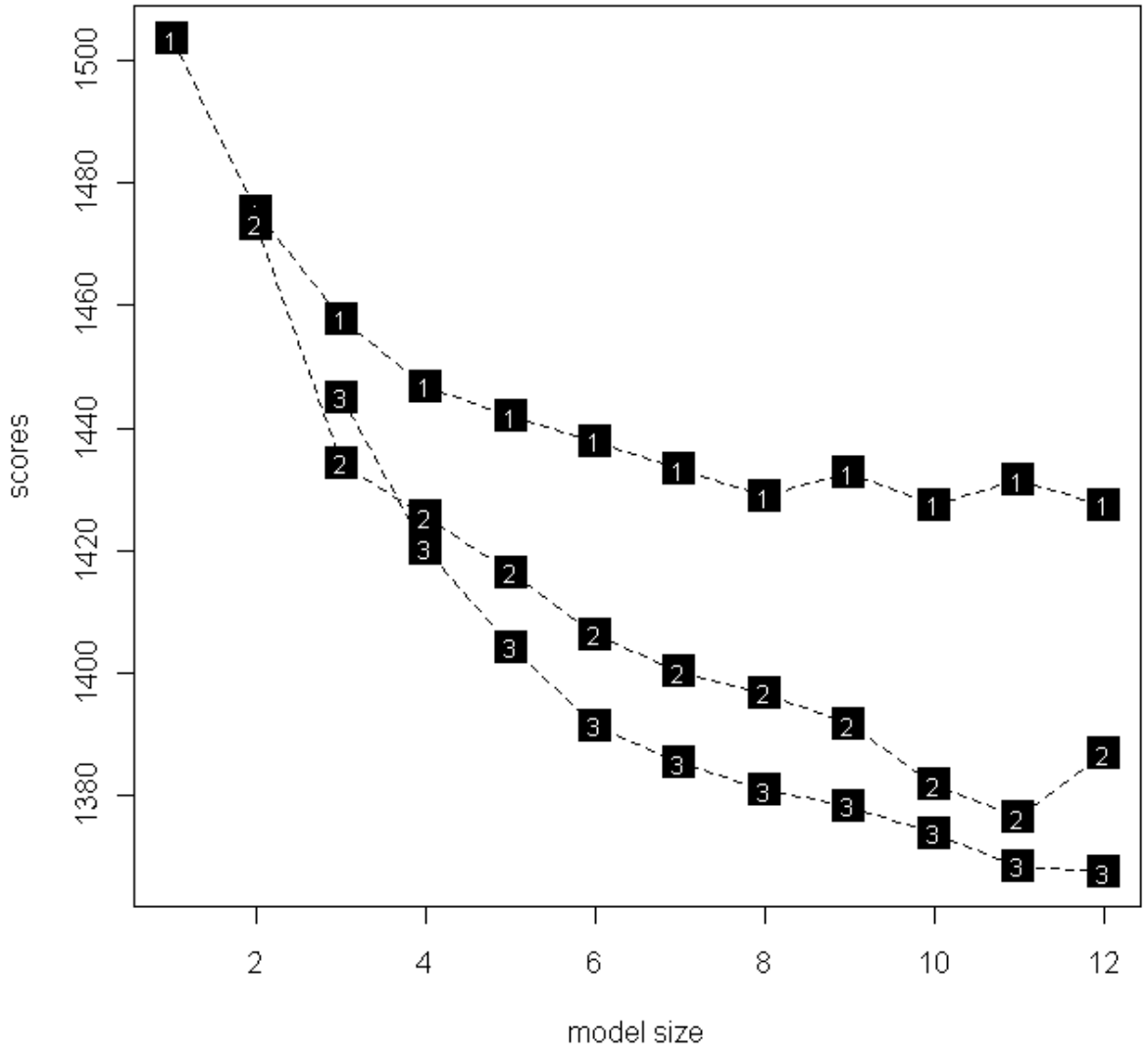
Figure 4.6. The scores of the null randomization test. The score of the null model was obtained by fitting an intercept, the score of the best model was obtained from a model with three trees allowed



Since the best model found with the simulation annealing algorithm typically overfits the data, I proceeded with several tests to select the optimum model of size *s*. The scores of all the considered

models, with one to three trees and one to 12 leaves, are presented in Figure 4.7. The models with one tree had clearly worse scores than models with two or three trees, while from a first look it seems that models with two and three trees had similar scores. As expected, the model with the best score is that with the maximum number of trees and leaves allowed (three trees and 12 leaves).

Figure 4.7. The scores of the best linear models with various numbers of leaves and trees. The number of trees allowed in the linear model is indicated by the white number super-imposed on the black squares



The cross-validated scores of the investigated models are presented in Figure 4.8. It may be noticed that models with three trees and five to nine leaves had better cross-validation scores than all the other models. Basing just on the results from cross-validation, the optimum model would be that with three trees and five leaves. This model is drawn in Figure 4.9 and included just phenotypic characteristics and no *MC1R* variant. Subjects with either brown eyes or more than 45 common naevi had a doubled risk of developing melanoma than subjects with none of these two phenotypic characteristics (OR; 95%CI: 2.04; 1.58-2.63); subjects with atypical neavi had OR (95%CI)=2.49 (1.79-3.47) of developing melanoma compared to those with no atypical naevi; subjects with either freckles or brown hair had higher melanoma risk than subjects with none of these phenotypic characteristics: OR (95%CI): 2.72 (2.06-3.60).

Figure 4.8. The cross-validation scores of the linear models with various numbers of leaves and trees. The number of trees allowed in the linear model is indicated by the white number super-imposed on the black squares.

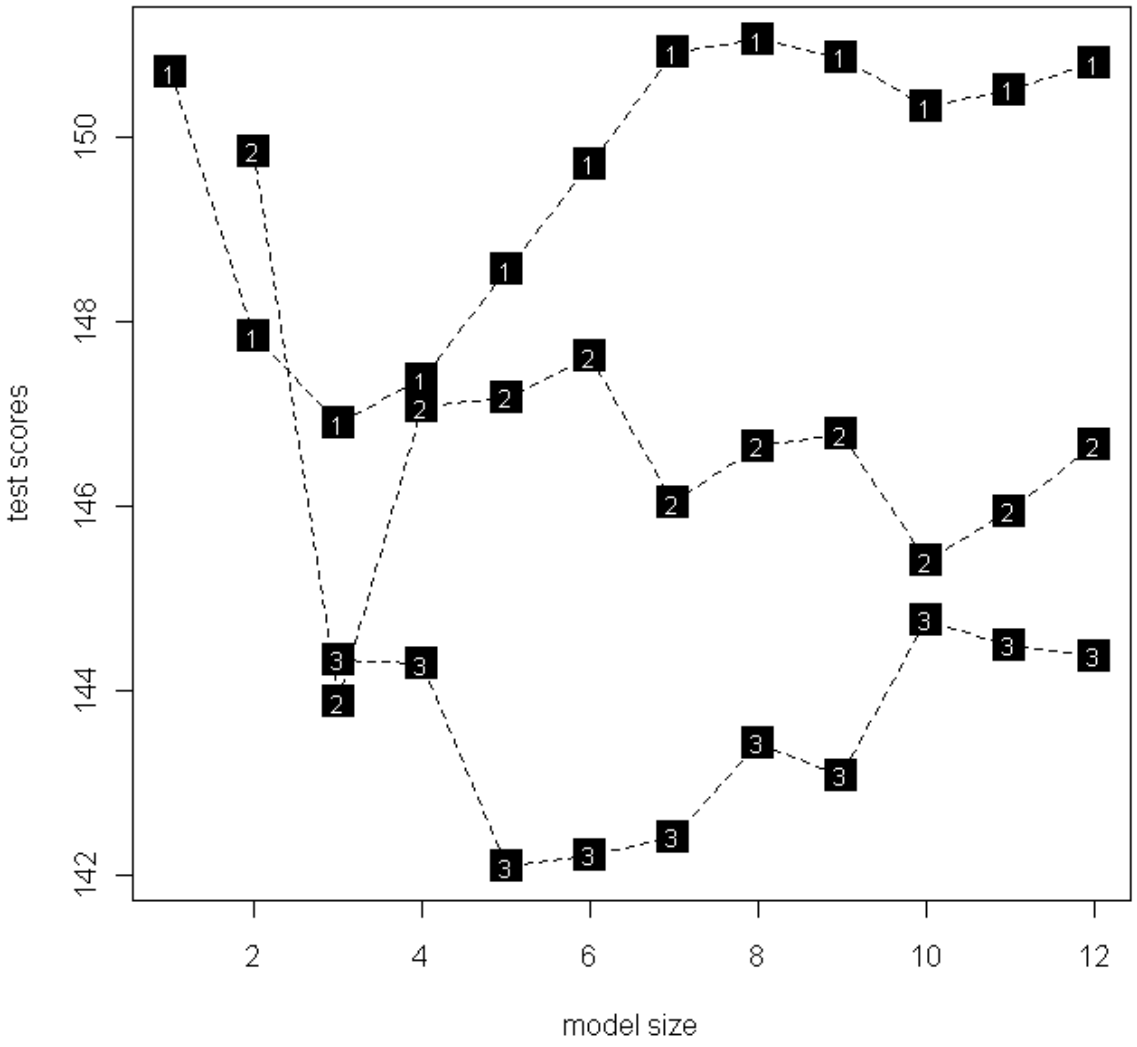
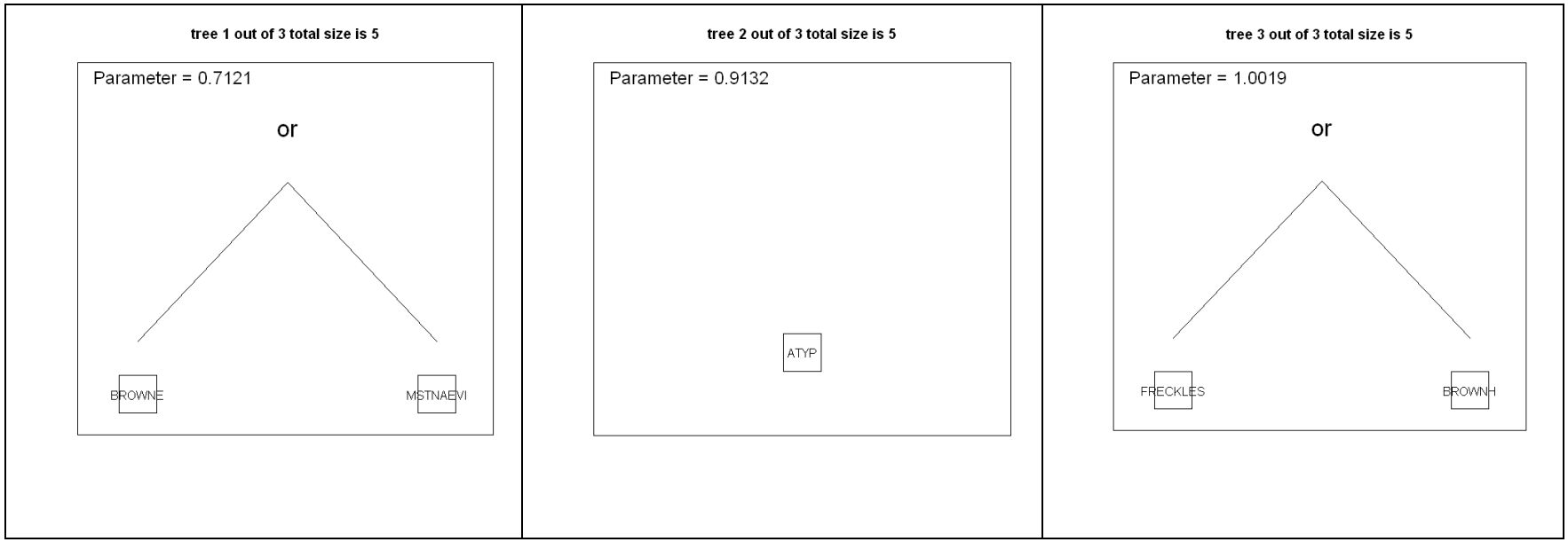


Figure 4.9. The logic trees of the optimum model obtained after cross-validation.



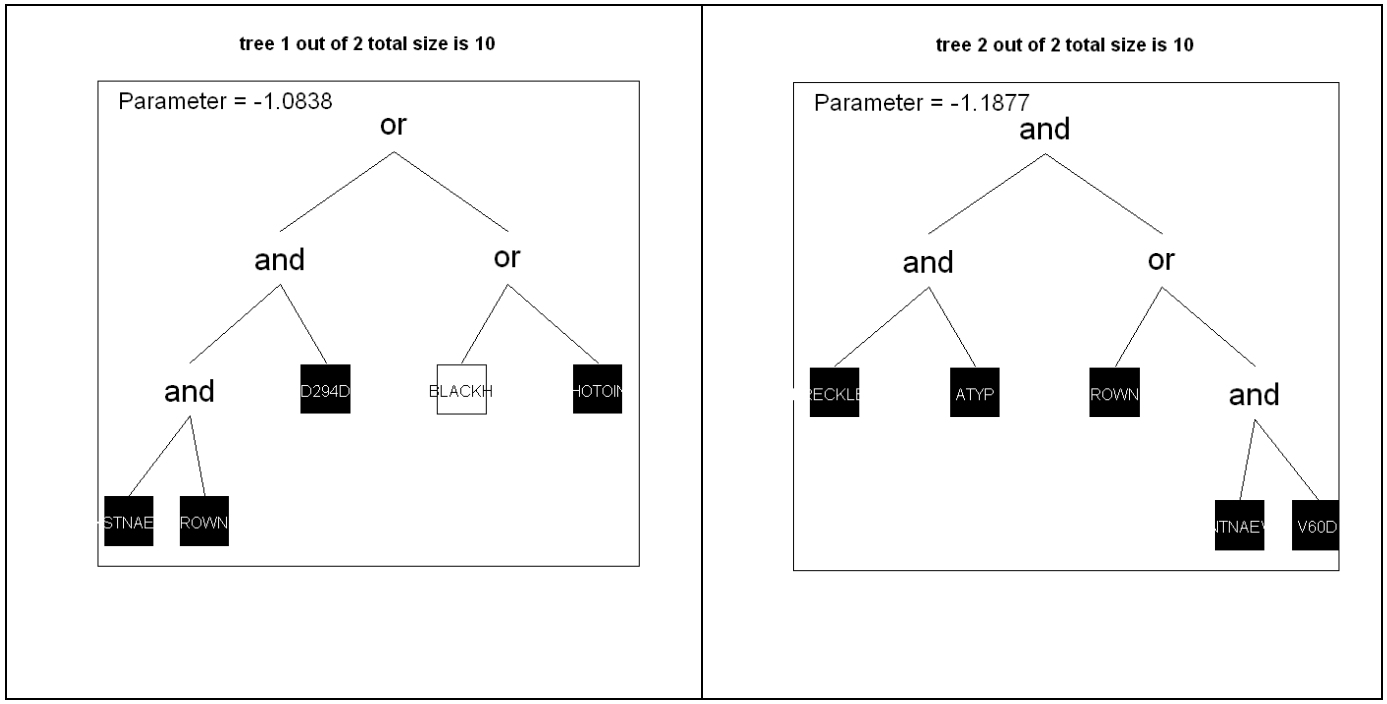
ATYP=atypical naevi; BROWNE=brown eyes; BROWNH=brown hair; MSTNAEVI=more than 45 common naevi

The results from randomization tests in order to verify the hypothesis that the optimum model size is *s*, with $s \in \{0,1,...,12\}$ are reported in Table 4.7. According to the results of randomization test, the acceptable models were those with two trees and ten to 12 leaves, and those with three trees and eight to 12 leaves. The optimum model would be the more simple, so that with two trees and ten leaves. This model is drawn in Figure 4.10 and included both phenotypic characteristics and the two *MC1R* variants V60L and D294H. Looking at gene-phenotype interaction, the *MC1R* variant V60L played a role in combination with common naevi count, while D294H interacted with both common naevi count and eye color. According with this model, subjects with no more than 45 common naevi, not brown eyes and not *MC1R* D294H variant or subjects with black hair or subjects with the extreme skin types I or IV had OR (95%CI)=0.34 (0.26-0.44) of developing melanoma compared with subjects without this combination of phenotypic and genetic characteristics. Subjects with no freckles and no atypical naevi with either not brown hair or both *MC1R* V60L wild type and extreme number of naevi (Ò10 or >45) had OR (95%CI)=0.31 (0.23-0.40) of developing melanoma compared with subjects without this combination of phenotypic and genetic characteristics.

Table 4.7. Distribution of scores of the randomization test for model selection, obtained conditioning on 0 to 12 leaves. The score of the null model (intercept only) is 1555.379 while the best overall score found using the original (non-randomized) data is 1327.852. The general rule is to choose the smallest model size where a fraction higher than 20% of the randomization scores have scores better than 1327.852 (bold)

Trees	Leaves	Minimum	1st quartile	Median	3rd quartile	Maximum	%<best
1	1	1413	1432	1438	1442	1452	0
1	2	1395	1407	1414	1420	1431	0
1	3	1375	1388	1396	1400	1407	0
1	4	1362	1375	1383	1388	1400	0
1	5	1374	1378	1382	1388	1394	0
1	6	1362	1378	1382	1390	1395	0
1	7	1365	1370	1379	1382	1394	0
1	8	1354	1375	1381	1386	1404	0
1	9	1362	1370	1377	1383	1404	0
1	10	1349	1377	1384	1388	1401	0
1	11	1364	1376	1379	1385	1395	0
1	12	1360	1368	1379	1388	1407	0
2	2	1391	1399	1408	1412	1434	0
2	3	1356	1367	1376	1383	1390	0
2	4	1355	1363	1368	1373	1390	0
2	5	1351	1357	1366	1368	1376	0
2	6	1322	1341	1349	1357	1369	4
2	7	1327	1337	1345	1351	1365	4
2	8	1330	1342	1348	1352	1363	0
2	9	1327	1340	1345	1347	1356	4
2	**10**	**1315**	**1326**	**1335**	**1340**	**1356**	**32**
2	11	1314	1324	1331	1338	1350	44
2	12	1315	1328	1338	1346	1353	28
3	3	1363	1369	1376	1382	1387	0
3	4	1329	1358	1366	1374	1386	0
3	5	1326	1344	1351	1354	1365	4
3	6	1326	1337	1342	1345	1358	4
3	7	1317	1333	1338	1341	1351	16
3	8	1318	1326	1339	1343	1356	28
3	9	1306	1326	1331	1336	1349	32
3	10	1305	1320	1328	1334	1344	48
3	11	1311	1320	1326	1331	1337	64
3	12	1298	1317	1323	1330	1338	60

Figure 4.10. The logic trees of the optimum model obtained after permutation tests



ATYP=atypical naevi; BLACKH=black hair; BROWNE=brown eyes; BROWNH=brown hair; INTNAEVI=intermediate number of common naevi (11-45),; MSTNAEVI=more than 45 common naevi; PHOTOINT=intermediate skin phototype (II or III)

By combining the results of cross-validation and permutation tests, the acceptable models were those with three trees and eight or nine leaves.

The frequency of models of any size with three trees visited by MCMC logic regression algorithm is presented in Figure 4.11. Models of size eight and nine were visited almost an equal number of times from the Monte Carlo algorithm and were the two model sizes with the highest frequency among all the considered model sizes. I therefore chose to select the less complex model with eight leaves as the optimum model for the data including in this study. The final model selected in this way is presented in Figure 4.12 and included brown hair, brown eye, intermediate skin types, common and atypical naevi, freckles and the two *MC1R* variants R151C and D294H. According to this finally selected model, the OR (95%CI) of developing melanoma for subjects with no atypical naevi and no *MC1R* D294H variant was 0.40 (0.29-0.55) compared to subjects with at least one of these risk factors; subjects with either brown eyes or more than 45 common naevi have a doubled melanoma risk (OR; 95%CI: 2.10; 1.63-2.72) than those with none of these phenotypic characteristics; subjects with freckles or subjects with both an intermediate skin type (II/III) and either brown hair or *MC1R* R151C variant had an OR (95%CI)=3.11 (2.35-4.12) compared to subjects without this combination of phenotypic and genetic characteristics (see Table 4.8).

Figure 4.11. Percent size distribution of regression models with three trees visited by the Monte Carlo algorithm

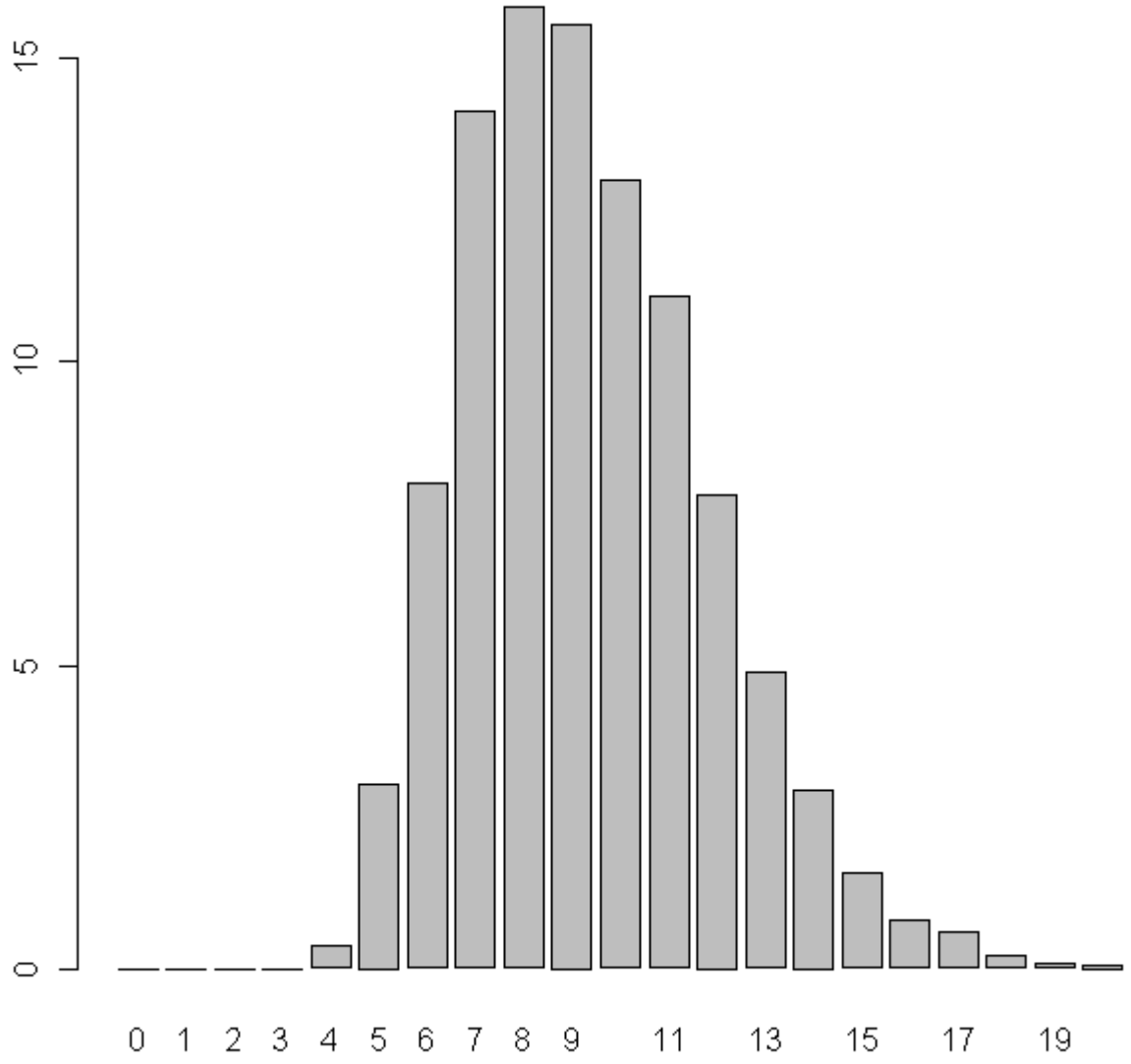
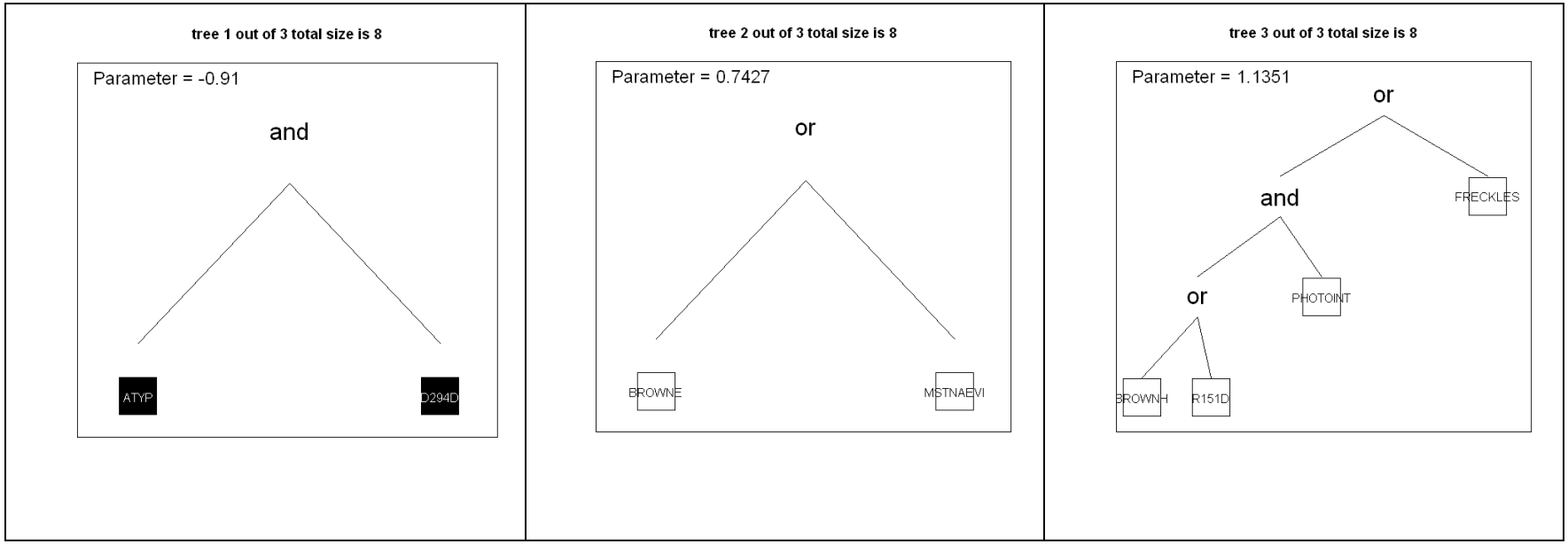


Figure 4.12. The logic trees of the final optimum model selected by a combination of cross-validation, randomization tests and Monte Carlo logic regression



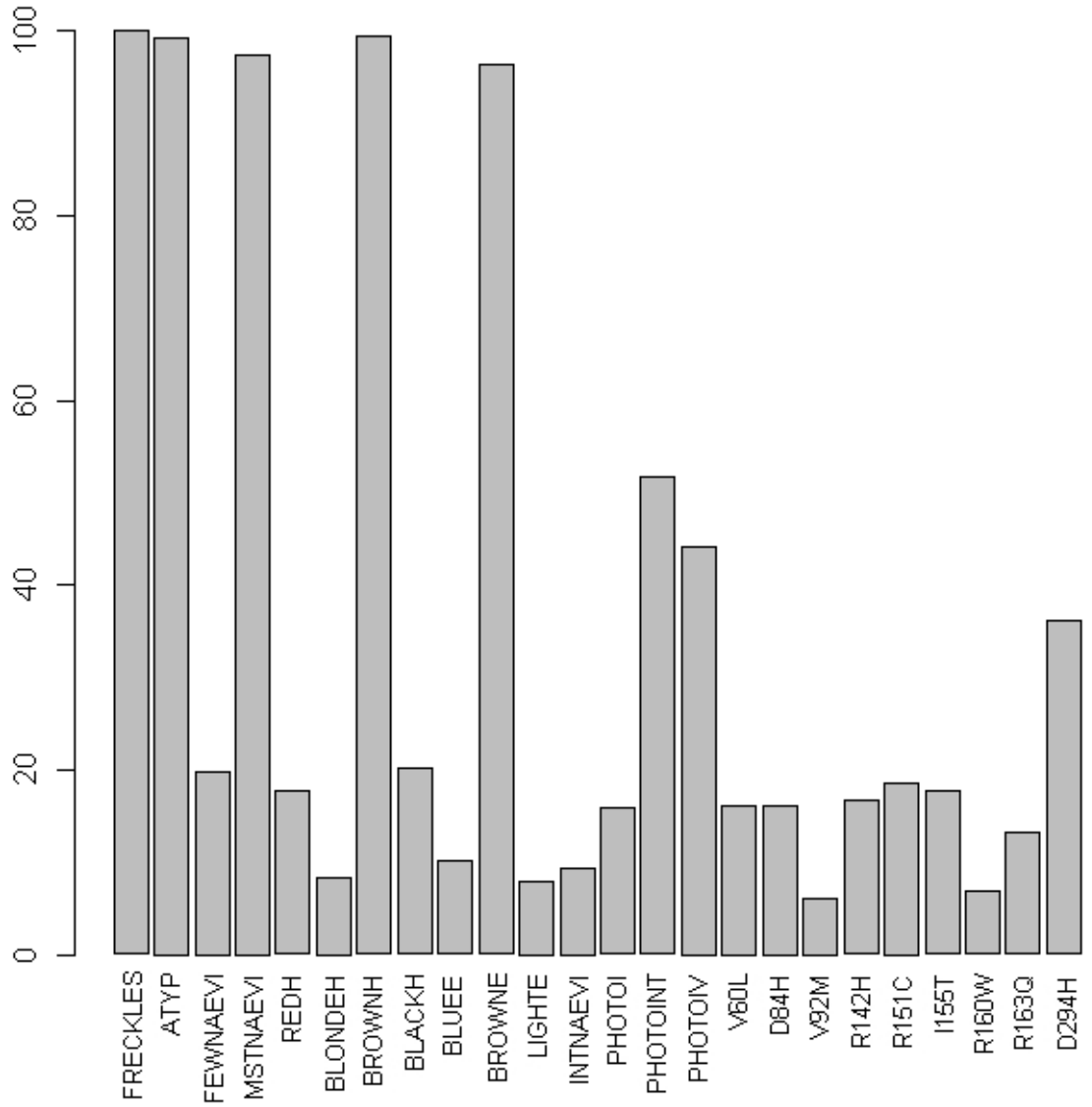
ATYP=atypical naevi; BROWNE=brown eye; BROWNH=brown hair; MSTNAEVI=more than 45 common naevi; PHOTOINT=intermediate skin phototype (II or III)

Table 4.8. Odds Ratios (OR) with 95%Confidence Intervals (CI) for the association between combinations of phenotypic characteristics and *MC1R* variants obtained from the final selected logic regression model

N tree	Gene-phenotype combination	OR (95%CI)
1	Any atypical naevi D294H variants Ó1	1.00 (ref)
	No atypical naevi + no D294H variant	0.40 (0.29-0.55)
2	Not brown eye + common naevi Ò45	1.00 (ref)
	Brown eye >45 common naevi	2.10 (1.63-2.72)
3	No freckles + photoype I/IV No freckles + phototype II/III + no brown hair + no R151C variant	1.00 (ref)
	Freckles Phototype II/III + brown hair Phototype II/III + R151C variants Ó1	3.11 (2.35-4.12)

The phenotypic characteristics most frequently included in models visited by the Monte Carlo algorithm were freckles, atypical naevi, more than 45 common_naevi, brown eyes and hair, and intermediate skin types (Figure 4.13). These are exactly the phenotypic characteristics included in the optimum selected model. The most frequent *MC1R* variant was D294H, followed by R151C, I155T and R142H. Again, this agrees with the inclusion of D294H and R151C in the optimum selected model.

Figure 4.13. Percent marginal frequency of being in the model for each variable according to Monte Carlo algorithm



ATYP=atypical naevi; BLACKH=black hair; BLONDEH=blonde hair; BLUEE=blue eyes; BROWNE=brown eyes; BROWNH=brown hair; FEWNAEVI=maximum of 10 common naevi; INTNAEVI=intermediate number of common naevi (11-45); LIGHTE=light eye color, other than blue (green, grey or hazel); MSTNAEVI=more than 45 common naevi; PHOTOI=skin phototype I; PHOTOINT=intermediate skin phototype (II or III); PHOTOIV=skin phototype IV; REDH=red hair

4.2.3. Classical model selection for the analysis of M-SKIP dataset

In order to compare the results obtained with logic regression with those obtained with classical methods of variables selection for logistic regression models, I performed a new analysis on the

same dataset with 496 cases and 639 controls from three studies. The aim was again to find combinations of phenotypic characteristics and *MC1R* variants mostly associated with melanoma.

Statistical analysis

The studied variables were the same as described in section 4.2.2. All the variables were binary. The reference categories for hair color, eye color, skin type and common naevi were, respectively: black hair, green/grey/hazel eyes, intermediate skin types II/III, intermediate number of common naevi (11-45). Since the reference categories for each phenotypic characteristic were linear combinations of the dummy variables, they were not included in the analysis and the final number of considered variables was therefore 20.

The main effect of each variable on melanoma development was estimated by unconditional logistic regression models. The best model including the variables mostly associated with melanoma was obtained by forward or backward selection, whichever had the smallest AIC. All the possible two by two interactions of the selected variables were defined and included in a new logistic regression model. The best model including the main effects and interactions mostly associated with melanoma development was obtained by forward or backward selection, whichever had the smallest AIC. Higher order interactions were not taken into account because they lead to difficultly interpretable models. The goodness of fitting data for classical model selection and logic regression was compared using the AIC obtained by the two final models.

Results

Using both forward and backward selection, the variables mostly associated with melanoma development were: red and brown hair, brown eyes, common naevi count Ò10 and >45, atypical naevi, freckles, and *MC1R* D294H variant. The OR with 95% CI for the main effect of these

variables were reported in Table 4.9. Subjects with at least one D294H variant had the highest risk of melanoma (OR; 95%CI: 2.32; 1.11-4.85), while subjects with few common naevi had the lowest risk (OR: 95%CI: 0.71; 0.54-0.94). Except few naevi count, all the other selected variables represented risk factors for melanoma development. With respect to the variables included in the best logic regression model, with classical methods two more variables were selected: red hair color and few common naevi count, otherwise skin type and *MC1R* R151C variant were not selected.

Table 4.9. Odds Ratios (OR) with 95%Confidence Intervals (CI) for the association between phenotypic characteristics and *MC1R* variants obtained by forward and backward selection applied to logistic regression models

Variable	Category	OR (95%CI)
Hair color	Black	1.00 (ref)
	Red	1.97 (1.12-3.48)
	Brown	1.93 (1.46-2.55)
Eye color	Green/grey/hazel	1.00 (ref)
	Brown	1.67 (1.26-2.20)
Common naevi count	Ò10	0.71 (0.54-0.94)
	11-45	1.00 (ref)
	>45	1.97 (1.24-3.12)
Atypical naevi	None	1.00 (ref)
	Any	2.13 (1.51-3.01)
Freckles	None	1.00 (ref)
	Any	1.88 (1.44-2.46)
D294H	No variant allele	1.00 (ref)
	1 or 2 variant alleles	2.32 (1.11-4.85)

When the two by two interactions were added to the logistic regression model, the variables selected as mostly associated with melanoma were: red and brown hair, brown eyes, common naevi count Ò10 and >45, atypical naevi, freckles. The interactions mostly associated with melanoma were: brown hair and eyes, brown hair and freckles, brown eyes and more than 45 common naevi, *MC1R* D294H and no more than 10 common naevi. This model was obtained by forward selection; the ORs with 95%CI were reported in Table 4.10. It may be noticed that in this model D294H seemed to play a role in melanoma development only in interaction with common naevi count, with

a significant synergic effect, somewhat nullifying the protective effect of having few common naevi.

Table 4.10. Odds Ratios (OR) with 95%Confidence Intervals (CI) for the association between phenotypic characteristics, *MC1R* D294H variant, and their interactions obtained by forward selection applied to logistic regression models

Variable	Category	OR (95%CI)
Hair color	Black	1.00 (ref)
	Red	1.76 (0.98-3.17)
	Brown	1.84 (1.64-3.77)
Eye color	Green/grey/hazel	1.00 (ref)
	Brown	1.84 (1.18-2.86)
Common naevi count	Ò10	0.69 (0.52-0.91)
	11-45	1.00 (ref)
	>45	3.48 (1.95-6.20)
Atypical naevi	None	1.00 (ref)
	Any	2.09 (1.48-2.97)
Freckles	None	1.00 (ref)
	Any	1.78 (1.90-4.08)
Hair and eye color	No brown hair and no brown eyes	1.00 (ref)
	Brown hair and brown eyes	1.09 (0.62-1.92)
Hair color and freckles	No brown hair and no freckles	1.00 (ref)
	Brown hair and freckles	0.47 (0.27-0.80)
Eye color and common naevi count	No brown eyes and Ò45 naevi	1.00 (ref)
	Brown eyes and >45 naevi	0.22 (0.09-0.52)
D294H and common naevi count	No variant allele and >10 naevi	1.00 (ref)
	1 or 2 variant alleles and Ò10 naevi	2.93 (1.12-7.63)

The AIC associated with the logic regression model selected in the section 4.2.2 was 1,388.722. The AIC associated with the logistic regression model obtained by forward selection was 1,401.665.

4.2.4. Discussion

Logic regression is a new tool useful for detecting subpopulations at high or low risk of disease, characterized by high-order interactions among covariates. The types of interactions identified by logic regression are not ñtraditionalò interactions, where one predictor modifies the effect of another predictor, but rather combinations of predictors that are associated with increased or decreased

disease risk. With respect to other regression models this allow to estimate disease risk for combinations including not only the operator ñANDò, but also the ñORò operator. With a single logic tree, such logic regression identifies a single group of persons at increased (decreased) risk; when the underlying risk profile is more complicated, additional logic trees may be needed. The logic trees provide easily interpretable description of subpopulations, and thus the methodology could be well applied to the study of complex diseases like cancer. In the study of gene-phenotype interaction in melanoma development, I could observe that logic regression generated a much simple characterization of the subsets of the population at high risk than did linear logistic regression, which depended on weighted averages of covariate values and of their interactions. Moreover logic regression takes into account even high-order interactions among covariates, while with classical logistic regression models this would lead to very difficultly interpretable models. A reason of the less complexity of logic regression approach is that it allows to compute the importance of variables interactions without using the interactions as input variables, thus using less degrees of freedom in the final model. In the application on M-SKIP dataset, the logic regression model had three degrees of freedom, while the classical logistic regression model had 11 degrees of freedom. This did not affect the model fitting; on the contrary the AIC for the logic regression model was smaller than that of logistic regression model. Thus it was possible to select by logic regression a model with a good fitting, not too complex and quite easily interpretable. Finally, since logic regression is a well-defined procedure, model selection and multiple-comparisons corrections for the significance level are implicit and do not require further resampling or bootstrapping.

The approach based on logic regression has, however, some limitations. First, the variable important measures are currently restricted to analyses of data with a binary outcome. If one wish to enter continuous predictors in the interactions, they would have to be dichotomized. In my application to M-SKIP dataset, I dichotomized categorical variables with dummy variables. This was easy and lead to a good interpretation of results, however other classifications (i.e. light vs dark hair color, skin types I/II versus III/IV) would had provide different results. My choice of

dummy variable creation was orientated by (1) having as much differentiation as possible in hair and eye color, and (2) separating the extreme classes of skin type and common naevi count by intermediate classes. This was done for clinical reasons and also because in pooled-analysis setting misclassification probably affects the intermediate classes of exposure more than the extreme ones [Gandini S et al. 2005c]. The computation time of the proposed approach depends not only on the number of iterations used in simulated annealing but also on the maximum number of variables and trees allowed in the models: 10-fold cross-validation and randomization tests, where models with three trees and 12 leaves were investigated, took several hours. In my application, I had missing data on a number of covariates, and chose to drop subjects with any missing values. The amount of missing data was huge, and the analysis was therefore restricted to 7% of the whole dataset. However, due to the very large amount of collected data in the M-SKIP project, this subgroup

In conclusion, logic regression seems an accurate method for identifying combinations of binary variables mostly associated with a response and its results are easily interpretable. The application of logic regression to the study of gene-phenotype interaction in melanoma risk let to identify three subgroups of patients characterized by a higher risk of melanoma, and suggested possible interaction between the two *MC1R* variants D294H and R151C with atypical naevi and skin type, respectively. Further investigation are warrant to better analyze gene-phenotype interaction and to assess the role of sun exposure on a larger M-SKIP dataset and within a pooled-analysis setting.

4.3. Validation analyses on gene-phenotype interaction

The previous analysis was carried out on a small subset of M-SKIP dataset (496 cases and 639 controls from three studies), characterized by no missing data on the studied phenotypic characteristics and *MC1R* variants. I then validated the previous results with a new analysis including, for each identified high-risk sub-population, all the studies in which the sub-population could be defined and taking into account the available confounders. The high risk sub-populations

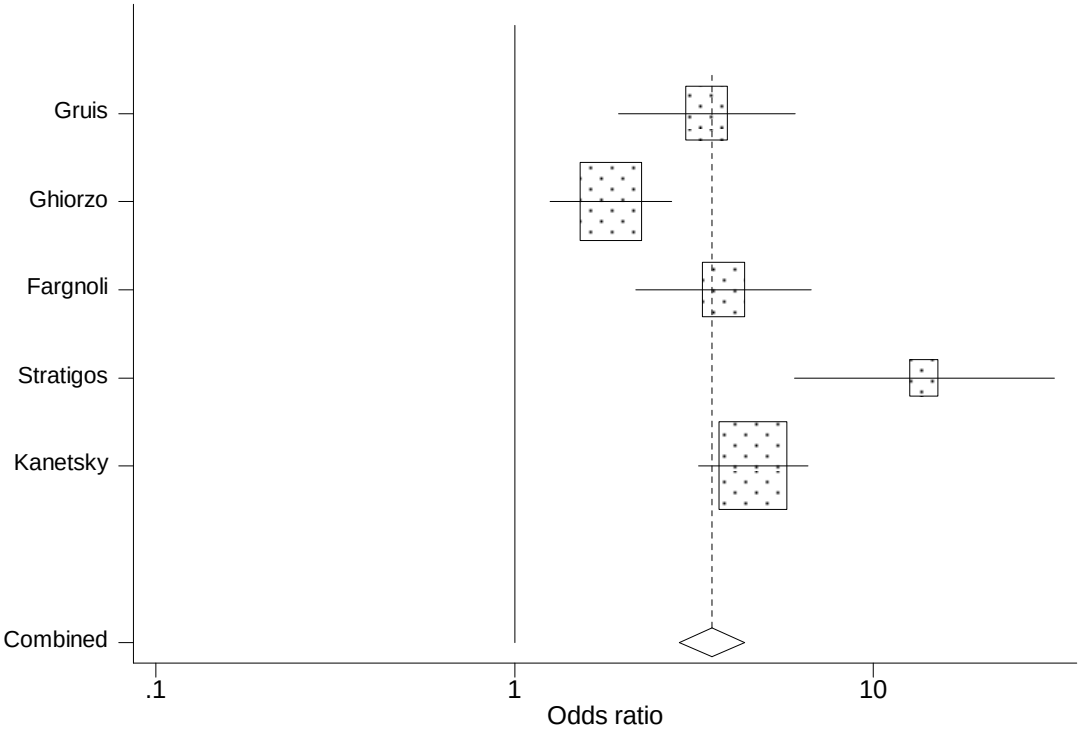
identified by logic regression, pooling subjects of three different studies (Gruis, Ghiorzo, Debniak), included subjects with 1) any atypical naevi or any D294H variant; 2) brown eye or more than 45 common naevi; 3) freckles or phototypes II/III and (brown hair or any R151C variant).

For this analysis I used a two-stage meta-analytic approach (section 3.2), by pooling, for each sub-population, the confounders-adjusted ORs of available case-control studies. I excluded case-control studies with more than 20% of missing data for the variable(s) used to define the high-risk sub-population.

4.3.1. Population one: atypical naevi or any D294H variant

The OR (95%CI) found with logic regression for this population was 2.50 (1.82-3.45). For this validation analysis I excluded the study by Debniak because there were more than 20% of missing data for atypical naevi variable. The validation analysis was carried out on five independent studies (Figure 4.14) including 1,360 cases and 1,220 controls. The pooled-OR (95%CI) to develop melanoma for population one was 4.08 (2.38-6.98), with evidence of heterogeneity between studies (I^2:82.7%, Q statistic p-value:<0.0001). However all the study-specific estimates were significantly higher than 1.00, therefore the association with melanoma for population one could be confirmed.

Figure 4.14. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between population one* and melanoma

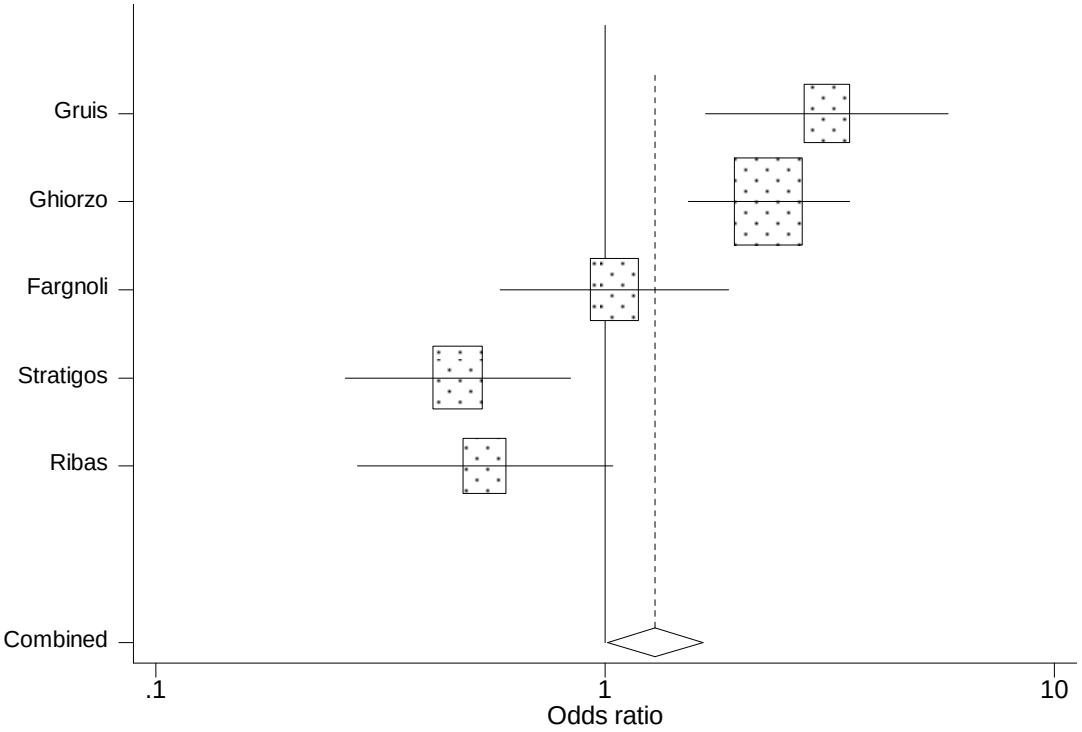


*subjects with atypical naevi or any D294H variant

4.3.2. Population two: brown eye or more than 45 common naevi

The OR (95%CI) found with logic regression for this population was 2.10 (1.63-2.72). For this validation analysis I excluded the study by Debniak because there were more than 20% of missing data for common naevi variable. The validation analysis was carried out on five independent studies (Figure 4.15) including 699 cases and 1,085 controls. The pooled-OR (95%CI) to develop melanoma for population two was 1.15 (0.55-2.40), with evidence of heterogeneity between studies (I^2:88.4%, Q statistic p-value:<0.0001). The significant association with melanoma for this population was indeed observed only in the two studies included in the logic regression analysis, while this was not confirmed by the further three studies included in the validation analysis. Therefore the association with melanoma for population two could not be confirmed.

Figure 4.15. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between population two* and melanoma

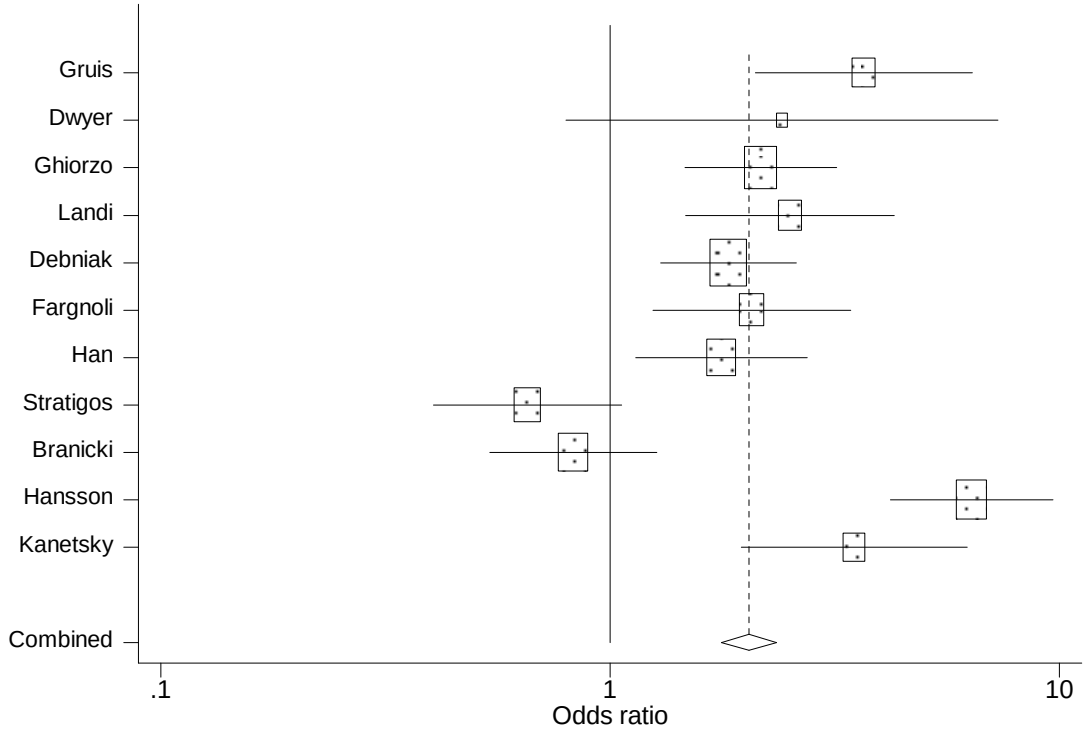


*subjects with brown eye or more than 45 common naevi

4.3.3. Population three: freckles or phototypes II/III and (brown hair or any R151C variant)

The OR (95%CI) found with logic regression for this population was 3.11 (2.35-4.12). The validation analysis was carried out on 11 independent studies (Figure 4.16) including 2,997 cases and 2,971 controls. The pooled-OR (95%CI) to develop melanoma for population two was 2.10 (1.41-3.13), with evidence of heterogeneity between studies (I^2:86.9%, Q statistic p-value:<0.0001). However all the study-specific estimates but two (Stratigos, Branicki) were higher than 1.00, with statistical significance reached for all the studies but one (Dwyer), therefore the association with melanoma for population three could be confirmed.

Figure 4.16. Study-specific and pooled-Odds Ratio (OR) with 95% Confidence Intervals (CI) for the association between population three* and melanoma



*subjects with freckles or phototypes II/III and (brown hair or any R151C variant)

Chapter 5

Discussion and conclusions

5.1. Main effect of *MC1R* gene variants on melanoma

I found a significant association with melanoma for the five *MC1R* variants D84E, R142H, R151C, R160W, and D294H by using both the standard two-stage analysis and the multivariate adaptation of the new approach proposed by Jackson [Jackson et al. 2009]. Our previous meta-analysis and earlier studies have already suggested that the three variants R151C, R160W, and D294H were significantly associated with melanoma risk, probably via pigmentary pathways [Raimondi et al. 2008; Palmer et al. 2000; Kennedy et al. 2001; Matichard et al. 2004; Landi et al. 2005; Debniak et al. 2006: Fargnoli et al. 2006b; Han et al. 2006]. The association of D84E and R142H with melanoma was controversial: in some studies these variants were found significantly associated with melanoma risk [Valverde et al. 1996; Smith et al. 1998; Kennedy et al. 2001; Mossner et al. 2007; Fargnoli et al. 2006b] but other studies [Palmer et al. 2000; Matichard et al. 2004; Stratigos et al. 2006] did not show a particular relationship between these variants and melanoma. The controversial results could be explained with the fact that these variants are less common and therefore large sample sizes are necessary to reach powerful results. In vitro expression studies on D84E, R151C, and R160W receptors revealed that they have reduced cell surface expression and a corresponding impairment in cAMP coupling. The R142H and D294H variants showed normal cell surface expression, but had reduced functional responses [Beaumont et al. 2007]. This could explain why these mutations are common in individuals with red hair and fair skin and therefore we can at least partly explain the association of these five *MC1R* variants with melanoma by pigmentary pathways: red hair and fair skin individuals are unable to increase melanin levels in the skin in

response to high exposure to UV light and therefore increase the levels of pheomelanin, which is mutagenic and cytotoxic [Harsanyi et al. 1980; Koch et al. 1986].

A small association between melanoma and V92M variant was suggested with the standard two-stage analysis (OR; 95%CI: 1.15; 1.00-1.31), but not confirmed by the analysis with Jackson approach (1.18; 0.99-1.42), although the two ORs and 95%CI were very similar. For this variant it is warranted to increase the statistical power of the pooled-analysis by including further studies: this may be done in a next step of the project, when new classical association studies and GWAS will be included and analyzed. Anyway, the lack of association observed with the Jacksonôs approach may be also explained with the lower precision of the CI with respect to the classical two-stage approach observed in the validation studies. In our previous meta-analysis [Raimondi et al. 2008], we found no association with melanoma for V92M variant. For these three variants, functional studies reported a marginal role in affecting cyclic adenosine monophosphate levels and therefore *MC1R* function [Beaumont et al. 2007].

No association between I155T variant and melanoma was found with the standard two-stage analysis (OR; 95%CI: 1.36; 0.97-1.90), while with the Jacksonôs approach I observed an OR significantly higher than 1.00 (2.84; 2.03-3.97). This may be explained with the fact that I155T variant is rare, and the Jacksonôs approach might therefore obtain false positive results, as happened in my validation study. An association between I155T variant and melanoma was previously observed [Debniak et al. 2006; Han et al. 2006; Raimondi et al. 2008], however the lack of association with red hair color and fair skin previously found [Raimondi et al. 2008] suggests that, for this variant, melanoma risk could be possibly increased via non-pigmentary pathways.

Finally, no association was observed between melanoma and the two *MC1R* variants V60L and R163Q by using both the standard two-stage analysis and the multivariate adaptation of the new approach proposed by Jackson. In our previous meta-analysis [Raimondi et al. 2008], we found no association with melanoma for V60L variant, while we observed a significantly higher risk of

melanoma for R163Q variant. For these two variants, functional studies reported a marginal role in affecting cyclic adenosine monophosphate levels and therefore *MC1R* function [Beaumont et al. 2007].

A significant heterogeneity among the study-specific estimates was found for the four *MC1R* variants V60L, R142H, R151C and R160W, but seemed to be attributable to single studies. With the exclusion, in a sensitivity analysis, of these studies with ñstrangeò risk estimates I obtained similar results than the main analysis.

A participation bias was suggested for R163Q variant: a blank area in the left-bottom part of the graph was observed, which would include small studies with low OR. This is a typical result for publication bias, indicating that results from small studies, with low statistical power, were published only if they suggest a significant association between the variant and melanoma. However, within the M-SKIP project, I asked all the available data from the identified studies, and therefore I received both published and unpublished data. A possibility is that certain investigators of small studies had deleted the data of R163Q variant after the observation of null results, also because functional studies did not demonstrated a role in melanoma development for this variant. This hypothesis, however, need to be directly verified with the investigators of small studies with no data on R163Q variant.

5.2. Gene-phenotype interaction

The independent role of *MC1R* variants on melanoma was investigated by taking into account several phenotypic characteristics that may increase, by themselves, the risk of melanoma., and that may be determined by *MC1R* variants.

Stratified analyses were performed for skin color, skin type, hair color, eye color and freckles. I found statistically significant lower ORs for red-haired subjects compared to subjects with other

hair colors for R142H variant (p-value: 0.02); higher ORs for subjects with dark eyes (brown or black) compared to subjects with light eyes (blue, green, grey or hazel) for D294H variant (p-value:0.02); higher ORs for subjects with no freckles compared to subjects with freckles for V92M and R160W variants (p-values: 0.05 and 0.02, respectively). Moreover, I generally observed higher ORs among darker pigmented subjects for almost all variants and pigmentation characteristics. Similar results were observed in a previous study and meta-analysis [Kanetsky et al. 2010], where association between some *MC1R* variants and melanoma was stronger in subjects with dark hair, dark eyes, skin type III/IV, and in subjects who reported low recreational sun exposure. These results seemed to suggest an important role of *MC1R* gene also via non-pigmentary pathways, therefore several *MC1R* variants could be associated with melanoma development independently by pigmentation characteristics. From a clinical point of view, this result suggests to screen the *MC1R* gene only in darker pigmented subjects, for which the presence of certain variants may significantly increase the risk of melanoma compared to dark pigmented subject without *MC1R* variants. For high risk subjects, specific approach for melanoma early detection may be developed and applied.

Since the sequencing of all *MC1R* gene is expensive, a further goal is to identify how combinations of *MC1R* variants and phenotypic characteristics are at higher risk of develop melanoma, in order to genotype only few *MC1R* variants, which were indeed associated with melanoma in combination with specific phenotypic characteristics. According with the results of the M-SKIP pooled-analysis on 496 cases and 639 controls from three studies (Gruis, Ghiorzo, Debniak), I found that three population subgroups were at higher risk of melanoma.

The first subgroup included subjects with either atypical naevi or *MC1R* D294H, who have a more than doubled risk of melanoma (OR=2.5) than subjects with none of these factors. The genetic risk factors for this group of patients could let identify it as a group with ñgenetic riskò. Atypical naevi was found as an important risk factor for melanoma in a previous meta-analysis [Gandini et al. 2005a] with a very high summary Relative Risk (RR) of 10.12 (95%CI: 5.04-20.2) for the presence

of any atypical naevus. Also the *MC1R* D294H variant was previously associated with melanoma [Raimondi et al. 2008] and was showed to have reduced functional responses. Interaction between these two risk factors, however, was never identified before. The higher risk of melanoma for this subgroup was confirmed by a further analysis on an extended dataset.

The second subgroup of high risk subjects included those with either brown eye or more than 45 common naevi. This group of subjects seemed to have a ñmixed genetic and environmental riskò. In fact brown eye color was not identified as a risk factor for melanoma, even if in a previous meta-analysis it was not analyzed alone, but aggregated with black eye color [Gandini et al. 2005c]. It would be possible that subjects with quite dark phenotypic characteristics like brown eyes and hair exposed more to sun and with less levels of filter protection than subjects with light pigmentation. This could increase their sun-related exposure risk. Otherwise, the association between high number of naevi and melanoma was previously suggested [Gandini et al. 2005a], with meta-analytic RR (95%CI) ranging from 2.24 (1.90-2.64) for the class 41-60 naevi to 6.89 (4.63-10.25) for the highest class of more than 100 common naevi. In the validation analysis performed on a larger M-SKIP dataset, the association with melanoma for this subgroup of patients was not confirmed. In particular, in most studies subjects with brown eyes were indeed protected by melanoma risk, according with the previous published results [Gandini et al. 2005c].

The last high risk subgroup again contained a such combinations of factors that characterized it as having ñmixed genetic and environmental riskò: subjects with freckles or with intermediate skin type and brown hair or with intermediate skin type and at least one *MC1R* R151C variant had a triple risk of melanoma than subjects without this combination of phenotypic and genetic factors. Freckles was found to be a risk factor for melanoma in a previous meta-analysis [Gandini et al. 2005c] with summary RR (95%CI) for high density of freckles: 2.10 (1.80ï 2.45). Subjects with both intermediate skin type and brown hair may had an increased risk of melanoma due to ñenvironmental reasonsò: as hypothesized above, they may exposed more to sun than high risk

subjects with skin type I and may not have sun protection as well as subjects with skin type IV. Similarly, the subjects with intermediate skin type and *MC1R* R151C variant may exposed to sun more than subjects with skin type I, having however a genetic predisposition to melanoma given by the *MC1R* variant R151C. If this hypothesis was true, the increased risk of melanoma in this subgroup of patients could be explained by a gene-environment interaction. This subgroup of subjects was confirmed to have high risk of melanoma also in the confirmatory analysis on an extended M-SKIP dataset.

5.3. Conclusions

In conclusion, taking into account the possible confounding effect of the main risk factors for melanoma (sun exposure, sunburns and naevi count) we found that the five *MC1R* variants D84E, R142H, R151C, R160W, and D294H resulted associated with melanoma risk, while no significant association with melanoma was found for the two not functional *MC1R* variants V60L and R163Q. Results for the association with melanoma of V92M and I55T variant were still controversial and further investigations is warranted.

Among the *MC1R* variants associated with melanoma, however, the two variants D294H and R151C seemed the two ones that could indeed increase melanoma risk in combination with phenotypic characteristics and that are warranted to screen. The suggestion is to genotype D294H variant in subjects without atypical naevi, and R151C variant in subjects with no freckles and intermediate skin type II/III, in order discriminate individuals with a higher risk of melanoma from those protected.

It would be worthwhile to validate the results of the present analysis with prospective studies.

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