FACULTAD DE CIENCIAS DE LA SALUD

THE GENETIC BASIS OF SUNLIGHT SENSITIVITY AND MELANOMA-RISK PIGMENTATION PHENOTYPES:

The role of sex-specific genetic effects, 3' untranslated regions and melanoma susceptibility genes

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“I have failed over and over in my life… That is why I succeed”

Michael Jordan

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Resumen

Los rasgos de pigmentación humana son los rasgos físicos más visibles y diferenciables entre individuos. La pigmentación basal es un rasgo de carácter poligénico con alta heredabilidad, influenciada por factores ambientales, genéticos y endocrinos. La exposición solar estimula la síntesis de melanina mediante la activación de las rutas de pigmentación humana, con el objetivo de proteger la piel de los efectos nocivos de la luz solar. La incidencia del cáncer de piel revela una clara relación entre los rasgos de pigmentación y los daños causados por la exposición solar, mostrando una mayor susceptibilidad a cáncer aquellos individuos con piel clara, ojos claros, cabello rojo o rubio, un elevado número de nevus o efélides, y que se broncean con dificultad. Además, estudios recientes muestran una diferencia tanto en las características de pigmentación así como en la prevalencia e incidencia de melanoma entre sexos, lo que sugiere que existe un factor relacionado con el sexo que contribuye a las diferencias observadas entre hombres y mujeres. Por lo tanto, el objetivo principal de esta tesis doctoral es ampliar el conocimiento actual sobre las bases moleculares de la pigmentación humana y la predisposición a cáncer de piel, especialmente a melanoma cutáneo.

Los resultados incluidos en esta tesis doctoral se organizan en cuatro capítulos, tres de ellos publicados en revistas científicas internacionales indexadas. En el **capítulo 1**, se realiza un análisis de asociación en una población de origen español con el objetivo de identificar las posibles diferencias entre hombres y mujeres en la pigmentación y el riesgo a melanoma. Los resultados revelan la existencia de efectos genéticos que influyen en la pigmentación humana según el sexo, con efectos mayores para rasgos de pigmentación más oscura en las mujeres en comparación con los hombres, así como para el riesgo de melanoma.

En el **capítulo 2**, mediante el análisis de varios genes de pigmentación en una población española, se buscó esclarecer la disparidad sexual en la epidemiología del melanoma, así

como en las diferencias en la capacidad de bronceado y la sensibilidad de la piel a la exposición solar observada entre sexos. Los resultados sugieren la existencia de diferencias en la capacidad de bronceado y sensibilidad a la luz solar entre mujeres y hombres en las poblaciones caucásicas. Un meta-análisis integrando datos de estudios previamente publicados confirmó nuestros resultados. Además, nuestros resultados sugieren que las diferencias en el fototipo de la piel entre sexos deberían ser en parte causadas por los efectos genéticos de gen *MC1R* y sus variantes.

En el **capítulo 3**, se investiga el impacto de polimorfismos localizados en la región 3'UTR en la pigmentación humana y en la sensibilidad solar. Dos SNPs en los genes *MLPH* y *WNT3A* mostraron una interesante asociación con número de nevus y con presencia de lentigos solares, respectivamente. A continuación, se analizaron los efectos de estas dos variantes génicas en la unión de microARNs mediante herramientas de predicción online. Los resultados de predicción obtenidos se validaron al confirmar que los microARNs que se unen a *MLPH* y *WNT3A* influyen en rutas relacionadas con la pigmentación humana y cáncer de piel: vía de señalización Wnt, vía de señalización MAPK, y carcinoma basocelular.

En el último capítulo (**capítulo 4**), se investiga si la herencia sinérgica de mutaciones en los genes *CDKN2A* y *MC1R* sensibiliza a los melanocitos frente a los efectos dañinos de la radiación ultravioleta (UV), aumentando así la posibilidad de su transformación maligna a melanoma. Como los melanocitos portadores de mutaciones en *CDKN2A* mostraron un comportamiento *in vitro* normal, podrían ser necesarias alteraciones genéticas y epigenéticas adicionales para transformar dichos melanocitos.

Abstract

Human pigmentation traits are some of the most visible and differentiable human characteristics. Basal cutaneous pigmentation is a polygenic quantitative trait with high heritability, being influenced by genetic, environmental and sex-endocrine factors. Ultraviolet (UV) exposure stimulates the synthesis of melanin in melanosomes via activation of human pigmentation pathways, with the aim of protecting skin from the harmful effects of sunlight. Skin cancer incidence reveals a clear relationship between genetically controlled pigmentation traits and sunlight damage, showing greater cancer susceptibility in those individuals with fair skin, lightly-coloured eyes, red and blond hair, high naevus count, freckles, and inability to tan. Besides, recent findings showing sexual disparity in skin pigmentation and melanoma incidence and outcome suggest that there is a sex-related factor contributing to the observed differences between males and females. Therefore, the general objective of this thesis is to expand the current knowledge on the molecular bases of human pigmentation and predisposition to skin cancer, mainly cutaneous melanoma.

The results included in this thesis are organized in four chapters, three of them having been published in indexed international scientific journals. In **chapter 1**, we perform an association analysis in a Spanish melanoma case-control population with the aim of shedding some light on the putative sex-related genetic differences in pigmentation phenotypes as well as in melanoma risk. Our results suggest that there are indeed sex-specific genetic effects in human pigmentation, with larger effects for darker pigmentation in females compared to males, as well as for melanoma risk.

In **chapter 2**, by analysing several pigmentation genes in a Spanish population, we try to clarify the presumed sex disparity in melanoma epidemiology, and the differences in tanning ability and skin sensitivity to UV-light exposure observed between sexes. Our results suggest that there are differences in tanning ability and sensitivity to sunlight

between females and males in Caucasian populations. These findings were validated by performing a meta-analysis with the results of several previously published studies. In addition, our study suggested that the sex-specific differences in skin phototype might be partly caused by sex-specific genetic effects mediated by the *MC1R* gene and its variants.

In **chapter 3**, we investigate the impact of 3'UTR SNPs in human pigmentation and response to sunlight. Two SNPs in the *MLPH* and *WNT3A* genes showed an interesting association with high naevus count and the presence of solar lentigines, respectively. The effects of these two SNPs in microRNA binding were then modelled using web-based prediction tools. To validate our prediction results, the microRNAs predicted to bind to both *MLPH* and *WNT3A* were confirmed through identification of their target pigmentation-related pathways: 'Wnt signalling', 'MAPK signalling' and 'basal cell carcinoma'.

In the last chapter (**chapter 4**), we investigated if the co-inheritance of germline mutations in *CDKN2A* and *MC1R* synergistically sensitizes melanocytes to the damaging effects of UV, and thus increases the chance for malignant transformation to melanoma. As p16-mutated melanocytes showed normal *in vitro* behaviour, additional somatic genetic or epigenetic changes may be needed to transform melanocytes.

Introduction

1. THE BIOLOGY OF HUMAN SKIN PIGMENTATION

1.1. The architecture of human skin

The skin is the largest organ of the human body, accounting for around 16% of the body's weight, and with a surface area of 1.5-2 m^2. As the outer covering of the human body, the skin plays an essential role in providing protection against damaging exogenous influences, such as ultraviolet (UV) radiation, toxicants, microorganisms or mechanical injuries, which may disturb the physiological status of the individual enclosed by the skin (1). In addition to this protecting function, the skin seems also to be involved in the regulation of the body temperature through transpiration, as well as in the synthesis of Vitamin D, an essential hormone needed for stable composition of bones and teeth, immune regulation, intestinal absorption of calcium, and for insulin secretion (2). The human skin consists of three layers: the epidermis, the layer in direct contact with the external environment; the dermis, the layer composed by connective tissue below the epidermis; and the hypodermis, the deeper skin layer consisting of fatty tissue that connects the dermis to musculoskeletal components (Figure 1).

1.1.1. Epidermis

The epidermis, the outermost layer of the human skin, is composed of distinct cell populations organized in four stratums (Figure 1). Regarding pigmentation, the main constituents of the epidermis are melanocytes and keratinocytes (whose specific role will be discussed below). Melanocytes, known as the pigment-producing cells, are located in the basal stratum of the epidermis. Keratinocytes, the predominant cell type in the epidermis, migrate towards the surface of the epidermis during their differentiation

process. Thus, the epidermis is composed of four functionally distinct stratums of keratinocytes at different differentiation stages. Other cell populations found in the epidermis are Merkel cells, essential cells for touch sensation transmission; and Langerhans' cells, antigen-presenting immune cells residing in the basal and suprabasal skin stratums (3). This cell population plays a critical role in immunological adaptive reactions and therefore in the protection against infections from pathogens and hazardous substances (4).

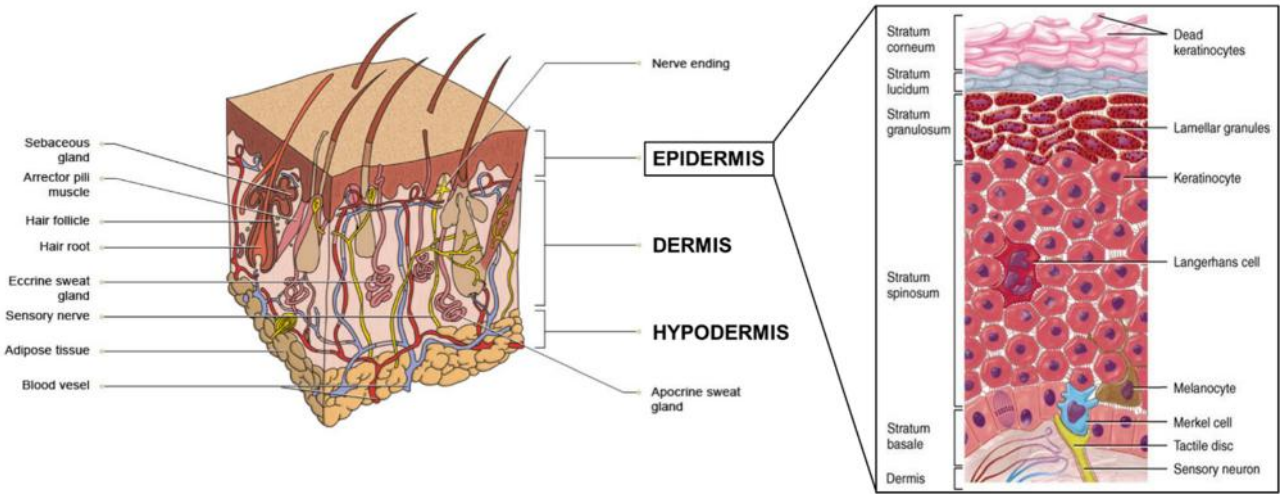

Figure 1. Structure of the human skin. The three different skin layers and components are schematically represented. The four epidermal layers are displayed in the box, showing the organization of the main cell populations within the epidermis. Figure retrieved and modified from http://author.webset-lms.com/repository/4431/0fcd5720-88de-44e0-8c09-9f03143bc201.jpg. Epidermal cross-section retrieved from https://s-media-cache-ak0.pinimg.com/originals/f7/e6/42/f7e6424a8e611f176cc33a1ac004d074.jpg.

1.1.2. Dermis

Lying below the epidermis is the dermis, a thick layer of connective tissue – made mostly of collagen, elastin and fibrillin – that provides flexibility and strength to the skin. The

dermis is mainly made up of fibroblasts, which are required to produce collagen, elastin and structural proteoglycans. This layer also hosts cells of the immune system such as macrophages and mast cells. Accommodated within fibrous dermal tissue are blood and lymphatic vessels, nerve endings, sweat and sebaceous glands, and hair follicles. Together with providing protection from mechanical injury, the main function of the dermis is to support the epidermis by bringing nutrients and oxygen, as well as by removing waste products from cell metabolism. It also assists in thermal regulation and includes receptors of sensory stimuli that transmit sensations of pain, irritation and pressure to the brain for interpretation (3).

1.2. The epidermal melanin unit

The epidermal melanin unit is a functional and structural biological complex within the epidermis denoting the symbiotic interaction between a melanocyte and a surrounding population of keratinocytes (5). It is now clear that the basic mechanism for human pigmentation and photoprotection involves the melanin synthesis within melanosomes, the transfer of these pigmented melanosomes from each melanocyte to its associated keratinocytes, and the distribution of these melanosomes into keratinocytes.

1.2.1. Melanocytes: the melanin producing cells

Melanocytes are specialized dendritic cells derived from the neural crest that reside in the basal stratum of the skin epidermis. These cells play an essential role in human pigmentation via their ability to produce melanin (6). Melanin, an irregular light-absorbing biopolymer, is the main determinant of skin, hair and eye colour. The two forms

of cutaneous melanin are eumelanin, an insoluble black-brown polymer, and phaeomelanin, a red-yellow polymer of benzothiazine units that is mostly responsible for red hair and freckles (7). Melanin is produced and enclosed within melanosomes, lysosome-related organelles that are transferred from melanocytes to keratinocytes through the latter's dendrites (8). All human races appear to have approximately the same amount of epidermal melanocytes, indicating that racial diversity in skin pigmentation depends on the amount and type of melanin within melanosomes, as well as on the number, size, and distribution of melanosomes in the epidermis (9).

1.2.1.1. Melanosome biogenesis, transport and transfer

Once established in the epidermal-dermal junction, melanocytes start producing melanosomes, extremely organized membrane-bound organelles wherein melanin synthesis occur. The development and maturation process of the melanosome takes place in the Golgi and endoplasmic reticulum, where it receives all enzymatic and structural proteins required for melanogenesis (Figure 2). Melanosomes are typically divided into four maturation stages (I–IV) depending on their structure and the quantity, nature, and disposition of the melanin produced (10). The type of melanin produced determines the appearance of mature melanosomes, with eumelanosomes being large and ellipsoidal and phaeomelanosomes small and spherical. This is because only eumelanosomes progress to maturation stages III and IV, while phaeomelanosomes are probably arrested in stage II having minimal tyrosinase activity – a critical enzyme in the synthesis of eumelanin (11). As soon as they mature, melanosomes are trafficked away toward the periphery of the cell within the dendrites. The molecular complex required for melanosome intracellular transport from microtubules to actin filaments at the cell periphery consists of Rab27a,

melanophilin and MyosinVa. Mutations in any one of these genes result in hypopigmentation of the skin and hair due to the accumulation of melanosomes at the perinuclear region of the melanocyte (12).

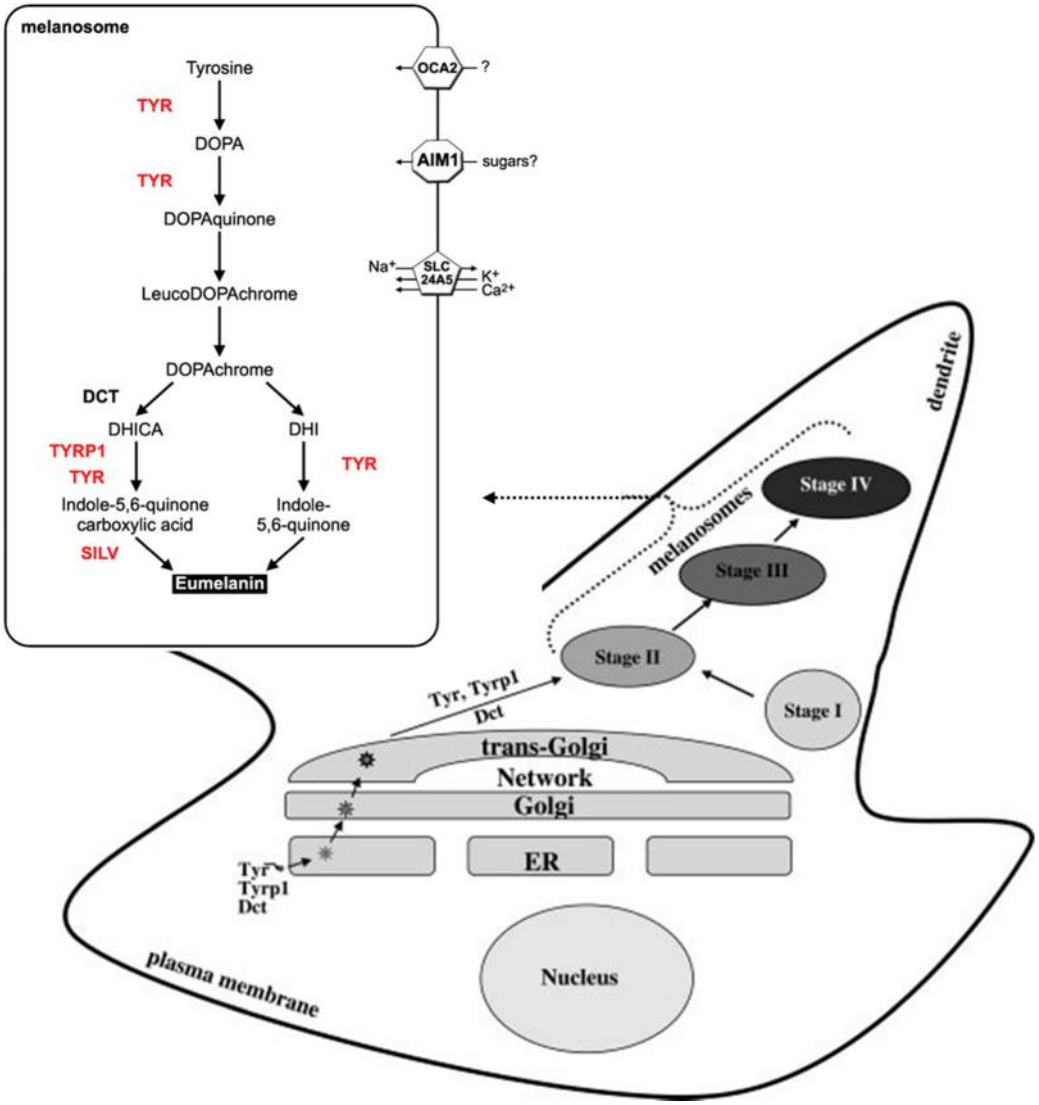

Figure 2. Schematic representation of melanosome biogenesis and maturation during melanin production by a melanocyte. The biochemical pathway of melanin synthesis is displayed in the box showing the principal enzymes involved. Figure based on and modified from Costin and Hearing, 2007 (11). Melanogenesis pathway in the box from Braasch and col, 2007 (13).

Mature melanosomes are finally packed in clusters enclosed by the melanocyte plasma membrane, and are known as pigment globules. These pigment globules containing multiple melanosomes are released into the extracellular space from various areas of the melanocyte dendrites, and then uptaken by keratinocytes via microvillus-associated phagocytosis. Once incorporated into keratinocytes, pigment globules are degraded and the multiple melanosomes are disseminated around the perinuclear area (8). In fair skin, melanosomes tend to cluster above keratinocyte nuclei, while heavily pigmented melanosomes in dark skin are distributed uniformly within the cells (Figure 3).

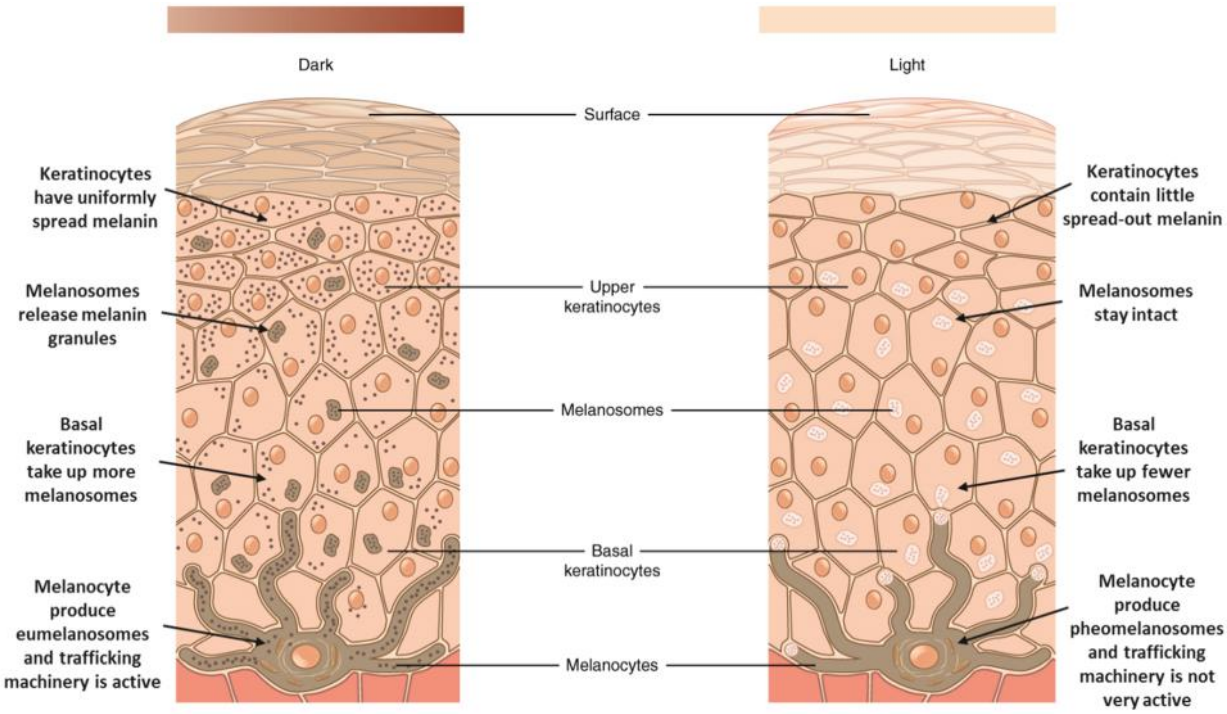

Figure 3. Variation in human skin pigmentation. The relative skin colouration depends on eumelanin/phaeomelanin ratio produced by melanocytes, the amount of melanosomes taken up by basal keratinocytes, and the distribution of melanosomes and melanin particles within keratinocytes in the outer skin layer. Figure retrieved and modified from https://opentextbc.ca/anatomyandphysiology/wp-content/uploads/sites/142/2016/03/504_Melanocytes.jpg.

1.2.1.2. Melanogenesis: melanin pigment production

Human pigmentation is a consequence of the enzymatic processes involved in the synthesis of melanin, and therefore it is mainly explained by the presence of melanin in epidermis, iris and hair (9,14). Melanogenesis consists of a series of reactions involving various melanocyte-specific enzymes (Figure 2). Both eumelanin and phaeomelanin derive from 3,4 di-hydroxyphenylalanine (L-DOPA). The hydroxylation of tyrosine to DOPA and subsequent oxidation to dopaquinone are the rate-limiting steps in melanogenesis catalysed by tyrosinase – a copper-containing membrane-bound protein located in melanosomes (15). The remaining eumelanin-producing reactions are catalysed by the tyrosine-related proteins 1 (TYRP1) and 2 (TYRP2, also known as dopachrome tautomerase, DCT).

The biochemical synthesis of phaeomelanin involves the production of cysteinyl-DOPA, via condensation of dopaquinone and the amino acid L-cysteine, followed by its oxidation to phaeomelanin. Melanogenesis produces mixtures of eumelanin and phaeomelanin at different mixed ratios. The eumelanin/phaeomelanin ratio is determined by tyrosinase activity, which is in fact dependent both on pH levels within melanosomes and L-cysteine concentration (16). The wide range of different skin colours is mainly explained by the type and levels of melanin in the epidermis, since the density of melanocytes in all types of skin is similar and constant (11). Therefore, individuals with melanocytes that synthetize more phaeomelanin than eumelanin tend to have lighter skin.

Additionally, several melanosome membrane proteins have been involved in the synthesis of melanin (17). For example, an active uptake of tyrosine by the transmembrane protein OCA2 is required for the activation of tyrosinase within melanosomes. Furthermore, the coupling of H^+, Na^+, Ca^{2+} and K^+ transport, which is

essential for controlling pH within melanosomes and thus for tyrosinase activity, is mediated by the V-ATP complex, SLC45A2, SLC24A5 and TPCN2 (see below for additional information).

1.2.2. The involvement of keratinocytes in human pigmentation

As previously discussed, the epidermal melanin unit is a biological complex within the epidermis consisting of two cell types: melanocytes and keratinocytes. The melanocyte-keratinocyte complex is able to respond to a wide range of environmental stimuli, often in paracrine and/or autocrine manners. After exposure to UV radiation, keratinocytes produce and release several factors involved in stimulating melanocyte growth, melanogenesis and melanin transfer to keratinocytes by melanocytes (11). Among these keratinocyte-derived factors, melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) have a pivotal role in melanogenesis and/or melanocyte dendrite formation (18). Both hormones bind to a melanocyte-specific receptor (melanocortin-1 receptor, MC1R) that switches on the cAMP signalling pathway, leading to the phosphorylation and activation of cAMP response element binding protein (CREB) through protein kinase A (PKA). CREB later binds to the cAMP response element (CRE) present in the promoter of the microphthalmia-associated transcription factor (MITF) gene. MITF ultimately up-regulates *TYR*, *TYRP1* and *TYRP2* gene expression, required for melanin synthesis (19,20). The agouti signalling protein (ASIP) acts as the antagonist of α-MSH for MC1R. The presence of ASIP impedes the binding of α-MSH to MC1R, promoting thus the production of phaeomelanin instead of eumelanin (21).

The melanocytic developmental process involves other keratinocyte-derived factors including prostaglandin E2 (PGE2), endothelin-1 (ET-1), basic fibroblast growth factor (bFGF), steel factor (SLF), stem cell factor (SCF), leukaemia inhibitory factor (LIF), hepatocyte growth factor (HGF), nerve growth factor (NGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (9). Therefore, human melanocyte proliferation and differentiation require the cross-talking of cAMP/PKA, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) signalling pathways (Figure 4). The keratinocyte-melanocyte crosstalk in human epidermis is fundamental in regulating cutaneous pigmentation in response to numerous intrinsic and extrinsic factors.

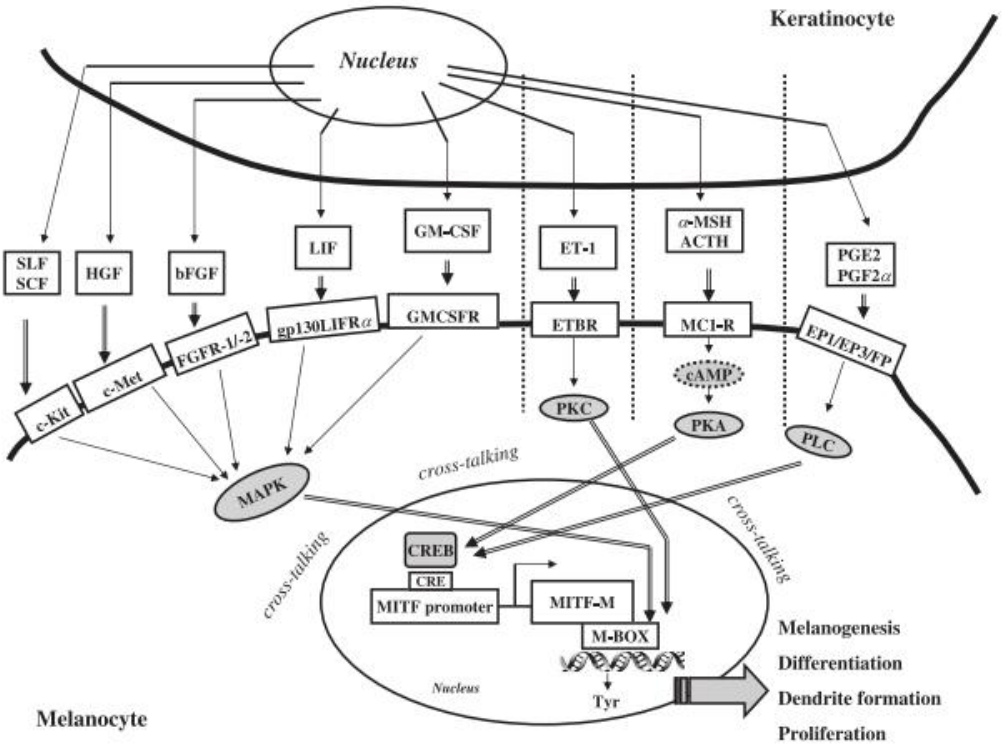

Figure 4. Schematic representation of keratinocyte-melanocyte crosstalk within the epidermal melanin unit. Keratinocyte-derived factors stimulate human melanocyte proliferation and differentiation, melanogenesis and dendrite formation via activating signalling pathways after binding to the corresponding receptor on the melanocyte surface (11).

2. FACTORS CONTRIBUTING TO HUMAN SKIN PIGMENTATION

Basal pigmentation seems to be mostly genetically determined, being altered by numerous intrinsic and extrinsic factors affecting the epidermal melanin unit network (22). The focus of this section is to describe some of the most important or well-studied factors that modulate human skin pigmentation, including ethnicity and gender differences, variable hormone-responsiveness, genetic variations within genes controlling pigmentation pathways, and sunlight exposure.

2.1. Genetics of human pigmentation

Genetic variations in the genes encoding proteins involved in the human pigmentation pathway have been associated with phenotypic differences in skin, hair and eye colour, freckling, tanning ability and sunlight sensitivity, but also with the risk of developing skin cancer (9,23). Pigmentation traits appear to follow a complex polygenic inheritance model, that is, they are influenced by many genes with relatively small effects, with a few major genes (24).

The genetic basis explaining pigmentation diversity between individuals has been the subject of intense research. Pigmentation-related genes were initially discovered by performing comparative genomics of candidate genes related to congenital pigmentation disorders, such as oculocutaneous albinisms (OCA). Genetic polymorphisms in *TYR* (linked to OCA type I, OMIM #203100), *OCA2* (responsible for OCA type II, OMIM #203200), *TYRP1* (related to OCA type III, OMIM #203290) and *SLC45A2* (linked to OCA type IV, OMIM #606574) have been largely associated with normal variation in

skin, hair and eye colour. Other major genes affecting constitutive pigmentation are *SLC24A5*, *MC1R*, *ASIP*, *KITLG*, *HERC2*, *SLC24A4*, *IRF4*, *TPCN2*, *LYST*, and *BNC2* (23). Complementing early candidate gene studies, specific allele and/or genome wide association approaches in individuals with a well-defined phenotype have resulted in the establishment of a growing list of single nucleotide polymorphism (SNP) markers that contribute to variation in pigmentation phenotypes seen in human populations (25–31). A list of all known loci influencing pigmentation is available at www.espcr.org/micemut (32), which currently lists 378 loci described in mice and their human and zebrafish homologues.

2.1.1. Melanocortin-1 receptor

The melanocortin-1 receptor (*MC1R*) gene encodes a seven-pass transmembrane G-protein coupled receptor that is usually located in the cell surface of cutaneous and follicle melanocytes (Figure 5). Activation of human MC1R by its ligands (α-MSH and ACTH) leads to the activation of the cAMP signalling pathway, which in turn leads to a stimulation of melanogenesis and a switch from the synthesis of phaeomelanins to the production of eumelanins (33). However, the binding of its antagonist ligand (ASIP) switches back to phaeomelanin production.

Genetic variation within the *MC1R* gene is the main contributor to the diversity of human pigmentation (34). Sequencing of the gene encoding this receptor has allowed the identification of more than 100 non-conservative allelic variants in Caucasians (35). Most of its variants are relatively rare, but their frequencies vary among populations, being more common in European populations (36). Six of these *MC1R* variants have been traditionally associated with fair skin, red hair and freckling phenotype (RHC alleles):

D84E, R142H, R151C, I155T, R160W and D294H (37,38). Functional analyses have demonstrated that several *MC1R* genetic variants reduce the stimulation of the pigmentation pathway due to incomplete binding between the receptor and its ligand (α-MSH), resulting in an increased synthesis of phaeomelanin (instead of eumelanin) in melanocytes (24,33,39). In addition, three other frequent variants (V60L, V92M and R163Q), not found to be associated with the RHC phenotype (known as non-RHC alleles), have been shown to influence melanoma risk in darkly-pigmented Caucasian populations, such as Italians, Greeks and Spaniards (35,40).

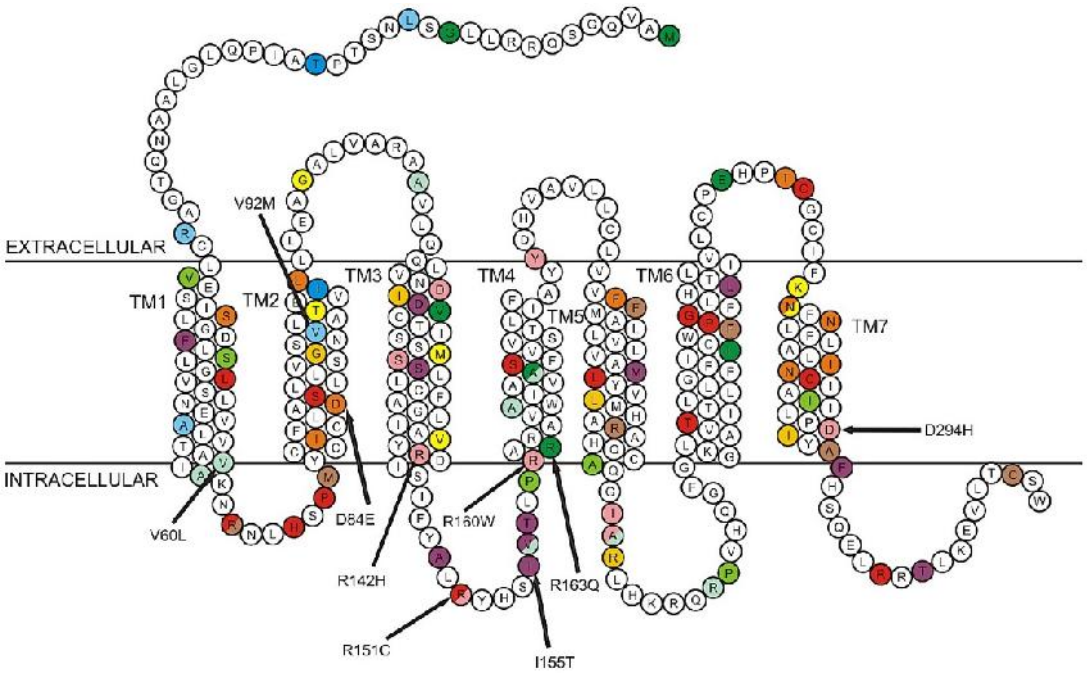

Figure 5. Two-dimensional structure of the MC1R protein and location of genetic variants. Loss-of-function variants of MC1R, both red hair colour (RHC) and non-RHC alleles, are indicated by arrows. TM, transmembrane domain. Figure based on and modified from Hepp and cols., 2015 (41).

The *MC1R* gene has been the subject of intense research because of its clear and direct association with melanoma and non-melanoma skin cancer (9,29,42,43). The risk for

melanoma attributable to the *MC1R* genotype may be determined by the ability to response to UV exposure (burning *versus* tanning). However, in addition to its influence on human pigmentation and on susceptibility to different types of skin cancer, *MC1R* variants also appear to modify the penetrance of mutations in the *CDKN2A* gene – the major genetic locus implicated in familial melanoma (44,45).

2.1.2. Agouti signalling protein

The agouti signalling protein *(ASIP)* gene encodes a 32-aminoacid paracrine-signalling peptide that acts as an antagonist of α-MSH precluding its binding to MC1R, and thus leading to a down-regulation of eumelanogenesis and an up-regulation of phaeomelanogenesis (21). Contrary to *MC1R*, non-synonymous genetic variations in the *ASIP* gene are unusual. A polymorphism located in the 3'-untranslated region of the gene (A8818G) has been significantly associated with dark hair, brown eyes and dark skin (46,47). Functional studies have shown that carrying the G allele decreases agouti protein levels compared to the ancestral allele, via mRNA instability and premature degradation of the peptide. Consequently, the inhibitory effect of agouti is diminished, promoting the synthesis of eumelanin and thus favouring dark pigmentation (48).

2.1.3. Tyrosinase

Tyrosinase is a copper-dependent enzyme that catalyses the first two steps of melanogenesis (see Figure 2). As explained above, the generation of eumelanin occurs after an increase in the tyrosinase activity. It has been shown that melanocytes derived from dark individuals display a tenfold increased enzymatic activity of tyrosinase,

although the amount of this enzyme within the melanosomes appears to be similar in white- and black-skin melanocytes (49).

Mutations in the *TYR* gene have been related to variations in normal pigmentation of eye, hair and skin colour, as well as to pigmentation disorders. At least 36 mutations in *TYR* cause oculocutaneous albinism type 1 (OCA1) (50), an autosomal recessive disorder characterized by the absence of melanin in skin, hair and eyes. Two non-synonymous variants in the *TYR* locus (S192Y and R402Q) have been linked with fair pigmentation, sensitivity to sunlight, and susceptibility to melanoma and non-melanoma skin cancer (51). These genetic variants encode a form of tyrosinase with a reduced catalytic activity compared to the wild-type protein (52).

2.1.4. Tyrosinase-related proteins

Tyrosinase-related proteins 1 and 2 (TYRP1 and TYRP2) are melanosomal proteins involved in the production of eumelanin, but not phaeomelanin (see Figure 2). Mutations in *TYRP1* are responsible for oculocutaneous albinism type III (OCA3), a hypopigmentation disorder in southern African populations characterized by having bright red-copper colouration of hair and skin and brown iris colour (53). In European populations, the polymorphic variant rs1408799 has been consistently associated with variation in iris pigmentation, as well as with risk of cutaneous melanoma (54–56). Besides, a cysteine-to-arginine change in *TYRP1* seems to be responsible for the high prevalence of blond hair in Melanesian populations, which differs from the general trend of darker skin and hair pigmentation in populations living near the equator with higher UV radiation (57). Genetic variations in the *TYRP2* locus seems also to be correlated with eye colour diversity in Caucasians (55).

2.1.5. OCA2 Melanosomal Transmembrane Protein

Another gene with a major contribution to human pigmentation is the oculocutaneous albinism II (*OCA2)* gene. *OCA2* encodes the P protein, a transmembrane protein involved in the transport of tyrosine, the precursor to melanin synthesis, to the inside of the melanosome. Abnormalities in this gene result in oculocutaneous albinism type II (the most common form of albinism), as well as with some cases of ocular albinism (58).

In European populations, *OCA2* is a highly polymorphic gene influencing pigmentation traits, particularly eye colour. *OCA2* has been defined as the major human iris colouration gene, since its genotype appears to explain up to 75% of eye colour variation in Europeans (59). The derived allele of rs12913832, a variant located upstream of the *OCA2* promoter within an intron of the contiguous *HERC2* gene, shows an association with blue eye colour attributable to a silencing of *OCA2* expression (60). Interestingly, a missense mutation (rs1800401, R305W) in the *OCA2* gene seems to modulate the penetrance of *MC1R* RHC variants (61). On the other hand, several studies focused on determining the susceptibility to cutaneous cancer according to the *OCA2* genotype have shown that carriers of certain variants, such as R419Q, have a significantly higher risk for melanoma and basal cell carcinoma (26,54).

2.1.6. Potassium-dependent sodium/calcium exchangers

The *SLC24A4* and *SLC24A5* genes (solute carrier family 24, members 4 and 5) encode two potassium-dependent sodium/calcium exchanger proteins located in the melanosomal membrane. These ion transporters are involved in the regulation of the melanosome environment and may contribute to the melanosome maturation process.

Variations in the *SLC24A4* and *SLC24A5* genes have also been significantly associated with variation in human pigmentation and sun sensitivity traits. *SLC24A5* is considered one of the major human pigmentation genes since its A111T variant (rs1426654) accounts for about 25-40% of the difference in skin colour between Europeans and Africans. The ancestral allele (A) of this polymorphism is largely fixed in populations of African origin, whereas the derived allele (G) is extremely common in fair-skinned European individuals. Thus, carriers of AG or GG genotypes present a fairer skin colour, because of lower eumelanin levels, compared to homozygous individuals for the ancestral allele (62,63). The SNP rs12896399 in the *SLC24A4* gene has been associated with eye and hair colour, skin sensitivity to sunlight, as well as susceptibility to malignant melanoma (31,54,64). Besides, interactions between *HERC2* and *SLC24A4* may also affect determination of blue eye colour (65).

2.1.7. Membrane-Associated Transporter Protein

Another melanosomal transmembrane solute carrier involved in the pigmentation pathway includes SLC45A2 (solute carrier family 45, member 2), also known as MATP (membrane-associated transporter protein). This protein may be involved in arranging melanogenic enzymes during melanosome maturation. In humans, pathogenic mutations in the *SLC45A2* gene cause oculocutaneous albinism type IV (OCA4), where both tyrosinase processing and post-Golgi enzyme trafficking are disrupted (66,67). Other variants, located in both the promoter region and the coding region of the *SLC45A2* gene, have been significantly associated with dark pigmentation in hair, epidermis and iris and with protection against melanoma in southern European populations (28,68).

2.1.8. Interferon regulatory factor 4

The interferon regulatory factor 4 is a transcription factor that negatively regulates signalling via Toll-like receptors – a key process for activation of the innate and adaptive immune system response. It has been shown that IRF4 interacts with MITF, which in turn regulates the expression of the melanogenic enzymes and several differentiation factors (69). A polymorphism located in the intronic region of *IRF4* (rs12203592) has been associated with hair, skin and eye colour, naevus number, freckling and tanning ability after sunlight exposure (27,31,61). This variant seems to be only polymorphic in European-origin populations, showing a north-south gradient across Europe (70). Interestingly, the T allele of rs12203592 has been recently associated with hair greying in Latin Americans (71).

2.1.9. Other pigmentation-related genes

Apart from the major pigmentation-related genes listed above, other genes have also been involved in normal variation of pigmentation traits. The *KITLG* gene encodes the ligand of the tyrosine-kinase receptor encoded by the *KIT* locus. After binding to its receptor, KITLG promotes migration, survival and proliferation of melanocytes. Although no non-synonymous polymorphisms have been found in the *KITLG* gene of different human populations, this ligand is expressed at significantly higher levels in keratinocytes of Africans than Europeans (72). Two polymorphisms in the upstream region of *KITLG* (rs12821256 and rs642742) have been shown to contribute to normal variation to pigmentation (31,73), perhaps via regulatory changes in gene expression.

The *BNC2* (basonuclin 2) gene encodes a potential transcriptional regulator specific for skin keratinocytes. In humans, variants in *BNC2* have been associated with freckling (74), and with skin colour (75). Recently, a polymorphism (rs62543565*C) located 30 kb upstream from this gene has been significantly associated with facial pigmented spots independently of skin colour (30).

Association studies performed in the last years have suggested other candidate genes associated with human pigmentation traits, including *TPCN2* (two-pore segment channel 2), *LYST* (lysosomal trafficking regulator) or *UGT1A* (UDP glucuronosyltransferase 1 family, polypeptide A complex), among others (27,56,75). However, larger association studies in different populations should be needed to validate the significance of these observations. Besides, functional analysis of these genes would elucidate the role of their genetic variants in pigmentation variability.

2.2. Sunlight influence on human pigmentation

Cutaneous pigmentation is the main photoprotective mechanism against sun-induced damage. UV radiation received at the surface of the Earth from the sun is divided in UVA (320-400nm), which can penetrate deeply into the basal layer of the epidermis, and UVB (280-320 nm), with less depth but greater damaging potential (76). UVB produces photochemical damage to DNA by creating cyclobutylpyrimidine dimers (CPDs) and (6–4) photoproducts, and it also generates reactive oxygen species (ROS) by peroxidation of lipids from cellular membranes (77).

The skin responds to UV radiation by creating a melanin coating in the epidermal layer of the skin, with the aim of absorbing UV radiation and thus impeding UV-induced injury.

The photobiological response that increases skin pigmentation over the basal constitutive level is called tanning (78,79). This protective mechanism is orchestrated by both the melanocyte and the surrounding keratinocytes of the epidermal melanin unit. After exposure to UV radiation, keratinocytes increase production of ET-1, nitric oxide (NO) and proopiomelanocortin (POMC; precursor of α-MSH and ACTH), which act as paracrine factors activating melanogenesis in melanocytes (Figure 6). Tanning involves increased number of melanocytes and keratinocytes in the epidermis, as well as increased levels of TYR activity, increased production of melanosomes, and higher number of melanosomes transferred to surrounding keratinocytes (80,81). It is shown that tanning ability has genetic determinants and is generally more noticeable in individuals with darker constitutive pigmentation. Furthermore, eumelanin presents higher photoprotective properties than phaeomelanin because of its resistance to photodegradation and its ability to scavenge ROS (82,83). Therefore, individuals with light skin colour and poor tanning ability, who are skin phototype I-II according to the Fitzpatrick classification, are more susceptible to UV-induced damage, having higher risk of skin cancer, both melanoma and non-melanoma (9).

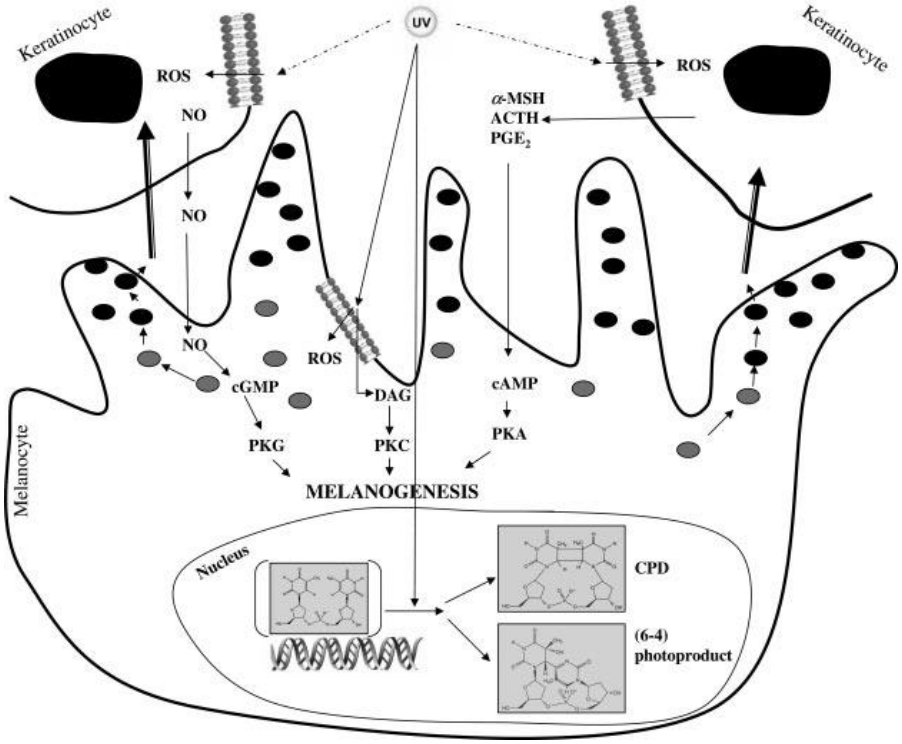

Figure 6. Mechanisms involved in UV-induced pigmentation. The tanning response is orchestrated by both melanocytes and surrounding keratinocytes, the two cell populations forming the epidermal melanin unit (11).

2.3. The action of oestrogens on skin pigmentation

Pigmentation can be modulated by oestrogens and androgens through the regulation of melanin synthesis. In fact, cutaneous hyperpigmentation (tanning, dark spots, *linia nigra* and/or melasma/chloasma) is common in pregnant women (84,85). It has been shown that pregnancy-related hormones – oestrogen, progesterone and α-MSH – induce the activation and expression of genes involved in melanin synthesis in melanocytes (84), while androgens inhibit tyrosinase activity (86). Besides, the use of oestrogen-containing oral contraceptives, certain cosmetics and oestrogen-progesterone therapies have also been associated with hyperpigmentation (11).

2.4. Human pigmentation diversity

Natural variation in skin colouration depends on both geographical location and ethnic origin, suggesting that genetic adaptation to the intensity and duration of solar radiation play a very important role in the historical evolution of the variation in cutaneous pigmentation (87). The most widespread evolutionary hypothesis for skin colour variation among populations suggests that skin pigmentation is determined by a balance between the amount and intensity of UV radiation received, which induces melanogenesis as a protective mechanism to avoid photodamage; and the necessity of absorbing UV for adequate synthesis of vitamin D in the skin (88). Conversely, dark skin in UV-intense regions is needed to avoid the destruction of folates and other micronutrients (flavine, tocopherol, carotenoids) by photodegradation. Consequently, dark skin colouration is observed in latitudes near the equator where solar radiation is more intense, while fair-skinned individuals would have a biological advantage over dark-skinned individuals in regions distant from the equator where solar radiation levels are lower (Figure 7) (89). Thereby, historical migration of human populations from Africa to other regions at higher latitudes seems to provide the basis of human genetic adaptation, according to local levels of solar radiation. That is, the frequency of genetic variants clarifying skin colour increased in Europeans in order to ensure the UV absorption needed for vitamin D synthesis (90).

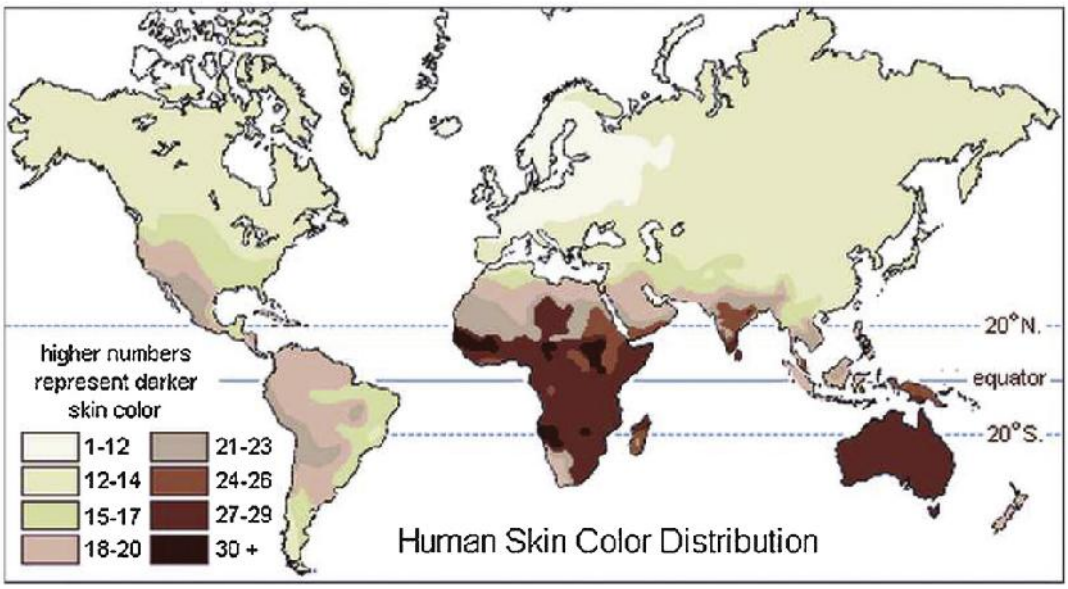

Figure 7. Worldwide distribution of human skin colouration. World map showing that darker skin colours are found predominantly between 20º north and south of the equator (23).

3. BENIGN HYPERPIGMENTED LESIONS

Hyperpigmentation of the skin occurs commonly in a variety of different forms. Hyperpigmentation is typically a harmless condition in which zones of the skin become darker than the normal surrounding skin areas. This darkening results from an increased deposit of melanin in the skin, arising either from increased melanin synthesis in existing melanocytes or from increased number of active melanocytes. In this section, I will focus on explaining those benign hyperpigmented lesions induced or exacerbated by sun exposure, including ephelides (freckles), solar lentigines, melasma, and melanocytic naevi (moles) (Figure 8) (11,91–93).

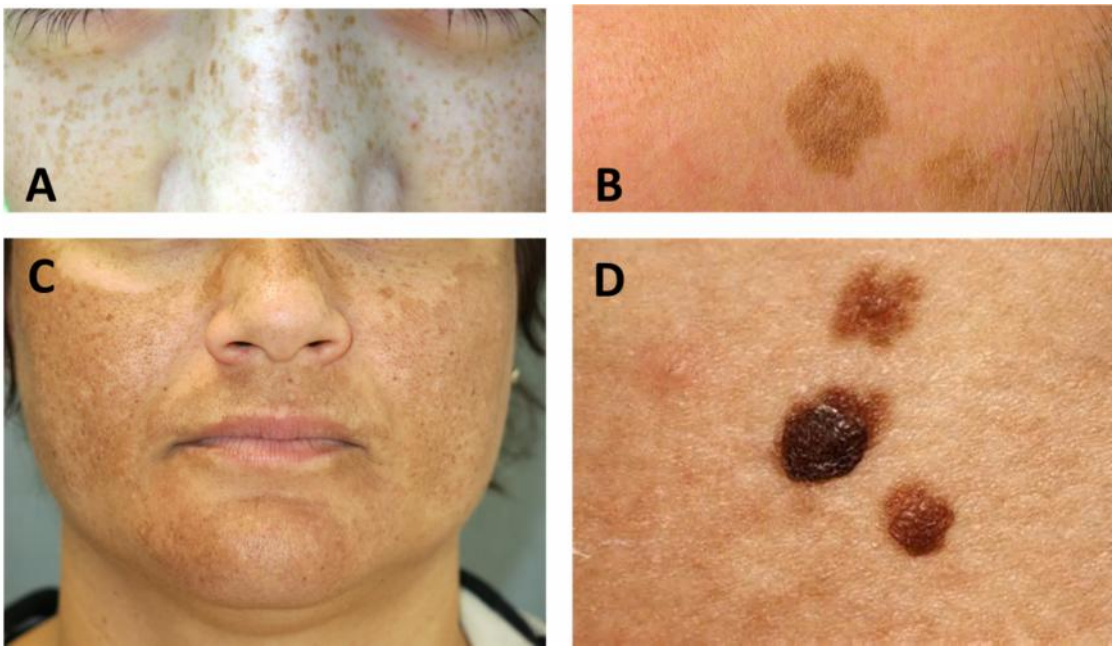

Figure 8. Pictures of common benign hyperpigmented lesions. A) Ephelides on the face. Image retrieved and modified from https://upload.wikimedia.org/wikipedia/commons/e/ea/Vesnuschki.jpg. **B)** Solar lentigines on the face. Image retrieved and modified from http://doctorv.ca/wp-content/uploads/2013/04/14458735194e471006f37ec1347571610060-e1365028691357.jpg). **C)** Melasma found on the cheeks and upper lip of a pregnant woman. This picture was retrieved from http://www.britishskinfoundation.org.uk/Portals/0/melasma.JPG. **D)** Melanocytic naevi. Image retrieved from http://images.digopaul.com/wp-content/uploads/related_images/2015/09/08/naevi_2.jpg.

3.1. Ephelides

Ephelides (also known as freckles) are small, flat, pale-brown spots commonly observed in fair-skinned and/or red-haired individuals (Figure 8A). Ephelides appear early in childhood, then increase during adolescence and partly disappear with age. The formation of these hyperpigmented spots can be triggered by exposure to sunlight through increased melanin production by melanocytes. That is, ephelides become more pigmented, and therefore more visible, during summertime (93). At the histological level, it is shown that freckled areas present highly pigmented melanocytes with large melanosomes (identical

to those found in dark-skinned individuals), while adjacent non-freckled areas exhibit poorly pigmented melanocytes with smaller melanosomes (94). This suggests regional differences in the activation of pigmentation pathways that lead to pigmented spots rather than hypopigmented skin (93).

The presence of ephelides is largely genetically determined. The *MC1R* gene seems to be the major contributor to the formation of freckles in European-origin individuals (34,37,95). Carriers of one or more RHC *MC1R* variants usually have a high number of ephelides. Nevertheless, individuals with no mutations in the *MC1R* gene sometimes also display freckles. Accordingly, other genes have been shown to contribute to ephelides formation, including *IRF4*, *ASIP*, *TYR* and *BNC2* (31,56,74).

3.2. Lentigines

Solar lentigines are round, flat, irregular, brown-pigmented macules ranging in size from millimetres to centimetres in diameter (Figure 8B). They are commonly found on chronic sun-exposed skin (mostly on the face and back of the hands), and typically appear during middle-age and increase in number with age (93). Compared with unaffected surrounding skin areas, both melanocytes and keratinocytes proliferate excessively in solar lentigo lesions. Besides, there is an increase in the production of ET-1 by keratinocytes, stimulating melanocytes to produce melanin due to higher tyrosinase activity (96).

Solar lentigines are mostly environmentally determined, as they are a clear sign of accumulated photodamage in the skin. However, some of the pigmentation-related genes have also been implicated in the formation of solar lentigines. Loss-of-function *MC1R* alleles has been associated with the presence of solar lentigines, suggesting that

melanocytes with reduced MC1R activity are expected to form these benign hyperpigmented lesions (37,97). Genetic variants in the *SLC45A2* gene have also been found to be implicated in solar lentigines development (98), although this correlation was not found in a GWAS study using Caucasian individuals (30). This high-powered GWAS study demonstrated significant associations between variants in four pigmentation-related genes (*IRF4*, *MC1R*, *ASIP* and *BNC2*) and facial pigmented spots, including solar lentigines among others (30).

3.3. Melasma

Melasma is an acquired hypermelanosis of the skin characterized by symmetrical dark- to light-brown patches (Figure 8C). It typically occurs on the face, particularly on the forehead, temples, cheeks and/or upper lip. Melasma is primarily associated with pregnancy, although other factors can exacerbate this hyperpigmented condition including UV exposure, oral contraceptives, certain cosmetics and drugs, endocrine dysfunction, or genetic influences (92). Melasma pigmentation normally improves in the winter and aggravates in summertime, where melanogenesis is stimulated by sunlight exposure. As stated above, pregnancy-related hormones lead to increased expression of tyrosinase and thus melanin production by melanocytes (11,84).

Genetics also appears to have an influence on melasma. Melasma is more prevalent in people with dark skin types (Fitzpatrick skin types III-V) (92). Besides, genetic alterations related to the stimulation of melanin production have been recently described. Upregulation of genes related to melanogenesis (*TYR*, *TYRP1*, *TYRP2* and *MITF*) and a subset of Wnt-pathway modulators, which play a critical role in melanocyte development,

have been detected in melasma lesions (99). Besides, several keratinocyte-related factors (ET-1, SCF, c-KIT and GM-CSF) are more highly expressed in skin with melasma compared with adjacent skin areas (100,101), stimulating the epidermal-melanin unit in the hyperpigmented areas. The basal membrane in skin areas with melasma appears disrupted and disorganized due to down-regulation of genes related to lipid metabolism, highlighting the key role of epidermal-dermal cross-talk in this pigmentation disorder (102).

3.4. Melanocytic naevi

The term melanocytic naevi refers to a group of non-malignant melanocytic cells formed by clonal proliferation (Figure 8D) (103). The development of melanocytic naevi is a multifactorial and heterogeneous biologic process in which basal skin pigmentation, genetics, sex and UV radiation have been shown to have an influence on. It has been unequivocally demonstrated that UV radiation is a triggering agent in the development of acquired naevi (104). In this regard, some studies have suggested an association between childhood sunburns and larger number and size of naevi (105–107). Naevi seem to be concentrated on the face and neck in males, compared with the upper arms and thighs in females (106). These sex differences seem to be explained by sociological reasons, and not by sex-differentiated genetic effects (108). Additionally, the variation in naevus count between individuals appears to be determined by genetic factors (109). Individuals with fair skin, a tendency to sunburn and poor tanning ability have increased number of melanocytic nevi (110), which in turn has been shown to be a risk factor for cutaneous melanoma, since up to 30% of melanomas arise from pre-existing naevi (111). Recent GWAS approaches have identified several germline variants associated with naevus

count in European populations (112,113), although validation of these naevus-susceptibility loci in larger studies is required.

4. SKIN CANCER

Skin cancer is one of the more common neoplasms worldwide, especially in Caucasian populations (114). Skin cancers are classified in two main categories: cutaneous melanoma (CM), which initiates via the malignant transformation of melanocytes; and non-melanoma skin cancer (NMSC), which arises from other epidermal cells, principally keratinocytes. According to the epidermal layer of origin, non-melanoma skin cancers can also be subdivided into basal cell carcinoma (BSC) and squamous cell carcinoma (SCC) (Figure 9). The principal aetiological factors associated with the predisposition to skin cancer include environmental (such as the geographic location and sunlight exposure) and genetic factors (inherited germline variants that influence in pigmentation-related phenotype diversity) (115,116).

4.1. Non-melanoma skin cancer

Non-melanoma skin cancer (NMSC) is one of the most common malignancies among white-skinned populations although, given its benign nature, it does not usually present clinical complications. Basal cell carcinoma, which accounts for 80-85% of all NMSC, rarely metastasizes to other organs. Although squamous cell carcinoma is more likely to invade other tissues, its mortality is very low (117).

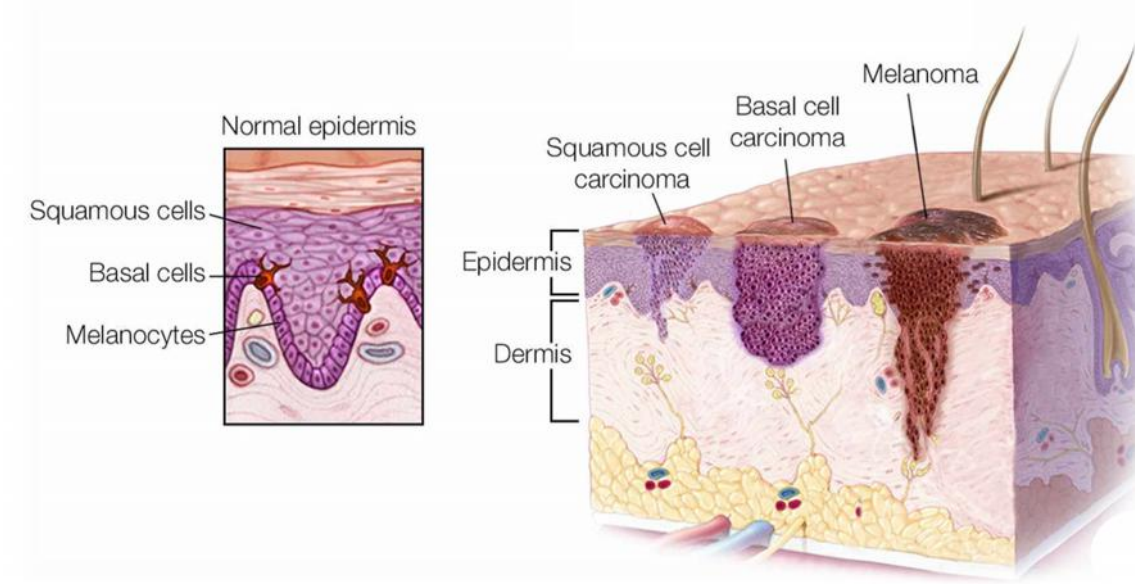

Figure 9. Types of skin cancer depending on the cell population from which the neoplasm arises. Image retrieved from http://healthlifemedia.com/healthy/wp-content/uploads/2016/05/EC3356125-001-0.jpg.

The primary cause of NMSC is exposure to UV radiation, since malignant lesions usually appear in sun-exposed areas of the body such as the face, neck or back of the hands and arms, and are more prevalent in outdoor workers (118). Epidemiologic studies demonstrate that the total amount of sunlight received over the years, particularly sunburn episodes, is the most important risk factor associated with NMSC (119). The harmful effects of UV radiation in the skin are caused by direct cell damage and impaired immune function, leading to erythema (inflammatory cutaneous lesion caused by sunburn), immunosuppression, genetic mutations and oxidative stress (120).

Genetically determined phenotypic characteristics that increase sensitivity to UV radiation are known to increase risk of NMSC (121). Burning and tanning response of skin (Fitzpatrick skin phototype) is essential in determining NMSC risk, since darker-skinned individuals with highly tolerance to sunlight exposure present a lower incidence

of NMSC (23,89,114). Polymorphism in the *MC1R* gene associated with fair skin and red hair independently contribute to the risk of NMSC (122). Other susceptibility genes for NMSC are involved in DNA repair, defence against oxidative stress and immune modulation (116,123,124).

4.2. Cutaneous melanoma

Cutaneous melanoma (CM), a malignancy arising from melanocytes, is one of the most malignant cancers among Caucasian populations (125). Although Spain has one of lowest melanoma incidence and mortality rates in Europe, melanoma incidence is currently increasing faster than that of any other malignancy in our country (1). Genetic, phenotypic and environmental factors contribute to melanoma predisposition, denoting that melanoma displays a complex aetiology.

As stated above, UV radiation is the leading environmental factor implicated in the development of skin cancer. Historically, incidence rates of CM in white populations have been inversely correlated with geographic latitude, being highest in equatorial regions and reducing with increasing distance from the equator (126,127). Nevertheless, the contribution of recurrent sun exposure in CM remains unclear. Melanoma appears more frequently in sporadically (rather than chronically) sun-exposed areas of the body, those that are usually covered by clothing. This seemingly paradoxical fact is attributable to injuries caused by an intermittent pattern of intense and acute sun exposure, which is associated with recreational and vacation activities (128). Accordingly, epidemiological studies reveal that outdoor workers, who are continuously exposed to UV radiation, have lower melanoma rates than those of indoor workers, supporting the role of intermittent

sunlight exposure in melanoma causation (129). Besides, melanoma incidence within Europe is lower in Mediterranean countries than in Nordic populations, even though these countries are at higher latitudes, having low UV radiation incidences over the year (excluding summer holidays) (130).

Sex-differences have also been consistently observed regarding CM. Females exhibit lower incidence, lower risk of metastases and significantly longer survival rates than males (130,131). Sex differences in pigmentation traits, tanning ability, and skin sensitivity to sunlight exposure may explain the sex disparity described in melanoma epidemiology (108,131,132). Additionally, individuals who are immunosuppressed, for example after an organ transplantation, are at higher risk of melanoma (133). However, smoking (a common carcinogen) has not been independently associated with melanoma (134).

Although the majority of genetic alterations triggering melanoma development occur as randomly-acquired mutations within melanocytes, the presence of inherited germline variants is an important factor in melanoma susceptibility (115). Melanoma predisposition is lower in dark-skinned individuals who tan easily and never burn (Fitzpatrick skin types III and IV) than in fair-skinned, blond or red-haired individuals who seldom tan and always burn (Fitzpatrick skin types I and II), suggesting that the genes determining skin colour are the primary genetic determinants of melanoma susceptibility (115,135). This fact is clearly observed in the USA, where European-origin Americans present up to 10 times higher incidence of melanoma compared to African-origin Americans (136). Frequent general-population polymorphisms in several pigmentation genes may confer low-to-moderate melanoma risk, including *MC1R* (42), *ASIP* (137), *TYR* (51), *SLC45A2* (51), *IRF4* (138), and *OCA2* (139).

The presence of naevi is a well-known phenotypic risk factor for CM, since sometimes they develop from a pre-exiting naevi (140). Individuals with familial atypical multiple mole and melanoma syndrome (FAMMM; OMIM #606719) or dysplastic naevus syndrome (DNS; OMIM #155600) present an almost guaranteed lifetime risk of CM. As described before, phenotypic naevi differences in count and density are in part genetically determined, and genetic variants predisposing to naevi are postulated to be low-to-medium penetrance susceptibility genes for CM (141–144). Additionally, several studies have focused on analysing the impact of genes involved in UV-induced DNA photoproduct repair (145,146). Individuals with *Xeroderma pigmentosum* – a genetic disorder in which nucleotide excision DNA repair (NER) machinery is impaired – are prone to develop melanoma as a result of having a genetic inability to repair UV-induced DNA damage and a propensity to accumulate somatic mutations (147).

Generally, a family history of melanoma appears to increase risk of melanoma. The major susceptibility gene implicated in familial melanoma is the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene, a tumour suppressor gene that negatively regulates cell cycle progression and promotes cellular senescence (115). Germline mutations in *CDKN2A* have been implicated in melanoma susceptibility and are present in approximately 40% of melanoma families (148). These loss-of-function mutations may restrict the preventing melanomagenesis function of CDKN2A. Although *CDKN2A* mutations confer substantial risk for melanoma, not all carriers of *CDKN2A* mutations develop melanoma, suggesting that other host, environmental and genetic factors modify melanoma risk in mutation carriers. For instance, penetrance of *CDKN2A* mutations is found to significantly differ depending on geographical location, likely correlating with sunlight exposure (148). Rarely inactivating mutations in high-risk susceptibility genes (*CDK4* and *BAP1*) have also been found in melanoma-prone families (149).

In addition to the evident impact of loss-of-function mutations within the *CDKN2A* gene, germline variants in the 3'-untranslated region (3'UTR) of *CDKN2A* gene have been associated with increased susceptibility for melanoma (150,151). The 3'UTR region plays a crucial role in regulating gene expression at posttranslational level. It is well-known that microRNAs (small non-coding RNAs) bind to the 3'UTR of the target gene modulating its expression by repressing mRNA translation or by destabilizing/degrading mRNAs in the cytoplasm. Therefore, natural mutations in the binding sequences of the target genes may affect the pathogenesis of melanoma (150,152). Recent studies have also shown an alteration in the expression pattern of miRNAs in melanoma samples compared to healthy melanocytes, altering several well-known oncogenic pathways and cellular processes (153).

Hypotheses and Objectives

This thesis aims to investigate the following hypotheses:

1. Females are more protected against melanoma than males, and this is attributable to increased skin pigmentation and decreased sensitivity to sunlight exposure (even in people of both sexes sharing the same genotype). This difference could be explained by sex-differences in the genetic effects of polymorphisms located on pigmentation- and melanoma-associated genes.

2. Genetic variations in the 3'UTR of genes associated with pigmentation and melanoma could have a potential role in the genesis of melanoma by modifying cutaneous pigmentation and tanning response.

3. Co-inheritance of loss-of-function germline mutations in *CDKN2A* and *MC1R* genes synergistically sensitizes melanocytes to the damaging effects of UV radiation, and thus increases the chance for malignant transformation to melanoma.

The general objective of this thesis is to expand the current knowledge of the molecular bases of human skin pigmentation and predisposition to cutaneous melanoma.

Specific objectives addressed

1. Asserting sex as a factor that influences the phenotypic differences in pigmentation between different individuals of the Spanish population, even though these individuals share the same genotypes (Chapter 1 and 2).

2. Identification of possible sex-specific genetic effects in human pigmentation explaining the differences in pigmentation phenotype, as well as in melanoma risk, between sexes in the Spanish population (Chapter 1 and 2).

3. Discovery of genetic variations in the 3'UTR regions of pigmentation and melanoma-associated genes involved in the genesis of melanoma by modifying the ability of microRNAs to target genes, resulting in differential gene expression (Chapter 3).

4. Investigation of the ability of primary cultures of human melanocytes with different *CDKN2A* and *MC1R* genotypes to proliferate and undergo stress-induced senescence, as a result of both serial passages in culture and UV exposure (Chapter 4).

Chapter 1

Sex-specific genetic effects associated with pigmentation, sensitivity to sunlight and melanoma in a population of Spanish origin

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1.1. INTRODUCTION

Human pigmentation traits are some of the most visible and differentiable human characteristics. Pigmentation in human tissue is attributable to the number, size and cellular distribution of melanosomes produced, and the type of melanin synthesised (the black-brown coloured eumelanin or the red-yellow coloured phaeomelanin), while the number of melanocytes is usually unchanged (23).

The type of melanin synthesised is influenced by sun exposure and is genetically controlled (14). Ultraviolet (UV) exposure plays a key role in the evolutionary selective pressure on human pigmentation. Geographic distribution of human skin pigmentation reflects an adaptation to latitude-dependent levels of UV radiation (87,154). The linear relationship between worldwide skin pigmentation variation, latitude and UV radiation levels is thought to result from the physiological role of melanin type in UV-mediated vitamin D synthesis, UV-induced photolysis of folate, and in the protection from exposure to UV, which can cause sunburn and skin cancer (89). However, the physiological role for eye and hair colour variations still remains unknown.

Variation in genes implicated in human pigmentation has been associated with phenotypic characteristics such as skin colour, hair colour, eye colour, freckling and sensitivity to sunlight (31), but also with the risk of various types of skin cancer (24,26,29,54,68,155–158). The proteins encoded by these genes have effects at various stages of the pigmentation pathway, ranging from melanogenesis, the stabilization and transport of enzymes in the melanin production pathway, the production and maintenance of melanosomes and the melanosomal environment, and the switch between the production of eumelanin and phaeomelanin. Other pigmentation-related proteins code for intrinsic factors that help in the regulation of pigmentation, such as factors produced by

fibroblasts in the dermis that affect overlying melanocytes and keratinocytes, endocrine factors from the blood supply, as well as neural factors and inflammation-related factors (27,31,159).

Melanin synthesis is also modulated, in part, by oestrogens and androgens (85). Physiological hyperpigmentation in various forms (tanning, dark spots, chloasma, *linia nigra* and/or melasma) is commonly seen in pregnant females due to an increase of the levels of pregnancy-related hormones (85). The increase of pregnancy-related hormones – oestrogen, progesterone and melanocyte-stimulating hormone (α-MSH) – during gestation induces the activation and expression of genes involved in melanin synthesis in melanocytes (84), while it has also been shown that androgens inhibit tyrosinase activity (86). In addition to sex-endocrine factors, the use of oestrogen-containing oral contraceptives, certain cosmetics and oestrogen-progesterone therapies has also been associated with hyperpigmentation (11).

Biological and behavioural gender differences likely contribute to the sexual disparity in skin aging, pigmentation and melanoma incidence and outcome (160,161). Recent studies point to Caucasian females having slightly darker eye colour (162,163) and skin colour (25) than Caucasian males. Regarding melanoma, females show lower melanoma predisposition and incidence, lower risk of metastases and longer melanoma-specific survival rates than males (130,131). Anatomic location of melanoma indeed tends to be different between sexes, being most commonly on the lower leg, hip, and thigh in females, and on the back, abdomen, and chest in males (130).

In order to reveal possible sex-related differences in pigmentation phenotype as well as in melanoma association, we investigated the influence of 363 polymorphisms from 65 gene regions — previously associated with pigmentation traits, congenital pigmentation

genetic syndromes, and/or skin cancer — in a melanoma case-control population of Spanish origin.

1.2. MATERIAL AND METHODS

1.2.1. Study Subjects and Data Collection

In this study, a total number of 599 females (316 melanoma cases and 283 cancer-free controls) and 458 males (234 melanoma cases and 224 cancer-free controls) were collected at several Spanish hospitals. We carefully selected all cases and controls included in the current study to account for confounding variables. All individuals were Caucasians of Spanish origin where, according to a previous work by Laayouni and cols, there is no evidence of genetic heterogeneity within different Spanish geographical regions (164). Controls were frequency-matched to the cases by age and place of birth.

A standardised questionnaire was used to collect information on sex, age, pigmentation characteristics (eye colour, hair colour, skin colour, number of naevi and presence of solar lentigines), history of childhood sunburns, Fitzpatrick's skin type classification, and personal and family history of cancer, to classify individuals as previously described (28). Forty melanoma cases from our previous work were excluded in the current analysis due to the absence of sex details.

All individuals gave written informed consent and the study was approved by the Ethics Committee of the Gregorio Marañon Hospital (Madrid, Spain) and the Biomedical Research Institute - INCLIVA (Valencia, Spain).

1.2.2. Gene, SNP Selection and Genotyping

Gene and SNP selection was performed as previously described (28). Sixty-five gene regions were included in this study. They covered a broad range of biological processes, mostly related to pigmentation. We genotyped a total number of 384 tag-SNPs from the selected genes ranging from the hypothetical promoter area (approximately 10 kb upstream) until approximately 5 kb downstream of the gene. SNP codes, locations, and frequencies were obtained from NCBI (www.ncbi.nlm.nih.gov/SNP), HapMap (www.hapmap.org) and Illumina databases. A minor allele frequency (MAF) threshold of 0.05 in the HapMap CEU population and an 'Illumina score' not lower than 0.6 (as recommended by manufacturer) were established to ensure high genotyping success rate of the SNPs selected.

SNP genotyping was done using the Golden Gate Assay according to manufacturer's protocol (Illumina, San Diego, CA, USA), as previously described (28).

1.2.3. Statistical Analysis

Quality control processes and allelic and genotypic association tests were performed using the SNPator software (www.snpator.com). Additional statistical analyses and plots were conducted using the R statistical framework. All genetic analyses were performed estimating the effect of the minor allele in the Spanish population.

For all polymorphisms studied, Fisher's exact test was used both to test for deviations from Hardy-Weinberg equilibrium (HWE) between sexes and to compare allele counts

between female and male individuals. Bonferroni correction was applied and *P*-values higher than $1.37x10^{-4}$ were considered in HWE.

Associations between the genotyped SNPs and various pigmentation and sun sensitivity traits were assessed via logistic regression, coded additively for each copy of the minor allele. This was done for males and females separately, with eye colour (blue/green *versus* brown/black), hair colour (brown/black *versus* blond/red), skin colour (fair *versus* dark), number of naevi (≥50 *versus* <50), presence of lentigines (yes *versus* no) and childhood sunburn (yes *versus* no) as the outcome variables. Genotype-related Odds Ratios (ORs), their corresponding 95% confidence intervals (CIs) and associated *P*-values were estimated. Results of the association analysis were represented using volcano plots, mapping significance (-log10 *P*-value) *versus* fold-change (log2 OR) for the comparison between individuals for eye colour, hair colour, skin colour, presence of lentigines, childhood sunburns and naevi number separately. *P*-values were two sided and those lower than 0.01 were considered statistically significant (since six pigmentation traits were studied separately, statistical significant threshold of *P*-value < 0.05/6 = 0.01).

In order to have an overview of all the significant estimates obtained in the sex-specific logistic regression analyses, we evaluated the differences in the number of polymorphisms associated both with protective and risk phenotypes between sex groups (*P*-values < 0.05), using 2x2 contingency tables and performing a Fisher's exact test.

Logistic regression was performed to re-assess associations between genotypes and melanoma risk done previously (28), but separating individuals by sex in order to estimate sex-specific ORs, 95% CIs and *P*-values. As mentioned above, the minor allele was also modelled under an additive model. Using the same criteria as in the analysis of

pigmentation traits, two-sided *P*-values lower than 0.01 were considered to constitute evidence of association.

Finally, we performed a sex-differentiated regression estimate test for each SNP for all phenotypic traits. We tested for equality of sex-specific allelic effects with the aim of obtaining sex-differentiated *P*-values (165), and a statistical significance threshold of sex-differentiated *P*-value < 0.05 was used. Briefly, for each sex-specific association test, sex-specific beta coefficients (log ORs) and the corresponding standard errors were evaluated using a Chi-square test with one degree of freedom. Then, a Chi-square test with two degrees of freedom was performed for each SNP, in which the previously calculated female-specific and male-specific Chi-square statistics were added up. Finally, a test of heterogeneity of allelic effects between males and females using a Chi-square test with one degree of freedom was conducted. A significant sex-specific and sex-differentiated *P*-value is required to verify a potential significance in allelic effect by sex, following the same criteria used by Kocarnik and cols. (166). Manhattan plots were used to display the strength of significant differences between male-only and female-only associated effects (-log10 sex-differentiated *P*-value) for each trait tested.

1.3. RESULTS AND DISCUSSION

Our sample set included 599 females and 458 males of Spanish ancestry. Median age was 45 years (range 18–92) for females and 47 years (range 18–92) for males. Regarding control individuals, mean age was 42 years (range 18–91) for females and 45 years (range 18–90) for males. Melanoma cases presented a median age of 52 years (range 18–92) for

females and 53 years (range 18–92) for males. Since age differences were not observed between sample subsets (*P*-value > 0.05), association analyses were not adjusted by age.

From an initial list of 384 tag-SNPs selected, 21 SNPs (5.4%) were discarded due to failed genotyping (no PCR amplification, insufficient intensity for cluster separation or poor cluster definition). All 363 remaining SNPs were in HWE after Bonferroni correction (Table S1.1). Minor allele frequencies estimated for each SNP were almost identical for females and males, with a remarkable linear correlation (R^2) of 0.982 (Figure S1.1).

1.3.1. Association with phenotypic characteristics by sex

In a previous study published by our group, the association of some genes with phenotypic characteristics was reported (28). However, analyses were performed without taking into account sex data. In the current study, samples were additionally stratified by sex to evaluate differences in pigmentation and sun response between males and females.

Thirty four SNPs showed association with at least one pigmentation trait, and 42 SNPs were associated with at least one sun response trait studied ($P < 0.01$) (Tables S1.2 and S1.3). Each of these polymorphisms displayed a moderate effect on pigmentation in our Spanish population dataset. Our results showed apparent differences in the direction of the association with the pigmentation characteristics, with variants showing ORs below 1.0 correlated with dark pigmentation and/or good tolerance to sunlight, and variants with ORs above 1.0 associated with light pigmentation and/or poor tolerance to sunlight. Variants in these genes most likely play important roles in the differences in pigmentation and tanning response among individuals of the Spanish population, and subsequently also in skin cancer risk (167).

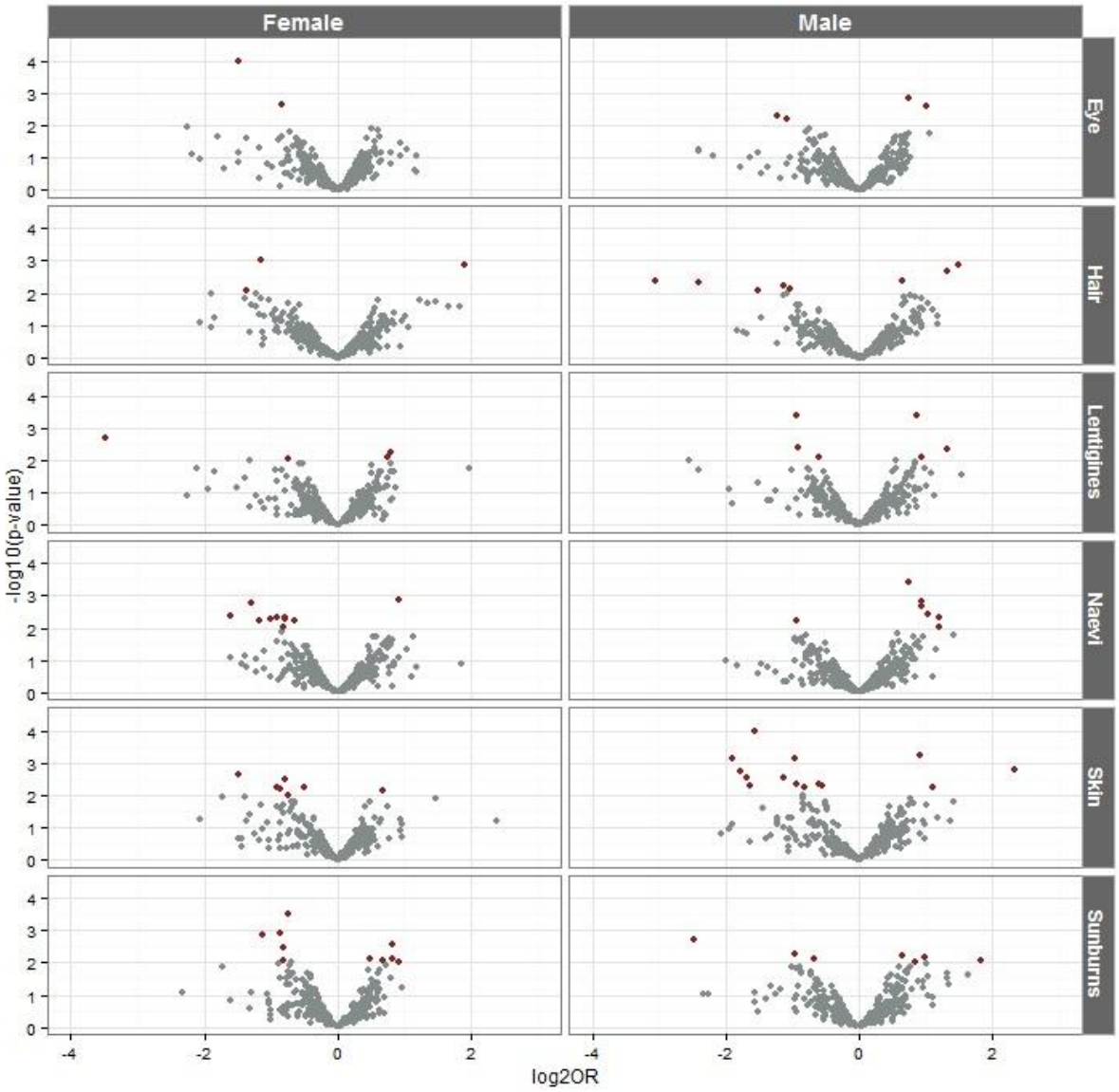

Figure 1.1. Volcano plots showing significance (-log10 *P*-value) *versus* fold change (log2 OR) for pigmentation and sun sensitivity traits separated by sex. Red dots indicate SNPs with a significant fold change (*P*-values < 0.01).

Representations of -log10 *P*-values *versus* log2 ORs comparing 599 female individuals to 458 males for eye colour, hair colour, skin colour, presence of lentigines, childhood sunburns and naevi number are shown in Figure 1.1. Detailed information on rs numbers,

genes, chromosome locations, minor alleles, ORs, 95% CIs, and *P*-values for pigmentation and sun response characteristics are summarised in Tables S1.2 and S1.3.

Sex-specific analyses in this study showed significant differences in the pattern of association with pigmentation and tanning response traits between male and female individuals. Out of all SNPs with significant sex-specific associations, we found significantly more SNPs associated with dark pigmentation or sun protection in female than in male individuals (107 vs. 75; $P = 2.32 \times 10^{-6}$), the latter being more commonly associated with light pigmentation and poor sun tolerance – traits highly associated with melanoma predisposition (26) (Figure 1.2). The percentage of SNPs associated with both dark eye and dark hair colour in females was higher than in males (72.72% vs. 40.74%, P=0.025; 78.57% vs. 48.28%, P = 0.018, respectively). This association pattern was also observed for skin colour, but without significance (66.67% vs. 41.94%, P = 0.068). In addition, female individuals presented more SNPs associated with both ≤50 naevi and absence of childhood sunburns than males (65.38% vs. 36.67%, P = 0.032; 61.11% vs. 36.11%, P = 0.034; respectively). On the other hand, a similar percentage of SNPs associated with absence of lentigines was observed in both female and male individuals (56.00% vs. 43.33%, P = 0.35). A representation of the distribution/count of polymorphisms associated with phenotype groups for each trait studied, separated by sex, is displayed in Figure 1.2.

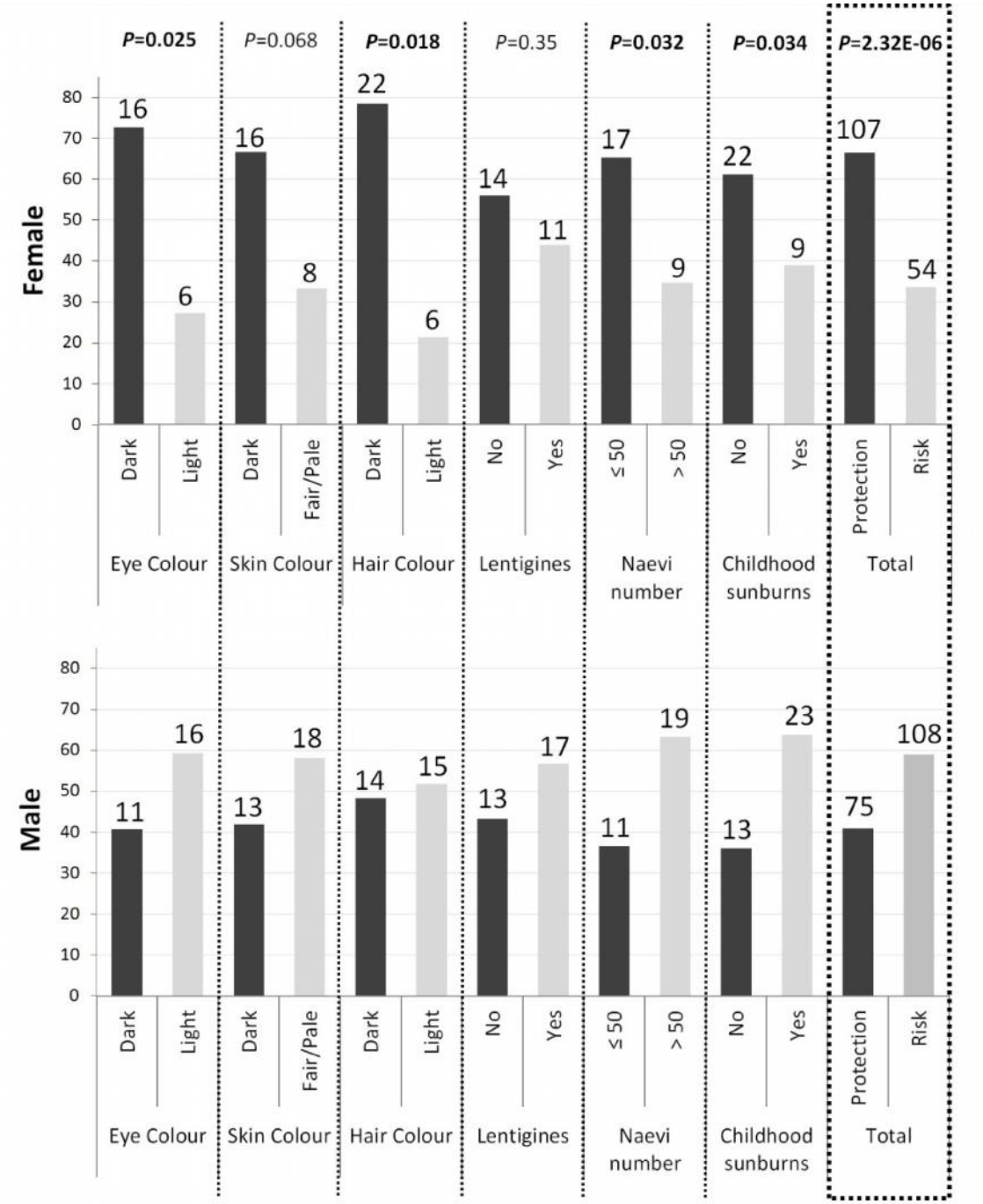

Figure 1.2. Distribution of the SNPs associated with pigmentation and sun sensitivity traits separated by sex. The percentage of each phenotype (protection or risk) is calculated taking into account the total number of significant SNPs associated in males and females (*P*-values<0.05). Percentages are represented by bars of the corresponding colour. The number on the top of each bar represents the count of associated SNPs in each category.

It is important to note that these associations do not reflect differences in the allelic frequencies of these pigmentation genes between males and females. These results basically indicate that, for a given genotype, the allelic effects on the phenotypic traits are shown to be significantly different in both sexes.

Additionally, sex-differentiated analysis was performed in order to test for equality between male-specific and female-specific regression estimates. Sex-differentiated *P*-values are represented in Figure 1.3. A significant sex-specific and sex-differentiated SNP association is required to establish a potential difference in effect for each polymorphism by sex. Three SNPs showed a strong potential sex-difference in eye colour effect, 10 SNPs in skin colour effect, 3 SNPs in hair colour effect, 4 SNPs in sunburns effect, 5 SNPs in lentigines effect, and 5 SNPs in naevi effect ($P < 0.01$). Among these SNPs, *PLDN* SNP rs12909221, *GPR143* SNP rs2521667, *POMC* SNP rs6734859, *AP3M2* SNP rs7009632, *BCL2* SNP rs1462129 and *TYRP1* SNP rs10809828 were associated with light pigmentation and poor sun tolerance in males. Only one polymorphism, rs2521578 on the *GPR143* gene, showed a high association with poor sun tolerance in females (Tables S1.2 and S1.3).

Promising differences in allelic effect by sex were also observed for *TYR* SNP rs1042602. Females and males showed statistically significant effects in opposite directions for this SNP, and this difference in effect by sex would remain hard to discriminate from chance. Indeed, a sex-differentiated *P*-value of 1.30×10^{-3} was estimated for rs1042602, as shown in Figure 1.3.

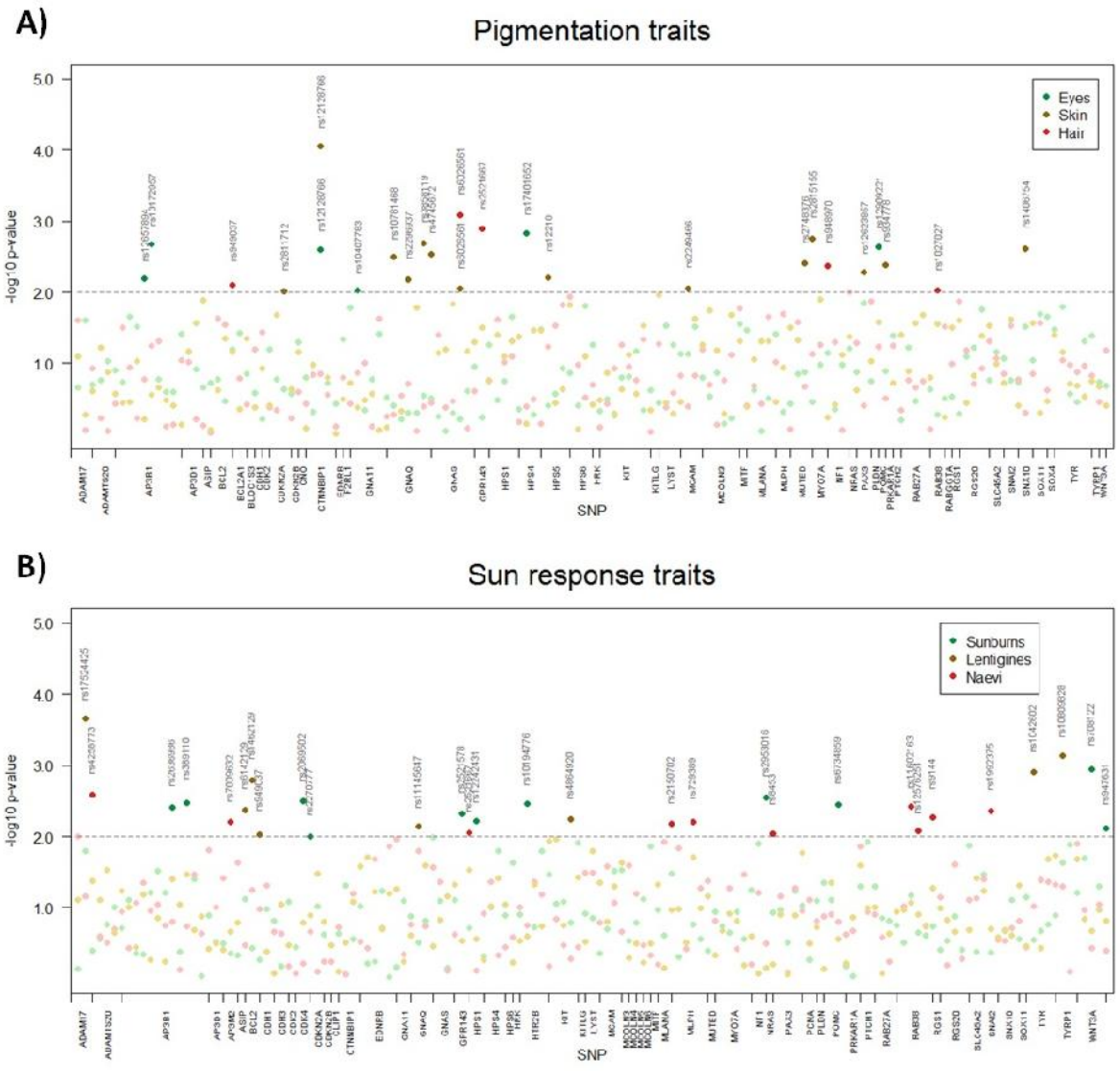

Figure 1.3. Manhattan plots displaying the strength of significant differences between male-only and female-only associated effects (-log10 sex-differentiated *P*-value) for pigmentation and sun sensitivity traits. Darker dots of the corresponding colour indicate SNPs with a significant fold change (sex-differentiated *P*-values < 0.01).

Polymorphisms showing potential differences in allelic effect by sex are located on genes that have functions related to melanocyte development, melanosome formation, maturation and transportation, as well as to skin cancer (26–28,31,54,56,158,168–170). Interestingly, we also found associations between pigmentation phenotypes and several

genes – *CDKN2A, GNA11, NRA and WNT3A* – involved in the up-regulation of melanogenic genes, the activation, survival and proliferation of the melanocyte, and/or the processes leading to carcinogenesis.

1.3.2. Associations with melanoma risk by sex

In a previous study published by our group, the association of 65 gene regions with melanoma risk was reported (28). However, at that time no sex stratification was applied to perform the association analysis. In this work, we have carried out an analysis of association between genotypes and melanoma risk for female and male individuals separately.

Sixteen SNPs located in 10 genes showed consistent male- or female-specific association with melanoma risk. Eleven of those SNPs showed potential differences in effect by sex, since *P*-values obtained in the sex-differentiated regression estimate test were lower than 0.05. Detailed information on rs numbers, genes, chromosome locations, minor alleles, ORs, 95% CIs and *P*-values for melanoma risk are summarised in Table 1.1.

Among these 11 SNPs, we found six SNPs located in 4 genes showing a strong difference in melanoma risk effect when samples were stratified by sex – sex-specific and sex-differentiated *P*-values lower than 0.01. *F2RL1* SNP rs2242991, *GPR143* SNPs rs2521667 and rs2732872, and *TYR* SNP rs5021654 increased melanoma predisposition in males as opposed to females. Additionally, a strong melanoma protective effect was displayed by rs2069398 on *CDK2/SILV* in females only. These SNPs were also associated with pigmentation and sun tolerance in opposite directions in males (ORs > 1, melanoma risk traits) *versus* females (ORs < 1, melanoma protective traits), supporting lower

melanoma predisposition and incidence in females than in males. Oppositely, rs1042602 on the *TYR* gene showed a melanoma protective effect in males compared to females. Therefore, these results are in accordance with the association between rs1042602 and dark pigmentation and good sun tolerance in males but not in females (Tables S1.2 and S1.3).

Table 1.1. SNPs highly associated with melanoma risk in sex-stratified analysis

				Melanoma				
				Female		Male		Sex-diff
Gene	**SNP ID**	**Chr**	**mA**	***P*-value**	**OR**	***P*-value**	**OR**	***P*-value**
AP3B1	rs11742673	5	A	**0.0020**	**1.43 (1.14-1.80)**	0.88	0.98 (0.75-1.28)	**0.0210**
CDK2/SILV	rs2069398	12	A	**3.03E-4**	**0.47 (0.31-0.71)**	0.06	0.60 (0.35-1.03)	**0.0016**
F2RL1	rs2242991	5	G	0.56	0.92 (0.68-1.22)	**0.0076**	**1.61 (1.13-2.29)**	**0.0067**
GPR143	rs2521667	X	G	0.46	0.89 (0.67-1.20)	**7.04E-4**	**1.89 (1.30-2.74)**	**7.85E-4**
	rs2732872	X	C	0.78	0.96 (0.73-1.27)	**8.43E-4**	**1.81 (1.27-2.57)**	**0.0052**
KIT	rs6554198	4	G	0.46	0.92 (0.73-1.15)	**0.0027**	**0.67 (0.51-0.87)**	0.07
MYO7A	rs3758708	11	A	0.20	1.30 (0.87-1.95)	**4.12E-4**	**2.38 (1.46-3.90)**	**0.0480**
RAB38	rs524121	11	C	**0.0086**	**0.51 (0.30-0.85)**	0.36	1.32 (0.73-2.38)	**0.0210**
RGS20	rs6981243	8	C	0.38	0.90 (0.71-1.14)	**0.0021**	**0.66 (0.51-0.86)**	**0.0490**
SLC45A2	rs35414	5	T	0.03	0.77 (0.61-0.97)	**0.0018**	**0.66 (0.51-0.85)**	0.39
	rs35415	5	A	0.03	0.78 (0.62-0.97)	**0.0080**	**0.70 (0.54-0.91)**	0.35
TYR	rs1042602	11	A	0.35	1.11 (0.89-1.40)	**0.0047**	**0.68 (0.53-0.89)**	**0.0081**
	rs12270717	11	C	0.60	1.07 (0.82-1.40)	**0.0032**	**1.56 (1.16-2.09)**	0.09
	rs17793678	11	T	0.32	1.14 (0.88-1.49)	**0.0036**	**1.55 (1.15-2.08)**	0.26
	rs2186640	11	G	0.39	0.90 (0.71-1.15)	**9.20E-4**	**1.57 (1.20-2.05)**	**0.0380**
	rs5021654	11	C	0.47	0.92 (0.72-1.16)	**2.03E-4**	**1.66 (1.27-2.17)**	**0.0014**

SNP, single nucleotide polymorphism; Chr, Chromosome; mA, Minor Allele; OR, Odds Ratio per minor allele; CI, Confidence Interval; Sex-diff, sex-differentiated meta-regression estimate test.
Bold indicates statistically significant results.

These genes with potential differences in melanoma risk effect by sex are graphically represented in Figure S1.2. The *F2RL1/PAR2* gene, expressed in keratinocytes but not in melanocytes, is a G-protein coupled receptor involved in melanosome transfer (171), and changes in its expression pattern are correlated with skin cancer progression (172). The *GPR143* gene, located in the X chromosome, encodes for a G-protein coupled receptor

for tyrosine, L-DOPA and dopamine localised on melanosomal membranes, and plays an important role in melanosome biogenesis, organization and transport. Ocular albinism type 1 (OA1; MIM300500) is caused by mutations in *GPR143*, and is transmitted as an X-linked trait. The *TYR* gene codes for another melanosomal membrane-bound enzyme involved in the rate-limiting steps of melanogenesis. Mutations in the *TYR* gene are associated with light pigmentation, freckling and sun sensitivity – well-recognised melanoma risk factors – as well as with melanoma (28,51). The *CDK2* gene, which overlaps with the melanocyte-specific gene *SILV*, is also important for melanoma growth and proliferation (173). SILV melanosomal matrix protein represents a melanoma specific antigen recognised by tumour infiltrating cytotoxic T lymphocytes (174).

A recent study is worth mentioning in this respect. According to Kocarnik and cols. (2014), *SLC45A2* SNP rs16891982, the non-synonymous mutation F374L located in exon 5, influenced melanoma risk differently by sex, with higher melanoma risk for males than females, probably through alterations in melanogenesis and pigmentation (166). In our study, two SNPs on the *SLC45A2* gene (rs35414 and rs35415) displayed associations with melanoma in both female-only and male-only analysis, although they do not present significant sex-differentiated *P*-values. It is important to note that the minor allele of these two SNPs showed a protective effect for melanoma, and that the allele frequencies for these protective minor alleles, causing a darker pigmentation, are actually quite common in the Spanish population, as opposed to Northern European populations. Subsequently, this association was stronger in males (rs35414: OR = 0.66, 95% CI 0.51-0.85, *P* = 0.0018; and rs35415: OR = 0.70, 95% CI 0.54-0.91, *P* = 0.008) than in females (rs35414: OR = 0.77, 95% CI 0.61-0.97, *P* = 0.026; and rs35415: OR = 0.78, 95% CI 0.62-0.97, *P* = 0.029). In the male-only but not in the female-only analysis, rs35414 and rs35415 tended to be also associated with dark pigmentation and the absence of childhood

sunburns ($P < 0.05$). It is important to state here that in the work by Kocarnik and cols., it was the major allele – in the Caucasian population – of the *SLC45A2* SNP that was in fact modelled as the purposed risk allele for melanoma, while in this study it is the minor alleles of the two *SLC45A2* SNPs that were actually used as reference to perform the analyses. Therefore, the genetic effect shown by the *SLC45A2* gene in our study exhibits the opposite direction that the one displayed by the Kocarnik and cols. work.

We are aware of the limitations of the current work. Firstly, the sample size was relatively restricted after dividing by sex the complete sample set, probably resulting in limited statistical power to detect modest effects for additional SNPs. Unfortunately, there are not previously published genome-wide studies presenting data stratified by sex, hindering chances of enlarging the sample size. Secondly, the subjective nature of the attributes considered may be another reason for misclassification. Thirdly, we presented two-sided unadjusted *P*-values for the associations considered; and the level of statistical significance considered was lower than the threshold required to declare unequivocally positive results. However, the results of this work – as well as other previous studies (162,163,166) – show that there is a strong tendency showing sex-differentiated genetic effects in pigmentary traits. Therefore, we believe that the work presented here is nonetheless reporting very interesting findings. For all these limitations, replication of our findings is essential before venturing on drawing firmer conclusions.

The results of this study suggest that there are indeed sex-specific genetic effects in human pigmentation, with larger effects for darker pigmentation in females compared to males. A plausible cause might be the differentially expressed melanogenic genes in females due to higher oestrogen levels. These sex-specific genetic effects would help explain the presence of darker eye and skin pigmentation in females, as well as the well-known higher melanoma risk displayed by males.

1.4. CONCLUSIONS

Overall, the results of this work reveal the presence of sex-specific effects in human pigmentation that might be important not only in skin colour and sensitivity to sunlight, but also in the higher incidence of melanoma described in males. These findings also show that, at times, sex-stratified analyses enrich genetic association studies with valuable information and knowledge.

1.5. SUPPLEMENTARY MATERIAL

- Table S1.1. List of 363 successfully genotyped SNPs, minor allele frequencies for all samples, males and females, and HWE *P*-values
- Table S1.2. List of SNPs associated with pigmentation traits in females and males
- Table S1.3. List of SNPs associated with sun response traits in females and males
- Figure S1.1. Comparison of minor allele frequencies, female *versus* male individuals
- Figure S1.2. A selection of genetic factors affecting pigmentation and sun sensitivity in human

Table S1.1. List of 363 successfully genotyped SNPs, Minor allele frequencies for all samples, males and females, and HWE *P*-values

					All samples			Males		Females	
Gene	**SNP rs#**	**Chr**	**Position on Chr (bp)**	**Location in Gene**	**mA**	**MAF**	**HWE *P*-value**	**MAF**	**HWE *P*-value**	**MAF**	**HWE *P*-value**
ADAM17	rs12473402	2	9550169	intron	C	0.370	0.34	0.374	0.28	0.366	0.97
ADAM17	rs12475630	2	9551832	intron	T	0.325	0.61	0.327	0.49	0.327	0.18
ADAM17	rs12992105	2	9581324	intron	C	0.165	0.13	0.169	0.84	0.162	0.09
ADAM17	rs17524425	2	9543455	3' downstream	G	0.213	0.76	0.202	0.78	0.221	0.74
ADAM17	rs4258773	2	9588663	intron	G	0.462	0.82	0.471	0.34	0.453	0.44
ADAMTS20	rs1510521	12	42193473	intron	G	0.342	0.90	0.321	0.72	0.356	0.82
ADAMTS20	rs17093450	12	42221612	intron	A	0.121	0.76	0.120	0.42	0.121	0.74
ADAMTS20	rs2048348	12	42215388	intron	A	0.105	0.89	0.123	0.98	0.092	0.81
ADAMTS20	rs2062731	12	42227223	intron	C	0.091	0.86	0.094	0.63	0.087	0.57
ADAMTS20	rs275630	12	42227033	intron	T	0.305	0.43	0.294	0.37	0.310	0.57
ADAMTS20	rs3764467	12	42105989	intron	T	0.093	0.94	0.084	0.46	0.099	0.68
ADAMTS20	rs7297057	12	42117952	intron	T	0.187	0.34	0.207	0.91	0.177	0.16
ADAMTS20	rs7960952	12	42212399	intron	C	0.420	0.94	0.407	0.88	0.431	0.61
ADAMTS20	rs9634256	12	42226764	intron	G	0.432	0.40	0.435	0.07	0.431	0.76
AP3B1	rs10514134	5	77355203	intron	A	0.094	0.96	0.098	0.93	0.090	0.79
AP3B1	rs10805919	5	77628680	5' upstream	C	0.237	0.72	0.258	0.27	0.218	0.69
AP3B1	rs11742673	5	77535322	intron	A	0.435	0.54	0.419	0.20	0.452	0.76
AP3B1	rs11746090	5	77375602	intron	A	0.249	0.91	0.230	0.97	0.261	0.91
AP3B1	rs12657894	5	77537609	intron	A	0.372	0.76	0.379	0.33	0.361	0.29
AP3B1	rs13172957	5	77611135	intron	G	0.329	0.72	0.330	0.22	0.324	0.56
AP3B1	rs17191796	5	77444232	intron	G	0.109	0.76	0.112	0.69	0.105	0.94
AP3B1	rs2636986	5	77421437	intron	T	0.215	0.41	0.223	0.81	0.210	0.34
AP3B1	rs34436	5	77394844	intron	G	0.106	0.97	0.109	0.88	0.107	0.70
AP3B1	rs389110	5	77408005	intron	A	0.215	0.36	0.223	0.81	0.211	0.28
AP3B1	rs4703747	5	77340718	intron	G	0.191	0.91	0.197	0.88	0.185	0.95
AP3B1	rs6453373	5	77460784	coding exon	A	0.075	0.61	0.073	0.88	0.077	0.73
AP3B1	rs6453374	5	77543915	intron	A	0.290	0.82	0.282	0.42	0.294	0.60
AP3D1	rs10413398	19	2057696	intron	C	0.201	0.43	0.204	0.42	0.197	0.75
AP3D1	rs17604954	19	2062382	intron	A	0.117	0.63	0.124	0.25	0.113	0.60
AP3D1	rs2238599	19	2091009	intron	C	0.460	0.65	0.474	0.29	0.449	0.16
AP3D1	rs2240655	19	2066790	intron	T	0.132	**0.05**	0.134	0.36	0.131	0.07
AP3D1	rs3786971	19	2092209	intron	T	0.438	0.19	0.428	0.91	0.447	0.15
AP3D1	rs7256735	19	2120121	5' upstream	G	0.086	0.89	0.088	0.70	0.088	0.72
AP3M2	rs2070713	8	42164812	3' downstream	T	0.417	0.61	0.423	0.45	0.408	0.93
AP3M2	rs7009632	8	42127343	5' upstream	G	0.422	0.14	0.423	0.27	0.421	0.19
AP3M2	rs7823824	8	42117935	5' upstream	A	0.433	0.48	0.447	0.24	0.424	0.75
AP3M2	rs8178890	8	42154511	3' downstream	T	0.082	0.91	0.071	0.71	0.088	0.59
ASIP	rs6142129	20	32283532	5' upstream	G	0.334	0.46	0.324	0.91	0.338	0.62
ASIP	rs819133	20	32333975	intron	T	0.126	0.60	0.135	0.69	0.124	0.73
BCL2	rs1462129	18	59131851	intron	T	0.467	0.91	0.480	0.27	0.460	0.19
BCL2	rs1564483	18	58945634	3' UTR	T	0.253	0.86	0.154	0.74	0.241	0.93
BCL2	rs4987852	18	58944901	3' UTR	C	0.069	0.74	0.057	0.88	0.077	0.56
BCL2	rs4987853	18	58944635	3' UTR	C	0.256	0.43	0.264	0.54	0.245	0.72
BCL2	rs949037	18	59129993	intron	A	0.437	0.59	0.445	0.76	0.432	0.15
BCL2A1	rs11636338	15	78032645	3' downstream	C	0.337	**0.03**	0.344	0.94	0.331	**0.03**
BCL2A1	rs17215263	15	78043311	intron	G	0.314	0.26	0.323	0.89	0.303	0.14
BCL2A1	rs6495460	15	78044410	intron	C	0.128	0.57	0.141	0.37	0.120	0.93
BLOC1S3	rs7253652	19	50372974	5' upstream	C	0.097	0.45	0.106	0.48	0.089	0.78
CDH1	rs11075699	16	67360288	intron	G	0.419	0.79	0.398	0.45	0.438	0.44

CDH1	rs16260	16	67328535	5' upstream	A	0.296	0.56	0.302	0.75	0.290	0.62
CDH1	rs1801552	16	67414942	coding exon	T	0.369	0.77	0.320	0.15	0.411	0.79
CDH1	rs2276329	16	67420815	intron	C	0.055	0.60	0.066	0.25	0.047	0.71
CDH1	rs2902186	16	67319398	5' upstream	G	0.097	0.55	0.110	0.74	0.090	0.25
CDH1	rs7188750	16	67400396	intron	A	0.153	0.73	0.168	0.97	0.142	0.69
CDH1	rs7203904	16	67420442	intron	C	0.218	0.53	0.242	0.23	0.200	**0.04**
CDH1	rs8059139	16	67402468	intron	G	0.061	0.85	0.068	0.98	0.056	0.76
CDH1	rs8061932	16	67420098	intron	C	0.174	0.27	0.197	0.96	0.158	0.10
CDH3	rs11075692	16	67239293	intron	G	0.378	0.35	0.370	0.30	0.387	0.69
CDH3	rs1124770	16	67263914	intron	G	0.150	0.54	0.153	0.93	0.146	0.49
CDH3	rs1886700	16	67243406	intron	T	0.128	0.37	0.130	0.70	0.127	0.14
CDH3	rs3114398	16	67264434	intron	G	0.283	0.69	0.291	0.96	0.277	0.71
CDH3	rs3118230	16	67266860	intron	T	0.093	0.46	0.103	0.97	0.087	0.32
CDK4	rs2069502	12	56430932	intron	T	0.237	0.85	0.253	0.42	0.235	0.73
CDK4	rs2270777	12	56431423	non-coding exon	C	0.469	0.97	0.490	0.33	0.459	0.51
CDKN2A	rs11515	9	21958199	3' UTR	G	0.170	0.46	0.170	0.81	0.170	0.30
CDKN2A	rs2518719	9	21960427	intron	G	0.158	0.43	0.175	0.84	0.146	0.53
CDKN2A	rs3731239	9	21964218	intron	G	0.317	0.21	0.308	0.55	0.324	0.36
CDKN2A	rs3731257	9	21956221	3' downstream	A	0.299	0.57	0.294	0.38	0.299	0.85
CDKN2B	rs1063192	9	21993367	3' UTR	G	0.357	0.20	0.365	0.50	0.354	0.20
CDKN2B	rs2811712	9	21988035	3' downstream	G	0.117	0.45	0.122	0.96	0.114	0.40
CDKN2B	rs3218009	9	21988757	3' downstream	C	0.085	0.88	0.077	0.93	0.087	0.73
CDKN2B	rs3218020	9	21987872	5' upstream	A	0.442	0.77	0.429	0.97	0.446	0.52
CDKN2B	rs495490	9	22000412	5' upstream	G	0.108	0.85	0.115	0.63	0.105	0.48
CLIP1	rs7388	12	121322137	intron	A	0.225	0.93	0.226	0.96	0.224	0.73
CNO	rs10937751	4	6762957	5' upstream	A	0.287	0.28	0.282	0.42	0.289	0.43
CNO	rs3172604	4	6769676	3' UTR	G	0.355	0.57	0.353	0.78	0.351	0.57
CNO	rs4689527	4	6771878	3' downstream	G	0.366	0.20	0.360	1.00	0.365	0.09
CTNNBIP1	rs11828	1	9909594	3' downstream	C	0.187	0.32	0.186	0.40	0.185	0.54
CTNNBIP1	rs12128766	1	9859628	intron	C	0.423	0.60	0.399	0.76	0.435	0.44
CTNNBIP1	rs1220392	1	9829390	3' downstream	A	0.150	0.24	0.155	0.99	0.144	0.15
CTNNBIP1	rs2379107	1	9833878	intron	G	0.167	0.63	0.149	0.41	0.179	0.83
CTNNBIP1	rs4846104	1	9900834	intron	G	0.064	0.78	0.060	0.92	0.063	0.69
DCT	rs2031527	13	93921918	intron	T	0.194	0.20	0.207	0.28	0.186	0.56
DCT	rs3782972	13	93901047	intron	C	0.165	0.21	0.163	0.70	0.167	0.13
DCT	rs3782973	13	93900698	intron	G	0.165	0.21	0.162	0.82	0.169	0.10
DCT	rs9301959	13	93908106	intron	C	0.451	0.55	0.455	0.90	0.447	0.32
DCT	rs9516418	13	93909510	intron	C	0.374	0.39	0.384	0.29	0.368	0.71
DCT	rs9524493	13	93899790	intron	G	0.460	0.07	0.451	0.12	0.464	0.39
EDNRB	rs11149080	13	77365761	3' downstream	G	0.424	0.45	0.425	0.86	0.421	0.30
EDNRB	rs3027110	13	77377230	intron	C	0.072	0.67	0.075	0.88	0.071	0.47
EDNRB	rs3027129	13	77384402	intron	T	0.060	0.84	0.051	0.96	0.068	0.77
EDNRB	rs3818416	13	77372469	intron	A	0.281	0.58	0.298	0.61	0.267	0.81
EDNRB	rs4885491	13	77368351	3' UTR	A	0.160	0.48	0.165	0.62	0.157	0.61
F2RL1	rs2242991	5	76150615	5' UTR	G	0.183	0.13	0.171	0.16	0.191	0.33
F2RL1	rs2243004	5	76152873	intron	G	0.174	0.80	0.177	0.74	0.172	0.89
F2RL1	rs2243010	5	76153524	intron	T	0.158	0.48	0.166	0.60	0.151	0.39
F2RL1	rs2243051	5	76161558	intron	G	0.449	0.38	0.459	0.96	0.441	0.19
F2RL1	rs639342	5	76148625	5' upstream	A	0.165	0.71	0.163	0.97	0.168	0.73
GNA11	rs10407783	19	3066124	intron	T	0.345	0.13	0.347	0.69	0.339	0.10
GNA11	rs1104737	19	3047788	intron	T	0.385	0.10	0.376	**0.04**	0.389	0.66
GNA11	rs2238625	19	3055652	intron	C	0.456	0.97	0.456	0.41	0.452	0.50
GNA11	rs308039	19	3046927	intron	T	0.175	0.54	0.178	0.77	0.173	0.27

GNA11	rs308054	19	3056987	intron	A	0.429	0.11	0.427	0.13	0.435	0.37
GNA11	rs3746069	19	3065864	intron	T	0.161	0.90	0.158	0.96	0.164	0.85
GNA11	rs3786947	19	3065229	intron	A	0.389	0.21	0.392	0.37	0.383	0.31
GNA11	rs404632	19	3065545	intron	T	0.122	0.25	0.120	0.57	0.123	0.23
GNA11	rs4806907	19	3044163	5' upstream	C	0.393	0.90	0.379	0.23	0.404	0.39
GNAQ	rs10781468	9	79690225	intron	T	0.256	0.15	0.249	0.59	0.261	0.15
GNAQ	rs10869977	9	79647096	intron	A	0.443	0.19	0.447	0.42	0.439	0.33
GNAQ	rs11145647	9	79801980	intron	C	0.201	0.69	0.204	0.69	0.200	0.50
GNAQ	rs12686139	9	79681047	intron	C	0.463	0.81	0.466	0.57	0.462	0.79
GNAQ	rs1328533	9	79674282	intron	G	0.341	0.51	0.340	0.77	0.342	0.58
GNAQ	rs1328534	9	79674191	intron	T	0.242	0.80	0.239	0.44	0.245	0.68
GNAQ	rs1410552	9	79691494	intron	T	0.291	0.34	0.289	0.24	0.294	0.79
GNAQ	rs17724885	9	79659385	intron	G	0.069	0.98	0.068	0.80	0.070	0.82
GNAQ	rs17786974	9	79733027	intron	T	0.083	0.80	0.086	0.93	0.081	0.83
GNAQ	rs2296937	9	79696407	intron	C	0.120	0.98	0.122	0.53	0.119	0.75
GNAQ	rs3780302	9	79576374	intron	T	0.121	0.88	0.129	0.89	0.117	0.87
GNAQ	rs3858119	9	79524896	3' UTR	C	0.300	0.06	0.303	0.14	0.296	0.35
GNAQ	rs4237275	9	79724578	intron	C	0.254	0.49	0.262	0.16	0.251	0.80
GNAQ	rs4745672	9	79688048	intron	T	0.489	0.39	0.491	0.37	0.486	0.82
GNAQ	rs7028873	9	79717352	intron	T	0.290	0.33	0.288	0.13	0.292	0.99
GNAS	rs12625436	20	56878608	intron	A	0.424	0.58	0.433	**0.05**	0.420	0.33
GNAS	rs13831	20	56908586	3' UTR	A	0.254	0.18	0.246	0.29	0.261	0.38
GNAS	rs2145288	20	56877604	intron	C	0.297	0.31	0.293	0.44	0.303	0.09
GNAS	rs234623	20	56922359	3' downstream	A	0.478	0.14	0.483	0.87	0.473	0.06
GNAS	rs234627	20	56920375	3' downstream	T	0.266	0.25	0.277	0.12	0.255	0.99
GNAS	rs6026561	20	56860527	5' upstream	C	0.386	0.42	0.401	0.21	0.377	0.99
GNAS	rs6026574	20	56889890	intron	T	0.304	0.55	0.289	0.57	0.318	0.66
GNAS	rs6064714	20	56847535	5' upstream	G	0.131	0.19	0.139	0.97	0.127	0.10
GNAS	rs6092704	20	56901873	intron	C	0.086	0.86	0.083	0.57	0.087	0.74
GNAS	rs6100269	20	56912671	intron	T	0.107	0.94	0.104	0.99	0.108	0.84
GNAS	rs6123832	20	56851466	intron	T	0.422	0.70	0.425	0.12	0.421	0.51
GNAS	rs919197	20	56914328	intron	T	0.468	0.28	0.477	0.43	0.461	**0.05**
GNAS	rs965808	20	56841821	5' upstream	C	0.255	0.27	0.247	0.73	0.263	0.15
GPR143	rs11095519	X	9667373	intron	A	0.141	***	0.141	***	0.140	0.52
GPR143	rs2521578	X	9655630	intron	T	0.135	***	0.165	***	0.112	0.92
GPR143	rs2521667	X	9690838	intron	G	0.167	***	0.155	***	0.179	0.22
GPR143	rs2732872	X	9688521	intron	C	0.197	***	0.179	***	0.213	0.86
GPR143	rs5979160	X	9650197	3' downstream	C	0.168	***	0.163	***	0.164	0.72
GPR143	rs6654731	X	9666637	intron	C	0.047	***	0.044	***	0.044	0.96
HPS1	rs1061135	10	100179128	intron	G	0.488	0.21	0.485	0.72	0.461	0.32
HPS1	rs10786422	10	100194717	intron	T	0.276	0.35	0.287	0.90	0.270	0.27
HPS1	rs10883094	10	100178096	intron	A	0.208	0.46	0.219	0.42	0.204	0.72
HPS1	rs12242431	10	100179066	intron	C	0.113	0.66	0.103	0.78	0.122	0.83
HPS1	rs1739	10	100166329	3' UTR	A	0.315	0.08	0.319	0.15	0.316	0.22
HPS1	rs17535384	10	100184592	intron	G	0.140	0.83	0.133	1.00	0.143	0.67
HPS1	rs1886728	10	100183669	intron	C	0.444	0.46	0.439	0.49	0.448	0.53
HPS1	rs3750605	10	100165644	3' downstream	T	0.202	0.41	0.218	0.51	0.193	0.50
HPS1	rs7075080	10	100202364	intron	G	0.408	0.86	0.404	0.96	0.414	0.77
HPS1	rs7921146	10	100176591	intron	A	0.209	0.41	0.225	0.56	0.199	0.45
HPS4	rs16982145	22	25191721	intron	C	0.038	0.93	0.030	0.52	0.046	0.72
HPS4	rs17401652	22	25198490	intron	T	0.104	0.66	0.104	0.82	0.099	0.55
HPS4	rs1894707	22	25189086	intron	C	0.372	0.71	0.362	0.72	0.376	0.80
HPS4	rs3213583	22	25191545	intron	A	0.109	0.45	0.105	0.29	0.111	0.82

HPS4	rs3747129	22	25192041	coding exon	A	0.145	0.21	0.135	0.61	0.154	0.24
HPS4	rs9608491	22	25200985	intron	G	0.187	0.98	0.204	0.68	0.174	0.58
HPS4	rs9613187	22	25205370	intron	T	0.112	0.98	0.123	0.98	0.104	0.90
HPS5	rs12218	11	18247897	coding exon	C	0.483	0.62	0.495	0.67	0.465	0.81
HPS5	rs2049129	11	18261909	intron	C	0.165	0.60	0.162	0.84	0.166	0.64
HPS5	rs2305564	11	18274034	intron	A	0.496	0.37	0.481	0.34	0.480	0.68
HPS5	rs4353250	11	18280093	intron	T	0.309	0.44	0.300	0.82	0.311	0.46
HPS5	rs4757637	11	18264151	intron	C	0.363	0.58	0.347	0.62	0.373	0.50
HPS5	rs7131332	11	18252099	3' downstream	G	0.318	0.25	0.314	0.05	0.320	0.97
HPS6	rs3737243	10	103815737	coding exon	A	0.102	0.41	0.097	0.42	0.107	0.71
HPS6	rs3816	10	103817727	3' UTR	G	0.220	0.81	0.233	0.64	0.212	0.32
HPS6	rs6584475	10	103796783	intron	C	0.381	0.45	0.366	0.75	0.384	0.24
HRK	rs10507275	12	115778887	3' downstream	A	0.158	0.96	0.161	0.86	0.158	0.99
HRK	rs1112700	12	115790588	intron	A	0.139	0.92	0.143	0.71	0.138	0.79
HRK	rs4767462	12	115781956	3' UTR	G	0.065	0.95	0.076	0.66	0.060	0.62
HRK	rs884378	12	115792581	intron	A	0.261	0.57	0.270	0.96	0.258	0.47
HTR2B	rs10194776	2	231688263	intron	T	0.396	0.99	0.391	0.38	0.399	0.61
HTR2B	rs17619600	2	231684704	intron	C	0.090	0.72	0.093	0.98	0.087	0.74
HTR2B	rs2161891	2	231662335	intron	G	0.345	0.79	0.330	0.77	0.357	0.84
HTR2B	rs4973377	2	231690236	intron	A	0.172	0.43	0.173	0.92	0.175	0.30
KIT	rs1008658	4	55294193	intron	T	0.356	0.97	0.361	0.71	0.350	0.86
KIT	rs11735550	4	55283476	intron	C	0.134	0.28	0.129	0.43	0.136	0.55
KIT	rs13135792	4	55247087	intron	C	0.355	0.15	0.348	0.48	0.355	0.25
KIT	rs2213180	4	55296084	intron	G	0.184	0.78	0.166	0.94	0.198	0.82
KIT	rs2237025	4	55236636	intron	T	0.457	0.72	0.465	0.88	0.455	0.63
KIT	rs2298976	4	55283293	intron	G	0.128	0.89	0.144	0.75	0.112	0.92
KIT	rs4864920	4	55285378	intron	T	0.211	0.86	0.223	0.50	0.199	0.76
KIT	rs6554198	4	55216917	5' upstream	G	0.425	0.94	0.423	0.32	0.427	0.57
KIT	rs759083	4	55232238	intron	G	0.370	0.30	0.365	0.37	0.368	0.76
KITLG	rs10858753	12	87434601	intron	T	0.051	0.87	0.053	0.93	0.050	0.89
KITLG	rs10858758	12	87468649	intron	G	0.232	0.27	0.231	0.78	0.228	0.16
KITLG	rs11104903	12	87407546	3' downstream	G	0.131	0.49	0.130	0.95	0.128	0.36
KITLG	rs11610915	12	87426172	intron	A	0.014	0.94	0.016	0.95	0.013	0.99
LYST	rs11810173	1	233899178	intron	T	0.170	0.96	0.163	0.42	0.176	0.66
LYST	rs17714318	1	234105797	intron	A	0.071	0.92	0.069	0.74	0.073	0.85
LYST	rs3754230	1	233893206	intron	A	0.114	0.97	0.114	0.99	0.111	0.90
LYST	rs3768051	1	233926337	intron	T	0.127	0.95	0.110	0.29	0.138	0.52
LYST	rs6429238	1	233921363	intron	T	0.347	0.96	0.354	0.86	0.346	0.66
LYST	rs6699717	1	233888473	3' downstream	T	0.500	0.43	0.495	0.26	0.497	0.93
LYST	rs7541057	1	233926963	intron	C	0.491	0.78	0.498	0.45	0.487	0.79
MCAM	rs2249466	11	118686380	coding exon	T	0.314	0.35	0.327	0.35	0.308	0.81
MCAM	rs2511837	11	118684217	3' downstream	T	0.476	0.92	0.469	0.38	0.476	0.38
MCAM	rs6589732	11	118690557	intron	A	0.407	0.83	0.398	0.40	0.407	0.48
MCOLN3	rs10518327	1	85260108	intron	A	0.272	0.99	0.259	0.64	0.279	0.71
MCOLN3	rs10782537	1	85257061	3' UTR	C	0.218	0.54	0.223	0.78	0.220	0.50
MCOLN3	rs10873682	1	85270400	intron	A	0.434	0.44	0.429	0.68	0.438	0.57
MCOLN3	rs12030837	1	85291971	5' upstream	T	0.125	0.87	0.127	0.89	0.125	0.80
MCOLN3	rs12735211	1	85262088	intron	A	0.076	0.51	0.074	0.49	0.075	0.94
MCOLN3	rs2304641	1	85285986	intron	T	0.173	0.80	0.184	0.93	0.168	0.89
MCOLN3	rs6674050	1	85288762	5' upstream	C	0.222	0.29	0.213	0.53	0.224	0.39
MCOLN3	rs7522239	1	85256954	3' UTR	A	0.143	0.52	0.137	0.75	0.150	0.50
MITF	rs11128152	3	70049623	intron	T	0.185	0.55	0.188	0.53	0.186	0.89
MITF	rs13072665	3	70076003	intron	A	0.090	0.86	0.079	0.97	0.096	0.75

MITF	rs2131025	3	70098337	3' UTR	C	0.070	0.96	0.076	0.87	0.069	0.96
MITF	rs3821364	3	70094111	intron	C	0.246	0.36	0.266	0.30	0.235	0.75
MITF	rs7623610	3	70087971	intron	G	0.480	0.19	0.487	**0.04**	0.481	0.97
MLANA	rs1056796	9	5899152	3' UTR	T	0.310	0.72	0.306	0.87	0.320	0.70
MLANA	rs10758717	9	5874281	5' upstream	C	0.244	0.91	0.247	0.53	0.249	0.51
MLANA	rs10815300	9	5874564	5' upstream	T	0.177	0.96	0.180	0.96	0.180	0.96
MLANA	rs10815303	9	5891100	intron	T	0.115	0.59	0.118	0.53	0.115	0.81
MLANA	rs10815304	9	5898217	intron	C	0.221	0.95	0.217	0.93	0.227	0.99
MLANA	rs10975339	9	5885586	intron	T	0.327	0.54	0.316	0.95	0.334	0.36
MLANA	rs2150702	9	5883861	intron	G	0.461	0.89	0.457	0.27	0.466	0.72
MLANA	rs7872509	9	5893555	intron	C	0.186	0.98	0.189	0.74	0.187	0.86
MLPH	rs10173589	2	238239684	intron	G	0.159	0.48	0.161	0.28	0.152	0.97
MLPH	rs13011946	2	238225025	intron	T	0.048	0.92	0.050	0.96	0.047	0.93
MLPH	rs13383648	2	238229295	intron	C	0.122	0.78	0.115	0.82	0.128	0.67
MLPH	rs2292881	2	238216249	coding exon	T	0.087	0.84	0.082	0.98	0.091	0.66
MLPH	rs729389	2	238207118	intron	A	0.129	0.79	0.130	0.76	0.128	0.97
MLPH	rs7606177	2	238176913	5' upstream	C	0.237	0.62	0.242	0.29	0.235	0.88
MLPH	rs880931	2	238178741	intron	A	0.101	0.36	0.109	0.55	0.095	0.44
MUTED	rs2743989	6	8009034	intron	A	0.393	0.36	0.398	0.30	0.391	0.80
MUTED	rs2748376	6	7960128	3' UTR	T	0.409	0.85	0.409	0.96	0.415	0.77
MUTED	rs2815155	6	8010229	5' upstream	C	0.427	0.75	0.420	0.91	0.425	0.32
MUTED	rs3734591	6	7960820	3' UTR	C	0.171	0.70	0.177	0.45	0.173	0.73
MUTED	rs9328451	6	7963071	intron	T	0.190	0.96	0.192	0.72	0.190	0.80
MYO7A	rs11237123	11	76600594	coding exon	A	0.189	0.71	0.210	0.45	0.173	0.87
MYO7A	rs11605022	11	76550062	intron	G	0.424	0.62	0.432	0.87	0.419	0.30
MYO7A	rs12793189	11	76598738	intron	G	0.488	0.36	0.465	0.41	0.493	0.60
MYO7A	rs12793619	11	76599006	intron	A	0.264	0.56	0.286	0.96	0.248	0.60
MYO7A	rs3740760	11	76531649	intron	C	0.252	0.94	0.260	0.85	0.246	0.98
MYO7A	rs3740763	11	76551268	intron	T	0.422	0.80	0.420	0.76	0.422	0.67
MYO7A	rs3758708	11	76572111	intron	T	0.109	0.90	0.112	0.92	0.108	0.85
MYO7A	rs7105374	11	76541520	intron	A	0.410	0.37	0.426	0.93	0.403	0.32
MYO7A	rs7123925	11	76598363	intron	G	0.450	0.61	0.442	0.74	0.454	0.74
MYO7A	rs762667	11	76546020	coding exon	C	0.329	0.55	0.327	0.72	0.331	0.21
MYO7A	rs883223	11	76602522	intron	A	0.331	0.58	0.333	0.63	0.327	0.84
MYO7A	rs885442	11	76597686	non-coding exon	T	0.434	0.51	0.444	0.68	0.425	0.53
MYO7A	rs948962	11	76597126	coding exon	A	0.472	0.90	0.467	0.65	0.476	0.63
MYO7A	rs948970	11	76563049	intron	G	0.464	0.87	0.465	0.66	0.465	0.44
NF1	rs1013948	17	26554835	intron	G	0.144	0.93	0.131	0.84	0.149	0.96
NF1	rs10438801	17	26717911	intron	G	0.362	0.77	0.351	0.36	0.369	0.68
NF1	rs2953014	17	26520021	intron	G	0.218	0.35	0.219	0.70	0.217	0.17
NF1	rs2953016	17	26518819	intron	G	0.196	0.25	0.200	0.24	0.192	0.59
NRAS	rs14804	1	115051366	3' UTR	A	0.229	0.40	0.224	0.82	0.237	0.46
NRAS	rs8453	1	115061122	5' upstream	T	0.113	0.99	0.115	0.99	0.111	0.90
NRAS	rs926938	1	115041339	5' upstream	G	0.476	0.70	0.468	0.19	0.477	0.36
NRAS	rs969273	1	115058192	intron	G	0.344	0.25	0.351	0.98	0.346	0.09
PAX3	rs12620338	2	222773012	3' UTR	A	0.203	0.50	0.212	0.95	0.196	0.56
PAX3	rs12623857	2	222870133	coding exon	A	0.145	0.41	0.160	0.43	0.131	0.82
PAX3	rs13405641	2	222869850	intron	A	0.261	0.27	0.286	0.07	0.239	0.99
PAX3	rs16863657	2	222872762	5' upstream	G	0.127	0.93	0.108	0.86	0.140	0.73
PAX3	rs2033806	2	222777323	intron	T	0.087	0.53	0.075	0.90	0.094	0.70
PAX3	rs2276630	2	222866283	intron	A	0.103	0.93	0.087	0.53	0.118	0.89
PAX3	rs3770210	2	222871006	intron	A	0.088	0.92	0.080	0.62	0.096	0.74
PAX3	rs7559271	2	222776530	intron	G	0.394	0.52	0.390	0.22	0.398	0.71

PCNA	rs17349	20	5047516	intron	A	0.130	0.82	0.130	0.94	0.130	0.87
PCNA	rs3729558	20	5043321	3' downstream	G	0.434	0.81	0.426	0.97	0.438	0.90
PLDN	rs12909221	15	43690071	3' downstream	C	0.440	0.68	0.458	0.82	0.422	0.50
PLDN	rs16945097	15	43667414	intron	T	0.134	0.70	0.118	0.89	0.146	0.60
POMC	rs1866146	2	25234077	3' downstream	G	0.331	**0.04**	0.336	0.17	0.326	0.09
POMC	rs6734859	2	25233412	3' UTR	T	0.148	0.87	0.149	0.69	0.146	0.74
POMC	rs7565877	2	25239568	intron	G	0.112	0.69	0.111	0.40	0.113	0.94
POMC	rs934778	2	25242728	intron	G	0.356	0.08	0.351	0.72	0.358	**0.03**
PRKAR1A	rs11651687	17	64029901	intron	A	0.190	0.40	0.190	0.13	0.193	0.96
PRKAR1A	rs4968898	17	64017451	5' upstream	G	0.201	0.81	0.204	0.42	0.202	0.70
PRKAR1A	rs8066131	17	64016614	intron	G	0.295	0.66	0.292	0.36	0.296	0.92
PRKAR1A	rs8076465	17	64024620	intron	A	0.317	0.83	0.305	0.79	0.323	0.71
PRKAR1A	rs8905	17	64039397	3' UTR	G	0.151	0.65	0.142	0.69	0.159	0.87
PTCH1	rs16909865	9	97247123	3' UTR	G	0.071	0.79	0.077	0.86	0.064	0.90
PTCH1	rs2297087	9	97282746	intron	G	0.201	0.10	0.208	0.40	0.199	0.20
PTCH1	rs4448343	9	97306191	intron	G	0.281	0.49	0.288	0.92	0.281	0.45
PTCH1	rs473902	9	95335790	intron	G	0.095	0.62	0.085	0.67	0.100	0.83
PTCH1	rs574688	9	95318745	intron	G	0.300	0.26	0.288	0.74	0.306	0.19
PTCH2	rs3795719	1	45061114	intron	G	0.137	0.44	0.137	0.57	0.137	0.13
PTCH2	rs7554177	1	45077697	intron	A	0.309	0.40	0.315	0.52	0.301	0.37
RAB27A	rs1007912	15	53360818	intron	A	0.082	0.54	0.071	0.64	0.089	0.61
RAB27A	rs1061824	15	53283255	3' UTR	C	0.184	1.00	0.177	0.80	0.192	0.72
RAB27A	rs11071175	15	53347717	intron	G	0.482	0.19	0.479	0.14	0.482	0.88
RAB27A	rs11855084	15	53368975	intron	A	0.067	0.86	0.076	0.87	0.061	0.95
RAB27A	rs12050885	15	53345916	intron	G	0.170	0.94	0.196	0.47	0.147	0.47
RAB27A	rs16976177	15	53296552	intron	G	0.155	**0.004**	0.150	**0.01**	0.161	**0.01**
RAB27A	rs16976194	15	53307051	intron	T	0.435	0.89	0.454	0.71	0.423	0.75
RAB27A	rs17238192	15	53302238	intron	T	0.108	0.21	0.115	0.49	0.106	0.27
RAB27A	rs7167572	15	53362172	intron	T	0.359	**0.05**	0.363	0.10	0.359	0.33
RAB27A	rs7496857	15	53342428	intron	T	0.253	0.74	0.273	0.56	0.237	0.91
RAB27A	rs9920165	15	53291631	intron	G	0.113	0.86	0.101	0.87	0.122	0.68
RAB38	rs1027027	11	87486711	3' UTR	A	0.240	0.59	0.252	0.97	0.231	0.61
RAB38	rs11602163	11	87548841	5' upstream	G	0.323	0.78	0.326	0.77	0.315	0.70
RAB38	rs12295107	11	87548384	5' upstream	A	0.077	0.80	0.081	0.79	0.074	0.94
RAB38	rs12576251	11	87521837	intron	G	0.335	0.68	0.336	0.78	0.328	0.57
RAB38	rs302646	11	87548096	coding exon	A	0.110	0.85	0.103	0.66	0.115	0.83
RAB38	rs524121	11	87487215	intron	C	0.053	0.55	0.051	0.57	0.054	0.74
RAB38	rs9144	11	87486405	3' UTR	T	0.408	0.74	0.422	0.85	0.392	0.61
RAB38	rs9666730	11	87547390	intron	T	0.159	0.75	0.166	0.76	0.155	0.75
RABGGTA	rs3940231	14	23818203	5' upstream	T	0.403	0.75	0.395	0.26	0.406	0.30
RABGGTA	rs941505	14	23803112	3' downstream	T	0.080	0.40	0.071	0.72	0.088	0.42
RGS1	rs10921202	1	190813675	intron	T	0.054	0.72	0.045	0.72	0.058	0.79
RGS1	rs1359062	1	190808095	5' upstream	G	0.181	0.60	0.188	0.99	0.177	0.35
RGS1	rs1923949	1	190814333	intron	G	0.273	0.93	0.276	0.19	0.273	0.35
RGS1	rs2816306	1	190814668	intron	C	0.111	0.50	0.112	0.53	0.113	0.60
RGS20	rs10958392	8	54917377	intron	T	0.429	0.91	0.425	0.49	0.437	0.36
RGS20	rs1123133	8	54942667	intron	G	0.034	0.76	0.031	0.86	0.035	0.81
RGS20	rs11783652	8	55021047	intron	A	0.359	0.51	0.353	0.18	0.359	0.78
RGS20	rs11783925	8	54933413	intron	T	0.239	0.57	0.246	0.74	0.232	0.59
RGS20	rs2220093	8	54963880	intron	C	0.104	0.89	0.114	0.83	0.100	0.99
RGS20	rs4738519	8	55031026	intron	C	0.138	0.76	0.140	0.38	0.137	0.81
RGS20	rs6473895	8	54925457	5' upstream	A	0.063	0.66	0.062	0.83	0.067	0.68
RGS20	rs6981243	8	55029044	intron	C	0.423	0.81	0.444	0.95	0.404	0.83

RGS20	rs7824575	8	54984872	intron	A	0.238	0.47	0.238	0.54	0.240	0.69
SILV	rs10783775	12	54625812	intron	G	0.195	0.86	0.185	0.63	0.198	0.68
SILV	rs2069398	12	54647143	5' upstream	A	0.079	0.83	0.067	0.99	0.089	0.77
SILV	rs2291615	12	54621417	intron	A	0.248	0.51	0.265	0.99	0.240	0.36
SLC45A2	rs35401	5	33977113	3' downstream	G	0.365	0.17	0.376	0.28	0.357	0.65
SLC45A2	rs35403	5	33979349	3' downstream	C	0.473	0.97	0.496	0.64	0.459	0.81
SLC45A2	rs35405	5	33981515	intron	T	0.455	0.32	0.457	0.24	0.450	0.68
SLC45A2	rs35414	5	34005385	intron	T	0.409	0.15	0.417	0.31	0.408	0.36
SLC45A2	rs35415	5	34008288	non-coding exon	A	0.429	**0.04**	0.438	0.54	0.427	0.05
SLC45A2	rs3756464	5	34005451	intron	A	0.438	0.46	0.428	0.94	0.446	0.29
SLC45A2	rs7718382	5	34004647	intron	A	0.197	0.83	0.196	0.62	0.199	0.64
SNAI2	rs1992375	8	50000397	5' upstream	A	0.492	0.62	0.483	0.98	0.477	0.48
SNAI2	rs2735455	8	49992695	3' downstream	T	0.075	0.72	0.074	0.69	0.075	0.93
SNX10	rs13222190	7	26102101	5' upstream	A	0.338	0.41	0.320	0.37	0.353	0.69
SNX10	rs1406754	7	26362723	intron	T	0.369	0.05	0.362	0.30	0.377	0.05
SNX10	rs1468286	7	26385234	3' downstream	T	0.429	0.35	0.436	0.79	0.429	0.17
SNX10	rs2699808	7	26175747	intron	C	0.470	0.65	0.487	0.58	0.455	0.29
SNX10	rs3801890	7	26159394	intron	C	0.349	0.99	0.343	0.89	0.347	0.94
SNX10	rs7782538	7	26174753	intron	T	0.192	0.77	0.189	0.90	0.200	0.61
SOX10	rs139879	7	36689052	3' downstream	T	0.386	0.49	0.394	0.41	0.381	0.99
SOX11	rs17362772	2	5748369	5' upstream	G	0.106	0.84	0.091	0.84	0.113	0.74
SOX11	rs3922853	2	5748050	5' upstream	A	0.175	0.85	0.155	0.69	0.191	0.97
SOX11	rs4371338	2	5788628	3' UTR	A	0.387	0.74	0.369	0.70	0.401	0.59
SOX11	rs4669779	2	5795648	3' downstream	C	0.422	0.78	0.406	0.84	0.435	0.99
SOX11	rs6432221	2	5746153	5' upstream	T	0.392	0.45	0.405	0.47	0.373	0.52
TYR	rs1042602	11	88551344	coding exon	A	0.459	0.75	0.452	0.34	0.470	0.26
TYR	rs11018535	11	88591995	intron	C	0.050	0.89	0.041	0.79	0.058	1.00
TYR	rs12270717	11	88551838	intron	C	0.259	0.77	0.272	0.14	0.245	0.62
TYR	rs17174064	11	88619139	intron	C	0.069	0.56	0.068	0.76	0.071	0.63
TYR	rs2186640	11	88615811	intron	G	0.377	0.57	0.392	0.18	0.362	0.11
TYR	rs5021654	11	88550237	5' upstream	C	0.373	0.48	0.386	0.21	0.361	0.10
TYRP1	rs10809828	9	12697861	intron	G	0.275	0.64	0.281	0.70	0.264	0.82
TYRP1	rs11791497	9	12677872	5' upstream	C	0.056	0.48	0.061	0.71	0.052	0.70
TYRP1	rs17346161	9	12695162	intron	T	0.053	0.83	0.047	1.00	0.060	0.76
TYRP1	rs683	9	12699305	3' UTR	C	0.411	0.70	0.424	0.69	0.396	0.99
WNT1/WNT10B	rs3782353	12	47645147	3' downstream	A	0.427	0.81	0.421	0.46	0.431	0.53
WNT1/WNT10B	rs7311091	12	47669474	3' downstream	T	0.079	0.47	0.071	0.85	0.084	0.39
WNT1/WNT10B	rs833820	12	47634086	3' downstream	C	0.404	0.46	0.402	0.36	0.405	0.99
WNT1/WNT10B	rs833839	12	47653157	5' upstream	T	0.468	0.31	0.464	0.18	0.470	0.90
WNT3A	rs697763	1	226259245	5' upstream	C	0.332	0.83	0.321	0.08	0.340	0.33
WNT3A	rs708122	1	226283620	intron	A	0.356	0.55	0.365	0.45	0.350	0.17
WNT3A	rs766972	1	226311447	intron	G	0.298	0.33	0.284	0.07	0.310	0.80
WNT3A	rs947631	1	226290170	intron	C	0.398	0.42	0.378	**0.04**	0.413	0.74

Chr, Chromosome; mA, Minor Allele; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium
Bold indicates statistically significant results. * *P*-value significant after Bonferroni correction. *** SNPs located in chromosome X

Table S1.2. List of SNPs associated with pigmentation traits in females and males

				Eye Colour				Skin Colour				Hair Colour			
				Female		Male		Female		Male		Female		Male	
Gene	SNP #rs	Chr	mA	*P*-value	OR	*P*-value	OR	*P*-value	OR	p-value	OR	*P*-value	OR	*P*-value	OR
ADAM17	rs12473402	2	C	0.22	1.26 (0.86-1.85)	0.27	1.26 (0.83-1.90)	0.45	1.30 (0.66-2.56)	0.76	1.09 (0.63-1.86)	0.54	0.92 (0.71-1.19)	**0.027**	0.72 (0.54-0.97)
	rs12992105	2	C	0.39	0.86 (0.61-1.22)	**0.022**	1.54 (1.07-2.24)	0.81	0.89 (0.36-2.20)	0.47	1.17 (0.77-1.77)	0.63	1.11 (0.73-1.70)	0.56	1.18 (0.68-2.05)
	rs4258773	2	G	0.45	1.17 (0.78-1.76)	**0.048**	1.34 (1.00-1.80)	0.62	0.94 (0.73-1.20)	0.14	0.72 (0.47-1.11)	0.13	1.54 (0.89-2.68)	0.25	1.43 (0.79-2.60)
ADAMTS20	rs1510521	12	C	**0.025**	1.36 (1.04-1.78)	0.24	1.27 (0.85-1.90)	0.18	0.70 (0.42-1.17)	0.19	1.29 (0.88-1.90)	0.19	1.26 (0.89-1.78)	0.081	1.60 (0.94-2.72)
	rs275630	12	A	**0.012**	1.42 (1.08-1.87)	0.73	0.88 (0.44-1.77)	0.15	0.66 (0.37-1.16)	0.53	0.81 (0.42-1.57)	0.35	1.18 (0.83-1.68)	0.21	1.28 (0.87-1.88)
	rs7297057	12	T	**0.030**	0.67 (0.46-0.97)	0.69	0.81 (0.27-2.37)	0.26	0.49 (0.14-1.75)	0.42	0.87 (0.62-1.22)	0.051	0.61 (0.37-1.02)	0.37	0.81 (0.50-1.30)
AP3B1	rs10514134	5	A	0.46	0.83 (0.51-1.36)	0.25	1.32 (0.82-2.12)	0.43	0.49 (0.08-2.96)	0.54	0.48 (0.04-5.34)	0.36	0.76 (0.41-1.41)	**0.048**	1.86 (1.03-3.38)
	rs11742673	5	A	0.16	0.75 (0.51-1.11)	0.077	1.61 (0.95-2.73)	0.54	0.93 (0.72-1.18)	0.45	1.23 (0.73-2.07)	0.23	1.42 (0.81-2.48)	**0.022**	1.56 (1.06-2.30)
	rs11746090	5	A	0.41	0.86 (0.59-1.24)	**0.023**	1.47 (1.05-2.05)	0.15	0.60 (0.30-1.20)	0.30	1.61 (0.65-3.97)	0.19	1.28 (0.88-1.85)	0.1	1.42 (0.94-2.15)
	rs12657894	5	A	0.12	1.23 (0.95-1.59)	**0.027**	0.63 (0.42-0.95)	0.75	1.06 (0.75-1.51)	0.62	1.10 (0.74-1.65)	0.41	1.15 (0.82-1.61)	0.27	0.74 (0.44-1.26)
	rs13172957	5	G	0.068	1.41 (0.97-2.06)	**0.012**	0.60 (0.40-0.89)	0.37	0.78 (0.46-1.33)	0.55	0.89 (0.60-1.31)	0.08	1.54 (0.94-2.52)	0.38	0.79 (0.47-1.33)
	rs17191796	5	G	0.60	0.88 (0.56-1.40)	0.15	1.38 (0.89-2.16)	0.43	0.49 (0.08-2.96)	0.29	0.32 (0.03-3.11)	0.65	0.88 (0.50-1.54)	**0.028**	1.93 (1.09-3.42)
	rs2636986	5	A	0.61	0.91 (0.62-1.33)	0.29	0.58 (0.21-1.63)	0.41	0.73 (0.34-1.55)	0.59	0.78 (0.32-1.92)	**0.021**	0.60 (0.38-0.95)	0.15	0.71 (0.44-1.14)
	rs389110	5	A	0.61	0.91 (0.62-1.33)	0.29	0.58 (0.21-1.63)	0.52	0.78 (0.37-1.65)	0.59	0.78 (0.32-1.92)	**0.035**	0.63 (0.40-0.99)	0.15	0.71 (0.44-1.14)
	rs6453374	5	A	0.27	0.81 (0.56-1.17)	0.072	1.34 (0.97-1.84)	0.72	0.94 (0.67-1.32)	0.89	0.97 (0.66-1.43)	**0.017**	2.34 (1.20-4.57)	0.3	1.24 (0.83-1.87)
AP3D1	rs10413398	19	C	0.17	0.77 (0.30-1.98)	**0.032**	1.45 (1.03-2.03)	**0.038**	0.40 (0.17-0.97)	0.55	1.31 (0.54-3.17)	0.08	0.24 (0.03-1.83)	0.34	1.23 (0.81-1.89)
	rs17604954	19	A	0.29	0.78 (0.50-1.24)	0.2600	1.27 (0.84-1.90)	**0.019**	0.63 (0.43-0.93)	0.41	1.18 (0.80-1.75)	0.45	0.81 (0.46-1.42)	0.96	0.98 (0.53-1.84)
	rs7256735	19	G	0.46	0.84 (0.52-1.34)	0.32	1.31 (0.77-2.22)	**0.0058**	0.53 (0.34-0.83)	0.5	1.18 (0.73-1.90)	0.69	0.88 (0.46-1.67)	0.81	0.92 (0.48-1.79)
ASIP	rs819133	20	T	0.26	0.78 (0.50-1.21)	0.058	0.66 (0.43-1.01)	**0.046**	0.70 (0.49-0.99)	0.23	0.79 (0.53-1.17)	0.11	0.65 (0.37-1.13)	**0.012**	0.46 (0.23-0.89)
BCL2	rs1462129	18	T	0.17	0.84 (0.65-1.08)	0.52	1.10 (0.82-1.47)	0.42	1.17 (0.80-1.70)	0.30	1.27 (0.80-2.03)	**0.014**	0.45 (0.22-0.89)	0.50	1.24 (0.67-2.26)
	rs1564483	18	A	0.11	1.27 (0.95-1.69)	0.30	1.24 (0.83-1.85)	0.11	1.26 (0.95-1.67)	0.13	0.49 (0.19-1.26)	**0.021**	2.55 (1.21-5.39)	0.66	1.12 (0.66-1.88)
	rs949037	18	T	0.2	0.85 (0.65-1.09)	0.16	1.43 (0.88-2.34)	0.18	1.28 (0.89-1.85)	0.075	1.56 (0.95-2.54)	**0.0083**	0.39 (0.18-0.84)	0.21	1.49 (0.81-2.74)
BCL2A1	rs11636338	15	C	**0.0021**	0.56 (0.39-0.81)	0.87	0.95 (0.52-1.75)	0.38	0.89 (0.68-1.16)	0.25	0.71 (0.39-1.28)	0.81	0.94 (0.58-1.52)	0.17	1.67 (0.83-3.38)
	rs17215263	15	G	**0.033**	0.71 (0.52-0.98)	0.89	0.98 (0.72-1.33)	**0.0023**	0.36 (0.18-0.70)	0.37	0.75 (0.39-1.42)	0.85	1.04 (0.71-1.52)	0.37	0.79 (0.46-1.33)
BLOC1S3	rs7253652	19	C	0.61	0.87 (0.52-1.46)	0.22	1.31 (0.85-2.03)	0.24	1.30 (0.84-2.02)	**0.034**	1.60 (1.03-2.48)	0.54	0.81 (0.41-1.60)	**0.020**	2.04 (1.14-3.66)
CDH1	rs1801552	16	T	0.72	0.95 (0.71-1.27)	0.39	0.83 (0.54-1.27)	0.16	0.76 (0.51-1.11)	0.44	1.13 (0.82-1.56)	0.69	0.90 (0.53-1.52)	**0.012**	1.71 (1.13-2.58)
CDK2	rs2069398	12	A	**0.023**	0.56 (0.32-0.97)	0.6	0.86 (0.48-1.53)	0.42	0.83 (0.53-1.30)	0.79	1.08 (0.62-1.86)	0.72	0.93 (0.51-1.67)	0.19	0.57 (0.24-1.38)
CDKN2A	rs2518719	9	G	0.11	1.33 (0.94-1.87)	0.65	0.92 (0.63-1.33)	0.81	1.04 (0.74-1.45)	**0.0006**	1.87 (1.29-2.69)	0.63	1.12 (0.72-1.74)	0.52	1.17 (0.74-1.84)
	rs2811712	9	G	0.077	0.22 (0.03-1.71)	0.54	0.88 (0.57-1.35)	0.35	1.23 (0.80-1.89)	**0.0044**	0.52 (0.33-0.82)	0.26	0.73 (0.41-1.29)		
	rs3218020	9	T	0.27	1.29 (0.82-2.03)	0.1	1.27 (0.95-1.69)	0.7	1.05 (0.82-1.35)	0.14	1.36 (0.91-2.05)	**0.029**	1.46 (1.04-2.06)	0.059	1.75 (0.96-3.20)
CDKN2B	rs495490	9	C	0.29	1.24 (0.83-1.84)	0.088	0.67 (0.42-1.07)	0.11	1.44 (0.91-2.26)	**0.017**	1.75 (1.10-2.79)	0.65	1.15 (0.64-2.06)	0.21	0.67 (0.35-1.28)
CNO	rs4689527	4	G	0.50	1.22 (0.69-2.14)	**0.048**	1.34 (1.00-1.80)	0.80	1.06 (0.65-1.74)	0.38	1.18 (0.81-1.73)	0.32	1.32 (0.76-2.30)	0.56	1.12 (0.76-1.66)
CTNNBIP1	rs11828	1	G	0.31	0.60 (0.22-1.66)	0.54	0.89 (0.63-1.28)	0.11	0.75 (0.52-1.07)	0.079	0.43 (0.16-1.14)	0.77	0.83 (0.24-2.87)	**0.036**	0.53 (0.29-0.98)

	rs12128766	1	C	0.14	1.35 (0.90-2.02)	**0.0049**	0.43 (0.23-0.80)	**0.047**	0.64 (0.42-0.99)	**0.0001**	0.34 (0.19-0.60)	0.4	0.81 (0.49-1.32)	**0.035**	0.66 (0.44-0.98)
	rs2379107	1	G	0.38	1.19 (0.81-1.75)	0.3	0.82 (0.55-1.21)	0.41	0.63 (0.21-1.90)	0.24	0.52 (0.17-1.58)	0.49	0.84 (0.50-1.40)	**0.024**	0.52 (0.28-0.96)
	rs4846104	1	G	**0.029**	0.54 (0.30-0.97)	0.62	0.86 (0.48-1.56)	0.68	0.90 (0.54-1.50)	0.78	0.92 (0.53-1.62)	0.67	0.85 (0.40-1.79)	0.17	0.54 (0.21-1.39)
EDNRB	rs3818416	13	T	0.15	0.57 (0.25-1.27)	0.14	1.35 (0.90-2.01)	0.78	0.91 (0.47-1.77)	0.28	1.18 (0.88-1.58)	0.16	0.46 (0.14-1.53)	**0.004**	0.12 (0.02-0.92)
F2RL1	rs2243010	5	T	0.17	1.32 (0.89-1.97)	**0.035**	0.64 (0.42-0.98)	0.43	0.55 (0.12-2.47)	0.23	0.38 (0.07-1.99)	0.44	1.22 (0.73-2.04)	0.64	0.88 (0.52-1.50)
GNA11	rs10407783	19	T	0.12	0.59 (0.29-1.19)	**0.017**	2.08 (1.14-3.80)	0.49	1.13 (0.80-1.60)	0.67	0.94 (0.70-1.25)	0.26	1.32 (0.81-2.15)	0.16	1.48 (0.85-2.56)
	rs2238625	19	C	0.92	1.01 (0.78-1.32)	0.28	1.27 (0.82-1.97)	0.39	1.22 (0.78-1.91)	0.62	0.94 (0.72-1.22)	0.094	1.59 (0.91-2.79)	**0.031**	1.94 (1.03-3.63)
	rs308039	19	T	0.55	0.90 (0.65-1.26)	**0.016**	0.58 (0.37-0.91)	0.88	0.98 (0.72-1.33)	0.16	1.29 (0.90-1.86)	**0.040**	0.57 (0.32-0.99)	0.3	0.77 (0.46-1.29)
	rs3786947	19	T	0.24	0.72 (0.41-1.27)	0.062	1.32 (0.99-1.78)	0.91	0.98 (0.69-1.40)	0.81	0.97 (0.65-1.45)	0.06	1.40 (0.99-1.98)	**0.0021**	2.52 (1.35-4.70)
	rs404632	19	T	0.24	0.77 (0.49-1.20)	**0.027**	0.60 (0.37-0.96)	0.39	1.17 (0.82-1.66)	0.16	1.40 (0.87-2.25	0.094	0.60 (0.32-1.12)	0.28	0.72 (0.38-1.34)
GNAQ	rs10781468	9	T	0.57	1.21 (0.64-2.28)	0.43	1.18 (0.79-1.75)	0.77	1.04 (0.80-1.36)	**0.0018**	0.29 (0.13-0.67)	0.088	1.36 (0.96-1.93)	0.26	1.34 (0.80-2.26)
	rs17786974	9	T	0.71	0.91 (0.55-1.51)	0.71	0.91 (0.55-1.51)	0.92	0.98 (0.61-1.56)	0.29	1.24 (0.83-1.85)	**0.014**	0.38 (0.16-0.90)	0.17	0.61 (0.28-1.30)
	rs2296937	9	G	0.15	1.33 (0.90-1.97)	**0.038**	1.64 (1.03-2.61)	0.66	0.73 (0.18-2.96)	**0.0048**	0.32 (0.14-0.74)	0.89	0.96 (0.57-1.63)	0.13	1.56 (0.90-2.71)
	rs3780302	9	T	0.49	0.85 (0.54-1.34)	0.87	0.97 (0.63-1.48)	0.35	1.20 (0.82-1.77)	**0.021**	0.57 (0.35-0.92)	0.72	1.10 (0.66-1.83)	0.65	1.14 (0.66-1.95)
	rs3858119	9	G	0.91	1.04 (0.53-2.01)	0.28	0.65 (0.30-1.45)	0.38	0.86 (0.61-1.21)	**0.0007**	0.27 (0.12-0.61)	0.28	1.30 (0.81-2.09)	0.67	1.12 (0.66-1.89)
	rs4745672	9	T	0.22	0.85 (0.65-1.10)	0.084	0.64 (0.39-1.07)	0.23	1.27 (0.86-1.87)	**0.0029**	0.31 (0.13-0.70)	0.47	0.82 (0.49-1.39)	0.49	0.81 (0.45-1.45)
GNAS	rs12625436	20	A	0.48	1.10 (0.85-1.42)	0.67	1.13 (0.65-1.96)	**0.022**	1.33 (1.04-1.70)	0.65	0.94 (0.70-1.25)	0.064	1.64 (0.96-2.82)	0.18	0.59 (0.26-1.34)
	rs13831	20	T	0.35	0.84 (0.58-1.21)	0.29	0.84 (0.61-1.16)	0.24	1.47 (0.77-2.81)	**0.015**	2.69 (1.16-6.23)	0.73	0.87 (0.36-2.13)	0.3	1.24 (0.83-1.85)
	rs234623	20	G	0.17	1.21 (0.92-1.59)	0.74	1.05 (0.79-1.39)	0.5	1.09 (0.85-1.41)	**0.012**	0.56 (0.35-0.88)	0.39	1.27 (0.72-2.24)	0.54	0.82 (0.43-1.55)
	rs6026561	20	C	0.53	0.84 (0.49-1.45)	0.42	1.26 (0.72-2.19)	**0.0031**	0.58 (0.41-0.84)	0.95	0.99 (0.66-1.48)	**0.0010**	0.45 (0.28-0.73)	0.32	0.82 (0.55-1.21)
	rs6092704	20	C	0.36	0.79 (0.48-1.31)	0.057	0.58 (0.32-1.03)	**0.024**	0.62 (0.41-0.94)	0.64	1.13 (0.68-1.86)	0.14	0.62 (0.32-1.21)	0.21	0.62 (0.29-1.36)
	rs6123832	20	T	0.49	0.84 (0.52-1.37)	**0.038**	0.63 (0.41-0.97)	**0.016**	0.64 (0.44-0.92)	0.71	1.08 (0.71-1.64)	**0.023**	0.67 (0.47-0.95)	0.35	0.77 (0.45-1.33)
GPR143	rs2521667	X	G	0.54	1.13 (0.76-1.68)	0.86	0.95 (0.54-1.67)	0.17	0.55 (0.24-1.29)	0.062	1.66 (0.97-2.86)	0.1	0.68 (0.43-1.10)	**0.0014**	2.80 (1.53-5.13)
	rs2732872	X	C	**0.022**	0.29 (0.09-0.98)	0.80	0.94 (0.55-1.58)	0.26	0.84 (0.63-1.13)	0.5	1.09 (0.85-1.40)	0.26	0.47 (0.11-2.02)	**0.004**	1.57 (1.17-2.11)
HPS1	rs1061135	10	T	0.65	0.91 (0.61-1.36)	0.38	1.23 (0.78-1.95)	**0.015**	0.62 (0.42-0.91)	0.38	0.82 (0.52-1.28)	0.11	0.66 (0.40-1.09)	0.1	0.74 (0.51-1.07)
	rs1739	10	C	0.12	0.61 (0.32-1.17)	0.65	1.15 (0.62-2.12)	0.13	0.66 (0.38-1.14)	0.18	0.66 (0.37-1.21)	0.49	0.75 (0.33-1.73)	**0.0046**	0.19 (0.05-0.82)
	rs1886728	10	C	0.08	1.49 (0.96-2.32)	0.13	0.66 (0.38-1.15)	0.36	0.82 (0.53-1.25)	**0.027**	0.56 (0.34-0.94)	0.54	1.18 (0.69-2.02)	0.094	0.63 (0.37-1.08)
	rs7921146	10	A	0.3	0.60 (0.22-1.65)	0.85	1.03 (0.74-1.44)	**0.017**	0.64 (0.45-0.92)	0.69	1.07 (0.78-1.46)	0.17	0.70 (0.42-1.18)	0.27	0.78 (0.50-1.23)
HPS4	rs17401652	22	T	**0.0001**	0.36 (0.20-0.63)	0.31	1.27 (0.80-2.02)	0.72	0.92 (0.60-1.41)	0.85	0.95 (0.58-1.56)	0.47	0.79 (0.42-1.50)	0.35	0.71 (0.35-1.47)
	rs3747129	22	A	0.33	1.20 (0.83-1.73)	0.8	1.06 (0.69-1.61)	0.31	0.83 (0.57-1.20)	**0.040**	1.58 (1.02-2.46)	0.74	1.09 (0.66-1.81)	0.23	1.42 (0.81-2.49)
	rs9608491	22	G	**0.014**	1.53 (1.09-2.14)	0.23	0.55 (0.20-1.53)	0.26	0.54 (0.19-1.59)	**0.033**	0.65 (0.44-0.97)	0.72	1.10 (0.67-1.80)	0.84	0.88 (0.25-3.07)
HPS5	rs12218	11	G	0.64	0.91 (0.61-1.36)	0.46	1.19 (0.75-1.88)	**0.0061**	0.55 (0.36-0.85)	0.19	0.75 (0.48-1.16)	0.33	1.19 (0.84-1.67)	0.11	1.61 (0.91-2.83)
	rs2049129	11	G	0.84	1.04 (0.70-1.55)	0.11	1.36 (0.93-1.98)	0.14	1.32 (0.91-1.93)	0.43	1.16 (0.80-1.68)	0.64	0.90 (0.56-1.43)	**0.013**	1.80 (1.14-2.83)
	rs2305564	11	A	0.88	0.98 (0.75-1.27)	0.12	1.44 (0.91-2.26)	0.24	0.86 (0.67-1.10)	0.35	0.82 (0.54-1.25)	0.33	1.31 (0.75-2.28)	**0.030**	1.88 (1.08-3.27)
	rs7131332	11	G	0.51	0.88 (0.61-1.28)	0.19	1.49 (0.83-2.68)	**0.0069**	1.61 (1.14-2.28)	0.52	0.88 (0.60-1.29)	0.14	0.52 (0.20-1.34)	**0.033**	2.16 (1.10-4.24)
HPS6	rs3737243	10	A	0.22	0.74 (0.46-1.20)	0.40	0.80 (0.47-1.35)	0.55	1.13 (0.75-1.70)	0.47	1.19 (0.74-1.94)	**0.047**	0.51 (0.26-0.99)	0.64	1.17 (0.61-2.24)
	rs3816	10	G	0.091	2.28 (0.89-5.84)	**0.030**	0.63 (0.42-0.96)	0.19	1.96 (0.69-5.59)	0.19	0.58 (0.26-1.32)	0.28	0.77 (0.47-1.26)	0.65	0.91 (0.59-1.40)
	rs6584475	10	C	0.19	1.42 (0.85-2.39)	**0.020**	1.63 (1.08-2.48)	0.24	1.36 (0.81-2.28)	0.23	1.19 (0.90-1.58)	0.45	1.30 (0.67-2.49)	0.2	1.28 (0.88-1.87)
HRK	rs884378	12	T	0.49	0.77 (0.37-1.63)	0.39	0.87 (0.63-1.20)	0.55	1.09 (0.83-1.43)	0.41	1.36 (0.65-2.86)	**0.032**	0.64 (0.42-0.98)	0.19	0.75 (0.48-1.16)

KIT	rs1008658	4	A	0.24	0.80 (0.55-1.16)	0.89	1.04 (0.58-1.88)	0.24	1.37 (0.80-2.33)	**0.0210**	2.01 (1.10-3.68)	0.44	0.74 (0.34-1.62)	0.63	1.20 (0.57-2.53)
	rs13135792	4	C	0.74	1.09 (0.65-1.83)	0.57	1.12 (0.75-1.68)	0.66	1.06 (0.82-1.35)	0.38	1.29 (0.73-2.29)	0.075	1.76 (0.97-3.21)	**0.013**	1.60 (1.11-2.32)
	rs2237025	4	A	0.16	0.75 (0.51-1.12)	0.33	0.78 (0.47-1.29)	0.35	1.20 (0.82-1.74)	0.43	0.89 (0.68-1.18)	0.36	0.75 (0.40-1.42)	**0.0057**	0.46 (0.27-0.79)
	rs2298976	4	C	0.088	0.68 (0.43-1.07)	0.62	0.89 (0.57-1.40)	0.96	1.01 (0.68-1.50)	**0.020**	1.68 (1.08-2.61)	0.059	0.55 (0.29-1.06)	0.72	1.10 (0.66-1.82)
	rs4864920	4	T	**0.047**	0.68 (0.46-0.99)	0.31	1.55 (0.68-3.54)	0.38	1.51 (0.60-3.79)	0.29	1.19 (0.86-1.65)	0.06	0.65 (0.41-1.04)	0.53	1.40 (0.50-3.89)
	rs6554198	4	G	0.79	0.95 (0.64-1.40)	0.46	1.17 (0.77-1.79)	0.18	1.28 (0.89-1.84)	0.077	0.65 (0.40-1.05)	**0.026**	0.43 (0.19-0.97)	**0.008**	0.35 (0.15-0.84)
	rs759083	4	G	0.45	1.11 (0.85-1.45)	0.36	0.75 (0.40-1.40)	0.52	0.85 (0.52-1.39)	0.63	1.10 (0.74-1.63)	**0.016**	1.52 (1.08-2.14)	0.054	1.45 (0.99-2.13)
KITLG	rs10858753	12	T	0.62	1.16 (0.65-2.06)	0.092	1.70 (0.92-3.15)	**0.022**	0.53 (0.30-0.91)	0.15	1.58 (0.84-2.96)	0.32	0.66 (0.28-1.57)	0.097	1.92 (0.92-4.04)
LYST	rs6429238	1	T	**0.024**	0.65 (0.45-0.95)	0.21	0.68 (0.37-1.26)	0.62	1.14 (0.67-1.96)	0.33	0.75 (0.43-1.33)	0.49	0.85 (0.53-1.36)	0.66	1.09 (0.75-1.58)
	rs6699717	1	A	**0.041**	0.65 (0.43-0.98)	0.26	1.31 (0.81-2.12)	0.44	1.16 (0.79-1.72)	0.85	0.96 (0.61-1.51)	0.76	1.09 (0.63-1.88)	0.22	1.27 (0.87-1.85)
	rs7541057	1	C	**0.043**	0.66 (0.44-0.98)	0.37	0.81 (0.50-1.30)	0.35	1.20 (0.82-1.76)	0.78	0.96 (0.73-1.27)	0.51	1.16 (0.67-2.00)	0.15	0.76 (0.52-1.11)
MCAM	rs2249466	11	T	0.42	1.19 (0.79-1.79)	**0.0014**	1.67 (1.22-2.31)	**0.019**	0.45 (0.23-0.88)	0.18	1.32 (0.88-2.00)	0.17	1.45 (0.85-2.47)	0.45	1.23 (0.71-2.13)
	rs2511837	11	T	0.32	1.14 (0.88-1.47)	**0.020**	0.71 (0.53-0.95)	0.94	1.02 (0.68-1.52)	**0.018**	0.71 (0.54-0.94)	0.087	1.64 (0.91-2.93)	0.68	0.92 (0.64-1.35)
	rs6589732	11	A	0.59	0.87 (0.53-1.43)	0.11	0.79 (0.58-1.06)	0.41	1.11 (0.87-1.42)	**0.019**	0.71 (0.53-0.95)	0.052	0.47 (0.22-1.02)	0.53	1.25 (0.63-2.49)
MCOLN3	rs10782537	1	C	0.73	0.86 (0.35-2.09)	0.30	0.61 (0.24-1.59)	0.21	1.70 (0.73-3.99)	**0.025**	0.37 (0.15-0.92)	0.51	1.42 (0.52-3.91)	0.25	0.77 (0.49-1.22)
	rs12030837	1	T	**0.045**	0.63 (0.41-0.98)	0.69	0.72 (0.14-3.74)	0.063	5.24 (0.64-42.93)	0.27	0.80 (0.53-1.19)	0.57	0.84 (0.47-1.52)	0.84	0.95 (0.55-1.64)
	rs12735211	1	A	0.28	1.32 (0.80-2.19)	0.46	1.21 (0.73-2.01)	0.23	1.35 (0.82-2.23)	0.31	1.30 (0.78-2.15)	0.67	1.15 (0.60-2.21)	**0.029**	1.93 (1.10-3.41)
	rs2304641	1	A	0.76	0.94 (0.63-1.41)	0.56	0.71 (0.22-2.30)	**0.0027**	9.93 (1.29-76.46)	0.38	0.86 (0.60-1.21)	0.45	1.69 (0.46-6.29)	0.087	0.61 (0.34-1.09)
	rs6674050	1	A	0.087	1.91 (0.92-3.98)	**0.036**	1.42 (1.02-1.97)	0.38	0.85 (0.60-1.21)	0.054	1.37 (0.99-1.90)	0.11	2.08 (0.89-4.82)	**0.019**	1.63 (1.09-2.44)
MITF	rs11128152	3	T	**0.016**	0.61 (0.41-0.92)	0.87	1.03 (0.71-1.49)	0.54	1.37 (0.50-3.75)	0.58	0.89 (0.60-1.33)	0.32	0.77 (0.46-1.30)	0.49	0.84 (0.51-1.38)
MLANA	rs1056796	9	T	0.23	0.68 (0.35-1.30)	0.73	0.88 (0.44-1.78)	0.58	1.08 (0.83-1.40)	**0.026**	1.55 (1.05-2.28)	**0.043**	1.44 (1.01-2.05)	0.76	1.08 (0.64-1.83)
	rs10758717	9	C	0.79	1.04 (0.77-1.42)	0.74	1.05 (0.77-1.45)	0.19	1.26 (0.89-1.78)	**0.030**	1.54 (1.04-2.27)	0.41	1.50 (0.59-3.81)	0.48	1.21 (0.72-2.03)
	rs2150702	9	C	0.66	0.91 (0.61-1.37)	0.32	1.15 (0.87-1.51)	0.67	1.10 (0.72-1.68)	**0.012**	1.71 (1.12-2.62)	0.16	0.78 (0.55-1.10)	0.058	1.79 (0.96-3.36)
	rs7872509	9	C	0.17	0.76 (0.51-1.13)	0.11	1.32 (0.94-1.87)	0.28	1.22 (0.85-1.76)	0.15	1.29 (0.92-1.81)	0.87	0.96 (0.58-1.59)	**0.035**	1.77 (1.05-3.00)
MLPH	rs10173589	2	G	0.66	0.90 (0.60-1.36)	0.52	0.86 (0.55-1.35)	0.37	0.86 (0.61-1.20)	0.18	1.28 (0.89-1.84)	**0.016**	0.49 (0.27-0.91)	0.43	1.21 (0.76-1.92)
	rs13383648	2	C	0.33	1.24 (0.81-1.88)	0.88	1.04 (0.64-1.68)	0.41	1.19 (0.79-1.78)	**0.029**	0.62 (0.40-0.96)	0.73	0.92 (0.55-1.52)	0.97	1.01 (0.57-1.80)
MUTED	rs2743989	6	T	0.68	0.95 (0.72-1.24)	0.14	1.37 (0.90-2.08)	0.28	0.77 (0.47-1.24)	0.086	1.41 (0.95-2.10)	**0.023**	0.41 (0.17-0.98)	0.31	1.21 (0.84-1.74)
	rs2748376	6	T	0.41	1.19 (0.74-1.93)	0.17	0.75 (0.49-1.13)	**0.032**	0.61 (0.38-0.96)	**0.038**	1.34 (1.01-1.78)	0.11	1.31 (0.94-1.84)	0.16	1.61 (0.85-3.06)
	rs2815155	6	C	0.45	1.20 (0.75-1.93)	0.55	0.92 (0.69-1.22)	0.21	1.26 (0.88-1.83)	**0.003**	0.46 (0.27-0.77)	0.31	0.71 (0.36-1.40)	**0.034**	0.66 (0.45-0.98)
MYO7A	rs762667	11	C	0.43	0.86 (0.60-1.25)	0.13	1.64 (0.87-3.10)	**0.035**	0.57 (0.34-0.96)	0.18	1.54 (0.81-2.93)	0.44	0.74 (0.34-1.62)	0.055	1.46 (0.99-2.15)
	rs948970	11	G	0.13	0.74 (0.50-1.09)	0.25	0.75 (0.46-1.24)	0.57	0.89 (0.59-1.34)	0.86	0.98 (0.74-1.28)	**0.010**	0.43 (0.22-0.87)	0.11	0.57 (0.28-1.16)
NF1	rs1013948	17	G	**0.032**	1.47 (1.04-2.08)	0.25	1.27 (0.85-1.91)	0.34	1.21 (0.82-1.78)	0.14	1.39 (0.89-2.18)	0.41	1.21 (0.77-1.88)	0.055	0.53 (0.27-1.05)
	rs10438801	17	G	0.51	1.09 (0.84-1.43)	**0.032**	1.56 (1.04-2.35)	0.84	0.97 (0.76-1.25)	**0.0220**	1.38 (1.05-1.83)	0.88	1.03 (0.73-1.45)	0.89	0.95 (0.44-2.04)
	rs2953016	17	G	0.078	1.33 (0.97-1.82)	0.54	1.31 (0.57-3.02)	0.33	1.16 (0.86-1.58)	0.069	1.35 (0.97-1.87)	0.28	1.25 (0.84-1.85)	**0.011**	0.47 (0.25-0.87)
NRAS	rs14804	1	T	0.054	2.05 (1.00-4.21)	0.71	1.06 (0.76-1.48)	0.083	1.96 (0.89-4.32)	0.093	1.32 (0.95-1.82)	**0.0013**	3.77 (1.76-8.05)	0.12	1.39 (0.92-2.11)
PAX3	rs12623857	2	A	**0.021**	1.55 (1.07-2.23)	0.30	0.36 (0.04-3.09)	0.39	0.85 (0.60-1.22)	**0.0007**	0.51 (0.34-0.76)	0.52	1.17 (0.73-1.88)	0.88	0.96 (0.54-1.69)
	rs16863657	2	G	0.2	0.77 (0.52-1.15)	0.46	1.20 (0.74-1.95)	0.68	0.92 (0.62-1.36)	**0.019**	0.60 (0.38-0.92)	0.079	0.62 (0.35-1.09)	0.067	1.75 (0.98-3.15)

PLDN	rs12909221	15	C	0.36	0.88 (0.67-1.16)	**0.0026**	2.00 (1.26-3.18)	0.63	0.89 (0.55-1.44)	**0.018**	0.56 (0.35-0.91)	0.11	1.66 (0.91-3.02)	0.17	1.50 (0.82-2.75)
POMC	rs934778	2	C	0.48	1.15 (0.79-1.67)	0.12	1.39 (0.92-2.10)	0.17	1.28 (0.90-1.82)	**0.005**	0.66 (0.49-0.88)	0.31	1.29 (0.79-2.11)	0.43	1.24 (0.73-2.12)
PRKAR1A	rs8066131	17	G	**0.033**	1.93 (1.06-3.50)	0.48	0.75 (0.33-1.68)	0.30	1.39 (0.75-2.57)	0.24	1.21 (0.88-1.65)	0.073	1.96 (0.97-3.93)	0.25	1.36 (0.81-2.30)
PTCH2	rs3795719	1	G	0.12	0.69 (0.44-1.10)	0.25	0.78 (0.51-1.20)	0.88	1.03 (0.68-1.56)	**0.010**	0.56 (0.36-0.88)	0.41	1.26 (0.73-2.17)	0.88	0.96 (0.55-1.66)
RAB27A	rs11071175	15	G	0.17	0.75 (0.50-1.13)	0.16	0.70 (0.42-1.16)	**0.0058**	0.71 (0.56-0.91)	0.58	0.92 (0.70-1.22)	0.30	0.76 (0.46-1.27)	0.12	0.58 (0.29-1.19)
	rs17238192	15	T	0.37	0.81 (0.50-1.30)	0.69	1.09 (0.72-1.66)	**0.0095**	0.60 (0.41-0.89)	0.46	1.16 (0.78-1.75)	0.44	0.78 (0.41-1.48)	0.26	1.35 (0.81-2.24)
	rs7167572	15	T	0.13	1.54 (0.89-2.64)	0.073	0.55 (0.28-1.08)	**0.015**	1.39 (1.06-1.81)	0.18	0.66 (0.36-1.21)	0.22	1.54 (0.79-2.98)	0.41	1.38 (0.65-2.92)
	rs7496857	15	T	0.66	0.92 (0.63-1.34)	0.23	0.63 (0.28-1.38)	**0.011**	0.38 (0.18-0.82)	0.42	0.85 (0.58-1.25)	0.84	0.95 (0.59-1.54)	0.83	0.96 (0.63-1.44)
RAB38	rs1027027	11	A	0.3	1.17 (0.87-1.58)	0.41	1.39 (0.64-3.02)	**0.012**	2.78 (1.18-6.56)	0.27	1.55 (0.71-3.38)	**0.018**	2.76 (1.25-6.07)	0.29	1.32 (0.79-2.22)
	rs9666730	11	T	0.16	1.28 (0.91-1.80)	0.58	1.36 (0.46-3.99)	0.78	0.95 (0.64-1.39)	0.4	1.17 (0.82-1.67)	**0.026**	3.54 (1.25-10.02)	0.33	1.26 (0.80-2.00)
RABGGTA	rs3940231	14	A	0.29	0.81 (0.56-1.19)	0.14	0.81 (0.61-1.08)	0.73	0.96 (0.75-1.23)	**0.0059**	0.57 (0.38-0.85)	0.12	1.51 (0.89-2.56)	0.55	0.85 (0.50-1.44)
RGS1	rs1359062	1	G	0.54	0.73 (0.26-2.04)	0.74	0.94 (0.65-1.35)	**0.011**	0.30 (0.11-0.80)	0.47	0.86 (0.57-1.29)	**0.025**	3.19 (1.23-8.27)	0.4	0.81 (0.50-1.33)
RGS20	rs10958392	8	T	0.10	1.40 (0.93-2.11)	0.10	1.51 (0.92-2.49)	0.89	1.03 (0.71-1.49)	0.19	1.20 (0.91-1.57)	0.065	1.66 (0.95-2.91)	**0.049**	1.86 (1.02-3.37)
	rs11783925	8	T	0.34	1.50 (0.66-3.41)	**0.022**	0.67 (0.47-0.95)	0.47	0.88 (0.62-1.25)	0.14	0.50 (0.20-1.28)	0.64	0.75 (0.22-2.57)	0.091	0.63 (0.37-1.09)
	rs6981243	8	C	0.054	0.77 (0.58-1.01)	0.081	1.55 (0.95-2.52)	**0.020**	0.57 (0.35-0.91)	0.66	0.90 (0.56-1.45)	0.32	1.29 (0.78-2.15)	0.16	0.76 (0.52-1.12)
	rs7824575	8	A	0.18	1.29 (0.89-1.86)	0.58	0.79 (0.33-1.86)	0.29	1.17 (0.88-1.56)	0.051	0.43 (0.18-1.03)	0.056	1.59 (0.99-2.55)	**0.015**	1.92 (1.13-3.25)
SLC45A2	rs35414	5	T	0.30	0.82 (0.56-1.20)	0.075	0.76 (0.57-1.03)	0.20	0.79 (0.54-1.13)	**0.015**	0.70 (0.53-0.94)	0.27	0.82 (0.58-1.17)	**0.0078**	0.49 (0.29-0.82)
	rs35415	5	A	0.19	1.38 (0.86-2.22)	0.21	0.83 (0.62-1.11)	0.51	0.92 (0.71-1.19)	**0.022**	0.72 (0.54-0.95)	0.41	0.76 (0.38-1.49)	**0.024**	0.54 (0.32-0.91)
SNAI2	rs1992375	8	A	0.86	0.96 (0.64-1.45)	0.056	0.76 (0.57-1.01)	0.20	1.28 (0.88-1.88)	**0.0048**	0.68 (0.51-0.89)	0.34	0.75 (0.41-1.37)	**0.048**	0.57 (0.33-0.99)
	rs2735455	8	A	0.91	0.97 (0.60-1.58)	0.1	1.56 (0.92-2.65)	0.059	1.57 (0.97-2.54)	**0.0054**	2.15 (1.23-3.75)	0.90	1.04 (0.55-1.99)	0.085	1.80 (0.94-3.45)
SNX10	rs1406754	7	T	0.087	0.60 (0.33-1.10)	0.15	1.35 (0.89-2.04)	**0.024**	0.55 (0.33-0.93)	**0.044**	1.50 (1.01-2.22)	0.76	0.95 (0.66-1.36)	0.53	1.19 (0.69-2.03)
	rs2699808	7	C	0.17	1.38 (0.87-2.17)	0.33	0.80 (0.52-1.25)	**0.022**	1.68 (1.06-2.67)	0.65	0.94 (0.72-1.22)	0.81	1.08 (0.59-1.95)	0.14	0.65 (0.38-1.13)
SOX11	rs17362772	2	G	0.16	0.72 (0.46-1.15)	0.064	1.61 (0.98-2.65)	0.56	0.88 (0.58-1.34)	0.75	1.08 (0.68-1.72)	**0.031**	0.52 (0.27-0.99)	0.22	1.45 (0.81-2.59)
	rs6432221	2	T	**0.042**	0.68 (0.47-0.98)	0.28	1.17 (0.88-1.57)	0.27	0.82 (0.57-1.17)	0.86	1.04 (0.69-1.55)	0.22	0.80 (0.56-1.15)	0.65	1.09 (0.75-1.60)
SOX4	rs9368326	6	G	**0.011**	0.21 (0.05-0.92)	0.41	0.59 (0.16-2.21)	0.31	1.20 (0.84-1.73)	**0.0017**	5.00 (1.08-23.10)	0.33	1.22 (0.82-1.83)		
TYR	rs1042602	11	A	0.30	0.81 (0.54-1.21)	**0.0062**	0.47 (0.26-0.83)	0.61	1.03 (0.81-1.32)	0.056	0.62 (0.38-1.02)	0.095	1.58 (0.93-2.68)	0.52	0.80 (0.40-1.60)
	rs12270717	11	C	0.37	1.15 (0.85-1.54)	**0.037**	1.53 (1.02-2.29)	0.43	1.15 (0.81-1.63)	0.1	1.30 (0.95-1.79)	0.26	0.76 (0.47-1.23)	0.24	1.29 (0.85-1.95)
	rs17793678	11	T	0.35	1.15 (0.86-1.55)	**0.025**	1.45 (1.05-2.01)	0.42	1.15 (0.81-1.64)	0.087	1.32 (0.96-1.82)	0.39	0.81 (0.50-1.32)	0.18	1.34 (0.88-2.03)
	rs2186640	11	G	0.66	1.10 (0.66-1.84)	**0.025**	1.62 (1.06-2.49)	0.87	0.99 (0.77-1.27)	0.2	1.20 (0.91-1.60)	0.42	0.82 (0.51-1.32)	0.15	1.51 (0.85-2.67)
	rs5021654	11	C	0.71	1.10 (0.66-1.84)	**0.018**	1.67 (1.09-2.56)	0.94	0.99 (0.78-1.27)	0.12	1.37 (0.92-2.04)	0.49	0.89 (0.63-1.25)	0.19	1.45 (0.83-2.55)
TYRP1	rs10809828	9	G	**0.024**	0.39 (0.16-0.95)	0.56	0.91 (0.67-1.24)	0.61	1.08 (0.82-1.42)	0.13	1.66 (0.83-3.32)	0.53	1.13 (0.78-1.63)	0.40	1.44 (0.63-3.28)
WNT3A	rs708122	1	T	0.11	0.74 (0.51-1.07)	0.55	0.88 (0.59-1.33)	0.3	1.35 (0.77-2.37)	0.85	1.04 (0.70-1.53)	**0.010**	0.27 (0.08-0.89)	0.45	0.82 (0.49-1.38)

Chr, Chromosome; mA, Minor Allele; OR, Odds Ratio per minor allele; CI, Confidence Interval.
Bold indicates statistically significant results

Table S1.3. List of SNPs associated with sun response traits in females and males

				Sunburns				Lentigines				Naevi			
				Female		Male		Female		Male		Female		Male	
Gene	SNP ID	Chr	mA	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR
ADAM17	rs12473402	2	C	0.74	1.05 (0.80-1.36)	0.75	1.10 (0.60-2.03)	0.26	0.71 (0.40-1.28)	0.14	1.38 (0.90-2.13)	**0.0063**	0.64 (0.46-0.89)	0.23	1.33 (0.83-2.12)
	rs17524425	2	G	**0.022**	1.52 (1.06-2.18)	0.24	0.81 (0.58-1.15)	0.061	1.37 (0.98-1.92)	**0.0004**	0.52 (0.35-0.75)	0.14	1.30 (0.92-1.83)	0.26	0.80 (0.53-1.19)
	rs4258773	2	G	0.96	0.99 (0.77-1.28)	0.40	1.23 (0.76-2.00)	0.25	0.85 (0.64-1.12)	0.094	1.51 (0.93-2.43)	**0.0063**	0.44 (0.24-0.83)	0.11	1.53 (0.91-2.58)
ADAMTS20	rs1510521	12	C	0.43	0.81 (0.48-1.37)	0.36	1.15 (0.86-1.54)	0.52	0.91 (0.68-1.22)	0.077	1.88 (0.92-3.86)	**0.043**	0.72 (0.52-0.99)	0.19	0.74 (0.48-1.16)
	rs2048348	12	A	0.56	0.50 (0.05-5.54)	**0.035**	0.64 (0.41-0.97)	0.26	1.32 (0.81-2.15)	**0.047**	0.63 (0.40-1.00)	0.37	1.28 (0.75-2.17)	0.54	0.85 (0.50-1.45)
	rs2062731	12	G	**0.03**	0.59 (0.36-0.96)	0.82	1.05 (0.67-1.66)	0.69	1.11 (0.66-1.87)	0.23	0.71 (0.40-1.25)	0.16	1.44 (0.87-2.38)	0.69	0.90 (0.53-1.52)
	rs7960952	12	C	0.43	0.84 (0.54-1.30)	0.14	1.23 (0.93-1.63)	0.29	0.80 (0.53-1.21)	0.099	1.60 (0.91-2.84)	**0.036**	0.73 (0.54-0.98)	0.075	0.66 (0.42-1.04)
AP3B1	rs10514134	5	A	0.74	1.07 (0.71-1.63)	**0.0066**	1.98 (1.20-3.28)	0.27	0.40 (0.07-2.19)	0.16	1.46 (0.86-2.48)	0.61	0.87 (0.52-1.47)	0.48	1.22 (0.71-2.11)
	rs10805919	5	C	0.12	0.75 (0.52-1.07)	0.58	0.91 (0.67-1.25)	0.35	0.83 (0.55-1.24)	0.31	0.80 (0.53-1.23)	0.045	0.64 (0.41-0.99)	0.63	0.80 (0.31-2.04)
	rs11742673	5	A	0.79	1.05 (0.72-1.54)	0.41	1.25 (0.74-2.13)	**0.023**	1.74 (1.07-2.83)	0.56	1.19 (0.66-2.12)	**0.023**	1.78 (1.09-2.90)	0.73	0.92 (0.57-1.48)
	rs11746090	5	A	0.16	1.28 (0.90-1.83)	**0.0083**	3.57 (1.28-9.95)	0.47	1.12 (0.82-1.52)	0.11	1.33 (0.93-1.91)	0.17	1.71 (0.81-3.60)	0.42	0.65 (0.21-1.96)
	rs12657894	5	A	**0.016**	1.56 (1.09-2.23)	0.35	1.31 (0.74-2.32)	0.26	1.36 (0.79-2.34)	0.29	0.71 (0.38-1.33)	0.09	1.63 (0.94-2.82)	0.63	1.12 (0.70-1.78)
	rs13172957	5	G	0.057	1.41 (0.99-2.01)	0.4	1.32 (0.69-2.55)	0.42	0.78 (0.43-1.42)	0.83	0.97 (0.70-1.33)	**0.019**	1.44 (1.06-1.96)	0.59	1.13 (0.72-1.77)
	rs2636986	5	A	**0.01**	0.62 (0.43-0.89)	**0.024**	3.08 (1.09-8.71)	0.055	0.72 (0.52-1.01)	0.13	2.18 (0.76-6.26)	**0.0054**	0.58 (0.39-0.87)	0.61	0.90 (0.62-1.33)
	rs34436	5	G	0.089	0.69 (0.45-1.06)	**0.014**	0.54 (0.33-0.89)	0.53	1.17 (0.71-1.93)	0.058	0.63 (0.39-1.02)	0.79	1.07 (0.63-1.82)	0.56	1.15 (0.71-1.86)
	rs389110	5	A	**0.01**	0.62 (0.43-0.89)	**0.024**	3.08 (1.09-8.71)	**0.036**	0.38 (0.15-0.97)	0.13	2.18 (0.76-6.26)	**0.005**	0.58 (0.39-0.87)	0.61	0.90 (0.62-1.33)
	rs4703747	5	G	0.051	0.72 (0.52-1.00)	0.32	1.20 (0.84-1.72)	0.15	0.74 (0.49-1.11)	0.41	1.65 (0.49-5.60)	**0.014**	0.56 (0.35-0.90)	0.63	1.10 (0.74-1.64)
	rs6453373	5	A	**0.047**	0.61 (0.37-1.00)	**0.015**	0.52 (0.31-0.90)	0.41	1.28 (0.71-2.34)	0.17	0.67 (0.38-1.19)	0.86	0.95 (0.52-1.73)	0.12	1.56 (0.91-2.67)
	rs6453374	5	A	0.35	1.18 (0.83-1.68)	**0.022**	2.49 (1.10-5.61)	0.068	1.83 (0.94-3.57)	0.26	1.21 (0.86-1.71)	**0.019**	2.20 (1.18-4.09)	0.49	0.88 (0.61-1.27)
AP3D1	rs2240655	19	T	0.93	1.02 (0.68-1.52)	0.24	1.31 (0.84-2.06)	0.34	0.81 (0.53-1.24)	0.072	0.64 (0.40-1.04)	0.89	1.03 (0.65-1.65)	**0.031**	0.56 (0.32-0.96)
	rs3786971	19	TT	0.67	0.92 (0.63-1.35)	0.25	0.78 (0.51-1.19)	0.44	0.90 (0.68-1.18)	0.088	0.67 (0.42-1.06)	**0.032**	1.75 (1.06-2.87)	0.80	1.08 (0.61-1.90)
AP3M2	rs7009632	8	G	0.54	1.16 (0.73-1.85)	0.64	1.14 (0.67-1.94)	0.41	0.84 (0.56-1.27)	0.35	1.31 (0.74-2.29)	0.52	1.10 (0.82-1.50)	**0.0045**	2.29 (1.31-4.02)
	rs7823824	8	A	0.47	1.18 (0.75-1.87)	0.77	1.05 (0.78-1.40)	0.18	0.75 (0.50-1.14)	0.68	1.12 (0.65-1.93)	0.75	1.05 (0.78-1.41)	**0.0021**	1.91 (1.12-3.28)
ASIP	rs6142129	20	G	0.57	0.85 (0.48-1.50)	0.052	0.53 (0.27-1.02)	0.26	1.25 (0.84-1.86)	**0.0041**	0.53 (0.35-0.82)	0.64	0.90 (0.59-1.38)	0.4	1.16 (0.83-1.61)
BCL2	rs1462129	18	T	0.39	1.19 (0.80-1.75)	0.76	1.08 (0.67-1.72)	0.067	0.64 (0.39-1.03)	**0.0075**	1.93 (1.19-3.14)	0.19	0.70 (0.41-1.20)	0.06	0.58 (0.33-1.04)
	rs949037	18	T	0.61	0.89 (0.57-1.38)	0.73	1.09 (0.67-1.78)	0.16	0.70 (0.42-1.16)	**0.013**	1.78 (1.13-2.81)	0.069	0.60 (0.33-1.06)	0.11	0.77 (0.56-1.06)
CDH1	rs11075699	16	G	0.19	0.74 (0.48-1.16)	0.37	1.30 (0.75-2.27)	0.69	0.94 (0.71-1.25)	0.47	1.25 (0.68-2.27)	0.50	0.86 (0.55-1.34)	**0.031**	1.43 (1.03-1.98)
	rs2276329	16	G	0.87	1.05 (0.58-1.90)	**0.039**	1.96 (1.02-3.75)	0.48	1.29 (0.64-2.59)	0.13	1.59 (0.86-2.93)	0.20	0.59 (0.26-1.37)	0.58	0.56 (0.06-4.85)
CDH3	rs1124770	16	A	0.82	0.96 (0.67-1.38)	0.69	1.08 (0.74-1.57)	**0.038**	0.65 (0.44-0.98)	0.56	1.36 (0.32-5.77)	0.57	1.14 (0.72-1.82)	0.15	0.28 (0.03-2.18)
	rs1886700	16	A	0.25	1.22 (0.86-1.73)	0.096	0.21 (0.02-1.77)	**0.032**	1.52 (1.03-2.26)	0.72	1.08 (0.70-1.69)	0.74	0.93 (0.61-1.42)	0.57	0.56 (0.06-4.83)
CDK2	rs2069398	12	A	**0.028**	0.61 (0.39-0.95)	0.5	0.83 (0.47-1.44)	**0.031**	0.59 (0.36-0.96)			0.27	0.73 (0.40-1.31)	0.64	0.86 (0.45-1.65)
CDK4	rs2069502	12	A	0.090	0.73 (0.51-1.05)	**0.013**	1.52 (1.09-2.13)	0.063	0.74 (0.53-1.02)	0.37	0.82 (0.54-1.26)	0.57	0.88 (0.57-1.36)	0.64	0.86 (0.45-1.65)
	rs2270777	12	G	0.12	0.73 (0.50-1.09)	**0.037**	1.61 (1.03-2.53)	**0.037**	0.74 (0.56-0.98)	0.59	0.88 (0.55-1.40)	0.15	0.80 (0.59-1.08)	0.70	1.11 (0.67-1.84)
CDKN2A	rs3218020	9	T	**0.012**	1.65 (1.12-2.43)	0.16	1.44 (0.86-2.40)	0.12	0.68 (0.42-1.10)	0.11	0.64 (0.37-1.10)	0.81	0.94 (0.55-1.60)	0.88	0.96 (0.53-1.72)
	rs3731239	9	C	0.58	0.85 (0.47-1.52)	0.28	0.68 (0.33-1.37)	**0.021**	1.58 (1.07-2.34)	0.60	1.22 (0.58-2.55)	0.85	1.09 (0.55-2.15)	0.61	1.22 (0.58-2.55)
CDKN2B	rs495490	9	C	0.31	0.50 (0.12-2.01)	**0.049**	0.64 (0.41-1.00)	0.57	1.62 (0.29-8.94)	0.24	0.75 (0.47-1.21)	0.54	0.84 (0.49-1.46)	0.9	0.97 (0.59-1.59)
CLIP1	rs7388	12	A	0.27	1.18 (0.88-1.59)	**0.0063**	1.58 (1.13-2.21)	0.094	1.33 (0.95-1.85)	0.2	1.25 (0.88-1.78)	0.065	1.49 (0.98-2.27)	0.097	1.46 (0.93-2.29)

CTNNBIP1	rs11828	1	G	**0.041**	0.72 (0.52-0.99)	0.31	1.24 (0.82-1.89)	0.27	0.79 (0.53-1.20)	0.087	0.42 (0.15-1.16)	0.3	0.79 (0.50-1.24)	0.31	0.78 (0.48-1.26)
	rs12128766	1	C	0.2	0.85 (0.66-1.09)	0.75	1.05 (0.79-1.39)	0.86	0.96 (0.58-1.57)	0.086	0.60 (0.34-1.08)	**0.033**	0.62 (0.40-0.96)	0.37	1.24 (0.77-1.99)
	rs2379107	1	G	0.068	1.42 (0.97-2.07)	0.51	0.86 (0.55-1.34)	**0.0021**	0.09 (0.01-0.68)	0.13	1.37 (0.91-2.07)	0.17	2.28 (0.73-7.12)	0.25	1.28 (0.84-1.94)
EDNRB	rs11149080	13	G	0.81	0.94 (0.59-1.51)	0.58	1.08 (0.82-1.43)	**0.020**	0.56 (0.34-0.91)	0.43	1.20 (0.76-1.88)	0.69	1.06 (0.79-1.43)	0.1	1.31 (0.95-1.81)
	rs2242991	5	G	0.67	1.20 (0.53-2.73)	0.51	1.15 (0.76-1.76)	0.20	1.31 (0.86-2.00)	**0.038**	1.63 (1.02-2.59)	0.17	0.73 (0.46-1.15)	0.06	1.45 (0.99-2.12)
	rs2243010	5	T	0.088	0.20 (0.02-1.71)	**0.034**	0.65 (0.43-0.97)	0.39	0.84 (0.57-1.25)	**0.020**	0.60 (0.39-0.93)	0.34	0.80 (0.50-1.28)	0.33	2.16 (0.48-9.82)
	rs2243051	5	G	0.21	0.85 (0.65-1.10)	0.45	0.90 (0.68-1.19)	0.53	1.15 (0.75-1.75)	**0.036**	0.72 (0.53-0.98)	0.84	0.96 (0.60-1.51)	**0.0015**	1.91 (1.14-3.19)
	rs639342	5	A	0.68	1.08 (0.74-1.59)	0.86	0.96 (0.63-1.47)	0.18	0.77 (0.53-1.13)	0.16	1.40 (0.87-2.24)	0.35	1.21 (0.81-1.78)	**0.0058**	0.52 (0.32-0.85)
GNA11	rs308039	19	T	**0.012**	0.61 (0.42-0.90)	0.48	0.88 (0.61-1.27)	0.2	0.80 (0.57-1.13)	0.091	0.68 (0.43-1.07)	0.61	0.91 (0.62-1.33)	0.56	1.13 (0.75-1.70)
	rs404632	19	T	**0.018**	0.60 (0.39-0.92)	0.27	0.77 (0.47-1.23)	0.32	0.53 (0.15-1.89)	0.49	0.54 (0.09-3.26)	0.62	0.88 (0.53-1.47)	0.17	1.39 (0.88-2.19)
GNAQ	rs11145647	9	C	0.43	1.45 (0.54-3.86)	0.28	1.20 (0.86-1.68)	0.19	0.45 (0.13-1.55)	**0.011**	1.79 (1.14-2.80)	0.24	0.79 (0.54-1.17)	**0.026**	1.52 (1.05-2.19)
	rs3858119	9	G	0.19	1.26 (0.89-1.79)	0.45	0.76 (0.37-1.56)	**0.0081**	1.69 (1.14-2.50)	0.82	1.05 (0.69-1.60)	0.22	1.30 (0.85-1.98)	0.54	0.90 (0.63-1.28)
	rs4745672	9	T	**0.009**	0.57 (0.38-0.87)	0.72	1.09 (0.68-1.75)	0.30	0.78 (0.49-1.25)	0.74	0.91 (0.54-1.54)	0.20	0.72 (0.43-1.21)	0.068	1.62 (0.97-2.70)
GNAS	rs234623	20	G	0.50	0.87 (0.58-1.30)	0.072	1.52 (0.96-2.41)	0.37	1.22 (0.79-1.89)	0.17	1.42 (0.86-2.36)	0.53	0.86 (0.54-1.38)	**0.046**	1.70 (1.00-2.92)
	rs6064714	20	G	0.76	1.19 (0.39-3.58)	0.79	0.83 (0.22-3.15)	0.51	0.67 (0.20-2.22)	**0.025**	1.64 (1.05-2.56)	0.92	1.07 (0.29-3.95)	0.74	0.92 (0.55-1.54)
	rs6092704	20	C	**0.0014**	0.46 (0.29-0.75)	0.12	0.66 (0.38-1.12)	0.81	0.94 (0.56-1.57)	**0.019**	0.50 (0.28-0.90)	0.52	1.19 (0.70-2.04)	0.24	0.69 (0.37-1.30)
GPR143	rs2521578	X	A	**0.0077**	1.77 (1.15-2.72)	0.22	0.85 (0.65-1.11)	0.37	0.80 (0.50-1.30)	0.54	1.09 (0.82-1.46)	0.21	0.71 (0.40-1.23)		
	rs2521667	X	G	0.26	0.80 (0.55-1.17)	0.54	1.09 (0.83-1.43)	**0.021**	0.28 (0.09-0.89)	0.80	0.93 (0.52-1.66)	0.082	0.33 (0.08-1.41)	**0.0004**	1.68 (1.27-2.22)
HPS1	rs12242431	10	C	0.28	1.23 (0.84-1.79)	**0.0056**	0.51 (0.31-0.83)	0.21	0.75 (0.48-1.17)	0.11	0.64 (0.38-1.10)	0.82	1.05 (0.66-1.68)	0.67	0.90 (0.53-1.50)
	rs17535384	10	G	0.094	1.40 (0.94-2.09)	**0.020**	1.61 (1.07-2.43)	0.74	1.08 (0.69-1.67)	0.59	0.88 (0.54-1.42)	0.44	0.89 (0.66-1.20)	0.12	1.48 (0.90-2.43)
HPS4	rs1894707	22	C	0.32	0.88 (0.68-1.13)	0.18	1.31 (0.88-1.95)	0.34	1.31 (0.75-2.31)	0.07	1.48 (0.97-2.27)	0.36	1.15 (0.85-1.57)	**0.0094**	2.29 (1.24-4.23)
	rs9608491	22	G	0.28	0.55 (0.18-1.67)	**0.0078**	0.63 (0.45-0.89)	0.11	1.40 (0.92-2.11)	0.17	0.78 (0.55-1.12)	0.68	0.92 (0.62-1.37)	0.18	0.77 (0.52-1.14)
	rs9613187	22	T	0.71	0.75 (0.17-3.39)	**0.025**	0.61 (0.40-0.95)	0.37	1.25 (0.77-2.01)	0.15	0.69 (0.42-1.14)	0.18	0.71 (0.42-1.19)	0.52	0.84 (0.49-1.44)
HPS6	rs3737243	10	A	0.40	1.21 (0.78-1.87)	**0.021**	0.55 (0.33-0.92)	0.95	1.02 (0.62-1.67)	0.55	0.84 (0.48-1.47)	0.17	0.81 (0.60-1.10)	0.62	0.86 (0.48-1.55)
HRK	rs4767462	12	G	0.51	0.84 (0.50-1.41)	0.15	1.48 (0.87-2.53)	**0.020**	0.52 (0.29-0.91)	0.39	0.78 (0.44-1.38)	0.3	0.70 (0.35-1.40)	0.17	1.48 (0.85-2.59)
HTR2B	rs10194776	2	T	**0.031**	0.76 (0.59-0.98)	**0.034**	1.56 (1.03-2.37)	0.40	0.80 (0.48-1.34)	0.39	1.21 (0.78-1.89)	0.84	0.94 (0.54-1.67)	0.14	1.43 (0.89-2.31)
	rs17619600	2	C	0.077	0.65 (0.41-1.05)	0.71	0.91 (0.54-1.53)	0.27	0.74 (0.44-1.26)	0.062	1.68 (0.97-2.94)	0.9	0.97 (0.58-1.61)	**0.018**	1.95 (1.13-3.35)
	rs2161891	2	G	**0.021**	0.73 (0.56-0.96)	0.20	1.50 (0.80-2.80)	0.52	0.91 (0.68-1.21)	0.25	1.29 (0.84-1.97)	0.21	1.47 (0.82-2.66)	0.055	1.56 (0.99-2.48)
	rs4973377	2	A	**0.033**	1.42 (1.03-1.97)	0.14	0.38 (0.10-1.47)	0.28	1.21 (0.85-1.73)	**0.010**	0.17 (0.04-0.80)	0.65	0.92 (0.63-1.34)	0.22	0.76 (0.48-1.19)
KIT	rs1008658	4	A	**0.029**	0.55 (0.32-0.95)	0.56	1.19 (0.67-2.12)	**0.012**	0.69 (0.52-0.92)	0.19	1.50 (0.81-2.80)	0.69	0.94 (0.69-1.28)	0.16	1.57 (0.84-2.92)
	rs13135792	4	C	**0.030**	1.74 (1.05-2.87)	**0.049**	1.49 (1.00-2.21)	0.23	1.39 (0.81-2.38)	0.54	1.14 (0.75-1.75)	0.63	0.86 (0.46-1.61)	0.26	1.20 (0.87-1.65)
	rs4864920	4	T	0.074	0.75 (0.55-1.03)	0.13	0.73 (0.49-1.10)	0.066	0.68 (0.46-1.03)	**0.028**	2.90 (1.05-8.05)	0.23	0.79 (0.53-1.17)	0.39	0.82 (0.52-1.30)
	rs6554198	4	G	**0.011**	0.54 (0.33-0.87)	**0.02**	0.55 (0.33-0.92)	0.064	1.69 (0.96-2.98)	0.12	1.41 (0.91-2.20)	0.49	1.17 (0.74-1.84)	0.49	0.90 (0.66-1.22)
KITLG	rs10858758	12	G	0.64	0.92 (0.64-1.31)	0.066	1.45 (0.98-2.16)	0.64	1.09 (0.77-1.53)	0.35	0.64 (0.24-1.65)	0.35	1.19 (0.83-1.72)	**0.036**	0.61 (0.38-0.98)
LYST	rs11810173	1	T	0.032	0.69 (0.49-0.97)	0.93	1.02 (0.70-1.47)	0.18	1.28 (0.89-1.85)	**0.020**	0.19 (0.04-0.93)	0.23	1.32 (0.84-2.09)	0.052	0.64 (0.41-1.02)
	rs7541057	1	C	0.68	0.92 (0.61-1.38)	0.19	0.83 (0.62-1.10)	0.4	0.82 (0.52-1.30)	**0.017**	1.46 (1.07-2.01)	0.16	0.39 (0.09-1.70)	0.54	1.18 (0.69-2.00)
MCAM	rs2249466	11	T	0.51	1.26 (0.64-2.46)	0.19	1.56 (0.80-3.06)	0.18	1.25 (0.90-1.73)	0.13	1.40 (0.90-2.18)	0.87	0.97 (0.68-1.38)	**0.0039**	2.03 (1.25-3.32)
	rs2511837	11	T	**0.044**	1.53 (1.01-2.31)	0.40	0.83 (0.53-1.29)	0.14	1.40 (0.89-2.22)	0.19	0.71 (0.43-1.18)	0.062	0.65 (0.41-1.02)	0.96	0.99 (0.60-1.63)
	rs6589732	11	A	0.48	1.18 (0.74-1.89)	**0.013**	0.50 (0.28-0.87)	0.4	0.89 (0.68-1.17)	**0.034**	1.61 (1.04-2.51)	0.28	1.27 (0.81-2.00)	0.38	0.75 (0.39-1.44)
MCOLN3	rs10518327	1	A	0.56	1.11 (0.78-1.58)	0.38	0.70 (0.32-1.55)	0.53	1.26 (0.61-2.60)	0.87	0.97 (0.70-1.35)	0.34	1.44 (0.69-2.99)	**0.049**	2.24 (1.02-4.91)

	rs10782537	1	C	0.06	0.75 (0.55-1.01)	0.75	1.14 (0.49-2.66)	0.57	0.89 (0.60-1.33)	0.27	0.78 (0.51-1.21)	0.44	0.66 (0.22-1.97)	**0.045**	0.62 (0.39-1.00)
	rs12030837	1	T	**0.021**	0.63 (0.43-0.94)	0.78	1.06 (0.71-1.59)	0.59	0.88 (0.55-1.41)	0.84	1.05 (0.67-1.63)	0.79	1.07 (0.65-1.76)	0.34	0.77 (0.45-1.33)
	rs7522239	1	A	**0.0012**	0.55 (0.38-0.80)	0.58	1.12 (0.76-1.65)	0.33	0.80 (0.51-1.26)	0.69	1.09 (0.71-1.66)	0.82	0.95 (0.58-1.53)	0.45	0.83 (0.49-1.39)
MITF	rs13072665	3	A	0.41	1.19 (0.79-1.78)	**0.036**	1.85 (1.03-3.32)	0.57	1.14 (0.72-1.79)	**0.0043**	2.49 (1.29-4.78)	0.73	1.10 (0.65-1.85)	0.73	1.11 (0.60-2.04)
MLANA	rs10975339	9	T	0.60	1.15 (0.68-1.94)	0.24	1.27 (0.86-1.87)	0.42	0.85 (0.58-1.26)	0.49	0.89 (0.65-1.23)	**0.0044**	0.33 (0.14-0.78)	0.49	1.17 (0.75-1.83)
	rs2150702	9	C	0.16	0.75 (0.50-1.12)	0.09	1.45 (0.94-2.22)	0.80	0.94 (0.59-1.51)	0.064	0.62 (0.38-1.03)	**0.025**	0.53 (0.29-0.95)	0.064	1.61 (0.96-2.70)
MLPH	rs10173589	2	G	0.67	1.09 (0.74-1.61)	0.33	1.24 (0.80-1.92)	0.13	1.34 (0.91-1.97)	0.38	0.81 (0.51-1.29)	**0.0014**	1.89 (1.29-2.78)	0.43	1.58 (0.52-4.84)
	rs2292881	2	T			**0.011**	2.01 (1.16-3.47)	0.95	0.99 (0.60-1.63)	0.2	0.71 (0.43-1.19)	0.44	1.24 (0.73-2.09)	0.24	1.42 (0.80-2.52)
	rs729389	2	A	0.48	0.60 (0.14-2.53)	0.075	0.69 (0.46-1.04)	0.29	1.29 (0.80-2.06)	**0.017**	0.59 (0.38-0.91)	0.091	1.50 (0.94-2.41)	**0.020**	0.55 (0.32-0.94)
	rs7606177	2	C	0.29	1.55 (0.68-3.51)	0.75	1.13 (0.54-2.34)	0.16	0.52 (0.21-1.30)	**0.023**	0.69 (0.50-0.95)	0.14	1.37 (0.90-2.10)	0.2	0.79 (0.55-1.14)
	rs880931	2	T	0.25	0.40 (0.08-2.06)	0.53	0.86 (0.53-1.39)	0.5	1.18 (0.72-1.93)	**0.035**	0.62 (0.39-0.97)	0.27	1.32 (0.81-2.13)	0.066	0.61 (0.35-1.06)
MUTED	rs2748376	6	T	0.15	0.71 (0.45-1.13)	0.53	0.84 (0.50-1.43)	0.81	0.94 (0.57-1.56)	0.35	1.15 (0.85-1.56)	**0.038**	1.37 (1.02-1.84)	0.27	1.30 (0.81-2.10)
MYO7A	rs11605022	11	G	**0.0077**	1.40 (1.09-1.79)	0.56	1.09 (0.82-1.44)	0.1	1.25 (0.96-1.64)	0.81	0.96 (0.71-1.31)	0.51	1.19 (0.71-2.02)	0.25	0.83 (0.60-1.14)
	rs12793189	11	A	0.21	1.17 (0.91-1.49)	0.075	0.65 (0.40-1.05)	0.52	1.16 (0.74-1.80)	**0.036**	0.59 (0.35-0.97)	**0.021**	1.72 (1.09-2.70)	0.31	0.75 (0.43-1.31)
	rs7105374	11	AA	0.64	1.09 (0.76-1.57)	**0.023**	1.39 (1.04-1.85)	0.56	1.09 (0.83-1.43)	**0.010**	1.47 (1.08-2.01)	0.51	1.19 (0.71-2.02)	0.32	0.89 (0.62-1.28)
	rs7123925	11	G	0.30	0.82 (0.56-1.20)	0.12	0.80 (0.60-1.06)	0.38	0.88 (0.67-1.16)	0.17	0.81 (0.60-1.10)	**0.0049**	0.53 (0.34-0.82)	0.27	0.84 (0.61-1.15)
	rs762667	11	C	**0.016**	1.37 (1.06-1.77)	0.54	0.89 (0.60-1.31)	0.12	1.24 (0.94-1.64)	0.78	0.96 (0.69-1.32)	0.84	0.97 (0.72-1.31)	0.58	0.91 (0.65-1.27)
NF1	rs2953014	17	C	0.42	1.35 (0.64-2.85)	**0.002**	0.18 (0.05-0.64)	0.37	0.86 (0.62-1.19)	0.35	0.84 (0.58-1.21)	0.12	1.40 (0.91-2.13)	**0.038**	1.61 (1.03-2.52)
	rs2953016	17	G	**0.013**	0.30 (0.11-0.84)	0.052	1.39 (0.99-1.95)	0.69	0.92 (0.61-1.38)	0.93	1.02 (0.71-1.45)	0.32	0.56 (0.16-1.91)	0.37	0.81 (0.50-1.30)
NRAS	rs8453	1	A	0.15	0.33 (0.07-1.65)	0.36	1.22 (0.79-1.87)	0.5	0.60 (0.13-2.69	0.68	1.11 (0.66-1.86)	0.13	3.61 (0.72-18.13)	**0.025**	0.52 (0.28-0.95)
PAX3	rs12620338	2	A	0.14	0.76 (0.52-1.10)	0.05	0.67 (0.44-1.00)	**0.017**	0.23 (0.06-0.86)	0.23	0.79 (0.54-1.16)	0.14	0.75 (0.50-1.11)	0.21	1.34 (0.85-2.11)
	rs13405641	2	A	0.07	1.31 (0.98-1.76)	0.64	1.16 (0.61-2.21)	0.013	0.67 (0.48-0.92)	0.15	0.80 (0.58-1.09)	0.35	1.17 (0.84-1.64)	0.83	1.04 (0.74-1.45)
	rs16863657	2	G	**0.0033**	0.57 (0.38-0.83)	0.89	1.03 (0.67-1.59)	0.27	0.79 (0.52-1.20)	0.28	1.30 (0.80-2.11)	**0.0053**	0.50 (0.30-0.84)	0.69	1.11 (0.68-1.81)
	rs7559271	2	G	0.076	1.27 (0.97-1.64)	0.38	0.79 (0.47-1.33)	**0.014**	1.42 (1.07-1.89)	0.22	0.71 (0.41-1.23)	0.082	1.65 (0.95-2.86)	0.86	1.05 (0.59-1.88)
PCNA	rs17349	20	T	0.62	0.90 (0.60-1.36)	0.66	0.90 (0.57-1.42)	0.55	0.88 (0.59-1.33)	0.43	1.20 (0.76-1.88)	**0.029**	0.58 (0.35-0.97)	0.66	1.11 (0.70-1.77)
	rs3729558	20	G	**0.023**	0.75 (0.58-0.96)	0.13	0.67 (0.40-1.13)	0.42	1.23 (0.75-2.02)	0.29	0.74 (0.43-1.29)	0.44	1.20 (0.75-1.89)	0.21	0.68 (0.37-1.26)
PLDN	rs12909221	15	C	0.58	1.15 (0.70-1.89)	**0.022**	1.66 (1.07-2.56)	0.25	1.19 (0.89-1.59)	0.27	1.18 (0.88-1.60)	0.58	1.13 (0.72-1.77)	0.12	1.53 (0.90-2.59)
POMC	rs1866146	2	C	0.42	0.87 (0.61-1.23)	0.077	1.32 (0.97-1.79)	**0.026**	1.42 (1.04-1.95)	0.32	0.70 (0.34-1.42)	0.05	1.39 (1.00-1.92)	0.72	0.87 (0.41-1.85)
	rs6734859	2	T	0.13	0.76 (0.53-1.08)	**0.0095**	1.79 (1.15-2.79)	0.082	1.47 (0.95-2.27)	0.83	1.05 (0.68-1.61)	0.055	1.56 (1.00-2.44)	0.42	0.55 (0.12-2.57)
	rs7565877	2	G	0.37	0.83 (0.55-1.25)	0.78	0.94 (0.59-1.48)	0.38	0.81 (0.50-1.30)	0.65	0.89 (0.55-1.46)	0.078	0.63 (0.37-1.08)	**0.024**	0.51 (0.28-0.94)
PRKAR1A	rs4968898	17	G	0.23	1.22 (0.89-1.67)	0.2	1.25 (0.89-1.77)	**0.022**	1.54 (1.06-2.24)	0.42	1.51 (0.55-4.19)	0.42	0.85 (0.58-1.26)	0.13	0.70 (0.43-1.12)
	rs8066131	17	G	0.14	0.82 (0.63-1.07)	0.20	0.59 (0.27-1.33)	**0.0093**	0.60 (0.40-0.88)	0.69	0.92 (0.60-1.40)	0.77	0.94 (0.62-1.43)	**0.016**	2.67 (1.22-5.81)
PTCH1	rs2297087	9	G	0.32	0.67 (0.31-1.48)	**0.016**	1.66 (1.10-2.50)	0.17	0.80 (0.57-1.11)	0.16	0.52 (0.21-1.30)	**0.032**	1.60 (1.05-2.45)	0.25	1.30 (0.83-2.05)
	rs4448343	9	G	0.48	1.14 (0.79-1.65)	**0.013**	1.70 (1.12-2.59)	0.14	0.79 (0.57-1.08)	0.35	1.18 (0.83-1.69)	**0.026**	1.66 (1.06-2.59)	0.1	1.34 (0.94-1.92)
RAB27A	rs1061824	15	G	**0.048**	1.45 (1.00-2.11)	0.074	1.39 (0.97-2.00)	0.61	1.10 (0.77-1.56)	0.5	1.14 (0.78-1.68)	0.27	1.71 (0.68-4.29)	0.67	0.90 (0.56-1.45)
	rs16976177	15	G	0.089	1.41 (0.95-2.09)	**0.028**	1.48 (1.04-2.12)	0.98	0.99 (0.64-1.54)	0.14	1.45 (0.88-2.38)	0.54	1.12 (0.78-1.62)	0.56	1.32 (0.52-3.34)
	rs16976194	15	T	0.49	0.92 (0.72-1.17)	0.23	1.30 (0.85-2.00)	**0.022**	1.81 (1.08-3.03)	**0.022**	1.72 (1.08-2.76)	0.40	0.83 (0.54-1.28)	0.17	1.42 (0.86-2.36)
RAB38	rs1027027	11	A	0.59	1.08 (0.81-1.44)	0.35	1.45 (0.67-3.14)	0.14	1.27 (0.93-1.74)	0.42	0.87 (0.62-1.22)	**0.019**	1.49 (1.07-2.07)	0.78	0.94 (0.60-1.47)
	rs11602163	11	G	0.19	0.79 (0.56-1.13)	0.20	1.52 (0.80-2.88)	0.37	0.74 (0.38-1.43)	0.078	1.33 (0.97-1.85)	**0.009**	0.57 (0.38-0.87)	0.15	1.65 (0.84-3.25)
	rs12576251	11	G	0.45	1.25 (0.70-2.24)	0.27	1.42 (0.76-2.68)	0.82	0.97 (0.72-1.30)	0.06	1.36 (0.99-1.88)	**0.037**	0.71 (0.51-0.98)	0.09	1.80 (0.93-3.49)

	rs524121	11	C	0.21	0.70 (0.40-1.23)	0.65	1.16 (0.62-2.15)	**0.010**	0.40 (0.20-0.83)	0.31	0.70 (0.35-1.39)	0.32	0.70 (0.34-1.45)	0.36	1.34 (0.72-2.52)
	rs9144	11	C	0.26	1.32 (0.81-2.14)	0.13	1.47 (0.89-2.45)	0.41	0.80 (0.47-1.37)	**0.021**	1.42 (1.05-1.92)	0.072	0.76 (0.56-1.03)	**0.035**	1.80 (1.05-3.07)
RGS1	rs1359062	1	G	0.1	1.37 (0.94-2.00)	0.53	1.12 (0.79-1.60)	**0.017**	3.91 (1.11-13.83)	0.50	0.66 (0.20-2.21)	0.093	1.37 (0.95-1.96)	0.30	0.78 (0.48-1.26)
RGS20	rs1123133	8	G	0.59	0.82 (0.40-1.70)	0.35	1.47 (0.66-3.28)	0.16	1.76 (0.78-3.99)	0.47	0.73 (0.31-1.70)	0.14	1.79 (0.84-3.78)	0.32	1.55 (0.67-3.59)
	rs2220093	8	G	0.41	0.50 (0.09-2.74)	0.13	0.71 (0.45-1.12)	0.56	1.14 (0.74-1.76)	0.22	0.27 (0.03-2.58)	0.42	1.21 (0.76-1.92)	**0.022**	0.51 (0.28-0.93)
	rs6473895	8	A	0.35	1.28 (0.76-2.17)	0.82	0.93 (0.52-1.66)			**0.026**	2.12 (1.07-4.21)	0.37	1.30 (0.73-2.31)	0.26	1.46 (0.76-2.78)
	rs7824575	8	A	0.18	0.60 (0.28-1.29)	**0.029**	2.50 (1.06-5.90)	0.44	0.72 (0.32-1.63)	0.27	1.67 (0.66-4.24)	0.075	0.38 (0.11-1.27)	0.31	0.79 (0.50-1.24)
SLC45A2	rs35405	5	T	0.23	1.17 (0.91-1.50)	0.45	1.18 (0.76-1.84)	0.55	0.86 (0.54-1.39)	**0.018**	1.75 (1.10-2.79)	0.12	0.79 (0.58-1.07)	0.29	1.19 (0.86-1.65)
	rs35414	5	T	0.71	0.93 (0.64-1.35)	**0.02**	0.61 (0.40-0.93)	0.068	1.46 (0.97-2.20)	0.40	0.78 (0.44-1.39)	0.24	1.31 (0.83-2.06)	0.054	0.72 (0.52-1.01)
SNAI2	rs1992375	8	A	0.23	0.86 (0.67-1.10)	0.51	1.14 (0.72-1.81)	0.59	0.89 (0.58-1.37)	0.19	0.82 (0.61-1.10)	**0.0017**	0.41 (0.22-0.75)	0.39	0.80 (0.49-1.32)
	rs2735455	8	A	**0.0096**	1.89 (1.16-3.09)			0.16	1.45 (0.86-2.47)	0.72	0.89 (0.49-1.64)	0.46	1.23 (0.71-2.13)	0.42	0.78 (0.41-1.46)
SNX10	rs1406754	7	T	0.21	1.27 (0.88-1.82)	0.65	1.15 (0.62-2.13)	0.73	1.07 (0.72-1.60)	0.22	0.76 (0.50-1.18)	0.29	1.18 (0.86-1.62)	**0.039**	1.42 (1.02-1.99)
	rs1468286	7	T	0.67	0.91 (0.57-1.43)	**0.03**	1.36 (1.03-1.79)	0.25	1.27 (0.85-1.91)	0.87	0.96 (0.56-1.63)	0.18	0.82 (0.61-1.10)	0.58	1.09 (0.80-1.49)
	rs2699808	7	C	0.19	0.84 (0.65-1.09)	0.49	0.86 (0.55-1.33)	0.52	1.15 (0.75-1.76)	**0.032**	1.73 (1.04-2.89)	0.23	0.76 (0.49-1.18)	0.12	0.78 (0.58-1.07)
SOX11	rs17362772	2	G	**0.034**	0.65 (0.43-0.97)	0.53	1.18 (0.71-1.95)	0.56	0.87 (0.54-1.39)	0.44	1.23 (0.72-2.11)	0.24	0.73 (0.43-1.25)	0.26	0.71 (0.39-1.30)
TYR	rs1042602	11	A	0.62	0.90 (0.59-1.37)	0.20	0.72 (0.44-1.19)	**0.033**	1.68 (1.04-2.71)	**0.0082**	0.66 (0.48-0.90)	0.46	1.20 (0.74-1.95)	**0.031**	0.70 (0.50-0.97)
	rs17174064	11	C	0.7	1.10 (0.67-1.82)	0.21	0.69 (0.39-1.23)	0.28	0.74 (0.44-1.27)	0.86	1.06 (0.57-1.95)	0.47	0.80 (0.43-1.48)	**0.040**	1.87 (1.04-3.34)
	rs2186640	11	G	0.37	0.80 (0.49-1.31)	0.3	1.34 (0.77-2.35)	0.081	0.70 (0.47-1.05)	0.13	1.28 (0.93-1.75)	0.19	1.45 (0.84-2.53)	**0.015**	1.50 (1.08-2.09)
	rs5021654	11	C	0.40	0.81 (0.50-1.32)	0.18	1.47 (0.83-2.60)	0.12	0.73 (0.49-1.08)	0.062	1.35 (0.98-1.87)	0.19	1.46 (0.84-2.55)	**0.019**	1.77 (1.09-2.87)
TYRP1	rs10809828	9	G	0.18	0.82 (0.62-1.10)	0.062	1.94 (0.95-3.95)	0.19	0.81 (0.60-1.10)	**0.0004**	1.82 (1.29-2.57)	0.09	0.43 (0.15-1.25)	0.23	1.31 (0.84-2.05)
	rs11791497	9	C	0.21	0.70 (0.40-1.23)	**0.026**	2.05 (1.08-3.91)	0.41	0.77 (0.40-1.45)			0.54	0.81 (0.41-1.61)	0.78	0.91 (0.48-1.75)
	rs683	9	C	0.1	0.81 (0.63-1.05)	0.17	1.41 (0.86-2.33)	0.40	0.84 (0.56-1.26)	**0.018**	1.97 (1.12-3.47)	0.26	0.84 (0.62-1.14)	**0.027**	1.84 (1.08-3.13)
WNT3A	rs697763	1	C	**0.0086**	1.61 (1.13-2.30)	0.60	0.90 (0.61-1.33)	0.052	1.47 (1.00-2.18)	0.23	1.30 (0.85-1.98)	0.5	0.87 (0.57-1.32)	**0.017**	0.58 (0.37-0.91)
	rs708122	1	T	**0.0003**	0.60 (0.46-0.80)	0.14	1.56 (0.86-2.83)	**0.042**	0.53 (0.29-0.98)	0.49	0.90 (0.67-1.21)	0.80	1.06 (0.69-1.62)	0.15	1.27 (0.91-1.76)
	rs766972	1	G	**0.039**	1.45 (1.02-2.07)	0.59	0.83 (0.43-1.63)	**0.0054**	1.74 (1.17-2.57)	0.12	1.40 (0.92-2.14)	0.32	2.15 (0.51-9.16)	**0.036**	0.62 (0.39-0.97)
	rs947631	1	C	**0.0026**	1.77 (1.22-2.57)	0.16	1.46 (0.86-2.47)	0.13	1.37 (0.91-2.05)	0.072	1.49 (0.96-2.29)	0.35	0.81 (0.52-1.26)	0.15	0.79 (0.58-1.09)

Chr, Chromosome; mA, Minor Allele; OR, Odds Ratio per minor allele; CI, Confidence Interval.
Bold indicates statistically significant results

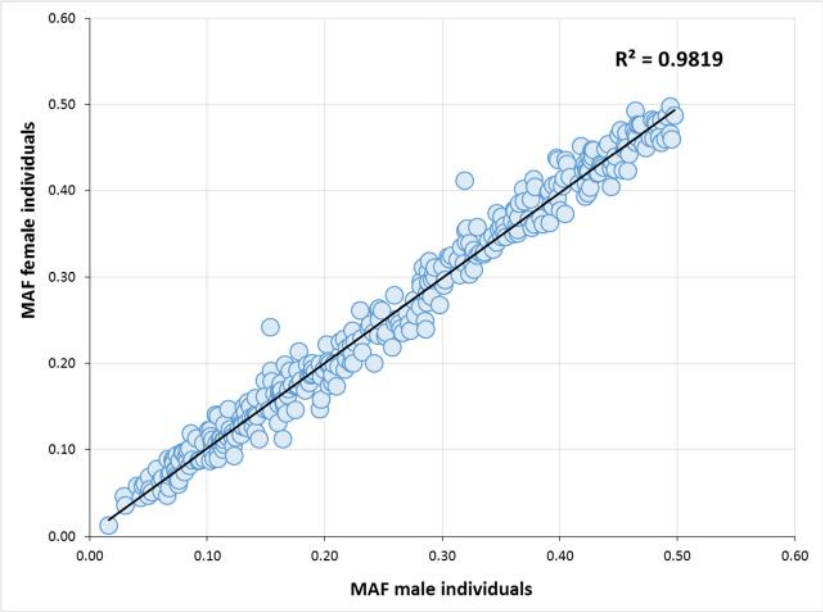

Figure S1.1. Comparison of minor allele frequencies, female *versus* male individuals. Minor allele frequencies were not significantly different between females and males after Bonferroni correction was applied.

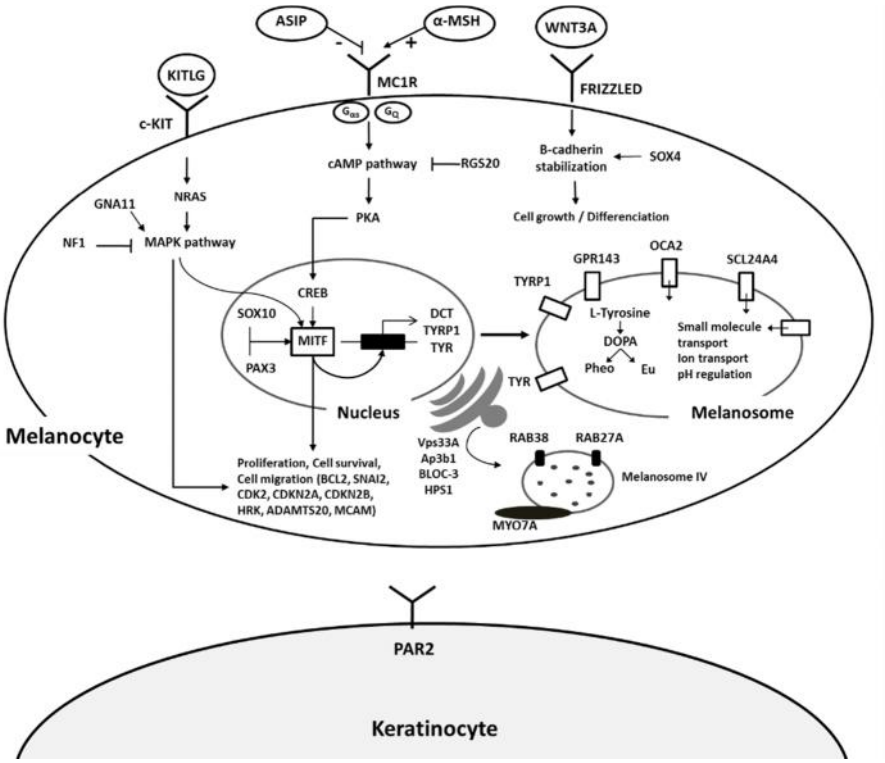

Figure S1.2. A selection of genetic factors affecting pigmentation and sun sensitivity in humans. Graph showing a selection of genes involved in melanocyte development, melanin synthesis, and melanosome biogenesis, transport and transfer.

Chapter 2

Sex and MC1R variants in human pigmentation: differences in tanning ability and sensitivity to sunlight between sexes

Hernando B, Ibarrola-Villava M, Peña-Chilet M, Alonso S, Ribas G, Martinez-Cadenas C.

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2.1. LETTER TO THE EDITOR

Human skin acts as a biological active barrier to the external environment, including exposure to UV radiation – clearly the major risk factor for melanoma. Sex differences are well-known regarding melanoma, with females presenting lower incidences, less metastases and better survival rates than males (131).

Both genetics and sex hormones contribute to sexual differences in skin aging, pigmentation, UV-light sensitivity, and melanoma incidence and outcome (11). Oestrogens accelerate wound healing, improve inflammatory disorders, increase skin thickness, protect from skin photoaging, and induce the activation and expression of genes involved in melanin synthesis (85).

Basal skin pigmentation, via melanin synthesis, darkens in response to sunlight, thus fulfilling its protective role against further irradiation-induced damage (11). Therefore, sex disparity in melanoma epidemiology might be explained by sex differences in tanning ability and skin sensitivity to UV-light exposure.

With the purpose of shedding some light on these questions, we evaluated a total of 1,112 individuals (515 males and 597 females) of Spanish origin for pigmentary traits related to tanning ability and sun sensitivity – skin phototype, history of sunburns, presence of solar lentigo and number of naevi. A brief summary of the materials and methods used in this work is available in the Supplementary Material.

When these phenotypic traits were analysed according to sex, the percentages of skin phototypes and naevus numbers appeared to be significantly different between the two sexes (Table S2.1). The percentage of phototypes I-II was notably higher in females than in males (48.06% vs. 38.17%, $P = 8.35 \times 10^{-3}$). Regarding naevus number, the percentage

of naevi was remarkably lower in females than in males (68.34% vs. 59.03%, $P = 1.25x10^{-3}$). No significant differences between the sexes were observed for history of sunburns and presence of lentigines.

A meta-analysis was performed to compare our results to previously published data (Figure 2.1). The guidelines followed to perform the meta-analysis are briefly explained in the Supplementary Material. We searched for studies conducted in Caucasian populations presenting phenotypic data stratified by sex ((31,61,106,175–178). When all individuals included in these studies were analysed together, the difference between females and males was extremely significant for both skin phototype (OR = 0.75, 95% CI: 0.68-0.83, $P = 1.90x10^{-9}$) and naevi (OR = 1.42, 95% CI: 1.30-1.55, $P = 1.11x10^{-15}$). The results obtained in this Spanish study were concordant with the results of the meta-analysis.

Our results are consistent with earlier anthropological studies indicating that females have less tanning ability, and therefore lower phototypes, than males in most populations, as males show greater pigmentation contrast between exposed and unexposed skin regions (61,87). Furthermore, Jacobs and cols. (2015) showed that females presented a much higher prevalence of facial sun spots than males, suggesting that females are more severely affected by sun exposure than males independently of genotype (30). These differences could be the result of socio-cultural reasons, as males tend to spend more time outdoors; physiological reasons, as males have thicker skin and increased number of blood vessels; differential tanning, as no sex difference in basal skin pigmentation has been shown; and hormonal factors, as oestrogens stimulate pigmentation while androgens have an inhibitory effect on melanocytes (61,87).

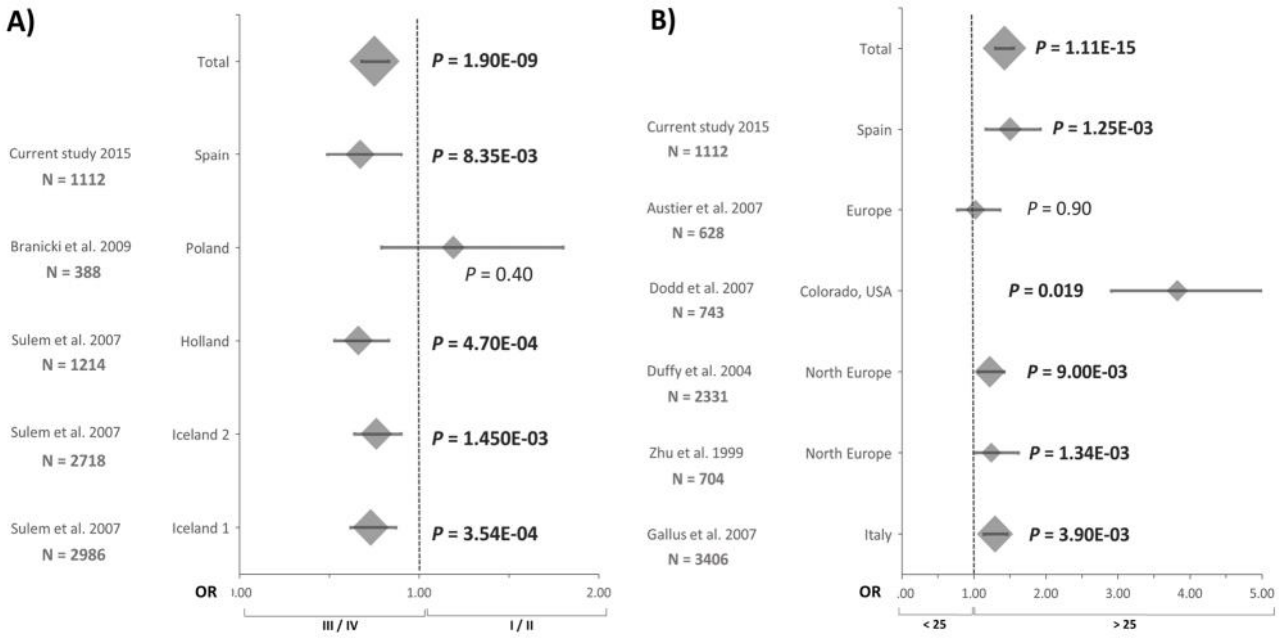

Figure 2.1. Sex-specific meta-analysis of (a) skin phototype and (b) naevus number in different Caucasian populations. Since femaleness was set as the reference, the results show male ORs. Diamond shapes represent odds ratio in each study and in the pooled analysis (Total). Diamond size is proportional to the number of individuals, and error bars represent 95% confidence intervals. Bold on *P*-values denotes statistically significant results. N refers to the total individuals analysed in each study. Total: results attained by taking into account all populations.

An elevated naevi number, a major predictor factor for melanoma occurrence, is directly correlated with high levels of sun exposure (106). As expected, females present fewer naevi than males. These results might be in apparent conflict with those obtained for skin phototype, since naevus prevalence has been significantly associated with the propensity to burn slightly and tan lightly (179). However, tanning degree – the difference between unexposed and exposed skin colour – has also been positively associated with naevus count (106). This inconsistency might arise as a consequence of the individual perception of overall darkness of tan when self-reported questionnaires are used to collect

information on pigmentation characteristics. As a result, this naevus count sex-specific difference might be attributable to higher acquired sun exposure levels in males than females, and not to genetic effects.

Previous studies have evidenced sex-differentiated genetic effects for anthropometric traits, serum metabolite concentrations, human pain inhibition, human pigmentation, and melanoma risk (162,163,166,180–183).

Considering the importance of genetics in UV-light response, we focused on identifying a possible genetic cause explaining phenotypic differences between sexes. We genotyped five SNPs involved in human pigmentation pathways: rs12913832 (located in the *HERC2/OCA2* region), rs1800407 (*OCA2* gene), rs16891982 (*SLC45A2* gene), rs1393350 (*TYR* gene), and rs12203592 (*IRF4* gene). The coding region of *MC1R* gene was also studied by direct sequencing, classifying *MC1R* functional variants as RHC (red hair colour) and non-RHC associated variants.

Genotype association analyses were performed via logistic regression for each SNP as well as for sex. To assess for possible confounding effects, regression estimates were adjusted by executing a multivariate logistic regression. After adjustment, skin phototypes I-II were significantly associated with *MC1R* RHC variants at Bonferroni-corrected level ($P = 2.96x10^{-4}$), but were also moderately associated with female sex ($P = 2.11x10^{-2}$). No association was observed between naevi number and any of the genetic variants studied. However, being male remained significantly associated with having ≥25 naevi ($P = 1.12x10^{-2}$), meaning that male sex might be a factor contributing to high naevus count (Table 2.1A).

Table 2.1

A) Genotypic association with phenotypic traits

			Skin Phototype I/II				Naevus number ≥25			
			Non-adjusted		Adjusted[a]		Non-adjusted		Adjusted[a]	
Gene	**SNP ID**	**Allele/Factor**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**
HERC2	rs12913832	C	0.89	1.02 (0.81-1.27)	0.57	1.07 (0.84-1.37)	0.44	1.07 (0.89-1.28)	0.76	1.03 (0.85-1.25)
OCA2	rs1800407	T	0.24	1.23 (0.87-1.73)	0.59	1.11 (0.76-1.63)	0.47	0.90 (0.68-1.19)	0.54	0.91 (0.67-1.24)
TYR	rs1393350	T	**3.32E-02**	**1.32 (1.02-1.69)**	0.06	1.29 (0.98-1.68)	0.34	0.90 (0.73-1.12)	0.31	0.89 (0.72-1.11)
SLC45A2	rs16891982	C	0.16	1.31 (0.90-1.90)	0.35	1.21 (0.81-1.78)	0.41	1.13 (0.85-1.50)	0.33	1.15 (0.89-1.54)
IRF4	rs12203592	A	0.82	0.97 (0.72-1.30)	0.75	1.05 (0.76-1.46)	0.72	1.05 (0.81-1.35)	0.89	0.98 (0.75-1.28)
MC1R	RHC variants	RHC	**3.00E-06***	**2.00 (1.49-2.69)**	**2.96E-04***	**1.89 (1.34-2.69)**	0.61	1.07 (0.83-1.38)	0.34	1.14 (0.87-1.48)
	Sex	Male	**8.57E-03**	**0.67 (0.49-0.90)**	**2.11E-02**	**0.65 (0.45-0.94)**	**1.28E-03***	**1.37 (1.05-1.78)**	**1.21E-02**	**1.45 (1.08-1.93)**
	Skin Colour	Fair/Pale	**6.11E-07***	**2.20 (1.61-3.00)**	**1.36E-04***	**2.03 (1.41-2.96)**	**5.98E-03**	**1.42 (1.11-1.83)**	**2.33E-02**	**1.40 (1.05-1.88)**
	Sunburn History	Yes	**5.12E-07***	**2.32 (1.67-3.22)**	**5.10E-05***	**2.20 (1.50-3.24)**	**4.32E-13***	**2.67 (2.05-3.48)**	**1.46E-12***	**2.94 (2.18-3.97)**

B) Sex differences in skin phototype within *MC1R* genotype

	***MC1R* Wild-type**				***MC1R* RHC variants**			
	Skin phototype[b]				**Skin phototype[b]**			
Sex	**III-IV**	**I-II**	**OR**	***P*-value[c]**	**III-IV**	**I-II**	**OR**	***P*-value[c]**
Male	65.70 %	34.30 %	reference		51.20 %	48.80 %	reference	
Female	58.00 %	42.00 %	1.39 (0.97-1.98)	0.071	34.80 %	65.20 %	**2.20 (1.13-4.29)**	**0.029**

Abbreviations: SNP, single nucleotide polymorphisms; OR, Odds Ratio; CI, Confidence Interval; RHC, red hair colour
RHC variants include both homozygotes and heterozygotes
Bold indicates statistically significant results
* *P*-value significant at Bonferroni-corrected threshold of 0.05/9 = 0.0055
[a] Multivariate logistic regression analysis. Results adjusted by including all the potential risk factors in the model, considering as risk factors all six SNPs, sex, skin colour and history of sunburns
[b] Percentages of the all individuals in each subgroup
[c] *P*-values for Fisher's exact test, estimating sex differences in skin phototype within each *MC1R* genotype

The protein encoded by the *MC1R* gene functions as a receptor for α-MSH, a hormone produced in the pituitary gland that depends on oestrogen levels. Interestingly, *MC1R* RHC variants presented differences in genetic effects by sex, with greater effects in skin phototype in females than in males (Table 2.1B). That is, females carrying an RHC variant tended to exhibit significant lower phototypes than males with the same *MC1R* genotypes (OR = 2.20, *P*=0.029). In a previous study, *MC1R* genotype revealed a significant greater influence on analgesia from pentazocine in females than in males (182). Furthermore,

mutations in another gene of the melanocortin receptor family, *MC4R*, presented about twice as stronger effect on body mass index in females than in males (180).

In summary, this study supports previous evidence that sex might be a factor explaining variations in tanning ability and sensitivity to sunlight between females and males in Caucasian populations. Additionally, we suggest that *MC1R* genetic effects might contribute to these sex-specific differences in skin phototype.

2.2. SUPPLEMENTARY MATERIAL

2.2.1. Material and Methods

Phenotypic traits were collected using a standardised questionnaire, under the supervision of a professional. All individuals were randomly selected and gave written informed consent. This study was approved by the Ethics Committee of the Biomedical Research Institute - INCLIVA (Valencia, Spain).

R statistical framework was used to conduct the quality control processes and statistical analyses. For all phenotypic traits, Fisher's exact test was used both to compare phenotypic frequencies between female and male individuals and to test for deviations from Hardy-Weinberg equilibrium (HWE) between sexes. Correction for multiple hypothesis testing was carried out using the Bonferroni method.

Then, we performed a fixed effect inverse-weighted meta-analysis to compare our results to previously published data. A literature search was carried out using PubMed and the terms 'skin phototype' or 'moles/naevi' and 'sex' or 'gender'. Additional articles were identified from cited references. After a full revision, we included those studies that were

conducted in Caucasian populations, permitted quantitative assessment of the association, and presented phenotypic data stratified by sex.

Study-specific association estimates were calculated and then combined to achieve overall effect estimates, setting female as the reference group. A Cochrane Q test was used for evaluating heterogeneity among all studies included. No evidence of heterogeneity was observed between studies. The methodological quality of the studies included in the meta-analysis was not assessed, but all of them account for environmental factors, age, sex as well as population stratification. Since each of these studies used a different dataset, the inherent sampling error variability among them was assumed. Nonetheless, the methodological approach followed in this work is in general accordance with MOOSE (Meta-analyses of Observational Studies in Epidemiology) group guidelines (184).

Finally, we analysed a possible genetic cause explaining phenotypic differences between sexes. Genotyping reactions were performed using Kaspar technology (KBiosciences, Hoddesdon, UK). For rs16891982, TaqMan technology was used (Applied Biosystems, Foster City, USA). Associations between the genotype and phenotypic traits were assessed via logistic regression, coded additively for each copy of the minor allele. The coding region of MC1R gene was analysed by direct sequencing using BigDye Terminator Cycle Sequencing kit and an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. All genotyped polymorphisms were in Hardy-Weinberg equilibrium after Bonferroni's correction (P-values < 0.0083). Genotype association analyses were performed via logistic regression for each SNP as well as for sex. To assess for possible confounding effects, regression estimates were adjusted by executing a multivariate logistic regression.

Table S2.1. Phenotype frequencies[a] by sex among sampled Spanish individuals

Trait	Phenotype	Total	Sex		*P*-value[b]
			Male	Female	
Skin Phototype	I/II	43.27 %	38.17 %	48.06 %	**8.35E-03**
	III/IV	56.73 %	61.93 %	52.04 %	
Naevi number	<25	35.97 %	59.03 %	68.34 %	**1.25E-03**
	≥25	64.03 %	41.07 %	31.76 %	
Presence of Solar Lentigines	No	44.35 %	42.23 %	46.10 %	0.20
	Yes	55.65 %	57.77 %	53.90 %	
History of Sunburns	No	46.51 %	47.11 %	46.00 %	0.72
	Yes	53.49 %	52.89 %	54.00 %	

Bold indicates statistically significant results

[a] Frequencies are presented as the percentages of total individuals in each subgroup (total, male and female)

[b] *P*-values for Fisher's exact test

Chapter 3

Genetic 3'UTR variation is associated with human pigmentation characteristics and sensitivity to sunlight

Hernando B, Peña-Chilet M, Ibarrola-Villava M, Martin-Gonzalez M, Gomez-Fernandez C, Ribas G, Martinez-Cadenas C.

Experimental Dermatology. 2017 Mar. doi: 10.1111/exd.13333.

3.1. INTRODUCTION

Cutaneous melanoma incidence is increasing rapidly among white-skinned populations (185). Melanoma incidence reveals a clear relationship between pigmentation traits and sunlight damage, with individuals with fair skin, green and blue eyes, red and blond hair, high naevus count, freckles, and inability to tan showing greater melanoma susceptibility (9). These phenotypic traits has been shown to be genetically determined by genes implicated in pigmentation and tanning ability (23,31), and genetic variations in these genes have been associated with the susceptibility to melanoma (26,28,29,54,68,157,168). Factors that are mainly involved in the aetiology of melanoma are not only of pigmentary/genetic nature, but also of environmental nature (186). Chronic sun exposure thus plays a key role in causing melanoma through DNA damage (120).

Ultraviolet (UV) exposure stimulates the synthesis of melanin in melanosomes via activation of human pigmentation pathways, with the aim of protecting skin from the harmful effects of sunlight (81). Gene expression can be regulated by a wide range of mechanisms. Recently, posttranscriptional regulatory processes – specifically controlled by mRNA-binding factors – have emerged as a fundamental and effective cellular mechanism to regulate gene expression, and alterations in these processes can cause numerous pathologies including immunological disease (187), neurodegeneration (188), and tumour development (189,190). Therefore, differential gene expression may be as important for disease susceptibility as non-synonymous coding changes.

Among the mRNA-binding factors, microRNAs (miRNAs) – short non-coding RNA molecules (22-24 nt) encoded by intronic or intergenic sequences – act as key gene regulators by repressing mRNA translation or by destabilizing/degrading mRNAs in the

cytoplasm, via perfect or imperfect binding to their complementary base pair sequence in the 3'untranslated region (3'UTR) of the mRNA target (191). Therefore, the 3'UTR region is emerging as critically important in regulating gene expression (189), and polymorphisms in the miRNA-binding sites of the 3'UTR of genes may alter the binding efficiency and miRNA-mRNA gene expression regulation. In support of this hypothesis, recent studies have identified variants in the 3'UTR of genes that increase the susceptibility for melanoma (150), lung (192), colorectal (193) and ovarian cancer (194) by affecting the ability of miRNAs to bind. In particular, two sequence changes in the 3'UTR of the *CDKN2A* gene have been significantly correlated with melanoma risk (195), but also with a shorter progression time from primary to metastatic melanoma (196).

Here, we hypothesise that differences identified in nucleotide composition of 3'UTRs SNP sites of genes previously associated with pigmentation and/or skin cancer can be a reason for causing differences in human pigmentation, sensitivity to sunlight, and thus in melanoma susceptibility. In the current study, we describe the role of 38 different 3'UTR polymorphisms from 38 different candidate pigmentation and melanoma susceptibility genes in a population of Spanish origin. Additionally, we use miRNA binding prediction tools to identify variants affecting putative miRNA-binding sites, and to predict their impact on miRNA-mRNA interaction.

3.2. METHODS

3.2.1. Study subjects and data collection

A total of 526 melanoma cases and 343 cancer-free controls were included in this study. Melanoma cases were recruited at the Departments of Dermatology of four Spanish hospitals: Gregorio Marañon General University Hospital (Madrid), La Paz University Hospital (Madrid), Ramon y Cajal University Hospital (Madrid) and Castellon Province Hospital (Castellon). Volunteer cancer-free control samples were recruited from the Madrid College of Lawyers, Gregorio Marañon Hospital, Valencia Clinic Hospital and Castellon Province Hospital. We carefully selected all cases and controls included in the current study to account for confounding variables. As far as it was possible, controls were frequency-matched to the cases by age, sex and place of birth. All individuals were Caucasians of Spanish origin with the same genetic background, since there is evidence of high genetic homogeneity within different Spanish geographical regions (164).

Each participant completed a standardised questionnaire to collect information on sex, age, pigmentation characteristics (eye colour, hair colour, skin colour, number of naevi and presence of solar lentigines), history of childhood sunburns, and personal and family cancer history.

Genomic DNA from cases and controls was isolated from peripheral blood lymphocytes using the traditional saline method or the DNAzol procedure (Invitrogen, Eugene, OR, USA) or the MagNA Pure LC Instrument according to the manufacturer's protocol (Roche Molecular Biochemicals AQ2, Mannheim, Germany). DNA concentration was quantified in samples before genotyping by using a Nanodrop 2000 spectrophotometer or Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Eugene, OR, USA). Genomic DNA

was amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Samples were diluted to a final solution of 50 ng/ml and stored at -20ºC.

The study was approved by the Ethics Committee of the Biomedical Research Institute - INCLIVA (Valencia, Spain). Written informed consent was obtained from all participants.

3.2.2. SNP Selection

Previous literature and information of public databases were used to perform our candidate gene list. We selected genes previously associated with pigmentation pathways and/or melanoma risk (28,157,168,197,198), preferably including direct targets of functional miRNA that happen to be deregulated in melanoma. Ensembl BioMart (http://www.ensembl.org/biomart/martview) was used to retrieve germline variants from all genes selected. Filters were used to ensure that all SNPs were located within the 3'UTRs. SNP codes, locations, minor and ancestral alleles and their frequencies, were obtained from the NCBI (www.ncbi.nlm.nih.gov/SNP), HapMap (www.hapmap.org) and Ensembl Variation (www.ensembl.org/info/genome/variation) databases. From the data retrieved, Haploview v4.2 was used to identify tag-SNPs that optimally capture allelic variation among SNPs, using a pairwise SNP approach with a minimum r^2 threshold of 0.8 (199). To ensure a high genotyping success rate, a minor allele frequency (MAF) threshold of 0.1 in the Caucasian population from the International 1000 Genomes Project (http://www.1000genomes.org/) was established in the SNP selection process. Forty-five tag-SNPs were finally selected.

3.2.3. Genotyping

SNPs genotyping was conducted by the Spanish National Genotyping Centre (CeGen-PRB2, Santiago de Compostela) as a contract service using the iPLEX Gold MassARRAY technology, according to manufacturer's protocol (Sequenom, San Diego, CA, USA). All assays were performed in 384-well plates, including a negative control and a trio of Coriell samples (Na10860, Na10861 and Na11984) for quality control. Genotyping specificity was assessed by adding three DNA duplicates (two intra-assays and one inter-assay) per plate, yielding 100% consistent replication results. In addition, cases and control samples were always included in the same run. SNPs with a genotyping rate lower than 90% (10% missing data) were excluded for further analysis.

3.2.3. Identification of potential microRNA binding sites

The potential effect of 3'UTR polymorphisms on miRNA binding was examined using MirSNP (http://cmbi.bjmu.edu.cn/mirsnp) (200) and miRNASNP (http://www.bioguo.org/miRNASNP/) (201).

MirSNP employs the miRanda target prediction algorithm (http://www.microrna.org)(202), with stringent 7-nt seed site pairing as major criteria for prediction consistency. To increase precision, we only considered target sites with an alignment score cutoff ≥ 140, energy cutoff ≤ −10 kcal/mol, and miRSVR score ≤ −0.1.

MiRNASNP uses two miRNA target prediction tools: TargetScanHuman (http://www.targetscan.org/) (203) and miRanda (202). MiRNASNP also incorporates RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) (204) to quantify the

binding energy changes in the interaction of miRNAs with the wild-type target sequence compared to the derived 3'UTR sequence. Only the duplexes with hybridization free energy ≤ −20 kcal/mol were chosen (205).

3.2.4. Identification of validated pathways targeted by *in silico* predicted microRNAs

In order to further investigate the miRNAs predicted to bind to the two 3'UTR SNPs highly associated with phenotypic traits (hsa-miR-149-5p, hsa-miR-892b, hsa-miR-185-3p and hsa-miR-762), we used DIANA-miRPath v2.0 (http://www.microrna.gr/miRPathv2) to identify the miRNA targeted pathways. The output provides intuitive heat maps and enriched KEGG pathway visualizations for easier inspection (206).

3.2.5. *In silico* quantitative analysis of tissue-specific expression

Data from the Genotype-Tissue Expression (GTEx) project (dbGaP accession No. phs000424.v6.p1) was used for external validation and to evaluate differential tissue-specific gene expression regarding 3'UTR SNP genotypes (http://www.gtexportal.org/home/).

3.2.6. Statistical Analysis

For each polymorphism studied, Fisher's exact test was used both to check for deviations from Hardy-Weinberg equilibrium (HWE) among controls and to compare differences in allele counts between cases and controls. In order to account for differences between populations, allele frequencies of our Spanish population were compared to those of both a North European population (CEU) and a Southern one from Tuscany (TSI) using Fisher's exact test.

Associations between the genotyped genes and various pigmentation characteristics were assessed via logistic regression. Association analyses were done for all samples pooled, with eye colour (blue/green *versus* brown/black), hair colour (brown/black *versus* blond/red), skin colour (fair *versus* brown), number of naevi (≥50 *versus* <50), presence of lentigines (yes *versus* no), and childhood sunburns (yes *versus* no) as the outcome variables. This was performed for four different patterns of inheritance: dominant (major homozygotes *versus* heterozygotes plus minor homozygotes), over-dominant (major homozygotes plus minor homozygotes *versus* heterozygotes), recessive (major homozygotes plus heterozygotes *versus* minor homozygotes), and additive (counting additively for each copy of minor allele). Genotype-related odds ratios (ORs), their corresponding 95% confidence intervals (CIs) and associated *P*-values were estimated. Association analyses with phenotypic traits were adjusted by sex, since sex-differentiated allelic effects for pigmentation traits, sensitivity to sunlight and melanoma have been previously shown (132,162,163).

In order to assess associations among genotypes and melanoma risk, genotype-related ORs, their corresponding 95% CIs and associated *P*-values were estimated via unconditional logistic regression. Multivariate logistic regression was also carried out

combining sex and all significant risk factors revealed in Table S3.1. This was also done for all four patterns of inheritance.

Statistical analyses and plots were conducted using R statistical framework (http://www.R-project.org). All genetic analyses were performed estimating the effect of the minor allele in the Spanish population. Unknown and missing values were excluded at each specific analysis. All *P*-values were two-sided, and those less than 0.05 were considered statistically significant.

3.3. RESULTS

The role of 38 polymorphisms in as many pigmentation and melanoma susceptibility genes was initially investigated. No evidence of departure from HWE for any of the 38 SNPs was found. Two 3'UTR polymorphisms revealed differences in minor allele frequencies (MAFs) between cases and controls: *ADAMTS20* rs6582463 and *HOXB7* rs15689. We did not observe differences in MAFs between cases and controls for any other SNP (Table S3.2).

We compared Spanish allele frequencies to those of CEU and TSI subjects, using the 1000 Genomes Project (phase 3) allele counts as the reference (Table S3.2). Spanish MAFs differed significantly from CEU frequencies in three SNPs (7.89%): rs4733967 (*ADAM9*), rs3212369 (*MC1R*), and rs1690916 (*MDM2*). Seven SNPs presented different allele frequencies from those reported in TSI population data: rs6582463 (*ADAMTS20*), rs742106 (*DTNBP1*), rs12952 (*EXOC2*) rs8022 (*KIT*), rs995030 (*KITLG*), rs14983 (*MMP7*), and rs1551306 (*TPCN2*). In spite of these differences, allele frequencies in Spain were very similar to those from both a North European population (CEU) and a

Southern one (TSI), with a high correlation (R^2) of 0.916 and 0.913, respectively (Figure S3.1).

3.3.1. Association analysis

Evidence of association with phenotypic characteristics for the thirty-eight 3'UTR SNPs was assessed. Considering a *P*-value threshold of 0.05, 17 SNPs were associated with at least one sun response trait, and 11 SNPs showed association with at least one pigmentation trait (Figure 3.1). Among them, we further investigated the 7 SNPs that presented the most potential allelic effects for phenotypic traits in the Spanish population (*P*-value < 0.01). The rs2325813 SNP, located in the *MLPH* gene, was correlated with the presence of more than 50 naevi (P=8.97x10^{-4}). Two SNPs, *HOXC8* rs4142680 and *WNT3A* rs752107, correlated with the presence of lentigines (P=6.57x10^{-3} and P=4.53x10^{-4}, respectively); while *LYST* rs6696123 showed association with an absence of lentigines (P=2.56x10^{-3}). Two more SNPs, rs10270 in the *CLIP1* gene and rs4980113 in the *KCNMA1* gene, were associated with dark hair colour (P=1.44x10^{-3} and P=2.67x10^{-3}, respectively). Finally, *KIT* rs8022 was correlated with light eye colour (P=8.88x10^{-3}) (Table 3.1).

Likewise, we carried out an association analysis between genotypes and melanoma risk. Five SNPs showed a tendency to correlate with melanoma susceptibility in the Spanish population. Among them, three SNPs (*HOXB7* rs1589, *MARCKS* rs28558559 and *ADAM9* rs4733967) showed a melanoma protective effect (OR<1). On the other hand, *PTCH2* rs41269085 and *ADAMTS20* rs6582463 displayed a melanoma risk effect (OR>1) (Table S3.3).

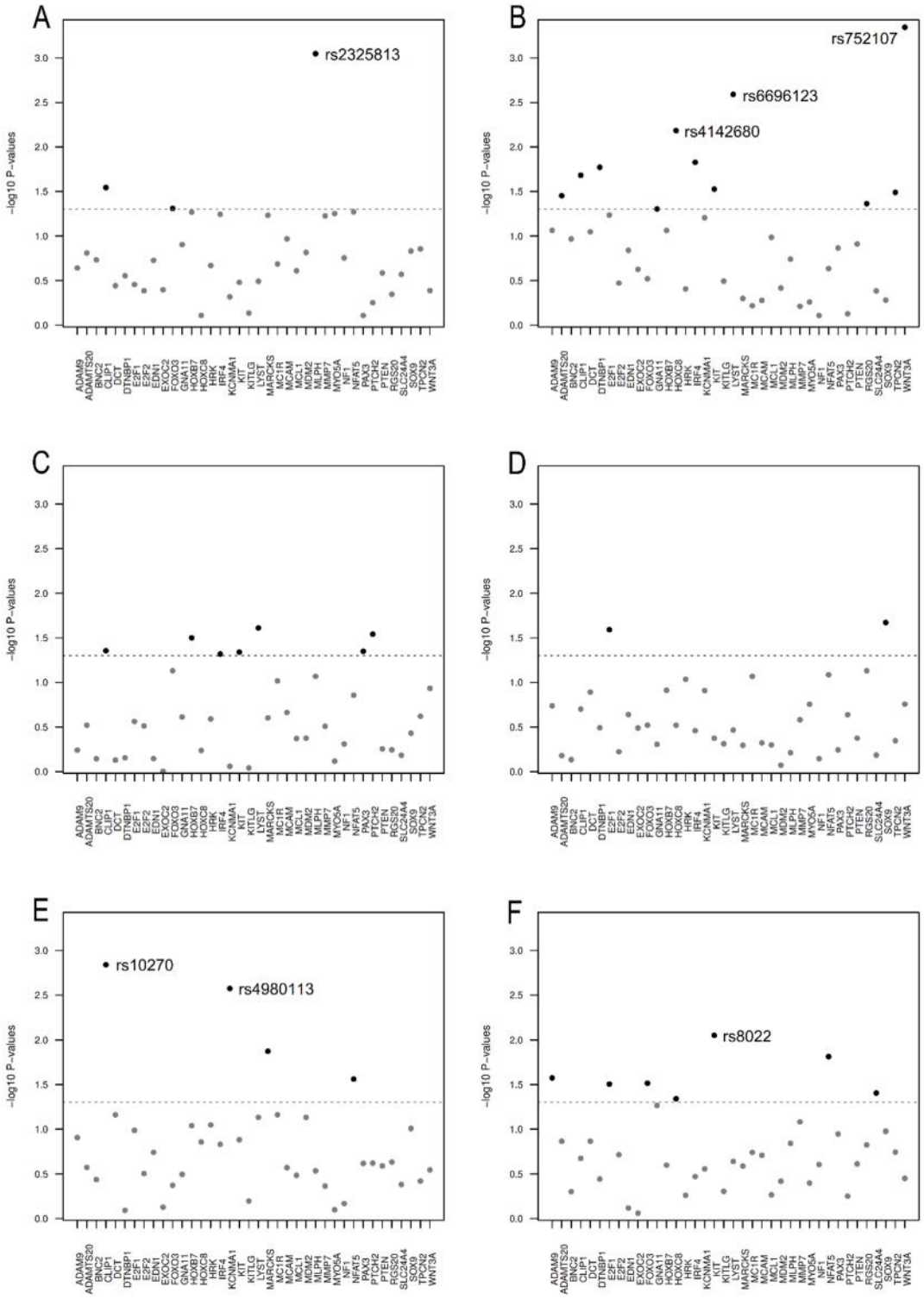

Figure 3.1. Manhattan plots display the significance of associated allelic effects (-log10 P-values) for each phenotypic trait. (A) naevus count, **(B)** solar lentigines, **(C)** childhood sunburns, **(D)** skin colour, **(E)** hair colour, and **(F)** eye colour. Each dot represents one of the 38 3'UTR SNPs genotyped. Black dots indicate SNPs with a significant fold change (*P*-values < 0.05). All rs numbers of polymorphisms highly associated with phenotypic traits are displayed next to the corresponding dot. All values displayed are from the most significant pattern of inheritance.

Table 3.1. 3'UTR variants highly associated with phenotypic traits in the Spanish population (*P*-value < 0.01)

Trait	Gene	SNP rs#	Genotype	Protective phenotype N (%)	Risk phenotype N (%)	Inheritance mode	OR (95% CI)	*P*-value
Naevi	*MLPH*	rs2325813	TT	591 (82.3)	75 (69.4)	Additive	2.03 (1.36-3.02)	8.97E-04
			CT	121 (16.9)	29 (26.9)	0 / C / CC		
			CC	6 (0.8)	4 (3.7)			
Lentigines	*WNT3A*	rs752107	CC	196 (56.2)	216 (45.3)	Over-dominant	1.66 (1.25-2.21)	4.53E-04
			CT	118 (33.8)	218 (45.7)	CC+TT / CT		
			TT	35 (10.0)	43 (9.0)			
Lentigines	*LYST*	rs6696123	TT	100 (28.6)	182 (38.1)	Additive	0.73 (0.60-0.90)	2.56E-03
			CT	184 (52.6)	231 (48.3)	0 / C / CC		
			CC	66 (18.9)	65 (13.6)			
Lentigines	*HOXC8*	rs4142680	TT	138 (39.4)	160 (33.6)	Over-dominant	1.47 (1.11-1.94)	6.57E-03
			CT	143 (40.9)	240 (50.4)	TT+CC / CT		
			CC	69 (19.7)	76 (16.0)			
Hair colour	*CLIP1*	rs10270	GG	328 (46.1)	83 (56.8)	Over-dominant	0.55 (0.37-0.80)	1.44E-03
			AG	321 (45.1)	45 (30.8)	GG+AA / AG		
			AA	63 (8.8)	18 (12.3)			
Hair colour	*KCNMA1*	rs4980113	GG	182 (25.5)	47 (32.2)	Over-dominant	0.57 (0.40-0.83)	2.67E-03
			CG	377 (52.9)	57 (39.0)	GG+CC / CG		
			CC	154 (21.6)	42 (28.8)			
Eye colour	*KIT*	rs8022	GG	416 (73.5)	229 (80.9)	Over-dominant	0.62 (0.43-0.89)	8.88E-03
			GT	139 (24.6)	48 (17.0)	GG+TT / GT		
			TT	11 (1.9)	6 (2.1)			

SNP, single nucleotide polymorphism; N, number of individuals; %, percentage of individuals per group among the total; OR, odds ratio per minor allele; CI, confidence interval

For the association results to be adjusted by the confounding variables, we performed a multivariate analysis including phenotypic risk factors (hair colour, solar lentigines and the presence of childhood sunburn) and sex as covariates. Polymorphisms located in *HOXB7, MARCKS, ADAM9* and *PTCH2* remained significant after the adjustment, with no substantial changes in allelic effects, confirming the putative role of these variants in melanoma susceptibility. Additionally, *KCNMA1* rs4980113 and *IRF4* rs9391997 were marginally associated with melanoma protection (Table S3.3).

3.3.2. Variants affecting microRNA binding sites in human pigmentation

All 3'UTR polymorphisms that presented association with phenotypic characteristics and/or melanoma were analysed by two specialized web-based programmes for predicting miRNA-binding sites in the 3'UTR.

Cross-prediction was required for verifying the predicted target sites. After applying all sequential filtering steps, eight of all 3'UTR polymorphisms evaluated had at least one miRNA predicted to bind (Table 3.2). Three 3′UTR variants interrupted miRNA-mRNA interaction or reduced miRNA-mRNA interaction by increasing the free energy of the corresponding duplexes after the minor allele introduction in the target sequence. Conversely, three variants created new miRNA target sequences or enhanced miRNA binding efficiency by decreasing hybridization free energy. Two variants both disrupted/decreased and created/enhanced multiple miRNA target sequences in the sequences studied (Table 3.2).

Once miRNAs of interest were identified using binding prediction tools, we used an *in silico* approach to identify pathways that are under the regulation of the predicted miRNA signature. The four selected miRNAs and the targeted KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways are displayed in Figure 3.2. Among all the significant targeted KEGG pathways, we identified three of them involved in pigmentation and skin cancer: "Wnt signalling pathway-hsa04310" (P=4.24x10^{-5}), "MAPK signalling pathway-hsa04010" (P=1.07x10^{-4}) and "Basal cell carcinoma-hsa05217" (P=2.52x10^{-3}). Figure S3.2 represents in detail these three KEGG pathways, highlighting the specific target genes of the selected miRNAs.

Table 3.2. Candidate microRNAs predicted to bind to 3'UTR SNPs showing association with pigmentation traits, sensitivity to sunlight and melanoma susceptibility

Gene	3'UTR SNP rs#	Allele change	miRNA predicted to bind to the target site [1]	Effect on miRNA binding [2]	Free energy of miRNA-mRNA binding for WT (kcal/mol) [3]	Free energy of miRNA-mRNA binding for MA (kcal/mol) [3]	Energy change (kcal/mol) [4]
DTNBP1	rs742106	G==>A	hsa-miR-1293	decrease	-26.40	-23.80	-2.60
		G==>A	hsa-miR-4782-5p	create	0.00	-21.30	21.30
E2F1	rs3213180	C==>G	hsa-miR-1182	break	-31.30	0.00	-31.30
FOXO3	rs9400241	A==>C	hsa-miR-2115-5p	break	-28.40	0.00	-28.40
		A==>C	hsa-miR-22-3p	create	0.00	-24.10	24.10
KIT	rs8022	G==>T	hsa-miR-548as-3p	create	0.00	-20.80	20.80
MLPH	rs2325813	T==>C	hsa-miR-185-3p	enhance	-29.00	-31.70	2.70
		T==>C	hsa-miR-762	enhance	-28.80	-31.50	2.70
MYO5A	rs7176482	A==>G	hsa-miR-198	break	-25.70	0.00	-25.70
		A==>G	hsa-miR-525-5p	break	-21.90	0.00	-21.90
SOX9	rs1042667	A==>C	hsa-miR-1181	create	0.00	-23.60	23.60
WNT3A	rs752107	C==>T	hsa-miR-149-5p	decrease	-29.90	-27.60	-2.30
		C==>T	hsa-miR-892b	decrease	-30.50	-28.20	-2.30

SNP, single nucleotide polymorphism; 3'UTR, 3'untranslated region; WT, wild-type target allele; MA, minor allele target allele

[1] The prediction of miRNA-binding sites was performed using MirSNP and miRNASNP

[2] The effect of the SNP on miRNA binding was given by MirSNP. These effects can be classified following four categories: a) decrease – reduction of the binding efficacy, b) enhance – increase of the binding efficacy, c) break – disruption of the binding site, or d) create – creation of a new binding site.

[3] The free energy value of miRNA-mRNA binding was obtained from miRNASNP

[4] Energy change (kcal/mol) indicates difference in minimum free energy of binding before and after introduction of the minor allele

We further evaluated the association between the genotype of both *MLPH* rs2325813 and *WNT3A* rs752107 and the gene expression levels in sun-exposed skin by using the GTEx portal. Individuals carrying rs752107*T allele, which was predicted to decrease miRNA-mRNA binding efficiency, seem to present increased expression of *WNT3A* in sun-exposed tissue (Figure S3.3). No changes in *MLPH* expression regarding genotype were observed.

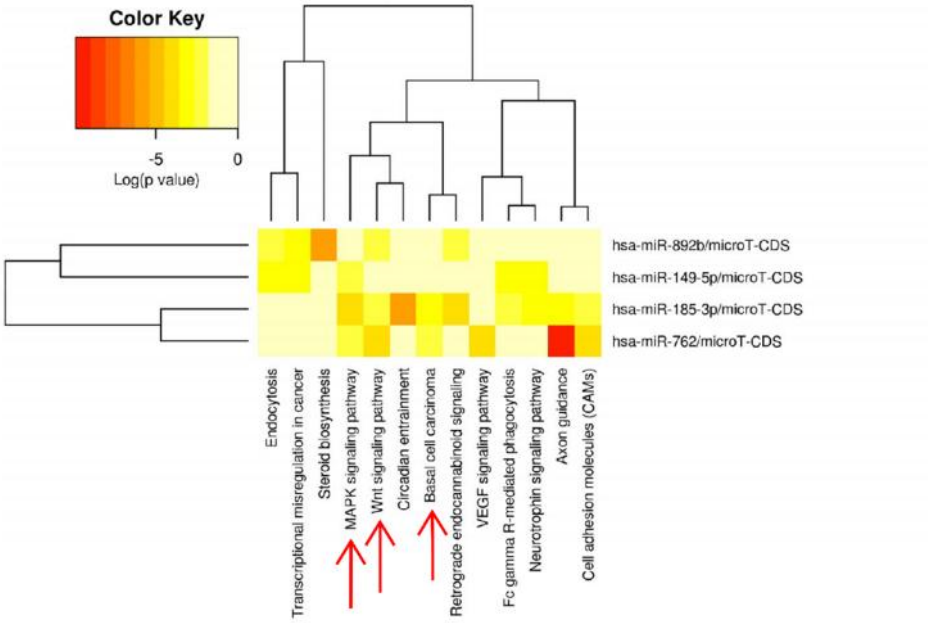

Figure 3.2. Heat map of selected miRNAs *versus* pathways. Darker colours represent higher significance. The attached dendrograms on both axes represent hierarchical clustering results for miRNAs (by exhibiting similar pathway targeting patterns) and pathways (by related miRNAs). Arrows indicate pathways involved in pigmentation and skin cancer.

3.4. DISCUSSION

In the current study, 38 tag-SNPs located in the 3'UTRs of pigmentation-related genes were successfully genotyped in 869 individuals from Spain, with the intention of detecting novel genetic variants with putative phenotypic implications. Since 3'UTRs are critical regulatory elements in gene expression (207), polymorphisms located in this region of genes associated with pigmentation pathways may contribute to pigmentation characteristics and sensitivity to sunlight, as well as to melanoma susceptibility.

This study allowed us to observe interesting associations between genotypic and phenotypic traits in our population. Despite detecting several candidate 3'UTR SNPs with a potential implication in pigmentation and sensitivity to sunlight, we could not validate

them since associations did not reach genome-wide nor candidate gene levels of statistical significance. Perhaps our restricted sample size resulted in limited statistical power to detect unequivocal associations for these SNPs. Replication of our findings in a larger study is therefore essential before drawing any firm conclusion. It is noted that adjusting analyses by sex has conferred strength to our results, excluding bias from the sexual disparity in pigmentation and melanoma incidence and outcome observed in previous studies (25,131,132,162,163).

The first interesting finding was the reasonably strong association of rs2325813, located in the 3'UTR of the *MLPH* gene, with high naevus count. The human *MLPH* gene (OMIM #606526) has been shown to be involved in mature melanosome transport within melanocyte before being transferred to keratinocytes. *MLPH* gene encodes a member of the exophilin subfamily of Rab effector proteins known as melanophilin, which acts as a link between the small GTPase melanosome-bound RAB27A and the actin-associated motor protein MYO5A (208). This protein complex plays a crucial role in the melanosome motility in melanocytes, and aberrations in any of the complex components has been shown to result in perinuclear localization of melanosomes and therefore failure to transfer mature melanosomes to adjacent keratinocytes, eventually causing hypopigmentation (209). Human individuals homozygous for a pathogenic *MLPH* mutation (c.102C>T; p.R35W) display Griscelli syndrome type 3, a pigmentary disorder characterized by a hypopigmented phenotype (209–211). The naevus-associated SNP in this work, rs2325813, is predicted to disrupt a binding site of two miRNAs (hsa-miR-185-3p and hsa-miR-762). The presence of the minor allele in the target sequence enhances miRNA binding efficiency, repressing mRNA translation of *MLPH*, and ultimately limiting the formation of RAB27A/Melanophilin/Myosin-5a complex. Thus, reduction of *MLPH* gene expression may cause an abnormal accumulation of mature

melanosomes around the nucleus of melanocytes, resulting in light pigmentation and poor tolerance to sunlight. Interestingly, our results are consistent with the well-known correlation between melanocytic naevus number, a main risk-prediction factor for melanoma incidence, and the propensity to burn, rather than tan, of light-skinned individuals (212). Therefore, genes implicated in functions related with melanosome trafficking, especially the RAB27A/Melanophilin/Myosin-5a membrane transport pathway, would be relevant candidates for additional investigation in further pigmentation and melanoma studies.

WNT/β-catenin signalling has a pivotal role in the formation of melanocytes, since this pathway has been implicated in promoting the development of neural crest-derived melanocytes (213,214). In humans, the WNT pathway is significantly up-regulated in solar lentigines, suggesting that overstimulation of melanocytes proliferation and differentiation play a crucial role in the pathogenic mechanism of solar lentigines (215). Interestingly, in this work we identify a polymorphism, rs7352107, located in the 3'UTR of the *WNT3A* gene that is strongly associated with the presence of solar lentigines. *WNT3A* (OMIM #606359) encodes a WNT ligand that acts through the WNT/β-catenin pathway promoting melanocyte differentiation, and may promote melanoma differentiation as well (213). Furthermore, the minor allele of rs7352107 is predicted to decrease the binding efficiency to the 3'UTR gene region of two microRNAs (hsa-miR-149-5p and hsa-miR-892b), leading to a weaker miRNA-mRNA interaction and therefore a higher level of secreted WNT3A ligand. This probably enhances the activation of the WNT/β-catenin signalling and subsequently the proliferation of melanocytes. These observations, together with the results from Yamada and cols. (2014) (215), suggest that abnormal regulation of melanogenesis via gene expression changes is expected to be involved in several pigmentary disorders and in melanoma risk phenotypes. Thus, studies

focusing on the regulation of WNT/β-catenin signalling could potentially clarify the causal mechanisms of pathogenic hyperpigmentation and hypopigmentation conditions.

The miRNAs predicted to bind to *MLPH* rs2325813 (hsa-miR-185-3p and hsa-miR-762) and to *WNT3A* rs7352107 (hsa-miR-149-5p and hsa-miR-892b) seem to target genes involved in pigmentation mechanisms and skin cancer. Remarkably, out of all significant pathways, "Wnt signalling pathway" and "MAPK signalling pathway" were the only ones targeted by three of the four miRNAs. Furthermore, "Basal cell carcinoma" pathway was also targeted by hsa-miR-185-3p and hsa-miR-762. These observations may corroborate the importance of these miRNAs in both human pigmentation and skin cancer pathways. Based on GTEx project data, genes encoding for these miRNAs, except for hsa-miR-892b, are expressed in sun-exposed skin (Figure S3.3), confirming the expression of these miRNAs in skin tissue, and suggesting a possible role of these miRNAs in skin regulation and function.

Additionally, five polymorphisms displayed a notable statistical association with phenotypic characteristics. Among these SNPs, we would like to highlight that the variant rs4142680, located in the 3'UTR of *HOXC8*, displays an interesting predisposition tendency towards sun-damaged phenotypes. The *HOXC8* gene has been shown to be massively up-regulated in melanoma cancerous cells as a consequence of diminished miR-196a levels, leading to an aggressive melanoma phenotype via the overexpression of several tumorigenic target genes (216). Curiously, the web-based miRNA binding prediction analysis in this work showed an intermediate free energy (-16.60 kcal/mol) for binding hsa-miR-4509 to the 3'UTR sequence containing the rs4142680*T allele, and predicted that presence of the C allele may break the putative binding site. Thus, the association between rs4142680*C and the presence of solar lentigines may be the result

of increased *HOXC8* expression that could be possibly promoting melanocyte proliferation.

In summary, we analysed the potential implications of 3'UTR polymorphisms in pigmentation, sensitivity to sunlight and skin cancer. A plausible cause of the action of these 3'UTR SNPs in the appearance of different sun-related benign pigmented skin lesions might be the differential gene expression attained by disrupting putative miRNA-binding sites. Specifically, we detected two potential associations with well-recognised skin cancer risk traits that modify miRNA-mRNA interactions: rs2325814 in the 3'UTR of the *MLPH* gene and rs752107 in the 3'UTR of the *WNT3A* gene. Future functional studies will be needed to determine the exact implications of these polymorphisms. In addition, we detected five genes that might contribute to pigmentation variation in our population. The fact that *MLPH*, *LYST* and *CLIP1* functions have been related to intracellular membrane trafficking and pigment disorders reinforces the need to explore more deeply the role of melanosome transport pathways in pigmentation and tanning ability. Similarity, the study of genes that are at least partially involved in melanocyte proliferation and differentiation, such as *WNT3A*, *KCNMA1*, *KIT* and *HOXC8*, may allow for the detection of novel low-penetrance genes involved in human pigmentation and in susceptibility to skin cancer.

3.5. SUPPLEMENTARY MATERIAL

Table S3.1. Classification of the Spanish individuals studied by age, sex and phenotype

		Controls (N=343)		Cases (N=526)		*P*-value[1]
		N	%	N	%	
Age	Mean ± SD	52.45 ± 15.95		52.63 ± 15.63		0.269
	< Mean	144	41.98	239	45.44	
	> Mean	143	41.69	280	53.23	
	Unknown	56	16.33	7	1.33	
Sex	Female	172	50.15	270	51.33	0.780
	Male	167	48.69	251	47.72	
	Unknown	4	1.17	5	0.95	
Eye Colour	Dark	239	69.68	337	64.07	0.102
	Light	101	29.45	183	34.79	
	Unknown	3	0.87	6	1.14	
Skin Colour	Dark	151	44.02	228	43.35	0.887
	Fair/Pale	185	53.94	287	54.56	
	Unknown	7	2.04	11	2.09	
Hair Colour	Dark	308	89.80	406	77.19	**4.60E-06**
	Light	33	9.62	113	21.48	
	Unknown	2	0.58	7	1.33	
Lentigines	No	180	52.48	170	32.32	**1.76E-12**
	Yes	131	38.19	347	65.97	
	Unknown	32	9.33	9	1.71	
Naevi number	≤ 50	271	79.01	447	84.98	0.395
	> 50	38	11.08	72	13.69	
	Unknown	34	9.91	7	1.33	
Childhood sunburns	No	220	64.14	170	32.32	**6.64E-27**
	Yes	91	26.53	347	65.97	
	Unknown	32	9.33	9	1.71	

N, number of individuals; %, percentage of individuals per group among the total

[1] Fisher's exact test *P*-value excluding unknown values at each specific analysis.
Significant results are presented in bold

Table S3.2. Minor allele frequencies in different European populations and Spanish cases and controls

Gene	SNP #rs	Chr	mA	Spanish population				CEU population		TSI population	
				HWE *P*-value	MAF Controls	MAF Cases	*P*-value[1]	MAF	*P*-value[2]	MAF	*P*-value[2]
ADAM9	rs4733967	8	T	0.364	0.234	0.209	0.234	0.146	**0.017**	0.262	0.164
ADAMTS20	rs6582463	15	C	0.411	0.270	0.317	**0.038**	0.389	0.155	0.243	**0.006**
BNC2	rs7035049	9	A	0.652	0.401	0.382	0.42	0.404	0.701	0.346	0.234
CLIP1	rs10270	12	A	0.896	0.290	0.320	0.917	0.318	0.691	0.322	0.759
DCT	rs17791924	14	G	0.326	0.449	0.447	0.961	0.465	0.652	0.439	0.827
DTNBP1	rs742106	6	A	1.000	0.359	0.389	0.222	0.354	0.536	0.299	**0.024**
E2F1	rs3213180	20	C	0.577	0.050	0.069	0.102	0.091	0.127	0.051	0.650
E2F2	rs3820028	4	G	0.829	0.469	0.485	0.554	0.520	0.293	0.477	1.000
EDN1	rs9296344	6	C	0.363	0.061	0.048	0.229	0.071	0.321	0.070	0.338
EXOC2	rs12952	6	G	0.414	0.273	0.292	0.384	0.273	0.803	0.383	**0.004**
FOXO3/FKHRL2	rs9400241	6	C	0.328	0.329	0.324	0.875	0.273	0.148	0.364	0.281
GNA11	rs397454	19	T	1.000	0.124	0.113	0.542	0.101	0.559	0.126	0.736
HOXB7	rs15689	17	G	1.000	0.284	0.237	**0.028**	0.247	0.863	0.210	0.156
HOXC8	rs4142680	15	C	0.658	0.426	0.395	0.21	0.394	0.190	0.425	0.482
HRK	rs10507275	12	A	0.102	0.159	0.163	0.841	0.136	0.412	0.131	0.275
IRF4	rs9391997	6	G	0.157	0.459	0.483	0.349	0.500	0.499	0.472	1.000
KCNMA1	rs4980113	10	C	0.589	0.496	0.533	0.128	0.490	0.454	0.490	0.169
KIT	rs8022	5	T	0.815	0.134	0.128	0.769	0.131	1.000	0.079	**0.037**
KITLG	rs995030	12	A	1.000	0.219	0.204	0.507	0.192	0.581	0.131	**0.007**
LYST	rs6696123	13	C	0.187	0.426	0.399	0.294	0.429	0.595	0.430	0.607
MARCKS	rs28558559	6	C	0.272	0.146	0.126	0.219	0.116	0.579	0.126	0.831
MC1R	rs3212369	16	G	1.000	0.187	0.195	0.064	0.146	**0.040**	0.206	0.929
MCAM	rs7914	11	A	1.000	0.224	0.240	0.451	0.263	0.378	0.201	0.302
MCL1	rs878471	22	G	0.269	0.424	0.446	0.373	0.424	0.762	0.421	0.662
MDM2	rs1690916	12	A	0.489	0.374	0.360	0.574	0.515	**0.001**	0.327	0.291
MLPH	rs2325813	1	C	1.000	0.093	0.110	0.295	0.131	0.225	0.117	0.555
MMP7	rs14983	11	A	0.640	0.224	0.227	0.525	0.212	0.593	0.168	**0.037**
MYO5A	rs7176482	9	G	0.573	0.400	0.412	0.88	0.343	0.108	0.472	0.056
NF1	rs1801052	17	G	0.063	0.254	0.246	0.734	0.308	0.085	0.262	0.677
NFAT5	rs7359387	16	G	1.000	0.150	0.137	0.513	0.101	0.391	0.140	0.143
PAX3	rs12620338	2	A	0.309	0.200	0.197	0.902	0.192	0.925	0.215	0.587
PTCH2	rs41269085	2	T	0.437	0.168	0.165	0.947	0.162	0.920	0.168	0.923
PTEN	rs701848	10	C	0.207	0.379	0.399	0.421	0.348	0.249	0.402	0.767
RGS20	rs72614663	8	G	0.269	0.140	0.143	0.888	0.101	0.127	0.187	0.082
SLC24A4	rs11160072	14	G	0.552	0.163	0.149	0.788	0.162	0.678	0.107	0.101
SOX9	rs1042667	11	C	0.653	0.394	0.386	0.801	0.394	0.939	0.336	0.137
TPCN2	rs1551306	11	A	0.829	0.465	0.448	0.49	0.465	0.821	0.542	**0.017**
WNT3A	rs752107	11	T	0.897	0.295	0.300	0.829	0.293	0.935	0.290	0.874

SNP, single nucleotide polymorphism; Chr, chromosome; mA, minor allele; MAF, minor allele frequency; CEU, Northern Europeans form Utah; TSI, Southern Europeans from Tuscany; HWE, Hardy-Weinberg equilibrium

[1] Fisher's exact test *P*-values for the comparison of minor allele frequencies between Spanish cases and controls

[2] Fisher's exact test *P*-values for the comparison of Spanish minor allele frequencies obtained from our sample to CEU and TSI frequencies

Significant results are presented in bold

Table S3.3. Association analysis between SNPs and melanoma susceptibility in the Spanish population

			Non adjusted		Adjusted [1]	
Gene	**SNP rs#**	**mA**	**OR (%95 CI)**	***P*-value**	**OR (%95 CI)**	***P*-value**
ADAM9	rs4733967	T	**0.34 (0.17-0.70)**	**0.0024**	**0.26 (0.10-0.63)**	**0.0026**
ADAMTS20	rs6582463	C	**1.25 (1.01-1.54)**	**0.0390**	1.16 (0.91-1.48)	0.2389
BNC2	rs7035049	A	0.77 (0.53-1.13)	0.1884	0.66 (0.42-1.04)	0.0732
CLIP1	rs10270	A	1.15 (0.93-1.42)	0.1857	1.18 (0.66-2.09)	0.5735
DCT	rs17791924	G	1.14 (0.86-1.49)	0.3618	0.74 (0.50-1.09)	0.1312
DTNBP1	rs742106	A	1.14 (0.93-1.39)	0.2126	1.16 (0.91-1.47)	0.2332
E2F1	rs3213180	C	1.42 (0.94-2.16)	0.0906	1.41 (0.86-2.32)	0.1652
E2F2	rs3820028	G	1.06 (0.88-1.29)	0.5283	0.99 (0.78-1.24)	0.9091
EDN1	rs9296344	C	0.78 (0.51-1.17)	0.2360	0.79 (0.46-1.36)	0.3961
EXOC2	rs12952	G	1.11 (0.89-1.38)	0.3580	1.11 (0.85-1.44)	0.4415
FOXO3	rs9400241	C	0.91 (0.60-1.40)	0.6783	0.84 (0.66-1.07)	0.1576
GNA11	rs397454	T	0.87 (0.62-1.22)	0.4240	0.68 (0.45-1.01)	0.0593
HOXB7	rs15689	G	**0.78 (0.63-0.97)**	**0.0264**	**0.77 (0.59-1.00)**	**0.0483**
HOXC8	rs4142680	C	0.83 (0.62-1.10)	0.1971	0.82 (0.65-1.04)	0.0968
HRK	rs10507275	A	0.44 (0.19-1.05)	0.0602	0.45 (0.18-1.12)	0.0826
IRF4	rs9391997	G	1.29 (0.92-1.80)	0.1422	**0.67 (0.48-0.93)**	**0.0152**
KCNMA1	rs4980113	C	0.86 (0.71-1.04)	0.1220	**0.79 (0.62-1.00)**	**0.0462**
KIT	rs8022	T	0.85 (0.62-1.19)	0.3474	1.63 (0.48-5.58)	0.4228
KITLG	rs995030	A	0.92 (0.72-1.16)	0.4719	0.84 (0.60-1.17)	0.3104
LYST	rs6696123	C	0.82 (0.61-1.09)	0.1655	0.85 (0.67-1.08)	0.1745
MARCKS	rs28558559	C	**0.32 (0.11-0.93)**	**0.0300**	**0.23 (0.06-0.81)**	**0.0164**
MC1R	rs3212369	G	1.06 (0.83-1.35)	0.6679	1.32 (0.59-2.95)	0.5016
MCAM	rs7914	A	1.14 (0.87-1.51)	0.3498	1.21 (0.86-1.69)	0.2695
MCL1	rs878471	G	1.20 (0.84-1.72)	0.3238	1.30 (0.85-2.00)	0.2261
MDM2	rs1690916	A	0.83 (0.56-1.24)	0.3688	0.90 (0.72-1.14)	0.4006
MLPH	rs2325813	C	1.20 (0.87-1.66)	0.2556	0.65 (0.15-2.85)	0.5742
MMP7	rs14983	A	1.37 (0.73-2.57)	0.3240	1.36 (0.64-2.88)	0.4216
MYO5A	rs7176482	G	1.13 (0.79-1.62)	0.5110	1.15 (0.75-1.75)	0.5168
NF1	rs1801052	G	0.92 (0.56-1.50)	0.7274	0.82 (0.45-1.50)	0.5239
NFAT5	rs7359387	G	0.87 (0.62-1.20)	0.3880	1.06 (0.72-1.55)	0.7821
PAX3	rs12620338	A	0.68 (0.35-1.34)	0.2656	0.47 (0.20-1.06)	0.0710
PTCH2	rs41269085	T	**2.29 (1.00-5.39)**	**0.0421**	**0.66 (0.46-0.95)**	**0.0263**
PTEN	rs701848	C	1.26 (0.96-1.65)	0.0998	1.04 (0.82-1.31)	0.7560
RGS20	rs72614663	G	2.15 (0.70-6.65)	0.1587	3.14 (0.79-12.46)	0.0818
SLC24A4	rs11160072	G	0.87 (0.65-1.17)	0.3649	0.86 (0.59-1.23)	0.4043
SOX9	rs1042667	C	0.96 (0.72-1.27)	0.7573	0.87 (0.69-1.10)	0.2360
TPCN2	rs1551306	A	0.86 (0.61-1.20)	0.3748	0.84 (0.59-1.20)	0.3419
WNT3A	rs752107	T	1.14 (0.71-1.82)	0.5913	0.72 (0.51-1.00)	0.0500

SNP, single nucleotide polymorphism; mA, minor allele; OR, odds ratio per minor allele; CI, confidence interval

Bold indicates significant *P*-values and their Odds Ratio according to the most significant model (dominant, over-dominant, recessive or additive)

[1] Adjusted for childhood sunburns, hair colour, lentigines and sex, via multivariate logistic regression

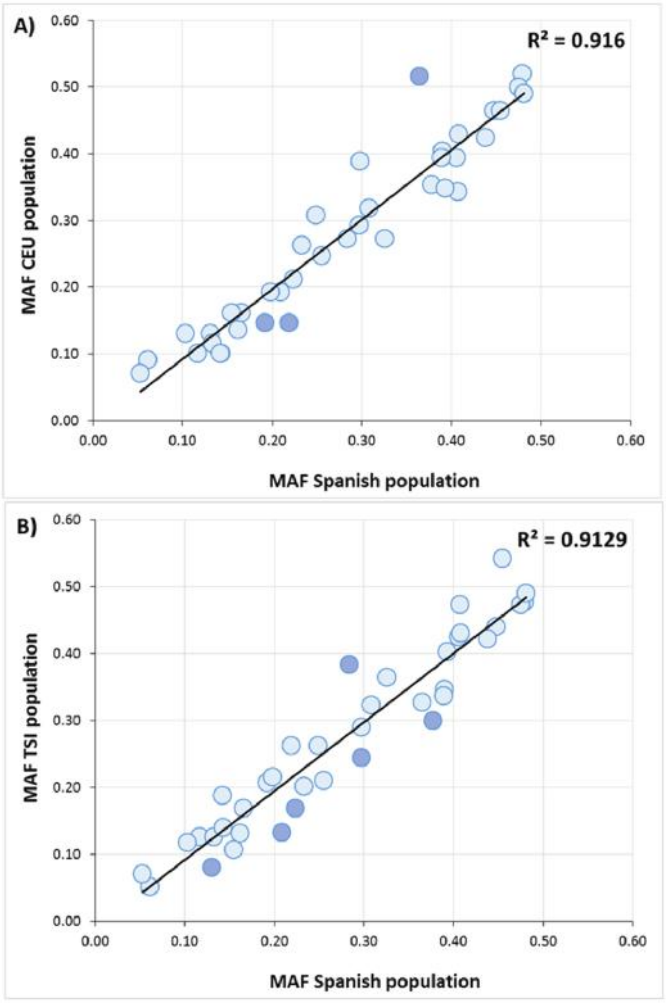

Figure S3.1. Comparison of minor allele frequencies between our Spanish sample and two different European populations

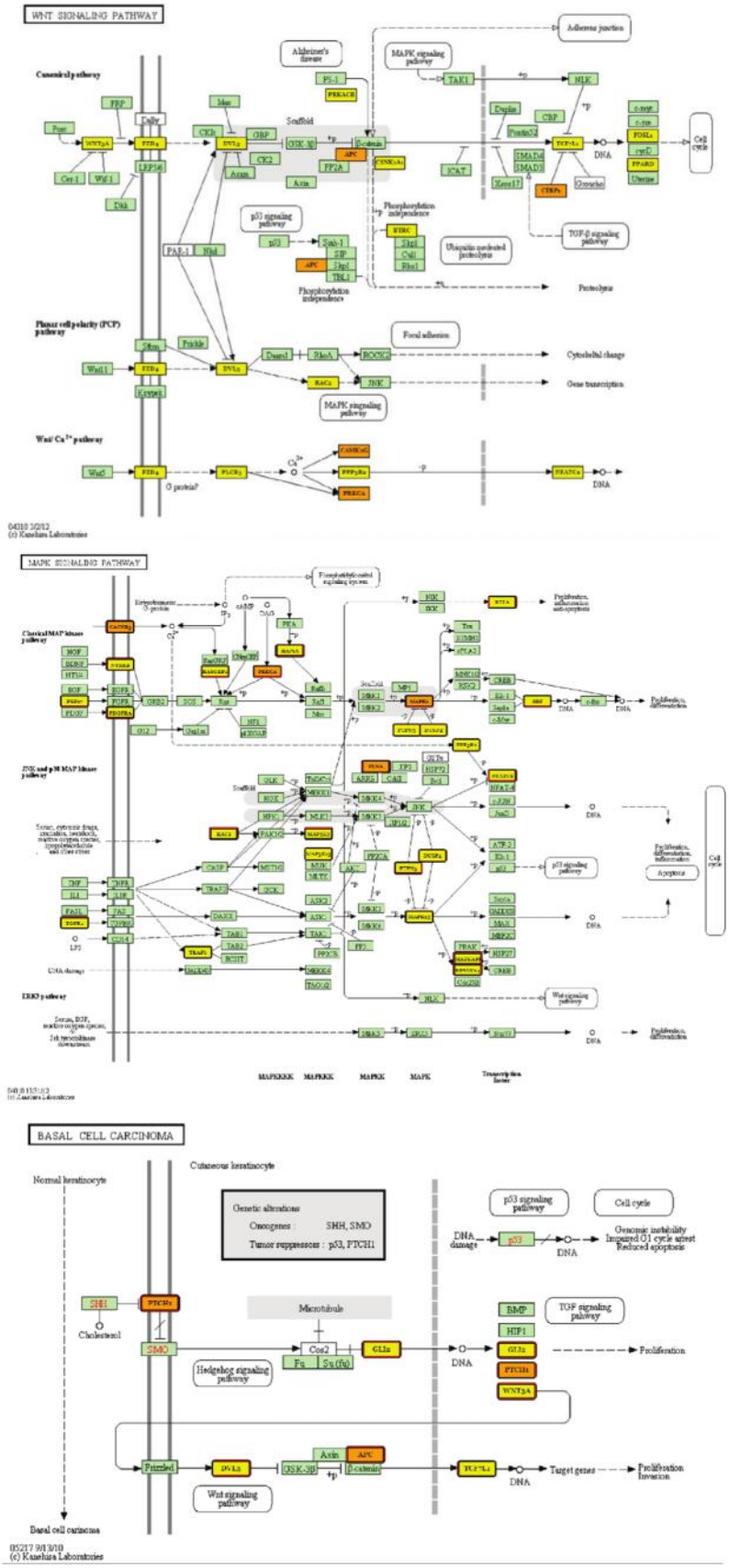

Figure S3.2. Enriched KEGG pathways involved in pigmentation and skin cancer risk that are targeted by miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs

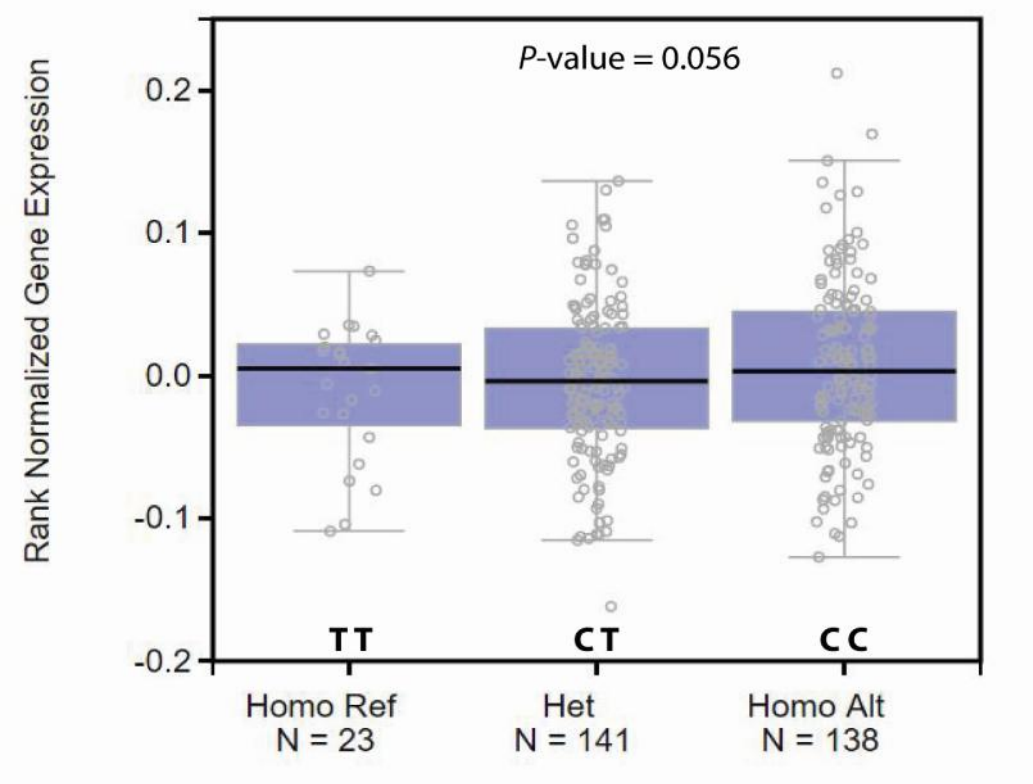

Figure S3.3. Box plot showing WNT3A expression according to SNP rs752107 genotype

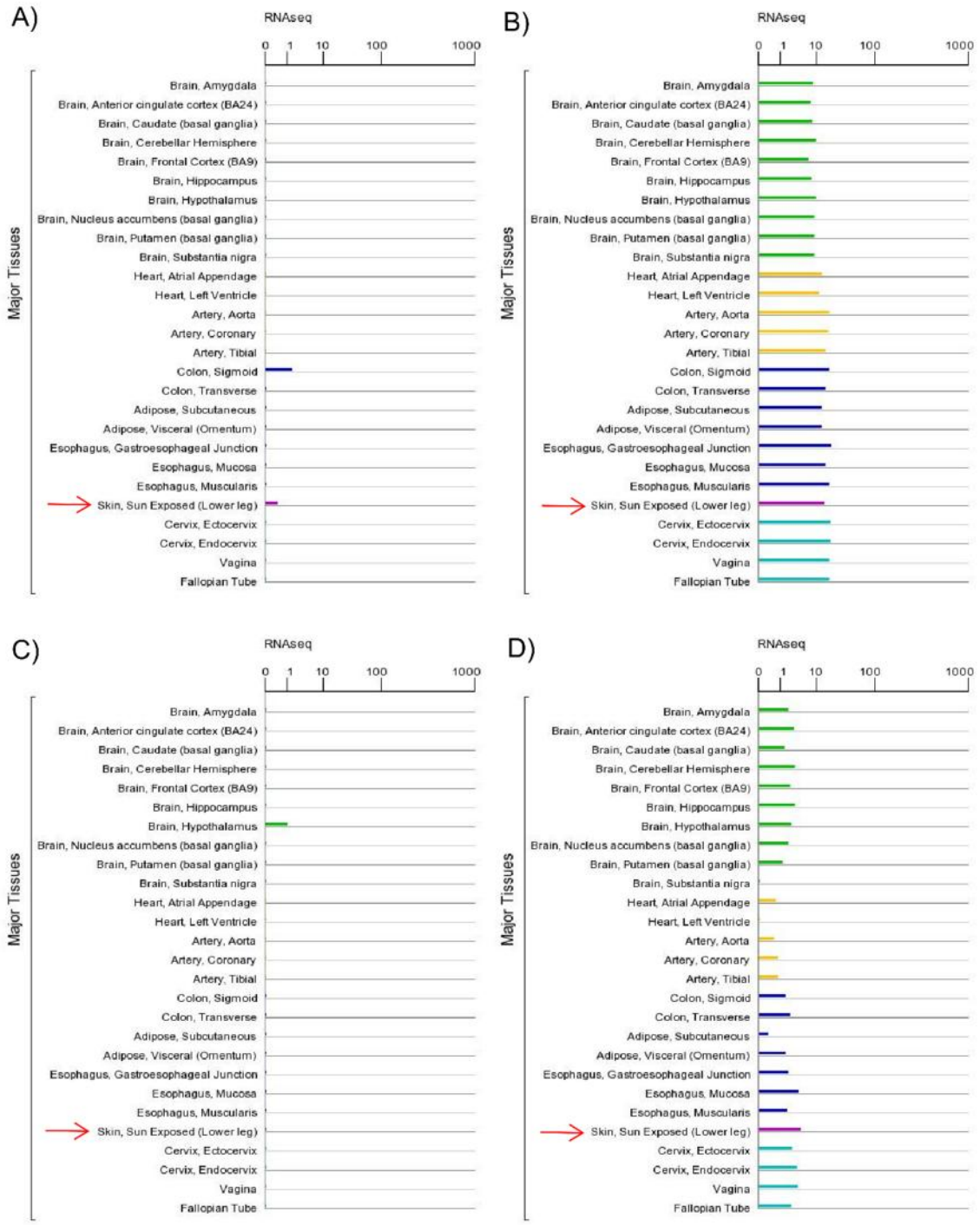

Figure S3.4. Expression in different tissues of the four miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs

Chapter 4

Capturing the synergistic effects of CDKN2A and MC1R germline mutations on human melanocyte proliferation and senescence, and on melanoma predisposition

This work was performed during a four-month stay in the Department of Dermatology of the University of Cincinnati under the supervision of Professor Zalfa Abdel-Malek

4.1. INTRODUCTION

Gene-environment interactions play a significant role in melanoma susceptibility. Random somatic mutations resulting mainly from exposure to solar ultraviolet radiation (UV-signature mutations) in key genes within melanocytes act as triggers that initiate sporadic melanoma and promote its progression (104). Additionally, the presence of heritable germline variations in high- and low-penetrance susceptibility genes is a well-known risk factor associated with both familial – which accounts for about 10% of all melanoma cases – and sporadic cutaneous melanoma (217). Therefore, germline mutations in these susceptibility genes may impact on the sensitivity of human melanocytes to solar UV and on the malignant transformation to melanoma.

To date, the major genetic locus implicated in familial melanoma is the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), located on chromosome 9p21. The *CDKN2A* locus codes for two important tumour suppressor proteins, $p16^{INK4A}$ (p16) and $p14^{ARF}$ (p14), which are transcribed from different reading frames (115). In response to DNA damage induced by chronic exposure to sunlight, p16 delays cell proliferation through inhibition of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and cyclin D1 (CCND1), thus preventing the phosphorylation of the retinoblastoma protein (Rb) and blocking cell cycle progression from G1 to S phase (115,149). This cell cycle arrest allows cells to repair DNA damage before resuming cell division. In addition, p16 induces cellular senescence – the acquired phenomenon whereby a proliferation-competent somatic cell undergoes inability to divide in response to stressful stimuli (218). Recently, p16 has been reported to play a role in counteracting intracellular oxidative stress in a manner independent of cell cycle arrest (219). Taking all these functions together, p16 prevents melanomagenesis by activating multiple innate mechanisms that enable melanocytes to evade malignant

transformation. p14 also restricts cell proliferation by stabilizing p53, which in turn induces the expression of $p21^{Cip1}$ (p21). This cyclin-dependent kinase inhibitor, encoded by the *CDKN1A* gene, inhibits cell cycle progression both through inhibition of CCND1 and CDK4/6, as well as CDK2 and cyclin E1 (CCNE1). Thus, both the p16/Rb and the p14/p53/p21 tumour suppressor pathways have been implicated in cell growth arrest, although in melanocytes the p16/Rb pathway seems to be the dominant senescence driver (220,221).

The *CDKN2A* gene is mutated, deleted or silenced in a wide range of human cancers (222). Germline mutations in *CDKN2A* have been implicated in melanoma susceptibility and are present in approximately 40% of melanoma families (148). Mutations occurring in p16 are predominantly loss-of-function missense mutations, with the V126D, G101W and 32ins24 mutations being the most common in familial melanoma pedigrees in North America (223). In rare instances, a germline *CDKN2A* promoter mutation (−34G>T), which causes a novel initiation codon that initiates translation of an aberrant protein, and decreases translation from the wild-type start codon, has also been identified in British-origin melanoma-prone families (223,224).

Despite other known high penetrance genes having been described in the literature up to now, the genetic basis for predisposition remains unexplained for a large percentage of familial melanoma cases. Indeed, the penetrance of *CDKN2A* mutations was found to vary among carriers, revealing that genetic backgrounds, host characteristics, and/or sun exposure may also contribute to melanoma risk (45,225). In this setting, it has been suggested that skin cancer is a polygenic mechanism of inheritance that includes multiple low-penetrance risk alleles, such as variants of the melanocortin-1 receptor (*MC1R*) gene. The *MC1R* gene encodes a seven-pass transmembrane G-protein coupled receptor that is expressed on the cell surface of cutaneous and follicle melanocytes (33). Stimulating of

human MC1R by its ligands (α-MSH and ACTH) leads to the activation of cAMP signalling pathway, which in turn triggers a wide range of responses in human melanocytes, including synthesis of eumelanin, melanocyte proliferation, reduction of UV-induced oxidative stress, and enhancement of DNA repair (33,34,226,227). The *MC1R* gene is highly polymorphic in populations of European ancestry, with more than 100 non-synonymous variants identified (35,40). These *MC1R* genetic variants are major determinants of human pigmentation diversity and, especially, sun sensitivity and tanning ability (34). Epidemiological studies have provided compelling evidence for the association of red hair colour (RHC) *MC1R* variants (R151C, R160W and D294H) with melanoma risk (43). The expression of one *MC1R* RHC allele was also reported to increase the risk for UV-signature somatic mutations (228), which underscores the significance of *MC1R* as a melanoma predisposition gene.

There is a strong epidemiological evidence that *MC1R* RHC variants, as well as certain non-RHC variants (V60L, V92M and T314T), significantly increase melanoma risk in *CDKN2A* mutation carriers of melanoma families, suggesting that these two genes interact synergistically to increase melanoma susceptibility (225,229–231). However, the molecular mechanisms by which mutations in these two genes interact to drive the malignant transformation of melanocytes remain unknown.

Stress-induced melanocyte senescence response may be the link between *CDKN2A* and *MC1R*, since melanocytes depleted of p16 exhibit extended replicative lifespan in the presence of replication-associated DNA damage (220), and chronic activation of the cAMP pathway enhances replicative senescence in melanocytes (232). Together, these findings raised the biological question of whether the co-expression of loss-of-function mutations in these two genes synergistically exacerbate the risk of melanoma

development by reducing the ability of UV-irradiated melanocytes to undergo senescence.

Other plausible mechanisms for overstated melanoma risk in carriers of both *p16* and *MC1R* mutations may be a reduced capacity to repair DNA damage and to initiate antioxidant responses. Loss of p16 impairs cells to repair UV-induced DNA damage (233), and is associated with increased oxidative stress (219). Also, activation of MC1R prevents the generation of reactive oxygen species (ROS) and enhances repair of DNA photoproducts and oxidative DNA damage in human melanocytes, while loss-of-function MC1R compromises NER and sustains oxidative stress (226,234,235).

To date, there are no studies on the dual action of *p16* and *MC1R* mutations on the maintenance of genomic stability of human melanocytes and their response to the damaging effects of UV exposure. Using primary cultures of human melanocytes with different *p16* and *MC1R* genotypes, which were established from skin biopsies of melanoma-prone family members, we have investigated the ability of these melanocytes to proliferate and undergo stress-induced senescence, as a result of both serial passages in culture and UV exposure.

4.2. MATERIALS AND METHODS

4.2.1. Primary melanocyte cultures

Primary human melanocyte cultures were established from discarded neonatal foreskins or adult skin from anonymous donors undergoing plastic surgery procedures. The protocol for obtaining these skin samples was considered exempt from approval by the

Institutional Review Board of the University of Cincinnati. Melanocyte cultures were also established from skin biopsies obtained from patients of the Melanoma Clinic at the Huntsman Cancer Institute, University of Utah, after informed consent. The p16-deficient melanocyte cultures from two rare individuals worldwide, carrying mutations in both copies of the *CDNK2A* locus, were obtained from St. George's Hospital Medical School (London, United Kingdom). All primary melanocyte cultures included in this study are described in Table 4.1.

Melanocytes were grown in a humidified atmosphere at 37 ºC with 5% carbon dioxide. All cell cultures, except p16-deficient melanocytes, were maintained in MCDB-153 media (Sigma-Aldrich, San Luis, MO, USA) supplemented with foetal bovine serum (FCS, 2%), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), penicillin/streptomycin (PS, 1%), human basic fibroblast growth factor (bFGF, 1 ng/ml), endothelin-1 (1 nM), and bovine pituitary extract (BPE, 28 μg/ml). Due to p16-deficient melanocytes having more stringent requirements for proliferation than p16 wild-type or heterozygous melanocytes (220), these primary cultures were maintained in our routine melanocyte growth medium enriched with 10% foetal bovine serum, twice the concentration of BPE, and 10 nM endothelin-1 (ET-1). For media comparison experiments, at least three weeks before performing all the experiments, routine growth medium was replaced by the modified melanocyte growth medium, or vice versa.

Table 4.1. List of primary human melanocyte cultures used in the current study

HM strains	Age	Sex	*CDKN2A* genotype	*MC1R* genotype
1n	54	F	Homozygotes for 266_244del19 (236)	ND
2d	23	–	M53T / D108N (237)	ND
3vw	28	M	V126D / +	+ / +
4vw	20	F	V126D / +	+ / +
5vR	42	M	V126D / +	R160W / R151C
6vR	35	F	V126D / +	R160W / +
7vR	31	F	V126D / +	D294H / +
8vR	18	M	V126D / +	D294H / +
9uR	21	M	-34G>T / +	R160W / +
10uR	18	M	-34G>T / +	R160W / +
11uR	24	M	-34G>T / +	D294H / +
12ur	22	M	-34G>T / +	V60L / +
13iR	32	F	32ins24 / +	R160W / V60L
14iR	22	M	32ins24 / +	R160W / +
15wR	23	F	+ / +	R151C / +
16wR	–	F	+ / +	V92M / R151C
17wR	–	M	+ / +	R151C / R151C
18wR	43	F	+ / +	R160W / +
19wR	13	M	+ / +	R160W / R160W
20wR	26	F	+ / +	D294H / +
21wR	35	F	+ / +	D294H / +
22wR	–	M	+ / +	D294H / V92M
23wr	–	–	+ / +	V92M / R142T
24ww	–	F	+ / +	+ / +
25ww	28	F	+ / +	+ / +
26ww	43	F	+ / +	+ / +
27ww	Neonatal	–	+ / +	+ / +
28ww	Neonatal	M	+ / +	+ / +
29ww	Neonatal	M	+ / +	+ / +

Strains name:

Digit refers to cell strain order

First letter refers to *CDKN2A* genotype (n, p16-null; d, p16-deficient; v, V126D carrier; u, -34G>T 5'UTR carrier; i, 32ins24 carrier; w, wild-type)

Second letter refers to *MC1R* genotype (R, RHC carrier; r, non-RHC carrier; w, wild-type)

Abbreviations: HM, human melanocyte; F, female; M, male; +, wild-type allele; –, no data; ND, not determined

4.2.2. Irradiation of human melanocytes with UV

Melanocytes were irradiated with a dose of 75 mJ/cm^2 using a bank of FS20 lamps with peak emission at 313 nm wavelength, with 75% UVB and 25% UVA emissions. A Kodacel filter was used to block UVC rays (Eastman Kodak, Rochester, NY, USA). Prior to irradiation, the culture medium was replaced by PBS. After UV exposure, PBS was removed and fresh medium was added.

4.2.3. Western blot analysis

Western blotting was carried out to compare protein expression of the human melanocyte strains with different genotypes. Protein extracts derived from melanocytes were obtained by using RIPA buffer supplemented with a combination of protease and phosphatase inhibitors, including sodium vanadate, microcystin, sodium fluoride, phenylmethylsulfonyl fluoride, leupeptin and aprotinin (Sigma-Aldrich). Protein concentration was quantified using the Pierce BCA Protein Assay kit (ThermoFisher Scientific), employing serial dilutions of bovine serum albumin (BSA) to generate the protein assay standard curve. Total protein of 50 μg from each sample was mixed with protein loading buffer and denatured using heating block at 95°C for 5 min. Then, proteins were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking of membranes in 5% skim milk for 1 hour at room temperature, primary antibodies against the following proteins were used: p16 (ab201980, 1:500 dilution, Abcam, Cambridge, UK), phospho-Rb Ser807/811 (#8516, 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), Rb (#9313S, 1:1000 dilution, Cell Signaling Technology), phospho-p53 Ser15 (#92845,

1:500 dilution, Cell Signaling Technology), p53 (sc-126, 1:500 dilution, Santa Cruz), p21 (#05-655, 1:2000 dilution, EMD Millipore, Billerica, MA, USA), phospho-JNK Thr183/Tyr185 (#9251, 1:1000 dilution, Cell Signaling Technology), JNK (#9252, 1:1000 dilution, Cell Signaling Technology), phospho-p38 Thr180/Tyr182 (#9211, 1:1000 dilution, Cell Signaling Technology), and p38 (#9212, 1:1000 dilution, Cell Signaling Technology). The membranes were incubated overnight at 4ºC, and washed three times for 5 min each with 0.1% TBST. Subsequently, the membranes were incubated with the appropriate HRP-conjugated anti-rabbit or anti-mouse immunoglobulin G (EMD Millipore) for 2 hours at room temperature. Following washing, as above, the membranes were exposed to Protoglow ECL (National Diagnostics, Atlanta, GA, USA) and bands were detected with the Molecular Imager VersaDoc System (Bio-Rad). Then, band densities were quantified by Image Lab software (Bio-Rad) and normalized to the loading control. As loading controls, antibodies against actin (#sc-1615) and vinculin (#sc-25336) were used (1:10000 dilution, Santa Cruz Biotechnology). Relative protein expression in UV-irradiated samples was calculated based on the signal intensity of the untreated (control) sample.

4.2.4. Determination of melanocyte proliferation

Melanocytes were seeded on 60 mm dishes at a density of $0.5x10^6$ cells/dish. On days 3, 5, 7, 9, 11, and 13 after seeding and/or UV exposure, human melanocytes were detached with trypsin, harvested, and counted in triplicates using the Z1 Brekman Coulter Counter (Brekman Coulter, Pasadena, CA, USA) to generate growth curves. Then, doubling times were calculated from the growth rate.

4.2.5. Detection of senescence-associated β-galactosidase activity

Cultured cells were seeded at a density of $2x10^4$ cells/well in 8-well chamber slides. After 2-3 days, β-galactosidase activity was detected using Senescence β-Galactosidase Staining Kit (#9860, Cell Signaling), following manufacturer's recommendations. Briefly, melanocytes were washed with PBS and fixed with Fixative Solution for 15 minutes at room temperature. Then, the cells were rinsed twice with PBS before incubating with X-gal Staining Solution for overnight at 37°C. Fluoromount G was used as a mounting medium (Southern Biotechnology, Birmingham, AL, USA). Melanocytes were examined under microscope for blue staining, and photographs were acquired using Spot Imaging software. Quadruplicate samples per cell line were evaluated, and data was expressed as the mean value ± SEM. At least 500 cells were analysed per group. Neonatal cell cultures were used as a negative control, while senescent cultured melanocytes gave a positive control.

4.2.6. Determination of melanocyte apoptosis

Forty-eight hours after UV irradiation, melanocytes were harvested and stained with APC-Annexin V (BD Pharmingen, San Diego, CA) and propidium iodide (PI; Sigma-Aldrich) following the recommended procedure. Samples were analysed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA). Triplicate samples were included per group, and 10,000 events were analysed per sample. The following control groups were used to set up flow cytometry analysis: unstained cells (negative control), cells stained with PI only (necrotic) and cells stained with Annexin only (early stage of apoptosis).

4.2.7. Cell cycle analysis

Cultured cells were seeded on 60 mm dishes at a density of $0.3x10^6$ cells/dish. Two days after plating, melanocytes were either untreated (control) or irradiated with UV75, harvested, and fixed after 0, 24, 48 and 72 h. Cell fixation was performed in ice-cold with 1% paraformaldehyde for 15 min followed by fixation in 70% cold ethanol for 1 hour. Cells were exposed to 10 μM 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) for 2 hours before the appropriate time point. Melanocytes were permeabilized and DNA denatured with PBS containing 0.5% Triton X-100 and 2N HCl for 30 min at room temperature. Nonspecific binding sites were blocked with 0.8 % foetal serum albumin (FSA) in PBS for 1 hour at room temperature. Then, cells were stained with anti-BrdU antibody (1:200 dilution, Cell Signalling Technology) for 1 hour at room temperature, followed by incubation with goat anti-mouse Alexa Fluor 488 IgG secondary antibody (1:200 dilution, ThermoFisher Scientific) for 45 min. Melanocytes were suspended in 1 mg/ml RNase and 5 μg/ml propidium iodide in PBS and analysed by flow cytometry. Triplicate samples per group were evaluated, and 10,000 events were collected per sample. BrdU-unstained cells were used as negative control.

4.2.8. Statistics analysis

Student's t-test was used to compare the effects of treatment/conditions on each individual melanocyte cultures. The responses to different treatment/conditions of melanocyte cultures with different genotypes were compared using ANOVA, followed by Tukey's test for pairwise comparisons.

4.3. RESULTS AND DISCUSSION

Twenty-nine primary cultures of human melanocytes with known *p16* and *MC1R* genotypes were established from skin biopsies of individuals (Table 4.1). Individuals with the *p16* mutations V126D, -34G>T, or 32ins24 were members of melanoma-prone families. Additionally, we tested two melanocyte cultures homozygous for *p16* mutations, one with a specific 19-pb deletion in exon 2 of the *CDKN2A* locus (266_244del19, also known as p16-Leiden), resulting in a severely truncated p16 protein (236); and the other being a compound heterozygote, carrying a different single base change in each copy of the gene (M53T and D108N). Although both point mutations impair p16 function, D108N variant presented some capacity for binding to CDK4/6, while the binding affinity was completely absent for M53T (237). Since familial melanoma patients are predominantly heterozygous for a *p16* mutation, these two cultures served as an ideal control for the remaining cultures harbouring one mutant *p16* allele.

4.3.1. Effect of *p16* genotype on melanocyte growth and senescence

Firstly, p16 basal expression was investigated by western blot in the established melanocyte cultures (Figure 4.1). Except for the strains established from donors carrying two p16 mutant alleles (1n and 2d), all cultures were maintained under identical growth conditions, and proteins were extracted at the same time. All cultures, regardless of genotype, were found to express p16 at different levels, with the exception of strain 1n, which showed no expression of p16 – confirming that these melanocytes are certainly p16-null. For the two melanocyte cultures established from 32ins24 carriers, the detection of a higher molecular weight band representing the aberrant protein confirmed the

expression of the mutant allele of the gene. No correlation between expression levels of p16 and *MC1R* genotype were observed.

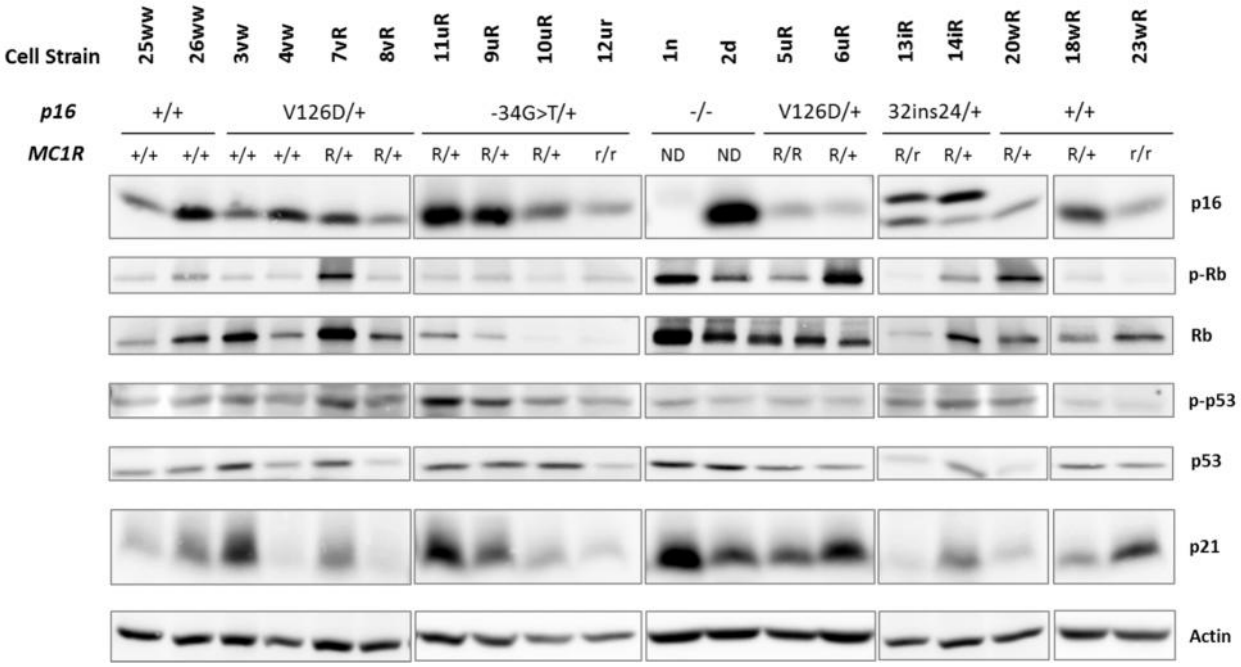

Figure 4.1. Basal protein level comparison of different tumour suppressor proteins in a panel of cultured human melanocytes with different *CDKN2A* and *MC1R* genotypes. Actin was used as loading control

Additionally, we examined the basal expression of other tumour suppressor proteins (Figure 4.1). Higher levels of phospho-Rb (p-Rb), an indicator of cell cycle progression, were observed in the most proliferative melanocyte cultures (6vR and 20wR), and were also correlated with the low p16 levels observed in these strains. Diploid p16-deficient melanocytes (2d) showed high levels of both p16 and p-Rb, suggesting that p16 was not functional in this cell strain, although some residual activity may remain as described previously (237). As expected, absence of p16 in p16-null melanocytes (1n) resulted in a hyperphosphorylation of Rb. The lower expression of p-Rb observed in V126D, 32ins24 and -34G>T heterozygous melanocytes, as well as those homozygous for the wild-type allele, suggested that p16 was functional in all these strains. All these cultures expressed

p53 at different levels, although the lowest p53 expression was found in the two wild-type melanocyte cultures (25ww and 26ww). The expression of p21 did not correlate with the levels of p53, suggesting that p21 expression is not entirely p53-dependent. Indeed, strains with high p21 expression (1n, 2d, 9uR, 11uR, 23wr, and 6vR) showed variable cell proliferation rates (Table 4.2). These results did not show a clear role for p21 in the arrest of cell growth, although it is possible that p21 expression is a compensatory mechanism for loss or reduced p16 function.

Table 4.2. Comparison of doubling times in days of human melanocyte cultures expressing different *CDKN2A* and *MC1R* genotypes at different passages

CDKN2A Genotype	Cell Strain	*MC1R* Genotype	Cell Passage	Doubling Time (days)
V126D / +	**4vw**	+ / +	P4	3.01
			P9	10.03
	3vw	+ / +	P5	5.32
			P11	NG
	8vR	D294H / +	P3	3.18
			P8	9.51
	7vR	D294H / +	P6	6.07
			P12	19.04
	6vR	R160W / +	P4	4.49
			P10	9.14
	5vR	R160W / R151C	P5	7.00
-34G>T / +	**12ur**	V60L / +	P4	13.41
	11uR	D294H / +	P4	2.76
			P9	10.47
	9uR	R160W / +	P4	7.31
			P9	15.54
32ins24 / +	**13iR**	R160W / V60L	P5	NG
	14iR	R160W / +	P4	10.60
+ / +	**18wR**	R160W / +	P2	3.71
	19wR	R160W / R160W	P7	12.31
	15wR	R151C / +	P7	7.22
	16wR	V92M / R151C	P3	9.12
	17wR	R151C / R151C	P9	11.31
	20wR	D294H / +	P9	3.98
	25ww	+ / +	P5	9.08
	26ww	+ / +	P3	7.01
	29ww	+ / +	P6	4.44
	28ww	+ / +	P9	3.86

Abbreviations: +, wild-type allele; P, cell passage number; NG, inability to growth (cell numbers remained the same throughout the course of the experiment)

We next compared the proliferation rates of early-passage melanocyte cultures to the late-passage cultures in all melanocyte strains (Table 4.2). The strains expressing one *p16* mutant allele showed an increase in the doubling time with passage in culture, suggesting that those melanocytes may undergo a senescence-like proliferation arrest in culture, known as replicative senescence. This behaviour differs from that of melanocytes from hemizygous mice (*CDKN2A*$^{+/-}$), which showed some deceleration of growth but did not senesce in culture (238). The proliferation rate of melanocytes from different donors varied widely, and did not correlate with the age of the donor. For example, primary cultures established from a 42-year old donor (5vR) presented the same doubling time than melanocytes from a 21-year old donor (9uR) and less proliferation rate than melanocytes from a 28-year old donor (25ww) at comparable passage number. Indeed, some adult melanocyte cultures even presented lower doubling time than neonatal melanocytes. Our results also revealed that proliferation rates were not affected by V126D and -34G>T mutations – other than 12ur –, regardless of *MC1R* genotype. However, the 32ins24 mutation resulted in a prolongation of the doubling time at early passages, with a notable growth arrest in the 13iR strain. Under the same growth conditions as the other melanocyte cultures, 1n and 2d had a very low proliferation rate, as previously reported (220). Despite lack of growth in our regular melanocyte growth media, cells did not look senescent (small, bipolar, and not or barely pigmented) (Figure 4.2B). In agreement with our results, senescence was absent in mouse fibroblasts and melanocytes homozygous for the same *CDKN2A* locus deletion (238,239).

In parallel with cell proliferation analysis, acidic β-galactosidase activity – a senescence biomarker also referred to as senescence-associated β-galactosidase (SA-β-Gal) – was determined in melanocytes over passage in culture (Figure 4.2). In accordance with growth rates, a significantly increased number of cells with SA-β-Gal activity at late

passage was found, as compared to early passage (Figure 4.2A). This was observed in all adult melanocyte cultures tested, with the exception of p16-null (1n) and p16-deficient (2d) strains. Despite their null growth rate, these strains had even a slightly lower percentage of SA-β-Gal positive cells than neonatal melanocytes, which were used as a negative control due to their high proliferation rate (Figure 4.2B). As a positive control, we used two melanocyte cultures (21wR and 22wR) that were in growth arrest for months, and those presented more than 90% of the melanocytes with an intense blue stain associated with SA-β-Gal activity. All together, these results support the critical role of p16 in melanocyte senescence, and confirm why *CDKN2A* is considered a melanoma suppressor gene (240).

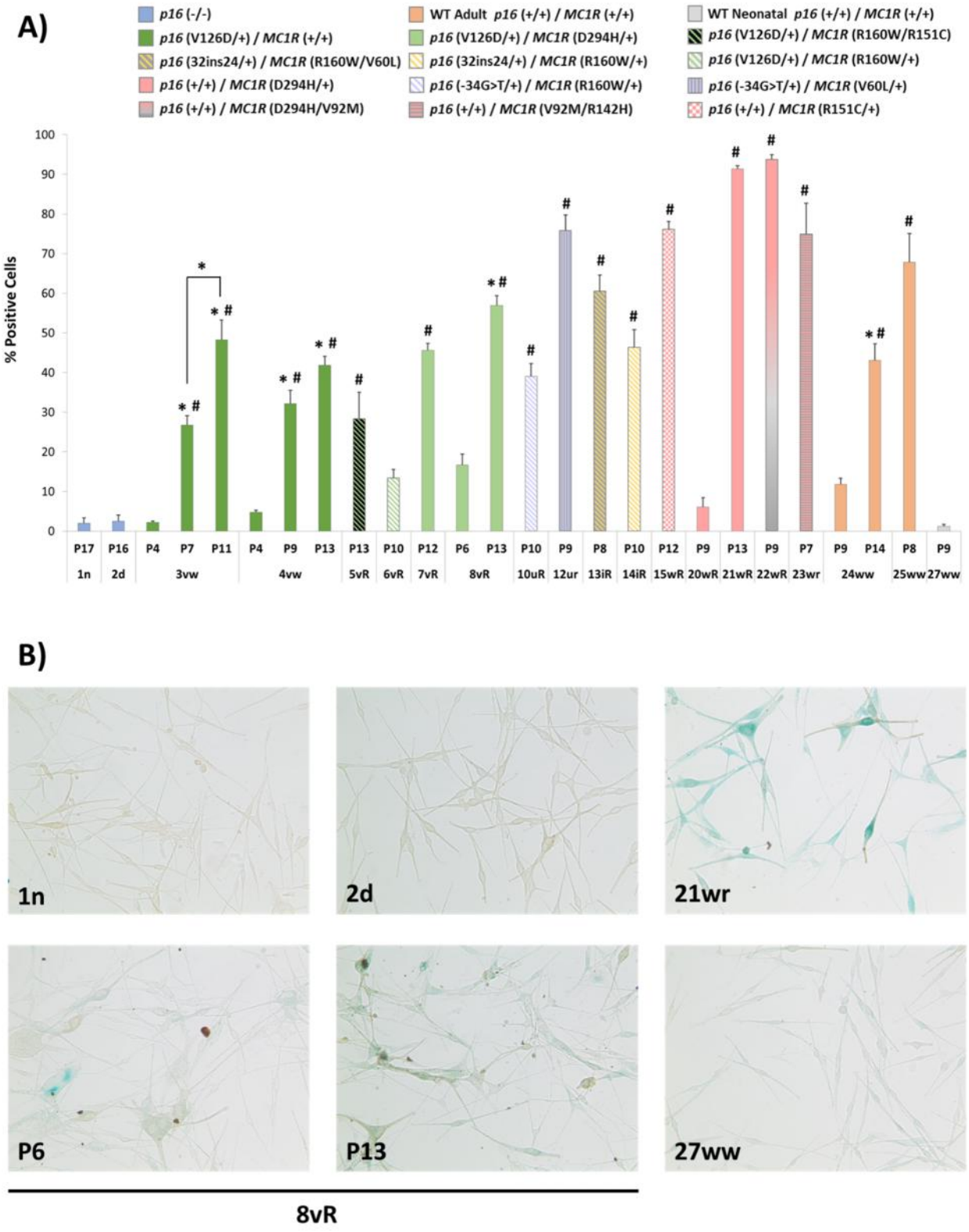

Figure 4.2. Senescence-associated β-galactosidase activity in melanocytes with different *CDKN2A* and *MC1R* genotypes at different cell passages. A) Quantification of the percentage of SA-β-Gal positive melanocytes over passage in culture. Each bar represents the mean ± SEM of at least 500 melanocytes counted in four determinations. **P*-value < 0.01 *vs.* early passage. # *P*-value < 0.01 *vs.* wild-type neonatal strain. **B)** Light-microscopy images of the melanocyte cultures stained for detecting SA-β-Gal activity. Pictures were performed at 20x magnification.

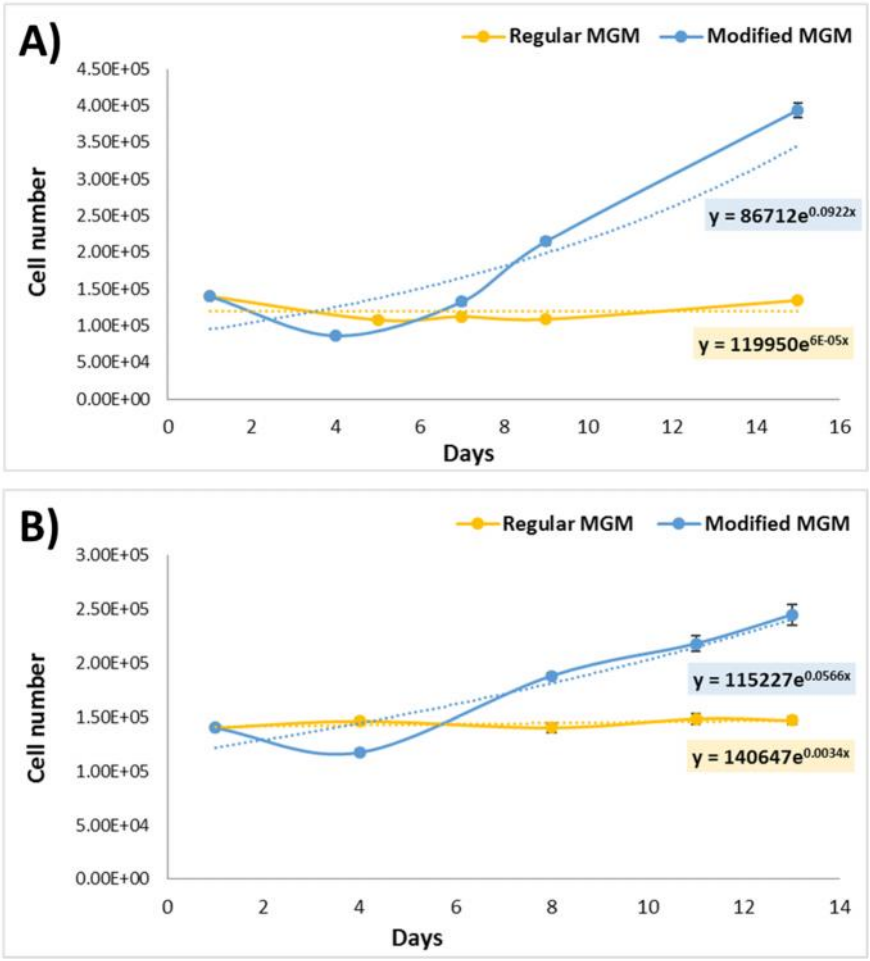

Figure 4.3. Comparison of the proliferation rates of A) p16-null and B) p16-deficient melanocyte cultures in regular *versus* modified melanocyte growth medium (MGM). The growth curves were generated by counting melanocytes every other day for a total of 13 days. Cells were plated at a density of $0.5x10^6$ cells/dish. Each data point represents the mean of three determinations. All standard deviations were less than 10%.

As stated earlier, 1n and 2d melanocytes had a very poor proliferation rate in the melanocyte culture medium routinely used, so they had to be grown in our enriched growth medium in an effort to enhance their proliferation. These melanocytes' growth improved dramatically in the enriched medium, showing a drastic reduction in their doubling time with remarkably short values at passages 14 and 17 (Figure 4.3). When high-passage melanocytes, with a poor proliferation capacity and prolonged doubling

time, were maintained in this enriched medium during at least 2-3 weeks, we observed formation of colonies denoting clonal expansion, reduction in doubling time, and decrease in the number of SA-β-Gal positive cells. However, no change in the percentage of SA-β-Gal positive cells was observed in strains failing to overcome their quiescent state in the enriched medium (Figure 4.4). These results provide evidence for the presence of a subpopulation of melanocytes that are in a state of reversible senescence caused by suboptimal growth conditions (241). Another possible explanation would be that a component of the enriched medium is causing melanocytes to become transformed, leading to senescence surpassing, an event needed to produce a progressively growing lesion as a melanoma (242).

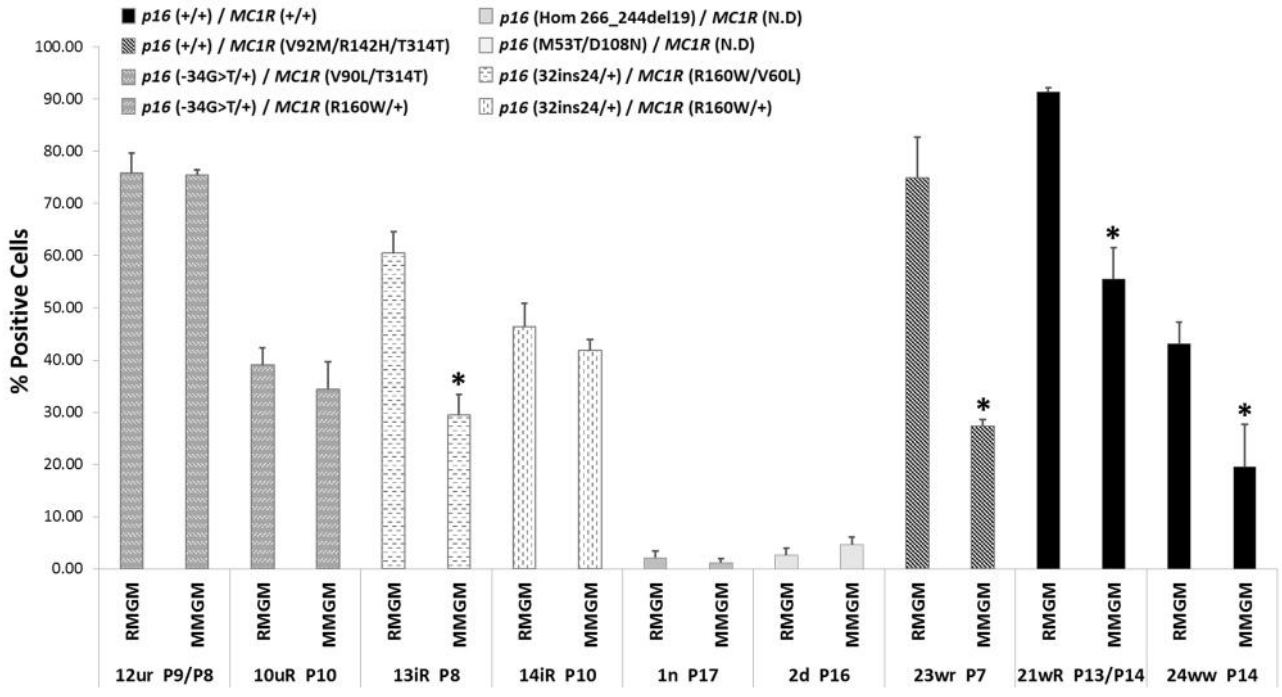

Figure 4.4. Comparison of the percent of senescence-associated β-galactosidase positive melanocytes maintained in regular (RMGM) *versus* modified melanocyte growth medium (MMGM). Each bar represents the mean ± SEM of at least 500 melanocytes counted in four determinations. * *P*-value < 0.01 *vs.* RMGM.

4.3.2. Effect of *p16* genotype on melanocyte response to UV

It is well-known that p16 is a cycling-dependent kinase inhibitor. In order to determine whether or not expression of V126D, -34G>T, or 32ins24 mutations prevents melanocytes from undergoing cell cycle arrest following irradiation with UV, we compared the doubling time and cell cycle profiles of UV-irradiated *versus* non-irradiated melanocytes. Irradiation with 75 mJ/cm^2 UV, a dose that did not induce significant apoptosis (Figure S4.1), resulted in a transient cell cycle arrest, which in turn led to a prolonged doubling time (Figure 4.5A). This is due to an induction of G1 and G2/M cell cycle arrest in response to UV irradiation, as cell cycle analyses show (Figure S4.2). We did not detect any difference between melanocytes heterozygous for one of the above *p16* mutations, and absence or co-expression of a RHC *MC1R* variant, as compared to wild-type melanocytes. Exposure to UV also resulted in a prolongation of doubling times, and therefore causing cell cycle arrest, in melanocytes from donors carrying two *p16* mutant alleles (Figure 4.5B).

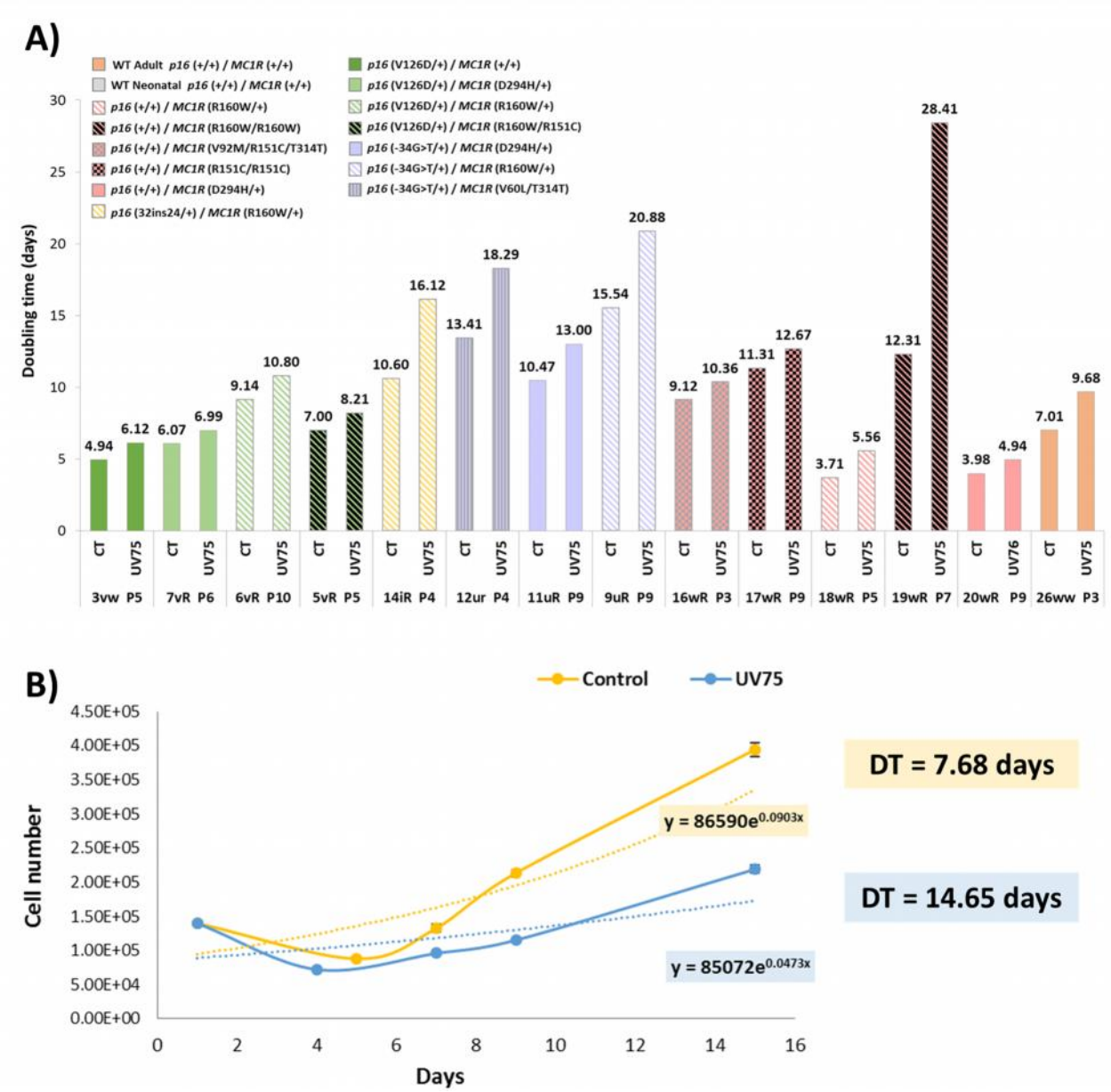

Figure 4.5. Effect of UV exposure on the proliferation rates of human melanocytes with different *CDKN2A* and *MC1R* genotypes. A) Bars show doubling times of UV-irradiated *versus* non-irradiated melanocytes. These cell strains were maintained in regular medium. **B)** Growth curves of UV-irradiated *versus* non-irradiated p16-null melanocytes (1n), which were maintained in modified melanocyte growth medium. Proliferation rates are expressed as doubling times, which represent the mean of three determinations. All standard deviations were less than 10%.

We further investigated the activation of the cellular transcriptional responses involved in cell cycle arrest. An accumulation of the cell cycle regulator p53 and its activated form

p-p53 was found at 6 h after UV exposure, and the effect was sustained for 24 h (Figure 4.6A and 4.6B). Phosphorylation of Rb, a tumour suppressor protein, was markedly decreased by 24 h post-UV. These results were in agreement with the cell cycle arrest induced on melanocytes by UV, regardless of the genotype (Figure 4.5 and S4.2). However, there was no significant up-regulation of p16 following irradiation with UV, casting doubts on the importance of p16 in the UV-induced DNA damage response. Western blotting indeed showed that irradiation of melanocytes rapidly resulted in the phosphorylation, hence activation, of stress-induced MAP kinases JNK and p38 – upstream activators of p53 involved in photoprotection against UV-induced DNA photoproducts via activation of nucleotide excision repair (NER) (Figure 4.6C and 4.6D). Minimal changes in the total protein levels of JNK and p38 were observed after UV irradiation, suggesting that UV affects phosphorylation, rather than synthesis or stability of these proteins, as previously shown (243) (Figure 4.6C). Levels of Gadd45a, a protein induced by p53-dependent and p53-independent mechanisms implicated in many biological processes related to maintenance of genomic stability and apoptosis, were significantly increased at 6 h and 24 h post-UV (Figure 4.6A and 4.6B). The activation of the DNA damage response following exposure to UV irradiation was observed in wild-type and *p16* heterozygous melanocytes, as well as in p16-null melanocytes. According to this, an alternative compensatory mechanism may cause cell cycle arrest in response to UV-induced DNA damage. In this regard, previous works suggest that the p53/p21 pathway may provide a secondary form of melanocyte senescence when p16 is dysfunctional or silenced (220,221). Additionally, the combined disruption of Rb/p16 and p53 pathways, but not the single loss of each protein function, accelerated tumorigenic growth in human fibroblasts (244).

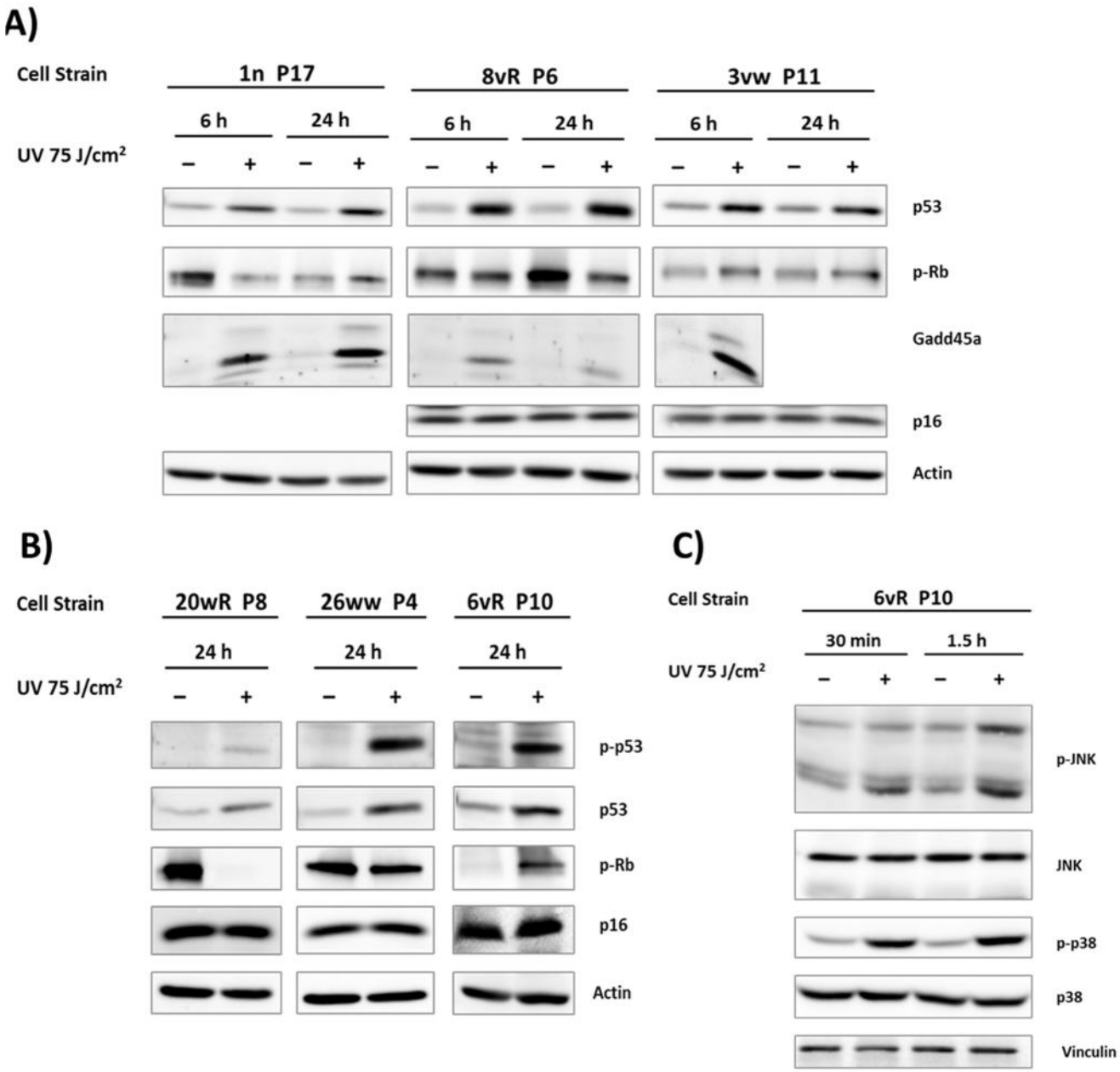

Figure 4.6. Western blotting showing a normal activation DNA damage response after UV irradiation of all cultured human melanocytes with different *CDKN2A* and *MC1R* genotypes. Actin and vinculin were used as loading controls. No detection of p16 protein was done for p16-null melanocytes (1n), since they do not express this protein. GADD45a was not detected at 24h for 3vw.

Taken together, our results suggest that the increased melanoma risk seen in *CDKN2A* mutation carriers, regardless of *MC1R* genotype, is not the result of significant intrinsic abnormalities in melanocytes. These p16-mutated melanocytes showed normal *in vitro* behaviour probably due to additional mechanisms of checkpoint control. Since carcinogenesis is driven by accumulating genetic alterations, additional somatic genetic or epigenetic changes may be needed to transform melanocytes. The spontaneous inactivation of p16 via small deletions, point mutations, promoter methylation or loss of heterozygosity is frequently observed in melanomas (245,246). Melanocytes derived from melanoma-prone individuals carrying *CDKN2A* mutations may be more susceptible to malignant transformation by activating mutations since only the spontaneous somatic inactivation of the remaining wild-type *CDKN2A* allele would be needed to overcome replicative senescence. Accordingly to this assumption, Ras-induced melanomas arising in p16 heterozygous mice exhibited a complete loss of heterozygosity for *CDKN2A* (247). Additionally, acquired somatic mutations in key melanoma driver genes, such as *BRAF* or *NRAS*, may cooperate with p16 inactivation to facilitate melanomagenesis (248,249). This is in line with data reporting that telomerase activation is sufficient to immortalize p16-null melanocytes but not normal human melanocytes (220,240), and with observations reporting the necessity of a combination of genetic alterations in human melanocytes (*NRAS* activation, telomerase expression and p16/Rb pathway ablation) to produce melanoma-like neoplasias (250).

4.4. CONCLUSION

Although germline mutations in the *CDKN2A* gene are the strongest melanoma risk factors identified to date, not all carriers develop melanoma. The different outcomes

observed in *CDKN2A* mutation carriers suggest that other genetic and environmental factors modulate melanoma risk in different family members even with a common high-risk genetic background. As a p16-deficient environment appears to be insufficient for melanoma development, better knowledge of spontaneous somatic mutations leading to clonal proliferation and immortalization will be helpful in increasing our understanding of melanomagenesis, as well as in designing targeted therapies.

4.5. SUPPLEMENTARY MATERIAL

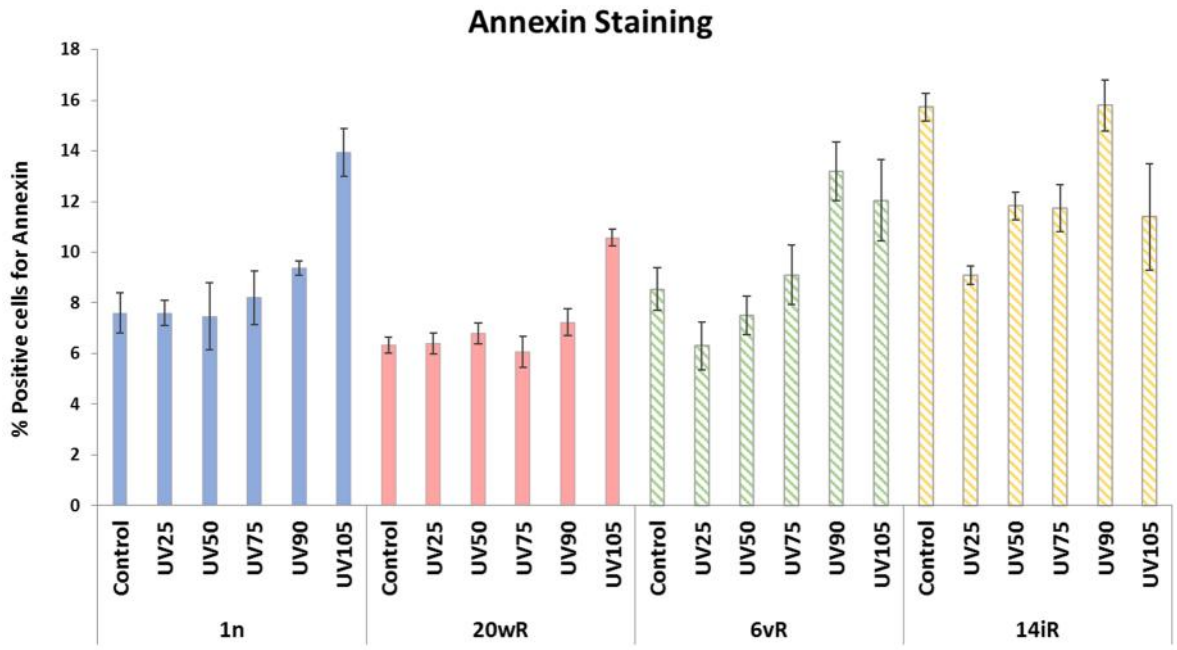

Figure S4.1. Percentage of apoptotic cells after irradiation with 25, 50, 75, 90 and 105 mJ/cm^2 UV. Annexin staining was performed 48h post-irradiation. Data show that the UV dose selected for irradiating melanocytes at all experiments (75 mJ/cm^2) did not induce significant apoptosis in human melanocyte strains. Bars represent the mean ± SEM of three samples. Ten thousand events were collected per sample.

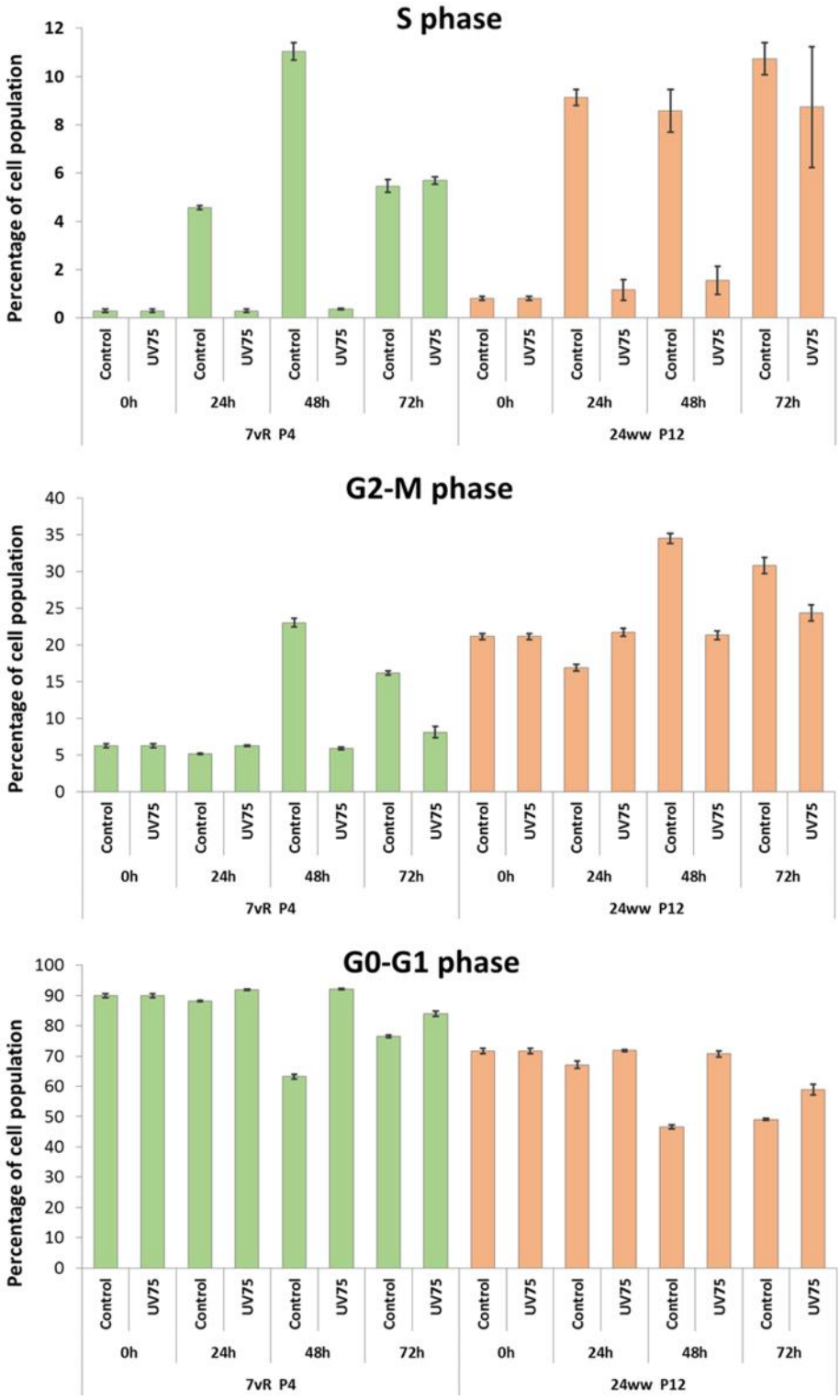

Figure S4.2. Cell cycle analysis of melanocytes with different *CDKN2A* and *MC1R* genotypes under control conditions or after UV irradiation. Bars represent the mean ± SEM of three samples. Ten thousand events were collected per sample.

Conclusions

1. **Sex might be a factor explaining variations in human pigmentation traits, tanning ability and sensitivity to sunlight between females and males in Caucasian populations**. These differences could be the result of socio-cultural reasons, as males tend to spend more time outdoors; physiological reasons, as males have thicker skin and increased number of blood vessels; differential tanning, as no sex difference in basal skin pigmentation has been shown; and hormonal factors, as oestrogens stimulate pigmentation while androgens have an inhibitory effect on melanocytes.

2. **There is a strong evidence for sex-differentiated genetic effects in pigmentation traits**. After stratifying all individuals by sex, the genotypic analysis reveals more polymorphisms associated with dark pigmentation and good sun tolerance in females than in males, who were instead associated with lighter pigmentation and poor sun tolerance.

   Additionally, RHC variants of the *MC1R* gene presented greater effects in skin phototype in females than in males. Therefore, *MC1R* genetic effects might contribute to the sex-specific differences described in skin phototype.

3. **There is a potential implication of two 3'UTR SNPs (rs2325813 in the *MLPH* gene and rs752107 in the *WNT3A* gene) in the appearance of different sun-related benign pigmented skin lesions.** *In silico* analyses show that these two SNPs disrupt a miRNA-binding site, possibly resulting in a differential gene expression through the disruption of putative miRNA-binding sites.

4. **The increased melanoma risk seen in *CDKN2A* mutation carriers, regardless of *MC1R* genotype, is not the result of significant intrinsic abnormalities in melanocytes.** These p16-mutated melanocytes showed normal *in vitro* behaviour, in terms of response to UV-induced damage and replicative senescence, probably due to additional mechanisms of checkpoint control.

   We propose that melanocytes derived from melanoma-prone individuals carrying *CDKN2A* mutations may be more susceptible to malignant transformation by activating mutations, since only the spontaneous somatic inactivation of the remaining wild-type *CDKN2A* allele would be needed to overcome replicative senescence.

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