UNIVERSITAT DE BARCELONA

Rol del gen de pigmentació *MC1R* en la susceptibilitat a malalties neurodegeneratives

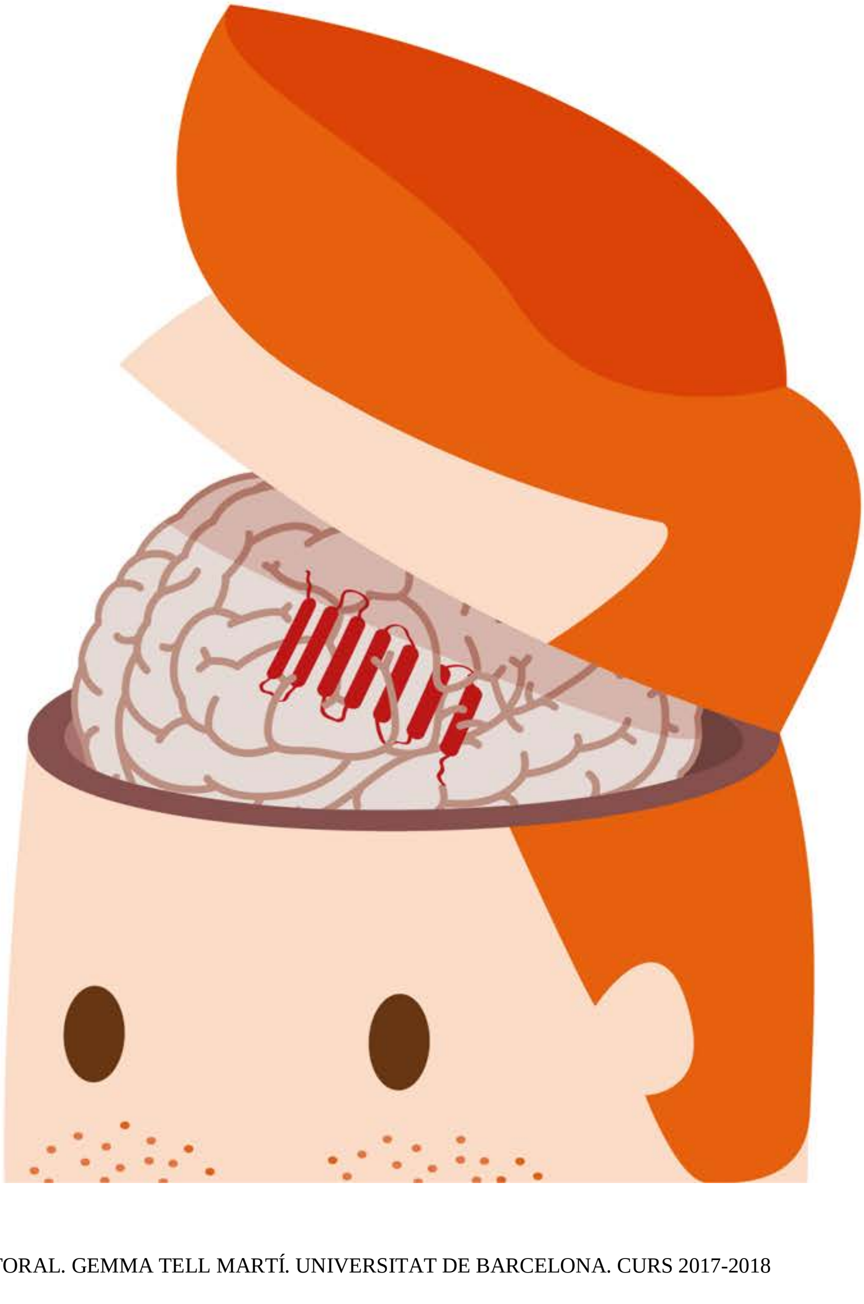
Gemma Tell Martí

ROL DEL GEN DE PIGMENTACIÓ *MC1R* EN LA SUSCEPTIBILITAT A MALALTIES NEURODEGENERATIVES

TESI DOCTORAL

Gemma Tell Martí

Directors
Dra. Susana Puig Sardà
Dr. Joan Anton Puig Butillé



BARCELONA 2018

Tesi Doctoral

Facultat de Biologia
Departament de Genètica, Microbiologia i Estadística
Programa de Doctorat de Genètica
Tutor: Francesc Mestres Naval

ROL DEL GEN DE PIGMENTACIÓ *MC1R* EN LA SUSCEPTIBILITAT A MALALTIES NEURODEGENERATIVES

Memòria presentada per **Gemma Tell Martí** per optar al grau de doctora per la Universitat de Barcelona

Tesi realitzada a la Unitat de Melanoma del IDIBAPS-Hospital Clínic de Barcelona, sota la direcció de la Dra. Susana Puig Sardà i el Dr. Joan Anton Puig Butillé

Barcelona, 2018

La codirectora	**El codirector**	**El tutor**	**La doctoranda**
Dra. S. Puig Sardà	Dr. JA. Puig Butillé	Dr. F. Mestres Naval	G. Tell Martí

Tot i que jo sóc la única signant de la tesi, aquesta no hagués estat possible sense la implicació i el coneixement de molta altre gent. Aprofito aquest espai per agrair-vos la feina feta i fer constar que aquesta tesi, en gran part, també és vostra.

De forma molt especial vull agrair als meus dos directors de tesi l'aprenentatge dels darrers anys i que m'hagin encoratjat a aconseguir aquest objectiu. A la Dra. Susana Puig li vull donar les gràcies per ensenyar-me que en ciència, i en la vida en general, les coses mai es poden mirar des d'una única perspectiva, i que si ets creatiu, descobriràs que un mateix fet es pot interpretar de moltes maneres. Al Dr. Joan Antón Puig li vull agrair la implicació en la meva formació, el seu coneixement i el seu esperit crític. Però especialment el seu suport. Quan els ànims queien una mica, tu sempre m'has animat a seguir endavant. A tots dos, gràcies per aquesta oportunitat.

Sens dubte, una de les millors coses d'aquesta etapa, ha estat sentir que formava part d'un gran equip. Vull agrair a tots els membres de la Unitat de Melanoma el treball fet durant aquests anys i la manera en la que em van acollir al grup des de el primer dia. Però sobretot, vull donar les gràcies als que per mi han estat "el nucli dur" del grup. Aquells amb qui hem treballat colze amb colze al laboratori, hem compartit fracassos experimentals i èxits científics, però sobretot hem format una verdadera pinya. Míriam, Pol, Estefi, Judit i Neus, us vull agrair la vostra humanitat, empatia i companyerisme, però per sobre de tot, em quedo amb la vostra amistat. Dona gust treballar amb vosaltres, mil gràcies per totes les bones estones que hem viscut junts i per totes aquelles que sé que viurem. Sou molt grans !!!!

A totes les estudiants de grau i màster amb les que hem compartit projectes: Aida, Marta, Ari, Mònica, Maria i Nerea, ha estat un paler coneixe-us i treballar amb vosaltres.

També vull agrair a tots els investigadors neuròlegs i genetistes amb els que he tingut la sort de col·laborar i que han format part activa en els treballs inclosos en aquesta tesi. En especial li vull agrair a la Dra. Montse Milà el tracte rebut durant aquests anys, i l'aprenentatge que he fet al seu costat. Gràcies per donar-me l'oportunitat de formar part de la família CIBERER i per haver comptat amb mi per treballar en el projecte UR-

DCat. Honestament, he rebut de tu molt més del que esperava i ha estat un plaer treballar al teu costat.

De tot cor, li vull agrair al Francesc Mestres que m'hagi volgut acompanyar en aquesta aventura com a tutor. Amb tu Francesc ja fa més de 10 anys que ens coneixem i la veritat que el llistat de coses que tinc a agrair-te comença a ser notablement llarg. Principalment et vull donar les gràcies per haver despertat en mi un gran interès per la genètica, però sobretot per obrir-me una porta que mai més has tancat. Sota la teva direcció vaig ser alumne interna del departament de genètica i això em va permetre conèixer com era el dia a dia en un laboratori i en que consistia "fer recerca". En els darrers anys he pogut comptar amb el teu parer cada vegada que havia de prendre una decisió acadèmica o professional, i ha estat per mi un orgull que t'hagis interessat en conèixer el treball de la meva tesi i que m'hagis ajudat a fer-la possible. Un GRÀCIES MAJÚSCUL!

Per últim, vull tenir unes paraules d'agraïment per totes aquelles persones que m'han acompanyat a nivell personal durant aquesta etapa:

Als meus amics, perquè sou la meva segona família, moltíssimes gràcies per totes les mostres d'interès en el meu treball i pel suport que m'heu donat per tirar endavant amb aquest projecte. Però principalment, gràcies per fer-me sentir tant estimada i per compartir amb mi les petites i grans coses de la vida.

Als meus pares, gràcies per TOT. Gràcies per haver cregut sempre en mi. De tu mama, he après, entre moltes d'altres coses, que el verdaderament important és no deixar mai de caminar. Que hi ha portes que s'obren i d'altres que es tanquen, però que amb esforç i perseverança sempre es tira endavant. A tu papa, que vas marxar quan aquesta experiència tot just començava, vull dir-te que m'has ajudat molt. I que encara que físicament ja no estem junts, jo acudeixo a la teva bondat, al teu amor i al teu sentit comú per prendre decisions en el dia a dia.

Als meus germans, Xavi i Mariona, gràcies per acompanyar-me en aquest i en tot els altres reptes que m'he plantejat a la vida. En general, gràcies per ser-hi sempre (i en el nostre cas és literal). Ja sabeu que per mi sou un tresor.

A tu Edu, gràcies per ser el millor company de viatge que es pot tenir. La teva generositat, empatia, sentit de l'humor i creativitat, m'han ajudat moltíssim a superar cada una de les dificultats que s'han presentat. Al teu costat, em sento capaç de tot. GRÀCIES INFINITES !!!

I a tots els membres de la meva nombrosa família (tiets/es, cosins/es, sogres i cunyat/da) que us heu interessat per conèixer la meva feina i m'heu encoratjat a tirar endavant amb la tesi, GRÀCIES de tot cor.

LLISTA D’ABREVIATURES

MC1R	Receptor de la melanocortina 1 (de l’anglès *melanocortin 1 receptor*)
RAPG	Receptor acoblat a proteïna G
nt	Nucleòtids
aa	Aminoàcids
ORF	Marc obert de lectura (de l’anglès *open reading frame*)
AC	Adenilat ciclasa
AMPc	Monofosfat d’adenosina cíclic (del castellà *adenosín monofosfato cíclico*)
MAF	Freqüència al·lèlica mínima (de l’anglès *minor allele frequency*)
SNP	Polimorfisme d’un sol nucleòtid (de l’anglès *single nucleotid polymorphism*)
LOF	Pèrdua de funció (de l’anglès *loss of function*)
RUV	Radiació ultraviolada
UVA	Ultraviolada tipus A
UVB	Ultraviolada tipus B
ADN	Àcid desoxiribonucleic
POMC	Proopiomelanocortina
α-MSH	Hormona estimuladora del melanòcit (de l’anglès *alpha melanocyte stimulating hormone*)
RHC	Color de cabell vermell (de l’anglès *red hair color*)
λ	Longitud d’ona
CPDs	Dímers de ciclobutiril pirimidina (de l’anglès *cyclobutanepyrimidine dimers*)
6-4PPs	Fotoproductes 6-4 pirimidina-pirimidona (de l’anglès *pyrimidine-(6-4)-pyrimidone photoproducts*)
ROS	Espècies reactives de l’oxigen (de l’anglès *reactive oxygen species*)
WT	*Wild type*
SNC	Sistema nerviós central
NF-KB	Factor nuclear potenciador de les cadenes lleugeres kappa de las cèl·lules B activades (de l’anglès nuclear factor of kappa light polypeptide gene enhancer in B cells)

GWAS	Estudi d’associació del genoma complet (de l’anglès *genome wide association study)*
NGS	Seqüenciació massiva (de l’anglès *next generation sequencing*)
NFT	Cabdells neurofibrilars (de l’anglès *neurofibrillary tangles*)
Aβ	Beta amiloide (de l’anglès *β-amyloid*)
APP	Proteïna precursora d’amiloide (de l’anglès *Amyloid precursor protein*)
P-tau	Tau fosforilada
T-tau	Tau total
LCR	Líquid cefaloraquidi
APOE	Apolipoproteïna E
APOEε4	Al·lel 4 de l’apolipoproteïna E
CAG	Trinucleòtid citosina-adenina-guanina
PoliQ	Poliglutamina
Htt	Huntingtina
PCR	Reacció en cadena de la polimerasa (de l’anglès *polymerase chain reaction*)
OR	Oportunitat relativa (de l’anglès *odds ratio*)
IC	Interval de confiança
PPI	Interacció proteïna-proteïna (de l’anglès *protein-protein interaction*)

INTRODUCCIÓ

1.- EL RECEPTOR DE LA MELANOCORTINA 1 (MC1R)

El receptor de la melanocortina 1 (MC1R) va ser identificat per primera vegada per dos grups independents: Chhajlani & Wikberg [1] i Mountjoy *et al*. [2] l'any 1992. Es tracta d'un receptor acoblat a proteïna G (RAPG) que s'expressa majoritàriament a la membrana dels melanòcits [3]. El gen *MC1R* (codi OMIM: #155555, codi Ensembl: ENSG00000258839) es localitza en el cromosoma 16q24.3 i a diferència de la majoria de gens que codifiquen per RAPGs presenta una estructura complexa amb diverses variants de *splicing.* Inicialment es va descriure com un gen sense introns [4], però en l'actualitat, s'ha establert que el gen està format per 4 exons i dóna lloc a diferents trànscrits com a resultat d'un *splicing* intra i intergènic [5]. El seu trànscrit majoritari MC1R-001 (codi Ensembl: ENST00000555147) està format pels exons 2, 3 i 4 i per 2 introns retinguts (Figura 1). Aquest trànscrit presenta una regió codificant de 951 nucleòtids (nt) que codifica per una proteïna de 317 aminoàcids (aa) (MC1R-001) considerada la forma canònica del receptor MC1R (NP_002377) (Figura 2).

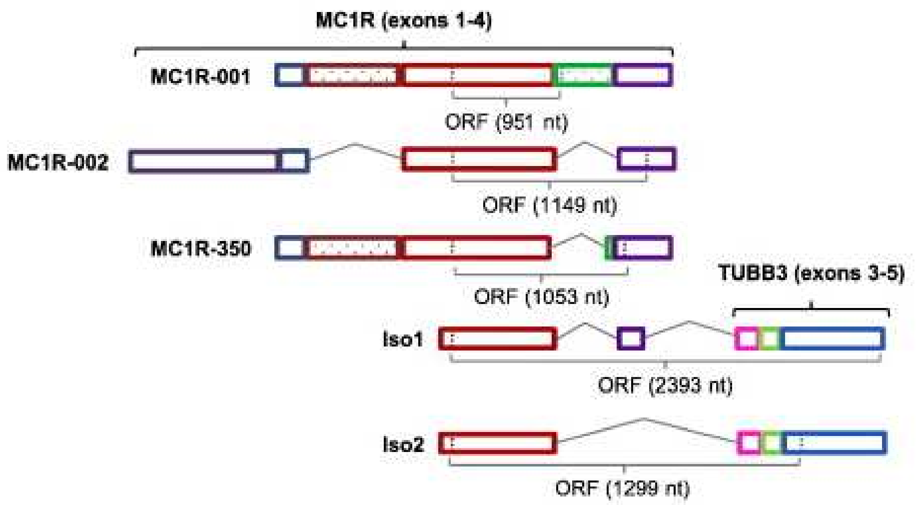


Figura 1. Estructura del gen *MC1R*. Organització exònica dels diferents trànscrits de *splicing* intragènic de *MC1R* (MC1R-001, MC1R-002 i MC1R-350) i de *splicing* intergènic de *MC1R-TUBB3*. Adaptada de Herraiz *et al.* [5].

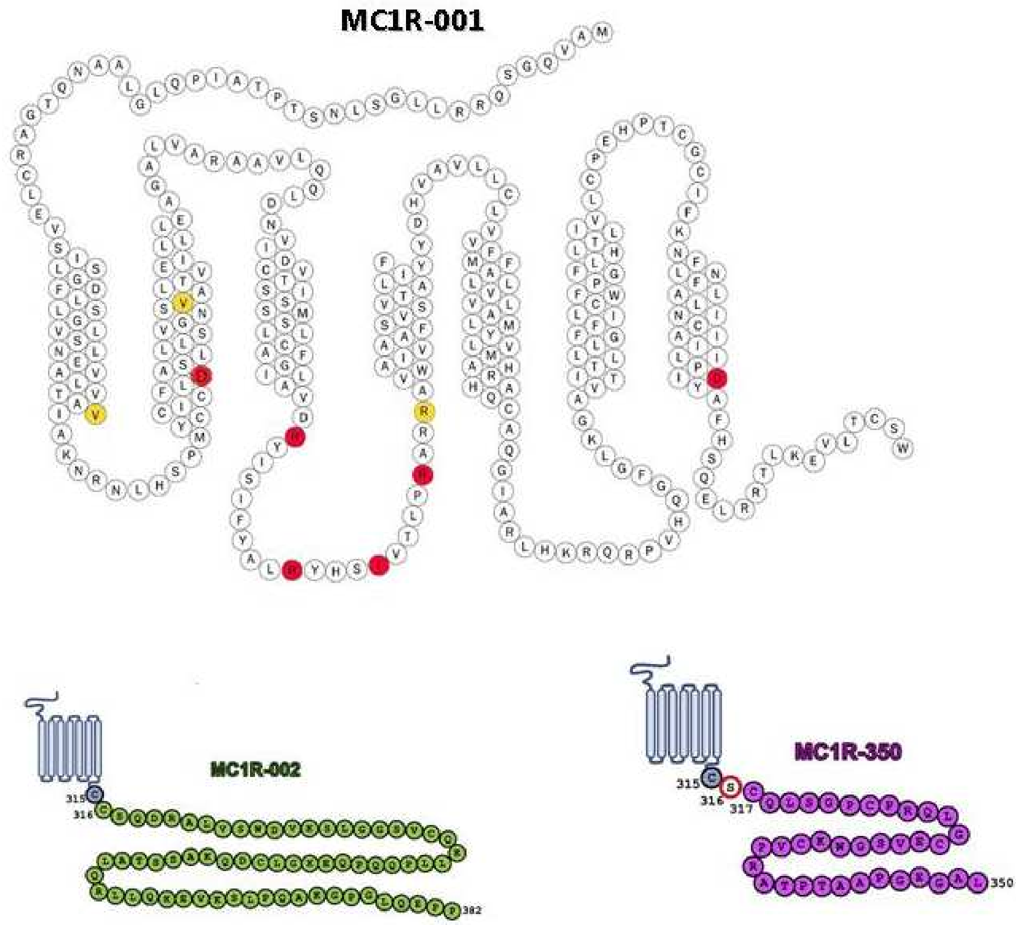


Figura 2. Representació de les diferents isoformes del MC1R. Adaptada de Herraiz *et al.* [5].

El trànscrit MC1R-002 (codi Ensembl: ENST00000555427) està format pels 4 exons i presenta una regió codificant de 1149 nt que codifica per una proteïna de 382 aa (MC1R-002). Aquesta isoforma és idèntica a la MC1R-001 fins a l'aa Ser316 i posteriorment incorpora una cua C-terminal de 66 aa (Figura 1 i 2). La isoforma MC1R-350 comparteix seqüència codificant amb la MC1R-001 i la MC1R-002 fins a l'aa Cys315 i posteriorment presenta una extensió C-terminal de 35 aa diferents (Figura 1 i 2) [5].

La regió 16q24 presenta una alta densitat en gens, de manera que les regions intergèniques entre *MC1R* i els gens veïns són curtes. Aquest factor, juntament amb la presència d'una poliadenilació inusual i ineficaç en la transcripció de *MC1R*, possibilita un *splicing* intergènic que dona lloc a la formació de dues proteïnes de fusió (ISO 1 i ISO 2), constituïdes a partir de la fusió entre trànscrits de *MC1R* i trànscrits del gen *TUBB3* (Figura 3) [6].

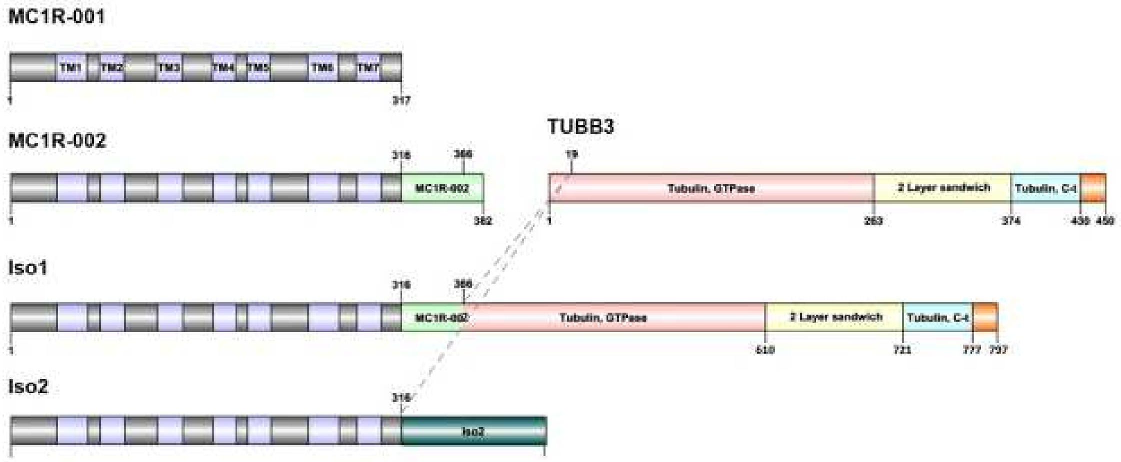


Figura 3. Representació estructural dels dominis que constitueixen la isoforma canònica del MC1R (MC1R-001) i les proteïnes de fusió ISO 1 i ISO 2. Adaptada de Herraiz *et al*. [6].

Els nivells dels diferents trànscrits alternatius poden estar modulats per senyals exògenes [7]. No obstant, calen més estudis per identificar quins poden ser aquests mecanismes de regulació i determinar la funció d'aquestes isoformes no canòniques [7].

L'activació de la forma canònica del MC1R (MC1R-001) comporta un acoblament amb l'adenilat ciclasa (AC), el posterior augment dels nivells de monofosfat d'adenosina cíclic (AMPc) i la transducció de la senyal d'activació *downstream* que genera un canvi fisiològic en la cèl·lula [8].

El gen *MC1R* és altament polimòrfic, fins a la data, s'han identificat prop de 200 variants en aquest gen i més de 100 són no sinònimes [5, 7, 8]. L'alteració funcional que provoquen la majoria d'aquestes variants en la proteïna és desconeguda [7]. No obstant, hi ha 9 polimorfismes d'un sol nucleòtid (SNP): rs1805005 (p.V60L), rs1805006 (p.D84E), rs2228479 (p.V92M), rs11547464 (p.R142H), rs1805007 (p.R151C), rs1110400 (p.I155T), rs1805008 (p.R160W), rs885479 (p.R163Q) i rs1805009 (p.D294H) que presenten una freqüència al·lèlica (MAF) > 0,01 en diferents poblacions caucàsiques i que han estat àmpliament estudiats tant a nivell epidemiològic com a nivell funcional [7-9]. Aquests disminueixen l'activitat del MC1R alterant diferents processos claus, com són: l'afinitat amb el seu lligand, l'acoblament amb l'AC i la senyalització a través del AMPc i/o el transport intracel·lular de la proteïna fins a la membrana [8]. Entre aquests SNPs, n'hi ha 6 (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W i p.D294H) que s'han associat amb una activitat residual del MC1R inferior al 50% i que es defineixen com a variants de pèrdua de funció (LOF) [5]. L'alteració funcional del MC1R causada per la presència d'aquestes variants al·lèliques és dosi dependent i segueix un model d'herència additiu [10, 11]. L'activitat del MC1R és pràcticament indetectable o nul·la en les cèl·lules homozigotes o heterozigotes compostes per aquestes variants LOF de *MC1R* [12, 13].

2. ROL DEL GEN *MC1R* EN LA PIGMENTACIÓ

El color de la pell, dels ulls i del cabell depèn principalment de la quantitat, el tipus i la distribució de la melanina que és el pigment que es sintetitza en els melanòcits i confereix protecció davant la radiació ultraviolada (RUV) [14, 15]. Els melanòcits són cèl·lules dendrítiques que es localitzen majoritàriament a l'epidermis i en el fol·licle pilós. En l'epidermis, els melanòcits es troben situats entre els queratinòcits de la làmina basal i cada melanòcit connecta a través de les dendrites amb aproximadament 36 queratinòcits adjacents, formant les unitats epidèrmiques de melanització (Figura 4) [16, 17].

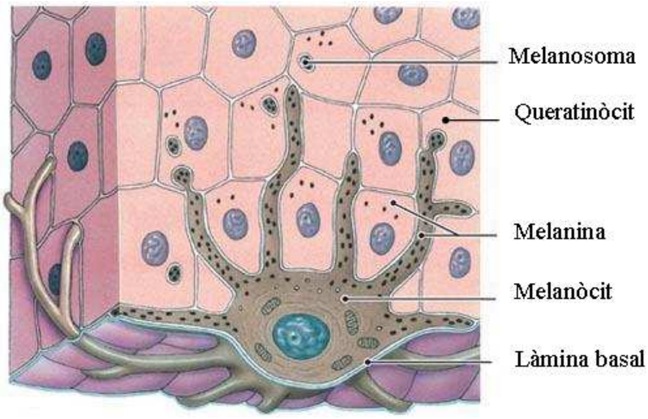


Figura 4. Representació d'una unitat epidèrmica de melanització. Disposició dels melanòcits i els queratinòcits en l'epidermis. Adaptada de [18].

El procés de síntesi de melanina conegut com a melanogènesi és un procés gradual que té lloc durant la maduració dels melanosomes. Aquests orgànuls citoplasmàtics es troben en els melanòcits i estan especialitzats en la síntesi, emmagatzematge i transport de la melanina fins als queratinòcits. Existeixen dos tipus de melanosomes que difereixen en mida, forma, estructura i composició química: els eumelanosomes, on es sintetitza el pigment eumelanina que és el pigment negre-marronós i cromòfor protector de l'ADN, i els feomelanosomas, on es sintetitza el pigment feomelanina que és el pigment groc-vermellós sense capacitat de protecció enfront a la RUV (Figura 5) [19].

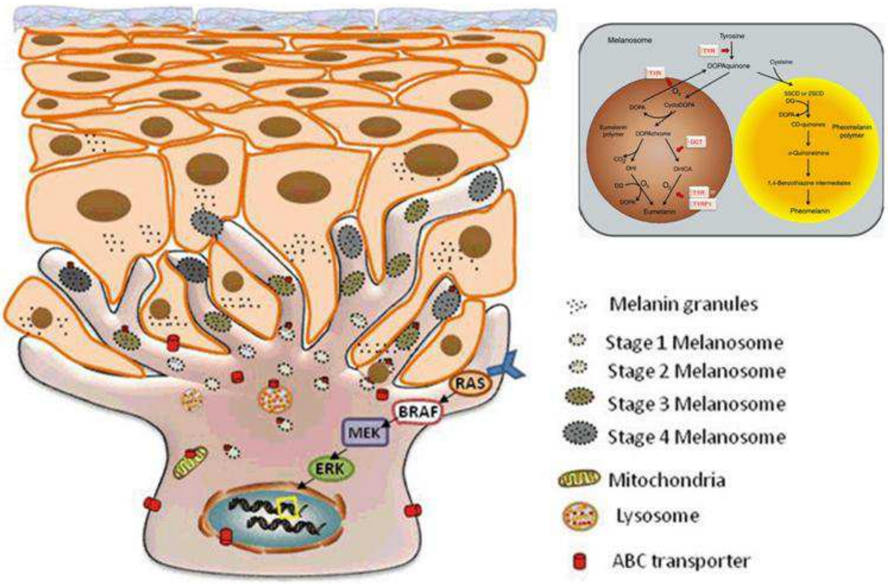


Figura 5. Maduració dels melanosomes i distribució de la melanina cap als queratinòcits. Representació del procés de distribució de la melanina cap als queratinòcits adjacents i representació dels eumelanosomes (eumelanina) i feomelanosomes (feomelanina). Adaptada de [20].

La pell té una determinada pigmentació constitutiva i una pigmentació facultativa la qual és induïda en resposta a la RUV, totes dues varien en funció de l'ètnia i l'individu [15]. L'exposició a la RUV causa dany genotòxic i estrès en les cèl·lules cutànies promovent l'activació de p53 i la síntesi de la proopiomelanocortina (POMC) en els queratinòcits. De la POMC deriva la hormona estimuladora del melanòcit (α-MSH) que activa la via de senyalització canònica del MC1R. Aquesta activació, comporta un augment d'AMPc, la posterior fosforilació de CREB i la transcripció de MITF que s'uneix a nivell de promotor i estimula la transcripció tant de gens de proliferació cel·lular i antiapoptòtics, com de gens relacionats amb la eumelanogènesi (síntesi d'eumelanina) (Figura 6) [21]. Així doncs, en les zones fotoexposades s'observa un augment del nombre de melanòcits i dels nivells d'eumelanina per tal de conferir fotoprotecció envers a la RUV [22, 23].

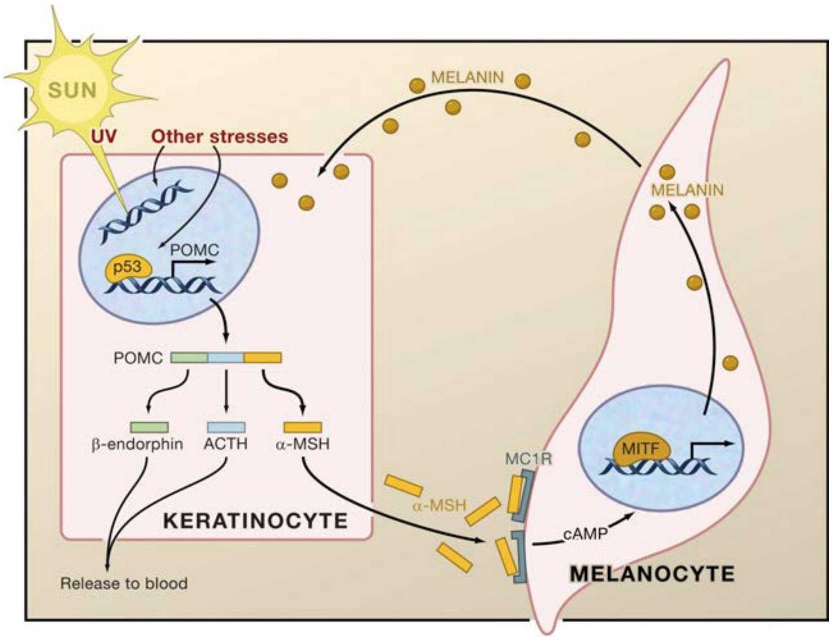


Figura 6. Activació de la síntesi de melanina en resposta a la RUV. Oren & Bartek [21].

La pigmentació és un caràcter complex i poligènic en el que es troben implicats més de 120 gens [8, 24]. Entre tots ells, el gen *MC1R* s'ha descrit com el gen clau en la regulació del color de la pell i el cabell i és el responsable de controlar la quantitat relativa de síntesi d'eumelanina i feomelanina [25].

El gran nombre de variants al·lèliques identificades en *MC1R* explica, en part, la diversitat fenotípica que existeix en quan al color del cabell i al fototip cutani (color de pell, sensibilitat a la RUV i capacitat de bronzejar-se) entre els humans [7, 26, 27]. Tot i que la majoria de variants identificades en *MC1R* són rares i la seva MAF és < 0,01, hi ha 9 SNPs *missense* que s'han identificat en diferents poblacions caucàsiques d'ètnia blanca amb una MAF > 0,01. Aquests SNPs s'han classificat en 2 grups segons la repercussió fenotípica que tenen en la pigmentació. Així doncs, les 6 variants LOF de *MC1R* (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W i p.D294H) que disminueixen notablement la capacitat de senyalització del MC1R a través de la via de l'AMPc i impedeixen la síntesi d'eumelanina, s'han definit com a variants 'R' (Figura 7) [28]. El individus homozigots o heterozigots compostos per a aquestes variants 'R' (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W i p.D294H) són incapaços de sintetitzar eumelanima i sintetitzen feomelanina (Figura 7) [25]. Aquests individus presenten una forta associació amb el fenotip "color de cabell vermell" (RHC) [28] que es caracteritza per una pell molt blanca, cabell pèl-roig, un elevat nombre de pigues, una baixa capacitat per a bronzejar-se i una elevada sensibilitat a la RUV (Figura 7) [29]. D'altra banda, els SNPs *missense* que disminueixen lleugerament la capacitat de senyalització del MC1R a través de la via de l'AMPc, però que no s'associen al fenotip RHC, s'han definit com a variants 'r'(p.V60L, p.V92M i p.R163Q) [28, 30]. El fenotip RHC s'ha associat amb un augment del risc a desenvolupar càncer cutani, tant melanoma [29] com carcinoma basocel·lular [31] i carcinoma escatós [32].

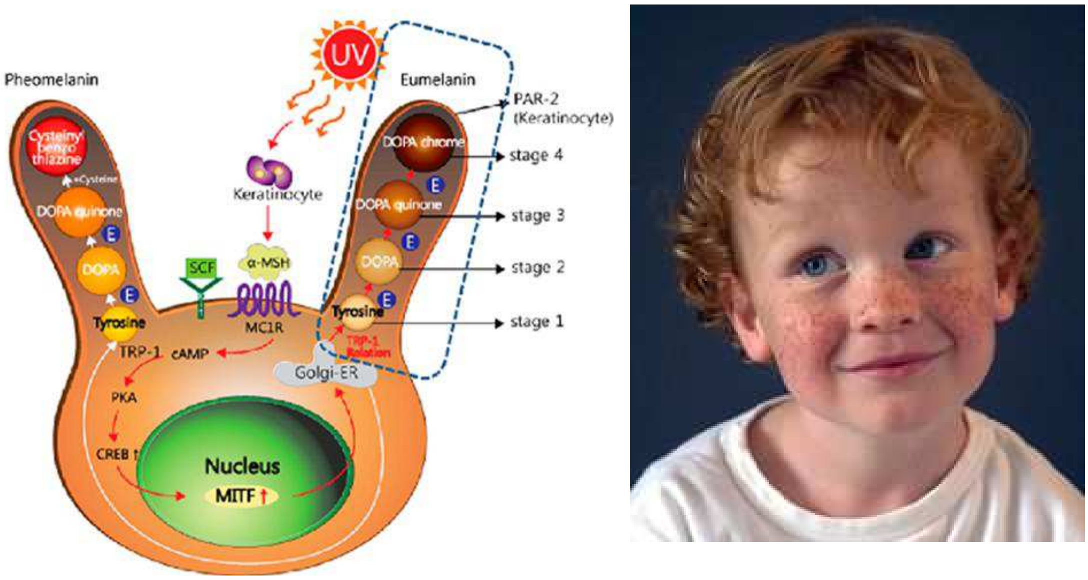


Figura 7. Via molecular de la síntesi de melanina depenent del MC1R i representació del fenotip RHC. Adaptada de [33].

3. ROL DEL GEN *MC1R* EN LA SUSCEPTIBILITAT A MELANOMA

El melanoma (codi OMIM: #155600) és una neoplàsia originada en els melanòcits. Tot i que representa menys d'un 5% dels tumors cutanis malignes, és el responsable del 75% de les morts causades per aquests tumors [34-36]. El melanoma és comú en les poblacions occidentals i la seva incidència mundial és de 15-25 per 100.000 habitants/any [35, 37] la qual representa aproximadament un 5,2% del total de casos de càncer diagnosticats a l'any [38].

Es tracta d'una malaltia complexa causada per la interacció entre factors ambientals, fenotípics i genètics [39]. La RUV és el principal factor ambiental i s'ha descrit una forta correlació entre una elevada exposició a la RUV i el risc a desenvolupar melanoma [40-42]. Per exemple, els individus que han patit més de 5 cremades amb butllofes al llarg de la vida presenten el doble de risc a melanoma [34, 43].

De la RUV que arriba a la terra, el 95% ho fa en forma de radiació UVA (λ 315-400 nm) i el 5% restant en forma de UVB (λ 280-315 nm) [44]. Inicialment, la radiació UVB s'havia definit com la única forma responsable de generar dímers de ciclobutiril pirimidina (CPDs) i fotoproductes 6-4 pirimidina-pirimidona (6-4PPs) que són els responsables d'originar en l'ADN una signatura mutagènica caracteritzada per la presència de múltiples canvis C per T o CC per TT [45]. Actualment, és coneix que la radiació UVA també està implicada en la formació de CPDs i en les cèl·lules epidèrmiques, especialment en els melanòcits [46], contribueix a la generació d'espècies reactives de l'oxigen (ROS).

El fenotip que s'associa a un augment del risc a melanoma està definit per: la presència de múltiples nevus melanocítics (100 o més), fototips clars que presenten dificultat per bronzejar-se i tendència a cremar-se (fototip I i II, segons l'escala de Fitzpatrick [47]), color clar d'ulls (verd o blau) i color clar de cabell (ros o pèl-roig) [48, 49].

Un estudi realitzat en diferents poblacions del nord d'Europa, ha reportat que un 58% del risc a desenvolupar melanoma és heretat, indicant que el melanoma és un dels càncers que presenta un major component genètic [50]. Aproximadament, un 5-10% dels casos de melanoma tenen lloc en un context familiar [51]. La susceptibilitat a desenvolupar la malaltia es manifesta amb la presència de múltiples casos de melanoma en familiars de primer o segon grau, o bé amb la presència de múltiples melanomes primaris en un mateix individu [52]. Un 20-40% d'aquestes famílies [39, 53] i entre un 8-16% dels pacients amb melanoma primari múltiple [54] presenten mutacions germinals en el gen *CDKN2A*, que és *el* principal gen d'alt risc a melanoma. Amb una menor freqüència, s'han identificat mutacions ens altres gens d'alt risc com: *CDK4*, *BAP1*, *POT1*, *ACD*, *TERF2IP* i *TERT* [55]. Tot i que les mutacions en aquests gens expliquen el 25% dels casos de melanoma familiar, la causa genètica en la resta de casos és desconeguda [39].

En els pacients amb melanomes esporàdics (90-95% dels casos) s'han identificat variants germinals de mig o baix risc en gens implicats en la regulació de característiques fenotípiques associades a melanoma [56]. Es tracta de variants genètiques relativament freqüents (MAF > 1%) i que confereixen un risc moderat o baix a desenvolupar melanoma (Figura 8) [57].

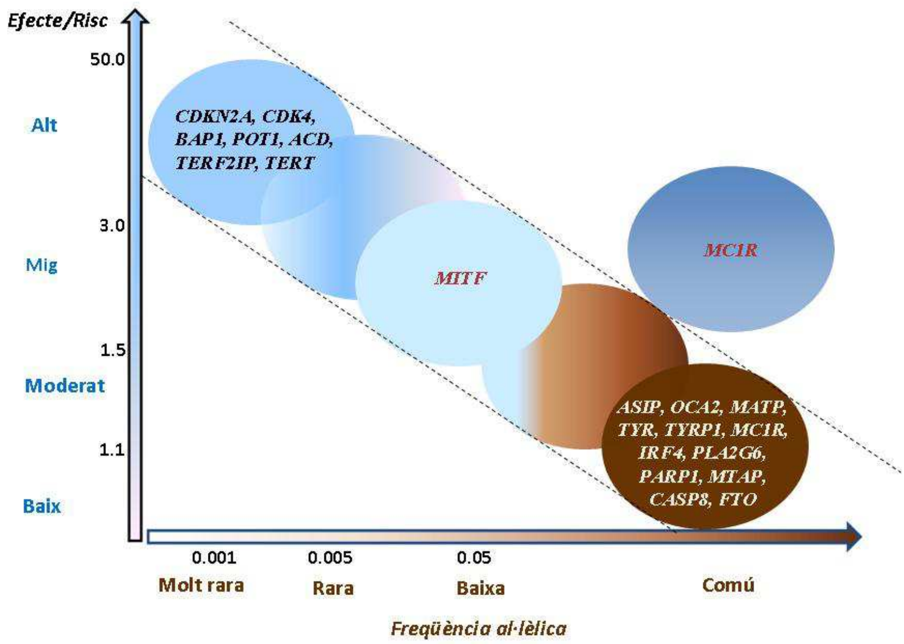


Figura 8. Gens implicats en la susceptibilitat a melanoma. Adaptada de Manolio *et al* [58].

El gen *MC1R* és un gen de risc mig que presenta variants genètiques comuns en la població i està implicat en la determinació d'un fenotip associat a melanoma [57]. A més, la presencia de variants genètiques en *MC1R* en individus portadors de mutacions en el gen *CDKN2A* augmenta del 50% al 84% la penetrància de la malaltia [59, 60] i disminueix 20 anys l'edat mitjana d'aparició del melanoma en aquests individus [29, 59].

Nombrosos estudis epidemiològics han reportat l'associació entre la presència de variants hipomòrfiques en el gen *MC1R* i un augment del risc a desenvolupar melanoma [28, 61] i altres carcinomes cutanis no melanoma [62, 63]. Els portadors de variants 'R' presenten més del doble de risc a desenvolupar melanoma (OR=2,44; 95% IC: 1,72-3,45) [28] i els portadors de variants 'r' presenten un lleuger increment d'aquest (OR=1,29; 95% IC: 1,10-1,51) [61]. Un metanàlisi que inclou 5.160 casos de melanoma i 12.119 controls va confirmar que l'augment del risc a desenvolupar melanoma (OR=1,66; 95% IC: 1,41-1,96) s'identifica en els portadors de qualsevol de les 9 variants genètiques més conegudes (p.V60L, p.D84E, p.V92M, p.R142H, p.R151C, p.I155T, p.R160W, p.R163Q i p.D294H) [64]. El treball confirmava l'existència d'un efecte dosi dependent, ja que en els portadors de 2 o més d'aquestes variants genètiques el risc a melanoma augmentava en més del doble (OR=2,51; 95% IC: 1,83-3,44) [64]. Els individus portadors de variants genètiques en *MC1R*, especialment variants 'R', presenten una disminució parcial o total dels nivells d'eumelanina, aquest fet explica la sensibilitat d'aquests individus a la RUV i l'augment de la susceptibilitat a melanoma. Ara bé, el fet que els portadors de variants en *MC1R* amb pell fosca, sense pigues i amb un color de cabell diferent al pèl-roig també siguin més susceptibles a desenvolupar melanoma [64], indica que l'efecte de *MC1R* com a factor de risc a la malaltia va més enllà del seu rol en la síntesi de pigment.

Diversos treballs han indicat que hi ha altres mecanismes biològics subjacents a aquesta associació. S'ha descrit que el MC1R està implicat en el control de la supervivència i proliferació del melanòcit i en la reparació del dany en l'ADN causat per la RUV [65-67]. L'activació del MC1R en resposta a la radiació UVB controla la supervivència i proliferació dels melanòcits a través de la interacció entre aquest i la proteïna PTEN. La seva unió impedeix la degradació de PTEN per WWP2 i condueix a la inactivació de la via de senyalització PI3K/AKT. Aquest mecanisme de regulació garanteix la supervivència del melanòcit i el control de la seva proliferació [65]. Per contra, una pèrdua de funció del MC1R provoca la degradació de PTEN i en conseqüència una hiperactivació de la via PI3K/AKT. L'efecte biològic que causa la hiperactivació d'aquesta via depèn molt del context cel·lular en el que té lloc. En els melanòcits primaris condueix a la senescència prematura, mentre que en els melanòcits portadors de mutacions en la via de les MAPkinases potencia una hiperproliferació i indueix a la transformació oncogènica d'aquests melanòcits (Figura 9A) [65]. A més, s'ha descrit que l'activació de la via canònica del MC1R comporta un augment de MITF i l'expressió de l'antiapoptòtic BCL-2 (Figura 9B) [66]. Els melanòcits presenten una baixa capacitat de proliferació, per tant, evitar-ne l'apoptosi i mantenir l'homeòstasi en les unitats epidèrmiques de melanització és essencial per a garantir la fotoprotecció. D'altra banda, s'ha identificat que l'activació del MC1R comporta l'activació de mecanismes de reparació del dany en l'ADN i la reducció de les ROS (Figura 9C) [67].

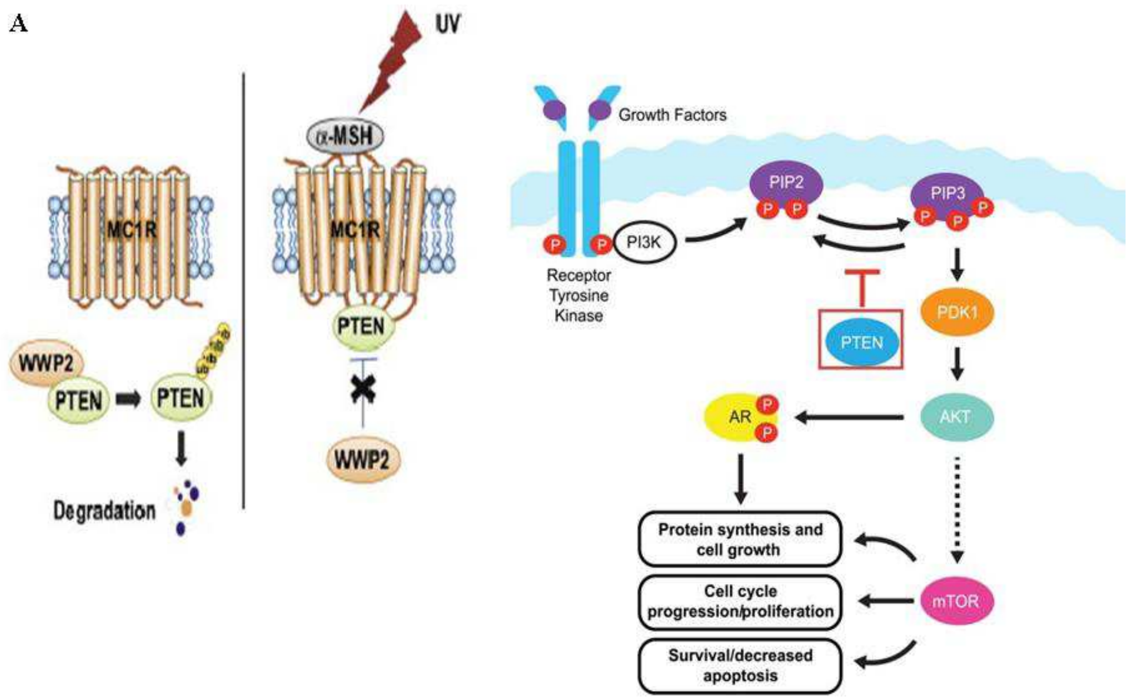


Figura 9A. Control de la degradació de PTEN en resposta a la radiació UVB i regulació de la via PI3K/AKT implicada en la proliferació i supervivència del melanòcit. Adaptada de Cao *et al.* [65] i de Phin *et al*. [68].

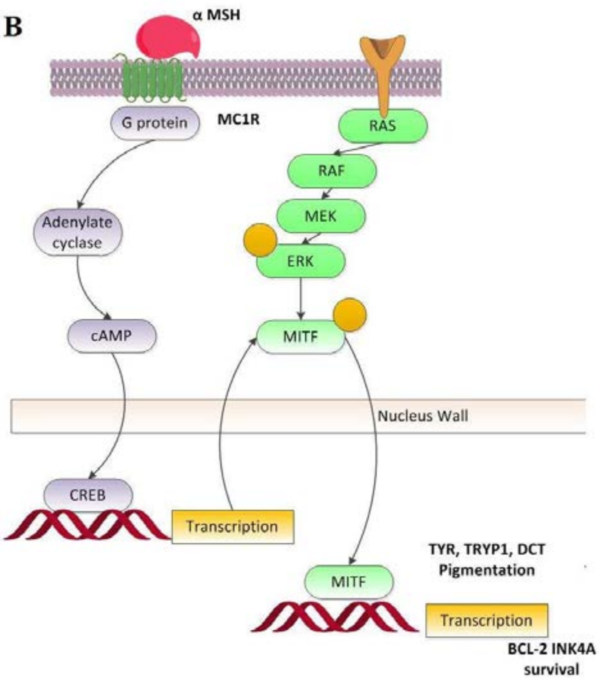


Figura 9B. Control dels nivells de l'antiapoptòtic BCL-2 a través de la transcripció de MITF. Adaptada de [69].

C

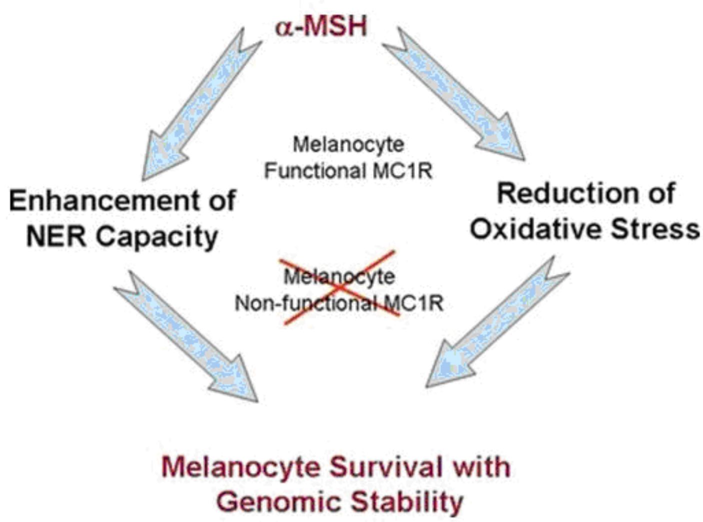


Figura 9C. Activació de mecanismes de reparació del dany en l'ADN (NER) i reducció de ROS. Abdel-Malek *et al.* [67].

Aquests mecanismes moleculars regulats pel MC1R contribueixen a mantenir l'homeòstasi cel·lular, l'estabilitat genòmica i a impedir la transformació oncogènica dels melanòcits. Per tant, l'alteració de tots aquests mecanismes regulats pel MC1R enfront a la RUV, expliquen la relació subjacent entre la presencia de variants genètiques en *MC1R* i l'augment del risc a desenvolupar melanoma.

Un treball realitzat amb models murins, modificats genèticament per desenvolupar melanoma, comparava la capacitat d'aquest desenvolupament i la taxa de supervivència entre ratolins negres ($Mc1r^{+/+}$), pèl-roigs ($Mc1r^{e/e}$) i albins (incapaços de sintetitzar melanina ($Tyr^{c/c}$) ($Mc1r^{+/+}$)) que no havien estat exposats a la RUV [70]. L'estudi va identificar que els ratolins pèl-roigs presentaven una major taxa de melanomes i una menor supervivència respecte als ratolins negres o albins. Aquests resultats indiquen que la pèrdua de funció del MC1R pot ser carcinogènica i contribuir al desenvolupament del melanoma independentment de la RUV (Figura 10) [70].

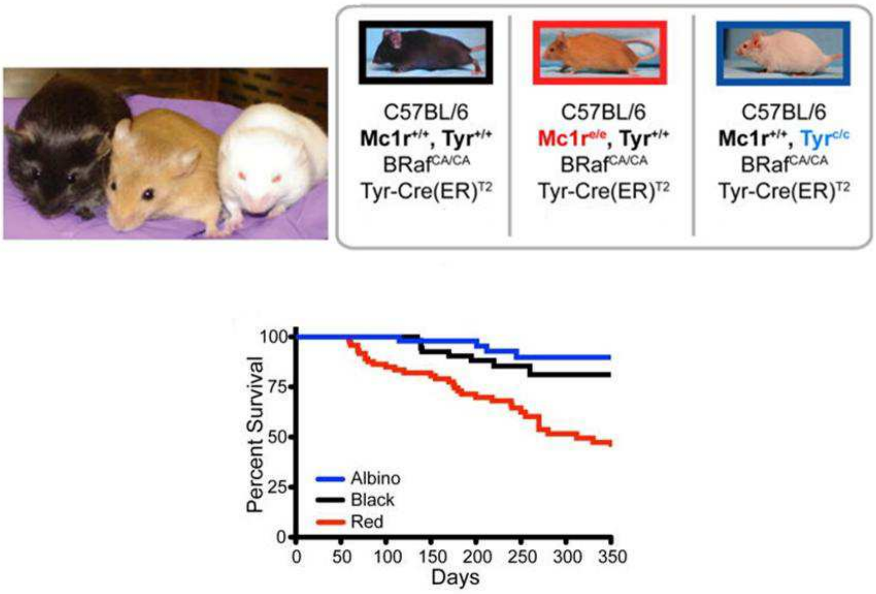


Figura 10. Els ratolins pèl-roigs desenvolupen un major nombre de melanomes i presenten una menor supervivència respecte als ratolins negres i albins independentment de la RUV. $Mc1r^{+/+}$ (WT) i $Mc1r^{e/e}$ (portadors d'un codó terminal prematur que codifica per una proteïna no funcional). Adaptada de Mitra *et al.* [70].

El treball atribuïa a la presència de feomelanina, la predisposició a desenvolupar melanomes observada en els ratolins pèl-roigs. Aquest treball indica que la síntesi de feomelanina a nivell basal contribueix a la formació de ROS i causa un increment del dany en l'ADN i de l'oxidació dels lípids en aquests ratolins [70]. Diversos treballs han confirmat un augment de l'estrès oxidatiu en les cèl·lules que contenen feomelanina i han destacat la capacitat de la eumelanina d'actuar com a receptor scavenger eliminant aquestes ROS [3, 71, 72].

Sorprenentment, un estudi realitzat amb models murins va mostrar un augment de CPDs en els melanòcits i queratinòcits hores desprès de ser exposats a la RUV [44]. Aquests CPDs generats "en la foscor" (d*ark* CPDs) constituïen la majoria de CPDs quantificats en les cèl·lules epidèrmiques dels ratolins. La generació d'aquests CPDs depenia de la presència de melanina (eumelanina o feomelanina), ja que aquests no s'observaven en els ratolins albins [44]. Els nivells més elevats de *dark* CPDs tenien lloc en les cèl·lules que contenien feomelanina [44, 73]. La generació d'aquests *dark* CPDs s'atribueix a l'excitació química que es produeix quan les ROS, generades a partir de la radiació UVA, es combinen amb els electrons de la melanina [44]. Aquesta excitació crea un triplet quàntic en la melanina que indueix la formació de CPDs transferint l'energia a l'ADN independentment de la RUV [73]. Aquests resultats suggereixen que els CPDs es podrien generar en totes les cèl·lules on aquestes ROS i la melanina hi siguin presents [73, 74]. Aquest mecanisme, juntament amb la incapacitat d'eliminar ROS que té la feomelanina, pot explicar perquè els portadors de ≥1 variant 'R' en *MC1R* presenten una major carrega de mutacions C-T en els seus melanomes [75] i un augment del risc a desenvolupar càncer cutani [29, 62].

Amb l'objectiu d'identificar l'impacte de les variants 'R' en les cèl·lules cutànies independentment de l'exposició a la RUV, es va analitzar el transcriptoma de co-cultius de queratinòcits i melanòcits [76] (Annex I). A partir de biòpsies cutànies de pell sana obtingudes d'individus portadors de variants 'R' en *MC1R* i individus *MC1R* WT, es van establir co-cultius de melanòcits i queratinòcits per tal d'emular les unitats epidèrmiques de melanització. El transcriptoma global d'aquestes cèl·lules sanes va ser capturat mitjançant micorarrays d'expressió (G4112F, Agilent, US). Al comprar el transcriptoma obtingut en els co-cultius de cèl·lules portadores de variants 'R' vs el de cèl·lules WT, es van identificar 3570 gens expressats diferencialment (p-valor ajustat < 0,05) (Annex I). Mitjançant un anàlisi bioinformàtic d'enriquiment funcional, que permetia posar en context biològic la signatura gènica identificada, es van identificar el conjunt de vies de senyalització expressades diferencialment en les cèl·lules 'R' respecte les WT (Taula 1). Aquest treball va confirmar que les cèl·lules portadores de variants 'R' mostren una infraexpressió de gens implicats en la pigmentació i la diferenciació melanocítica, i com a conseqüència una infraexpressió de les vies de senyalització implicades en l'endocitosi i la formació dels lisosomes. Aquests processos estan directament relacionats amb la maduració dels melanosomes i la transferència de pigment dels melanòcits als queratinòcits (Taula 1) [77, 78]. D'altra banda, es va identificar una sobreexpressió de gens implicats en la reparació del dany en l'ADN (reparació de malaparellament, reparació per excisió de nucleòtid, reparació per excisió de base i recombinació homòloga) i en processos biològics implicats en el manteniment de l'homeòstasi cel·lular, com són: la replicació de l'ADN, el control del cicle cel·lular i la fosforilació oxidativa, la qual comporta la formació de ROS (Taula 1). Aquests resultats indiquen que les cèl·lules dels individus portadors d'al·lels 'R' presenten un major dany en l'ADN i un increment de processos oxidatius no causats per la RUV.

De forma interessant, es va identificar una sobrexpressió de gens implicats en vies de senyalització associades a malalties neurodegeneratives, com són: la malaltia de Parkinson, la malaltia d'Alzheimer i la malaltia de Huntington (Taula 1) (Annex I). Tot i que la majoria d'aquests gens estan inclosos en les 3 vies de senyalització (hsa05012, hsa05016 i hsa05010), alguns d'ells es troben únicament associats a Parkinson (PARK7), a Alzheimer (NAE1, FAS) o a Huntington (AP2B1, CREB3L4, POLR2H i SOD1) (Annex I).

Taula 1: Vies de senyalització desregulades en els co-cultius de cèl·lules portadores d'al·lels 'R' de *MC1R.*

Via de Senyalització	Estat	Num. gens	Num. genoma	p-valor ajustat
Lisosoma (hsa04142)	Infraexpressada	123	125	4,70E-03

Endocitòsi (hsa04144)	Infraexpressada	211	211	3,83E-02
Reparació de malaparellament (hsa 03430)	Sobreexpressada	22	23	1,56E-06
Reparació per excisió de nucleòtid (hsa03420)	Sobreexpressada	49	49	7,01E-06
Reparació per excisió de base (hsa03410)	Sobreexpressada	34	34	1,07E-03
Recombinació homòloga (hsa03440)	Sobreexpresada	27	28	6,98E-04
Replicació de l'ADN (hsa03030)	Sobreexpressada	34	35	3,83E-14
Cicle cel·lular (hsa04110)	Sobreexpressada	116	119	7,87E-14
Fosforilació oxidativa (hsa00190)	Sobreexpressada	132	134	6,91E-05
Malaltia de Parkinson (hsa05012)	Sobreexpressada	126	128	3,10E-05
Malaltia de Huntington (hsa05016)	Sobreexpressada	171	174	5,25E-04
Malaltia d'Alzheimer (hsa05010)	Sobreexpressada	166	168	3,03E-03

Num. gens: Número de gens detectats en el *microarray*, Num. genoma: Número total de gens associats a cadascuna de les vies de senyalització. Sobreexpressada: via de senyalització sobrerepresentada en el grup de trànscrits sobreexpressats en les cèl·lules portadores d'un MC1R no funcional. Infraexpressada: via de senyalització sobrerepresentada en el grup de trànscrits infraexpressats en les cèl·lules portadores d'un MC1R no funcional.

De forma global, els resultats d'aquest treball van confirmar que les cèl·lules portadores d'un MC1R no funcional presenten un augment del dany en l'ADN i l'alteració de processos biològics claus per garantir l'homeòstasi cel·lular. Aquests resultats indiquen que el rol de *MC1R*, va més enllà del seu paper com a gen clau en la pigmentació i suggereixen que podria estar implicat en el desenvolupament de malalties neurodegeneratives.

4. EXPRESSIÓ I ROL DEL MC1R EN EL SISTEMA NERVIÓS CENTRAL

Tot i que l'expressió majoritària del MC1R té lloc en els melanòcits, tècniques d'hibridació *in situ* i immunohistoquímica han demostrat la seva expressió en el sistema nerviós central (SNC), concretament en les neurones de la substància gris periaqüeductal, astròcits i cèl·lules de Schwann [79-81]. Els melanòcits i les cèl·lules del SNC tenen un origen embrionari comú i deriven de la cresta neural [82].

Diversos estudis han suggerit una implicació del MC1R en la regulació de processos biològics a nivell del SNC. S'ha descrit la implicació del MC1R com a modulador de la resposta analgèsica a través dels receptors κ-opioides [83]. En els homes la regulació del dolor a través dels receptors κ-opioides està modulada per l'acció dels receptors de N-metil-D-aspartat. Mentre que en les dones, la regulació del dolor a través dels receptors κ-opioides està modulada per l'acció del MC1R [84]. Un estudi realitzat amb models murins mostrava que les femelles portadores de 2 al·lels 'R' de *Mc1r* presenten una major resposta analgèsica amb el consum de l'opiaci pentazocina que les femelles amb 1 o cap variant i que els mascles [85]. Tot i que el mecanisme biològic que explica la interacció entre el MC1R i els receptors κ-opioides no és ben conegut, es coneix que els dos subtipus de receptors senyalitzen a través de la via de l'AMPc [7, 86] i es localitzen en la substància gris periaqüeductal i en el *locus coeruleus*, dues regions cerebrals importants per a les dimensions sensorials i afectives del dolor [87]. En aquesta mateixa línia, estudis epidemiològics han reportat una associació entre la presència de variants al·lèliques de *MC1R* i una major por al dolor, por al tractament dental i un major requeriment anestèsic [88, 89].

L'expressió del MC1R en les cèl·lules de la glia (astròcits i cèl·lules de Schwann) i en les cèl·lules endotelials microvasculars [90] suggereix un possible rol immunomodulador i antiinflamatori del MC1R en el cervell. L'activació del MC1R en el cervell causa una infraregulació del factor nuclear potenciador de les cadenes lleugeres kappa de las cèl·lules B activades (NF-KB), a través de la qual modula la resposta inflamatòria que es produeix com a conseqüència d'una isquèmia, hemorràgia o dany cerebral [91]. Així doncs, l'activació del MC1R confereix neuroprotecció a través dels seus efectes antiinflamatoris [91].

Aquestes evidencies posen de manifest l'impacte del MC1R en el SNC i indiquen l'efecte pleiotròpic d'aquesta proteïna, ja que en funció del teixit en el que s'expressa modula diferents processos biològics.

5. MALALTIA DE PARKINSON

La malaltia de Parkinson (codi OMIM: #168600) va ser descrita per primera vegada per James Parkinson el 1817 en el seu assaig sobre la "paràlisi tremolosa" [92]. No obstant, la patologia de la malaltia no es va conèixer fins a principis del segle XX. El 1912 el patòleg alemany Frederick Lewy va identificar l'existència d'inclusions citoplasmàtiques neuronals en diverses regions cerebrals [93] i el 1919 Konstantin Tretiakoff va destacar que la pèrdua de neurones dopaminèrgiques en la *pars compacta* de la substància negra del mesencèfal és l'alteració més important en aquesta malaltia (Figura11) [94].

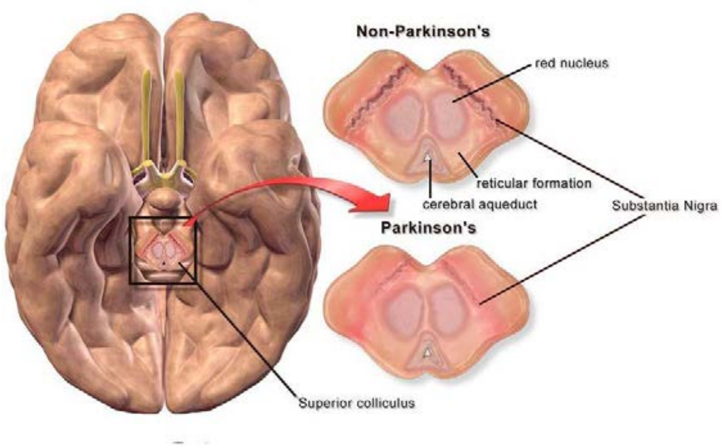


Figura 11. Pèrdua de neurones dopaminèrgiques en la substància negra del mesencèfal. Adaptada de [95].

La malaltia de Parkinson és la segona patologia neurodegenerativa més prevalent després de la malaltia d'Alzheimer [96], amb una incidència mundial estimada d'entre 5 a 35 casos per cada 100.000 persones/any [97]. La malaltia de Parkinson es caracteritza principalment per una pèrdua anormal de les neurones dopaminèrgiques i per agregacions anòmales de α-sinucleïna que formen els cossos de Lewy en la substancia negra del mesencèfal [98]. La malaltia és diagnostica clínicament amb l'aparició de la bradicinèsia i/o altres símptomes motors (rigidesa, tremolor, etc), i a més presenta molts altres símptomes no motors (depressió, ansietat, fatiga, dolor, apatia, deteriorament cognitiu, demència, etc) que contribueixen a la discapacitat general [97]. El Parkinson és una malaltia complexa causada per la interacció entre factors intrínsecs del hoste i factors ambientals [99]. El principal factor de risc és l'envelliment, la malaltia és poc freqüent abans dels 50 anys, però la seva incidència augmenta de 5 a 10 vegades entre la sisena i novena dècada de vida [100, 101]. La prevalença del Parkinson en els països industrialitzats és del 0,3% en la població general, del 1% entre els majors de 60 anys i d'un 3% entre els majors de 80 anys [102, 103]. La incidència de la malaltia és més alta en homes que en dones [101, 104]. En quan als factors ambientals, la malaltia de Parkinson és més freqüent en els països industrialitzats [105], no obstant, característiques pròpies de les zones rurals, com són: l'exposició als herbicides, als pesticides, el consum d'aigua de pou i de llet s'han associat a l'aparició de la malaltia [106, 107]. El consum de cafeïna i el tabaquisme s'han identificat com a factors protectors a desenvolupar Parkinson [108, 109]. L'ètnia, un historial familiar d'agregació a la malaltia i factors genètics, es consideren factors de risc a desenvolupar la malaltia de Parkinson [110, 111].

Un 60% del risc a desenvolupar la malaltia de Parkinson és heretat, mentre que la heretabilitat atribuïda a l'edat d'aparició del Parkinson augmenta fins a un 98% [112]. Aproximadament entre un 5-10% dels casos, són formes monogèniques causades per mutacions amb una elevada penetrància i que segreguen amb la malaltia dins de les famílies [113]. Fins a la data, s'han identificat 6 gens que s'associen amb aquestes formes, mutacions en 3 d'ells presenten un patró d'herència autosòmic dominant (*SNCA*, *LRRK2* i *VPS35*) i en els 3 restants aquestes s'hereten sota un model autosòmic recessiu (*Parkin*, *PINK-1* i *DJ-1*) [114]. Aquestes formes monogèniques de Parkinson mostren una edat d'aparició temprana (< 50 anys) [113, 115]. Aproximadament un 90% dels casos de Parkinson són esporàdics i presenten un etiologia complexa basada en la interacció entre múltiples variants de baix risc, factors ambientals i factors epigenètics. Els al·lels associats a les formes esporàdiques de Parkinson presenten una freqüència relativament comú (MAF>1%) en la població general i una baixa penetrància (Figura 12) [116].

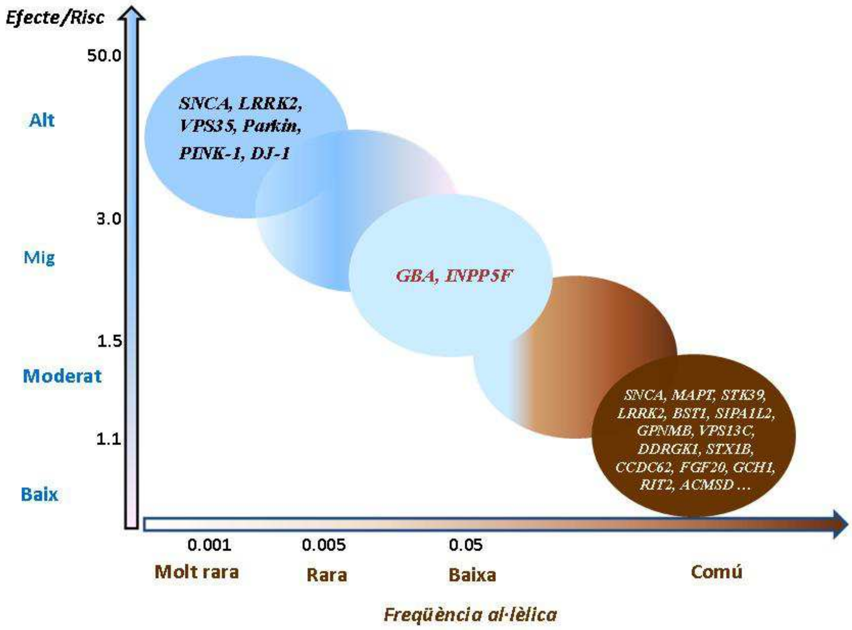


Figura 12. Gens implicats en la susceptibilitat a la malaltia de Parkinson. Adaptada de Manolio *et al*. [58].

Durant la darrera dècada, els esforços en investigació s'han centrat en identificar factors genètics associats a les formes esporàdiques del Parkinson [117]. Mitjançant estudis d'associació del genoma complet (GWAS) s'han identificat múltiples variants genètiques de risc mig o baix en més de 20 loci associats a la malaltia [114, 118]. Malgrat l'important progrés que ha suposat la identificació d'aquestes variants genètiques, el risc atribuït a aquestes no explica el percentatge total d'heretabilitat identificat en el Parkinson [114]. Aquest fet indica que encara queden variants genètiques pendents d'identificar implicades en la etiologia de la malaltia.

Donat que l'edat d'inici del Parkinson s'ha definit com un caràcter poligènic [112, 119] i es coneix que serveix com a predictor de la progressió i mortalitat [120], diversos treballs s'han centrat en avaluar si els gens de risc mig i baix a Parkinson poden actuar com a modificadors en l'edat d'aparició de la malaltia [117, 119, 121]. Diferents treballs han identificat que variants al·lèliques en 3 d'aquests gens: *SNCA*, *GBA* i *MAPT* modifiquen l'edat d'aparició de les formes esporàdiques de Parkinson [117, 121]. D'altra banda, variants al·lèliques en els gens *SNCA, MAPT* i *DNM3* modifiquen l'edat d'inici de la malaltia en els individus portadors de mutacions d'alta penetrància en el gen *LRRK2* [122-124]. No obstant, el percentatge de variació atribuït a aquestes variants genètiques no explica el percentatge total d'heretabilitat atribuït a l'edat d'inici de la malaltia de Parkinson [125].

Aquests resultats posen de manifest la complexitat genètica de la malaltia de Parkinson, ja que alguns gens contribueixen únicament al risc a desenvolupar la malaltia, altres confereixen risc i modifiquen l'edat d'aparició o la progressió motora dels símptomes i altres presenten un efecte modificador quan interaccionen amb d'altres variants genètiques [125]. Així doncs, són necessaris més estudis centrats en identificar nous gens i/o variants addicionals que ens permetin comprendre més bé la base genètica de la malaltia de Parkinson, per tal de millorar en el diagnòstic i/o identificar possibles dianes terapèutiques.

6. MALALTIA D'ALZHEIMER

La malaltia d'Alzheimer (codi OMIM: #104300) va ser descrita per primera vegada per Alois Alzheimer l'any 1906 [126]. Aquesta malaltia és el trastorn neurodegeneratiu més comú entre la gent gran [127] i es caracteritza per una pèrdua gradual de la memòria i les habilitats cognitives. L'Alzheimer representa més del 50% del total de casos de demència i actualment té una prevalença mundial superior als 27 milions de casos [128]. La incidència de la malaltia supera els 5 milions de casos/any i és del 1% entre les persones de 60-70 anys i del 6-8% entre les persones ≥ 85 anys [127]. S'espera que aquesta incidència augmenti en els propers anys degut a l'increment continuat de la longevitat en les societats actuals [129].

La malaltia d'Alzheimer es caracteritza principalment per la formació de plaques amiloides a l'espai extracel·lular i de cabdells neurofibrilars a l'interior de les neurones, la formació d'aquestes estructures comporta la mort neuronal i la pèrdua de sinapsis en amplies àrees del còrtex cerebral i l'hipocamp (Figura 13) [130]. La proteïna precursora d'amiloide (APP) pot ser escindida per 3 secretases diferents (α, β o γ), quan l'APP és escindida per la α-secretasa el producte resultant és un pèptid soluble que és eliminat amb facilitat per l'organisme. Per contra, si la proteïna APP és escindida per la β secretasa i posteriorment per la γ secretasa s'origina una proteïna beta amiloide (Aβ) de 42 residus ($A\beta_{42}$) que és altament insoluble i genera oligòmers i fibrilles que s'acumulen extracel·lularment donant lloc a la formació de plaques amiloides [127]. La formació d'aquests oligòmers i plaques amiloides augmenta els nivells de ROS i la toxicitat neuronal, fet que desencadena una hiperfosforilació de la proteïna tau que es dissocia dels microtúbuls i genera agregats de tau fosforilada (P-tau) que formen els cabdells neurofibrilars (Figura 13) [128, 130].

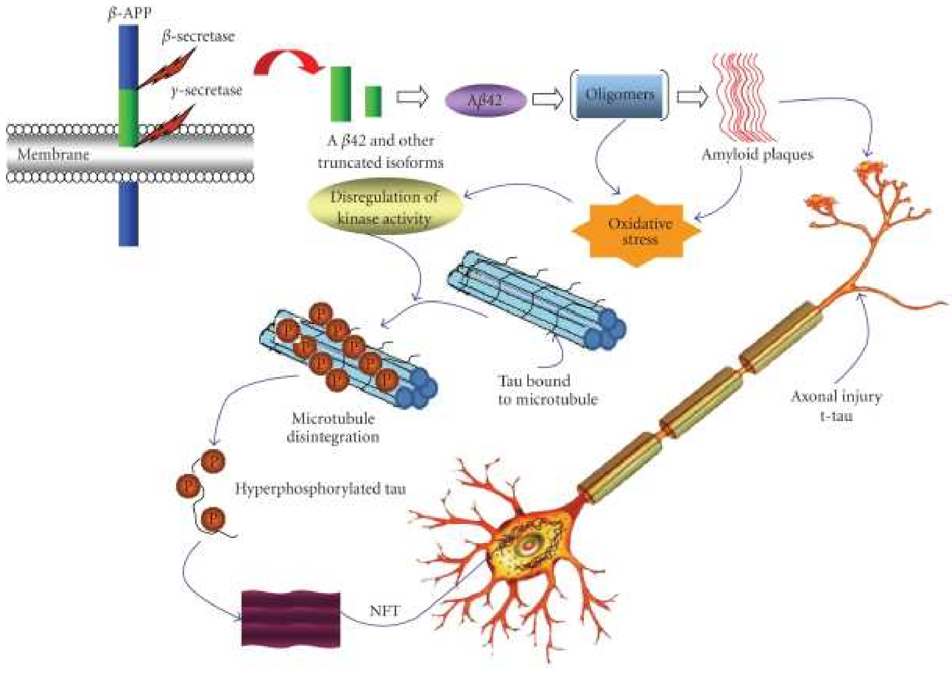


Figura 13. Cascada patològica i biomarcadors de la malaltia d'Alzheimer. Anoop *et al*. [128].

En l'actualitat, la malaltia d'Alzheimer és diagnostica en base a criteris exclusivament clínics [131]. No obstant, en els darrers anys s'ha suggerit l'ús de biomarcadors en líquid cefal·loràquidi (CSF) per a establir el diagnòstic [132, 133]. En els pacients amb Alzheimer s'observa una disminució dels nivells d' $A\beta_{42}$ (evidència de dipòsit d'amiloide) i un augment dels nivells de T-tau i P-tau (evidències de degeneració o lesió neuronal) en LCR [134]. El estudis on el diagnòstic es basa únicament en els criteris clínics presenten una sensibilitat del 85% i una especificitat del 70% [135], la inclusió dels nivells de biomarcadors en LCR incrementa la sensibilitat i l'especificitat diagnòstica fins a un 90% i 85%, respectivament [136, 137]. Degut a qüestions metodològiques i de disponibilitat de mostra no es pot recomanar l'ús generalitzat d'aquests biomarcadors en el diagnòstic de l'Alzheimer. A dia d'avui, el seu ús es veu restringit a la investigació, als assaigs clínics i al diagnòstic de determinats casos seleccionats pels neuròlegs especialistes.

L'Alzheimer és una malaltia complexa causada per la interacció entre factors intrínsecs del hoste i factors ambientals. Els 3 principals factors de risc són l'edat, el sexe femení i la presència de l'al·lel 4 de l'apolipoproteïna E (APOEε4) [138]. Tenir un baix nivell educatiu i factors de risc cardiovascular (hipertensió, tabaquisme, diabetis, hipercolesterolèmia, fibril·lació auricular, la obesitat i la inactivitat física) s'han associat, amb un menor consens, a un augment del risc a desenvolupar Alzheimer [139].

Un 1-5% dels casos d'Alzheimer són formes monogèniques causades per mutacions rares amb una elevada penetrància. Aquestes formes segreguen amb la malaltia amb un patró autosòmic dominant dins de les famílies i presenten una major velocitat de progressió [140]. S'han identificat 3 gens (*APP*, *PSEN1* i *PSEN2*) associats a aquestes formes d'herència mendelianes [141], tots ells codifiquen per proteïnes implicades en la degradació de la APP i la síntesi de la proteïna Aβ. Les mutacions en aquests gens s'associen a les formes d'Alzheimer d'inici temprà < 65 anys.

Un 95% dels casos d'Alzheimer són esporàdics i presenten la malaltia en edats ≥ 65 anys. Aquestes formes d'inici tardà són multifactorials i s'ha estimat que entre un 60-80% del risc a desenvolupar-les és genètic [140, 142]. Fins fa una dècada, la presència de l'al·lel 4 de l'apolipoproteïna E (APOEε4) era l'únic factor genètic de risc associat a les formes esporàdiques d'Alzheimer (≥ 65 anys). Els individus heterozigots per l'APOEε4 presenten 3 vegades més risc a desenvolupar la malaltia i aquest augmenta fins a 12 vegades en els individus homozigots (APOEε4/APOEε4) [143, 144]. L'APOE és el principal transportador de colesterol en el SNC i està implicat en l'eliminació de la proteïna Aβ, la presencia de l'APOEε4 s'ha associat a una menor eficiència en aquest procés d'eliminació [145]. El risc atribuït a l'APOEε4 representa menys del 50 % del risc genètic atribuït a desenvolupar Alzheimer [146]. En els darrers anys, mitjançant estudis GWAS i de seqüenciació massiva (NGS) s'han identificat variants de baix risc a desenvolupar la malaltia en 20 gens diferents [146-151], els quals es troben implicats en el control del sistema immunològic i la resposta inflamatòria, el metabolisme de lípids i colesterol i la endocitosi [141]. A més d'aquestes variants de baix risc, els estudis de NGS han identificat el gen *TREM2* com a gen de risc mig a desenvolupar la malaltia d'Alzheimer [152, 153] (Figura 14).

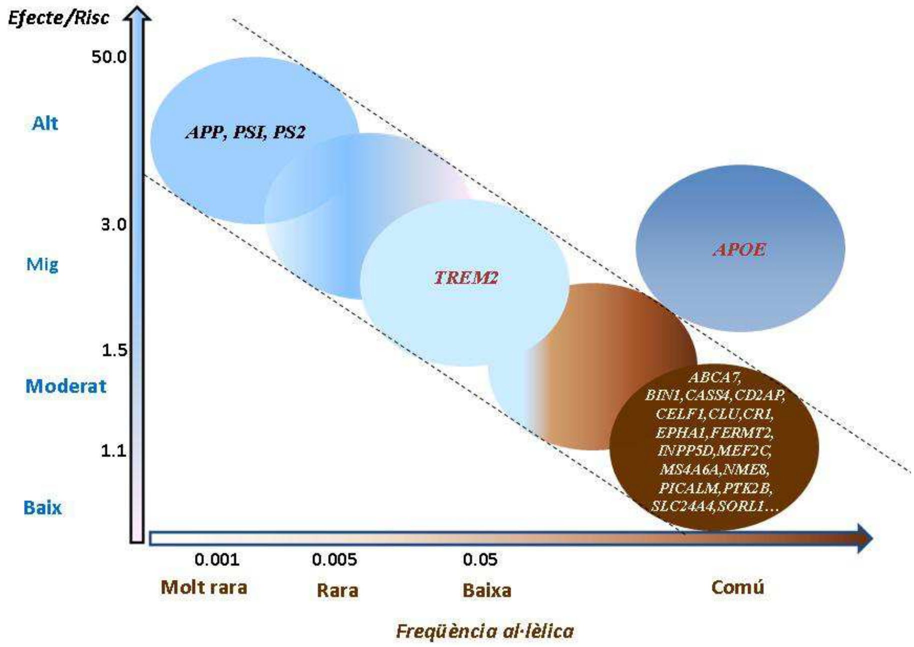


Figura 14. Gens implicats en la susceptibilitat a la malaltia d'Alzheimer. Adaptada de Manolio *et al.* [58].

El gen *TREM2* (codi OMIM: #605086) està implicat en l'activació de la resposta immunològica en macròfags i cèl·lules dendrítiques [152]. Els individus portadors de la variant rara p.R47H en *TREM2* presenten 4 vegades més risc a desenvolupar Alzheimer, aquest és similar al risc atribuït a la presència de l'APOEε4 [152, 153]. Malgrat l'èxit d'aquests treballs, el risc atribuïble a aquestes variants al·lèliques no explica el percentatge total d'heretabilitat identificat en aquesta malaltia [154] i suggereix que altres gens pendents d'identificar estan implicats en la etiologia de l'Alzheimer.

Malgrat l'edat d'aparició de la malaltia d'Alzheimer és un caràcter poligènic [155], únicament l'APOEε4 s'ha identificat, amb consens, com a variant al·lèlica modificadora de l'edat d'aparició de la malaltia [156-160]. Amb menor consens, els gens: *CR1*, *BIN1*, *PICALM*, *NCST* i *ZBTB4* s'han identificat com a gens modificadors de l'edat d'inici de les formes tardanes de la malaltia [155, 157]. No obstant, el percentatge de variació atribuït a aquestes variants genètiques no explica el percentatge total d'heretabilitat atribuït a l'edat d'aparició de la malaltia d'Alzheimer [155].

Així doncs, són necessaris més estudis centrats en revalidar els gens candidats prèviament identificats i en identificar nous gens i/o variants genètiques que ens permetin comprendre més bé la complexitat genètica d'aquesta malaltia. La identificació de factors genètics implicats en la patogènesi de la malaltia d'Alzheimer ens permetria millorar en el diagnòstic i/o identificar possibles dianes terapèutiques.

7. MALALTIA DE HUNTINGTON

La malaltia de Huntington (codi OMIM: #143100) va ser descrit per primera vegada per George Huntington l'any 1872 [161]. Aquesta malaltia és el trastorn neurològic monogènic més comú als països desenvolupats [162] i es caracteritza per l'aparició de moviments involuntaris, trastorns de conducta, trastorns psiquiàtrics i demència. Es tracta d'una malaltia progressiva que dura entre uns 15-20 anys i en la qual l'edat mitjana d'aparició és als 40 anys [163]. Els primers símptomes de la malaltia acostumen a aparèixer entre els 30-50 anys. No obstant, s'han reportat casos on la malaltia apareix en edats molt tempranes (2 anys) o en edats molt avançades (≥ 80 anys) [164]. La incidència de la malaltia és de 4,7-6,9 casos per milió de persones/any i la prevalença és de 10,3-13,7 persones per cada 100.000 habitants [165]. La malaltia de Huntington presenta un patró d'herència autosòmic dominant i està causada per l'expansió anòmala d'un trinucleòtid citosina-adenina-guanina (CAG) en l'exó 1 del gen *HTT* (codi OMIM: 613004), que es tradueix en una repetició anòmala de glutamines (polyQ) en l'extrem N-terminal de la proteïna Huntingtina (Htt) (NP_002102) [164]. Aquesta forma mutada de la proteïna Htt presenta propietats neurotòxiques i provoca una mort neuronal progressiva en el nucli estriat (Figura 15) [165].

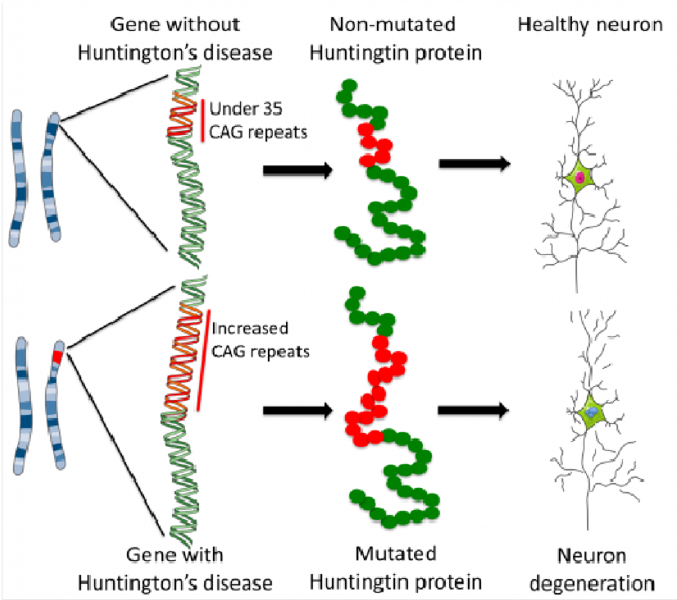


Figura 15. Representació de la mutació dinàmica CAG en el gen *HTT* i de la proteïna Htt mutada. Adaptada de [166].

Els al·lels que presenten < 27 repeticions del trinucleòtid CAG són normals i els que comprenen entre 27-35 repeticions s'anomenen al·lels intermedis. Aquests al·lels no són patogènics però poden expandir-se fins a rangs de patogenicitat (≥ 36 repeticions) en la transmissió de la línia germinal (predominantment per via paterna) i causar individus afectes en la descendència [167, 168]. Els al·lels patogènics són els que presenten entre 36-120 repeticions [169]. Dins d'aquest rang s'han identificat al·lels amb penetrància reduïda (36-39 repeticions), que poden trobar-se tant en individus afectes com en individus que no presenten simptomatologia en edats avançades (≥ 70-80 anys), i al·lels amb penetrància completa (≥ 40 CAG repeticions) [170].

En la malaltia de Huntington es dona un fenomen d'anticipació intergeneracional, ja que en les successives generacions s'observa una simptomatologia més severa i/o una anticipació en l'edat d'aparició de la malaltia. Degut a la inestabilitat i la capacitat d'expandir-se que presenten els triplets CAG durant l'espermatogènesi, aquest fenomen és més freqüent en la transmissió paterna [171].

Existeix una correlació inversa entre el nombre de repeticions CAG i l'edat d'aparició de la malaltia de Huntington. Aquesta correlació explica aproximadament entre un 50-

70% de la variació en l’edat d’inici i els valors màxims d’aquesta es donen en els extrems de l’interval de repeticions CAG [163, 172, 173]. Els individus portadors d’al·lels ≥ 60 CAG repeticions debuten majoritàriament ≤ 20 anys i és defineixen com a formes juvenils [174], mentre que els individus portadors de 36-39 CAG repeticions, en cas de presentar la malaltia, debuten en edats tardanes (> 60 anys). No obstant, aquesta correlació disminueix fins a un 50% en el rang de 40-50 repeticions que és el majoritari en les formes adultes [175]. Per tant, tot i que el nombre de repeticions CAG en el gen *HTT* és el principal factor determinant de l’edat d’inici de la malaltia, no la determina per complet.

Un 20% de la variació en l’edat d’aparició del Huntington no atribuïda a l’expansió CAG, s’ha atribuït a altres factors genètics [176, 177] i el percentatge restant a factors ambientals [178, 179]. En els darrers anys, nombrosos treballs han identificat factors genètics modificadors de l’edat d’inici del Huntington implicats en diferents processos biològics com: l'estrès oxidatiu (*MAP3K5,MAP2K6* i *PPARGC1A*) [180, 181], la transmissió sinàptica (*HAP1 i ADORA2*) [182, 183], la resposta glutamaèrgica (*GRIN2A i GRIN2B*) [184], l’autofàgia (*ATG7*) [185], la proteòlisis (*UCHL*) [186] i els mecanismes de reparació del dany en l’ADN (MLH1 i MLH3) [187]. Malgrat aquests avenços, el percentatge de variació en l’edat d’inici del Huntington atribuït a la majoria d’aquestes variants al·lèliques és aproximadament d’un 1% per a cadascuna d’elles, de manera que hi ha factors genètics modificadors de l’edat d’aparició de la malaltia pendents d’identificar.

La identificació de nous factors genètics modificadors de l’edat d’aparició de la malaltia de Huntington pot servir per realitzar un consell genètic més acurat i/o identificar possibles dianes terapèutiques per retardar o prevenir l'aparició de la simptomatologia clínica.

8. RELACIÓ EPIDEMIOLÒGICA ENTRE EL CÀNCER I LES MALALTIES NEURODEGENERATIVES

Estudis epidemiològics indiquen una associació inversa entre el risc a desenvolupar càncer i diverses malalties del SNC [188], especialment amb les malalties neurodegeneratives comunes associades a l’envelliment, com són la malaltia de Parkinson i la malaltia d’Alzheimer [189]. S’ha descrit, que els supervivents de càncer presenten una disminució d'un 20-50% del risc a desenvolupar Parkinson i Alzheimer, i que els malalts de Parkinson o Alzheimer presenten un risc notablement menor a desenvolupar càncer [190, 191]. Tot i que els processos biològics subjacents a aquesta relació inversa són força desconeguts, s'ha identificat que el càncer i les malalties neurodegeneratives comparteixen gens i vies de senyalització desregulades en sentits oposats, com la via de p53, de Pin1, de Wnt i de la ubiqüitina-proteosoma [189]. La infraregulació de p53 i la sobreregulació de Pin1, de Wnt i de la ubiqüitina-proteosoma afavoreix el desenvolupament de càncer i protegeix de la neurodegeneració, en el sentit invers succeeix el contrari.

De forma significativa, nombrosos treballs han reportat una associació bidireccional entre el risc a desenvolupar Parkinson i càncer cutani [192], especialment melanoma [193, 194]. Els pacients de melanoma presenten un augment del 50% del risc a desenvolupar Parkinson (OR=1,44; 95% IC: 1,03-2,01) [195] i els pacients de Parkinson presenten casi 2,5 vegades més risc a desenvolupar melanoma (OR=2,43; 95% IC: 1,77-3,32) [196, 197]. Un treball presentat l'any 1972, va hipotetitzar que l'augment de la incidència del melanoma entre els malalts de Parkinson es devia al tractament amb levodopa (L-dopa) que rebien aquests pacients [198]. Diversos estudis van donar suport a aquesta hipòtesi [199-201] basant-se en el fet de que la L-dopa és un producte intermediari tant en el procés de síntesi de melanina com de neuromelanina [202] (Figura 16), i que tant els nivells de melanina com els de neuromelanina es troben alterats en els melanomes i en els cervells de pacients amb Parkinson, respectivament [199, 200].

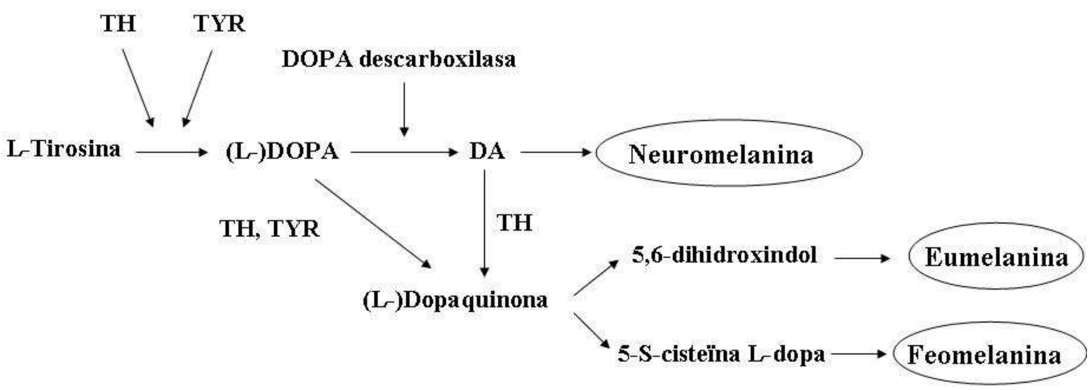


Figura 16. Biosíntesi de la neuromelanina i la melanina. Adaptada de Pan *et al.* [203].

Aquests estudis suggerien que l'augment de L-dopa en sang que es produeix en els pacients de Parkinson que reben tractament amb levodopa, pot comportar un augment de la producció de melanina en els melanòcits i en conseqüència el desenvolupament d'un melanoma [199-201]. No obstant, aquests treballs obviaven que són factors genètics els que provoquen una proliferació descontrolada dels melanòcits, i que l'augment de la síntesi de melanina en els melanomes és el resultat d'aquesta proliferació melanocítica descontrolada [34]. A més, tant la neuromelanina en les neurones dopaminèrgiques, com la melanina en els melanòcits, tenen un paper protector degut a la seva activitat *scavenger* enfront de les ROS [204, 205]. Paral·lelament es van publicar un seguit d'assaigs clínics i estudis prospectius que demostraven que la freqüència de casos de melanoma era més alta entre els pacients de Parkinson inclús abans que aquests debutessin amb els primers símptomes, i per descomptat, abans que iniciessin el tractament amb levodopa [206-208] Aquests resultats indicaven que l'associació entre aquest tractament i el melanoma és casual i no causal [209, 210].

D'altra banda, un treball que va avaluar l'associació epidemiològica entre presentar antecedents familiars de càncer i el risc a desenvolupar Parkinson, va identificar que els individus amb antecedents familiars de melanoma (en un familiar de primer grau) presentaven un major risc a desenvolupar la malaltia de Parkinson (RR=1,85; 95% IC: 1,2-2,8) [211]. El treball descartava una associació entre el risc a desenvolupar Parkinson i antecedents familiars de càncer de colon, mama, pulmó i pròstata. Per tant, els resultats d'aquest treball suggereixen que el melanoma i el Parkinson comparteixen factors genètics de risc [211].

La major taxa d'incidència del melanoma i del Parkinson s'observa en la població caucàsica, mentre que la taxa d'incidència més baixa per ambdues malalties s'observa en la població africana [212, 213]. En base a aquestes evidències es va hipotetitzar que gens implicats en la pigmentació podrien ser factors genètics de risc a desenvolupar tant melanoma com Parkinson [214]. En aquesta línia, un estudi cas-control que incloïa 539 casos de Parkinson, va identificar una associació significativa entre el color clar de cabell i un major risc a desenvolupar Parkinson. Concretament, els individus pèl-roigs presentaven dues vegades més risc a desenvolupar Parkinson (RR=1,93, 95% IC: 1,08-3,43) que els individus amb el cabell negre [215]. Aquests resultats indiquen que existeix una sobrerepresentació d'individus pèl-roigs entre els pacients de Parkinson, igual que succeeix entre els pacients de melanoma.

Ara bé, aquesta associació es veu restringida al Parkinson, ja que cap estudi ha identificat una associació epidemiològica entre el risc a Alzheimer i el risc a desenvolupar càncer cutani [216].

HIPÒTESI

OBJECTIUS

La hipòtesi de la present tesi doctoral és que el gen *MC1R* podria estar implicat en la patogènesi de les malalties neurodegeneratives.

Objectiu general:

Analitzar el paper del MC1R com a factor genètic de risc a desenvolupar malalties neurodegeneratives i/o com a factor modificador en l'edat d'aparició d'aquestes malalties.

Objectius concrets:

1. Avaluar el gen *MC1R* com a factor genètic de risc a desenvolupar la malaltia de Parkinson.

2. Avaluar el gen *MC1R* com a factor genètic de risc a desenvolupar la malaltia d'Alzheimer.

3. Avaluar el gen *MC1R* com a factor modificador de l'edat d'aparició de la malaltia de Parkinson, la malaltia d'Alzheimer i la malaltia de Huntington.

4. Identificar processos biològics alterats a nivell constitutiu en les cèl·lules cutànies portadores de variants de pèrdua de funció en *MC1R*, que en part expliquin, la patogènesi observada en les malalties neurodegeneratives.

INFORME DELS DIRECTORS

Títol de la tesi:

ROL DEL GEN DE PIGMENTACIÓ *MC1R* EN LA SUSCEPTIBILITAT A MALALTIES NEURODEGENERATIVES

Doctoranda: Gemma Tell Martí

Directors: Susana Puig Sardà i Joan Anton Puig Butillé

TREBALL I

Títol: The *MC1R* melanoma risk variant p.R160W is associated with Parkinson disease

Autors: Tell-Marti G, Puig-Butille JA, Potrony M, Badenas C, Milà M, Malvehy J, Martí MJ, Ezquerra M, Fernández-Santiago R, Puig S.

Publicació: Annals of Neurology. 77 (5), pp. 889 - 894. 2015. ISSN 1531-8249. Doi: 10.1002/ana.24373

Factor d'impacte (JCR Science Edition 2015): 9,638 (1er decil: Neurosciences)

Aportació de la doctoranda a l'article: La doctoranda va caracteritzar molecularment el gen *MC1R* en el conjunt total de mostres; mitjançant PCR d'amplificació, la posterior PCR de seqüenciació i l'anàlisi de les seqüències obtingudes. La doctoranda va dur a terme l'anàlisi estadístic de les dades i és responsable de la redacció del manuscrit.

TREBALL II

Títol: A Common Variant in the *MC1R* Gene (p.V92M) is associated with Alzheimer's Disease Risk

Autors: Tell-Marti G, Puig-Butille JA, Potrony M, Plana E, Badenas C, Antonell A, Sanchez-Valle R, Molinuevo JL, Lleó A, Alcolea D, Fortea J, Fernández-Santiago R, Clarimón J, Lladó A, Puig S.

Publicació: Journal of Alzheimer's disease : JAD. 56 (3), pp. 1065 - 1074. 2017. ISSN 1875-8908. Doi: 10.3233/JAD-161113.

Factor d'impacte (JCR Science Edition 2016): 3,731 (2n quartil: Neurosciences)

Aportació de la doctoranda a l'article: La doctoranda va caracteritzar molecularment el gen *MC1R* en el conjunt total de mostres; mitjançant PCR d'amplificació, la posterior PCR de seqüenciació i l'anàlisi de les seqüències obtingudes. La doctoranda va dur a terme l'anàlisi estadístic de les dades i és responsable de la redacció del manuscrit.

TREBALL III

Títol: The p.R151C Polymorphism in *MC1R* Gene Modifies the Age of Onset in Spanish Huntington's Disease Patients

Autors: Tell-Marti G, Puig-Butille JA, Gimenez-Xavier P, Segu-Roig A, Potrony M, Badenas C, Alvarez V, Millán JM, Trujillo-Tiebas MJ, Ramos-Arroyo MA, Milà M, Puig S.

Publicació: Molecular Neurobiology. 54 (5), pp. 3906 - 3910. 2017. ISSN 1559-1182. Doi: 10.1007/s12035-016-0305-5.

Factor d'impacte (JCR Science Edition 2016): 6,190 (1er decil: Neurosciences)

Aportació de la doctoranda a l'article: La doctoranda va caracteritzar molecularment el gen *MC1R* en el conjunt total de mostres; mitjançant PCR d'amplificació, la posterior

PCR de seqüenciació i l'anàlisi de les seqüències obtingudes. La doctoranda va dur a terme l'anàlisi estadístic de les dades i és responsable de la redacció del manuscrit.

TREBALL IV

Títol: Genomic expression differences between cutaneous cells from red hair color individuals and black hair color individuals based on bioinformatic analysis

Autors: Puig-Butille JA, Gimenez-Xavier P, Visconti A, Nsengimana J, García-García F, Tell-Marti G, Escamez MJ, Newton-Bishop J, Bataille V, Del Río M, Dopazo J, Falchi M, Puig S.

Publicació: Oncotarget. 8 (7), pp. 11589 - 11599. 2017. ISSN 1949-2553. Doi: 10.18632/oncotarget.14140.

Factor d'impacte (JCR Science Edition 2016): 5,168 (1er quartil: Oncology)

Aportació de la doctoranda a l'article: La doctoranda va participar en la interpretació: dels resultats obtinguts a partir de l'anàlisi de xarxes d'interacció proteïna-proteïna (PPI) i dels resultats obtinguts amb els diferents *sets* de revalidació. Va participar activament en la redacció del manuscrit.

Dra. Susana Puig Sardà — Dr. Joan Anton Puig Butillé

RESULTATS

TREBALL I

Treball realitzat per donar resposta als objectiu 1 i 3 de la present tesi doctoral.

Títol:

The *MC1R* melanoma risk variant p.R160W is associated with Parkinson disease

Doi: 10.1002/ana.24373

Resum:

En la darrera dècada, diferents treballs epidemiològics han indicat una associació bidireccional entre el risc a desenvolupar melanoma i el risc a desenvolupar Parkinson. Hi ha dades que indiquen que factors genètics poden estar implicats en aquesta associació, i més concretament, que aquests poden ser gens implicats en la regulació de la pigmentació. El fet que les cèl·lules cutànies portadores de variants LOF en *MC1R* mostrin una desregulació constitutiva de gens implicats en les vies de senyalització de malalties neurodegeneratives, suggereix que el gen *MC1R* podria estar implicat en la patogènesi de la malaltia de Parkinson.

L'objectiu principal d'aquest treball era avaluar el gen *MC1R* com a factor genètic de risc a desenvolupar la malaltia de Parkinson i com a factor modificador de l'edat d'aparició d'aquesta malaltia.

Per a donar resposta aquest objectiu, es va dissenyar un estudi cas-control que incloïa 870 pacients de Parkinson i 736 controls aparellats per edat i sexe, tots ells d'origen espanyol. El gen *MC1R* es va caracteritzar molecularment en el conjunt total de casos i controls, seqüenciant la regió codificant sencera del trànscrit (ENST0000555147) que codifica per la forma canònica del receptor MC1R (NP_002377).

De forma global es van identificar un total de 47 variants al·lèliques (13 sinònimes i 34 no sinònimes) en el gen *MC1R*. Entre aquestes, 38 variants (12 sinònimes i 26 no sinònimes) van ser detectades en pocs o en un únic individu i presentaven una MAF < 0,01. La freqüència global d'aquestes variants rares (MAF < 0,01) es va comparar entre casos

(0,053) i controls (0,043) i no es va detectar una diferència estadísticament significativa (p-valor = 0,383).

L'anàlisi d'associació genotípica cas-control es va dur a terme amb el conjunt de variants que complien els següents criteris: variants no sinònimes, en equilibri Hardy-Weinberg en la població control i amb una MAF > 0,01 en els pacients de Parkinson. Se'n van detectar 7: p.V60L (0,13), p.V92M (0,06), p.R151C (0,03), p.I155T (0,01), p.R160W (0,02), p.R163Q (0,02), i p.D294H (0,01). Tots els anàlisis d'associació genotípica cas-control es van ajustar per l'edat i el sexe.

Els resultats de l'anàlisi d'associació genotípica van mostrar que els individus portadors de l'al·lel p.R160W (rs1805008) presenten un augment del risc a desenvolupar la malaltia de Parkinson (OR=2,10; 95% IC: 1,18-3,73; p-valor ajustat = 0,009, p-valor corregit per Bonferroni = 0,063). Aquest resultat explica, en part, la relació epidemiològica bidireccional entre el risc a melanoma i el de Parkinson, ja que variants LOF en *MC1R* incrementen la susceptibilitat a desenvolupar ambdues malalties. El fet que els individus portadors de l'al·lel 'R' p.R160W s'associïn amb el fenotip RHC, explica la sobrerepresentació d'individus pèl-roigs que s'ha identificat entre els pacients de Parkinson. La resta de variants avaluades, tant 'r' (p.V60L, p.V92M i p.R163Q) com 'R' (p.R151C, p.I155T i p.D294H) no es van associar amb un augment del risc a desenvolupar Parkinson en aquesta cohort d'origen espanyol. Cap de les variants analitzades en *MC1R* es va associar a una variació en l'edat d'aparició de la malaltia de Parkinson, indicant que el gen *MC1R* no és un factor modificador de l'edat d'inici d'aquesta malaltia, almenys en aquesta cohort d'origen espanyol.

BRIEF COMMUNICATION

The *MC1R* Melanoma Risk Variant p.R160W Is Associated with Parkinson Disease

Gemma Tell-Marti, MSc,[1,2]
Joan Anton Puig-Butille, PhD,[2,3]
Miriam Potrony, MSc,[1]
Celia Badenas, PhD,[2,3]
Montserrat Milà, PhD,[2,3]
Josep Malvehy, MD, PhD,[1,2]
María José Martí, PhD,[4]
Mario Ezquerra, PhD,[4]
Rubén Fernández-Santiago, PhD,[4*] and
Susana Puig, MD, PhD[1,2,5*]

Epidemiological studies have reported the co-occurrence of Parkinson disease (PD) and melanoma. Common genetic variants in the *MC1R* (melanocortin 1 receptor) gene, which determines skin and hair color, are associated with melanoma. Here we investigated whether genetic variants in *MC1R* modulate the risk of PD by sequencing the entire gene in 870 PD patients and 736 controls ascertained from Spain. We found that the *MC1R* variant p.R160W (rs1805008) is marginally associated with PD (odds ratio = 2.10, gender- and age-adjusted p = 0.009, Bonferroni-corrected p = 0.063). Our results suggest that *MC1R* genetic variants modulate the risk of PD disease in the Spanish population.

ANN NEUROL 2015;00:000–000

Epidemiological studies have described an inverse association between cancer and age-related neurodegenerative diseases.[1] Thus, patients with Parkinson disease (PD) have a reduced incidence of most types of cancer excepting cutaneous melanoma (CM),[2] whose incidence is higher in PD.[3] In addition, CM patients have also shown an increased risk for PD.[3] Different hypotheses have been proposed to explain the co-occurrence of both diseases[4–6]; however, the underlying mechanism remains unknown. Genetic variants in the pigmentation genes could underlie the bidirectional link between both diseases, because a higher incidence of both diseases has been detected in Caucasians compared to black populations.[7] In addition, individuals with fair phototype (pale skin and light-colored hair) show an increased risk of developing CM[8] and PD.[9] Furthermore, skin cells from red hair color individuals have a constitutive deregulation of genetic pathways involved in neurodegenerative diseases.[10]

In the present study, we have investigated whether genetic variants in the *MC1R* (melanocortin 1 receptor) gene are associated with PD. The *MC1R* gene is the master regulator of pigmentation phenotype in humans.[11] Recessive *MC1R* loss-of-function genetic variants are responsible for the red hair color (RHC) phenotype (red hair, fair skin, and poor tanning response) and increase the risk of developing CM.[11] Six loss-of-function *MC1R* variants (R variants) are strongly associated with the RHC phenotye and a higher risk of CM (p.D84E, p.R142H, p.R151C, p.R160W, p.I155T, and p.D294H).[11] In addition, other *MC1R* variants with partial loss-of-function that are not associated with the RHC phenotypical characteristics (r variants) increase moderately the risk of CM (p.V60L, p.V92M, and p.R163Q).[12] To explore the role of *MC1R* in PD, we sequenced the entire coding region of *MC1R* in 870 PD cases and 736 gender- and age-matched controls ascertained from Spain, and analyzed all the common nonsynonymous R and r variants with an observed minor allele frequency (MAF) of at least 0.01.

Subjects and Methods

Subjects

We performed a case–control study of 870 PD patients (mean age ± standard deviation [SD] = 57.64 ± 11.89 years, male 60.5%/female 39.5%) and 736 gender- and age-matched healthy controls (mean age ± SD = 56.69 ± 12.74 years, male 56.1%/female 43.9%). All study subjects were Mediterranean Caucasians ascertained from Spain. All PD patients were recruited at the Parkinson's Disease and Movement Disorders Unit of the Hospital Clínic of Barcelona and diagnosed according to the United Kingdom Parkinson's Disease Society Brain Bank criteria.[13] The control group included healthy individuals without signs of neurodegenerative or psychiatric disorders

From the [1]Dermatology Department, Melanoma Unit, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS); [2]Center for Biomedical Network Research on Rare Diseases (CIBERER), Carlos III Health Institute (ISCIII); [3]Biochemical and Molecular Genetics Service, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS); [4]Laboratory of Neurodegenerative Disorders, Department of Clinical and Experimental Neurology, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Hospital Clínic of Barcelona, Center for Biomedical Network Research in Neurodegenerative Diseases (CIBERNED); and [5]Medicine Department, University of Barcelona (UB), Barcelona, Spain

Address correspondence to Dr Puig, Melanoma Unit, Dermatology Department, Hospital Clínic Barcelona, Villarroel 170. 08036, Barcelona, Spain. E-mail: susipuig@gmail.com; spuig@clinic.ub.es

R.F.-S and S.P. contributed equally to this work.

Received Nov 23, 2013, and in revised form Jan 9, 2015. Accepted for publication Jan 14, 2015.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.24373

obtained from 2 independent control series. Of these, 131 controls were spouses of patients examined by movement disorders specialists at the Hospital Clínic of Barcelona. The remaining 605 controls were a population-based series of healthy subjects recruited at the Spanish National Bank of DNA. These subjects passed a questionnaire on health status supervised by a physician and reported no signs, symptoms, or familial history of PD or of other neurological diseases. Dermatology and oncology patients were not included among these control subjects. All study participants gave written informed consent, and the study was approved by the local ethics committee of the Hospital Clínic of Barcelona.

MC1R Molecular Screening

Genomic DNA from blood samples was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The *MC1R* gene, which consists of a single exon encoding a 317-amino-acid protein (ENST00000555147), was sequenced using 50 to 100ng of total DNA per sample. Polymerase chain reaction (PCR) amplification was carried out as previously described[14] using an initial denaturizing step at 95°C for 5 minutes, followed by 35 PCR cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes) and a final extension at 72°C for 10 minutes. PCR products were purified using Multiscreen Filter plates (Millipore, Billerica, MA). We sequenced the entire coding region of *MC1R* using the internal primers TM-F 5′AACCTGCACTCACCC ATGTA3′ and TM-R 5′TTTAAGGCCAAAGCCCTGGT3′ and the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequences were run on an ABI3100 automatic sequencer (Applied Biosystems) and analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems, Ettenheim, Germany).

Statistical Analysis

The nonsynonymous *MC1R* variants were classified as red hair color (R) or non–red hair color (r) according to the classical criteria used in melanoma studies.[12] We focused on nonsynonymous *MC1R* variants with an observed MAF of at least 0.01 in PD patients. All variants were in Hardy–Weinberg equilibrium except p.R142H, which was excluded for further analysis. The allelic association analysis was performed using the 2-tail contingency χ_i^2 test. The genotypic association analysis was performed using multiple logistic regression models (codominant, dominant, recessive, overdominant, and log additive). The selection of the most suitable model of inheritance was performed based on both the Akaike information criterion and the Bayesian information criterion. Once the model was determined, differences in genotypic frequencies were calculated using a 2-tail contingency χ_i^2 test (Table 1). All statistical analyses were performed using the SNPStats software.[15] All reported odds ratios (ORs), 95% confidence intervals (CI), and probability values were adjusted for gender and age and also corrected using the Bonferroni correction for multiple comparisons.

Results

We detected 7 common nonsynonymous *MC1R* variants with an MAF ≥ 0.01 in PD patients: p.V60L (0.13), p.V92M (0.06), p.R151C (0.03), p.R160W (0.02), p.R163Q (0.02), p.I155T (0.01), and p.D294H (0.01; Fig) and compared their frequencies with those from controls. Results from the allelic and genotypic association analyses of these *MC1R* common variants are summarized in Table 1. We found that the red hair color variant p.R160W was associated with an increased risk of PD (gender- and age-adjusted OR = 2.10, 95% CI = 1.18–3.73, gender- and age-adjusted $p = 0.009$). After Bonferroni correction, this finding did not reach the threshold for statistical significance, yet showed a strong association trend (Bonferroni-corrected $p = 0.063$). The model of inheritance of p.R160W could not be determined, because homozygous carriers were not detected in our population and therefore the homozygous major allele genotype was compared only with the heterozygous genotype. In addition, we also identified a total of 38 rare variants with an MAF < 0.01 in *MC1R* (12 synonymous, 26 nonsynonymous) whose overall frequency distribution in PD patients (0.053) and controls (0.043) was not statistically different ($p = 0.383$; Table 2). A total of 18 of these rare variants were novel.

Discussion

We have investigated the association of common nonsynonymous genetic variants in *MC1R* with PD by sequencing the entire coding region of the gene in a case–control cohort from Spain. We found that the *MC1R* variant p.R160W (rs1805008) showed a strong trend of association with increased risk of PD (OR = 2.10, 95% CI = 1.18–3.73, gender-and age-adjusted $p = 0.009$, Bonferroni-corrected $p = 0.063$). Two other studies[9,16] have previously genotyped *MC1R* genetic variants in PD patients. The first study analyzed exclusively the loss-of-function variant p.R151C and found an increased risk of PD for this variant in 1 North American population.[9] Our study validates findings from this previous report indicating that loss-of-function *MC1R* variants modulate the susceptibility to PD. The second study specifically genotyped 2 loss-of-function *MC1R* variants, p.R151C and p.R160W, and reported no association with PD in a non-Hispanic white cohort from North America.[16] Population-specific allele frequencies of *MC1R* variants, which differ considerably across populations,[17] may underlie the different results observed between this study and our study. Furthermore, the different degree of genetic heterogeneity in this population and our population could also lead to different results. In our study, to minimize the effect of genetic heterogeneity, we analyzed a well-defined cohort consisting of ethnically homogeneous Mediterranean Caucasian cases

TABLE 1. Allelic and Genotypic Association Tests of Common Nonsynonymous *MC1R* Genetic Variants in PD

MC1R Variants	Amino Acid Change	Alleles	Minor Allele	MAF[a] PD, n = 870	MAF[a] Controls, n = 736	Adj. p^b	Adj. p^c	Genotype Frequency: PD Patients, n = 870: 1-1	1–2	2-2	Controls, n = 736: 1-1	1–2	2-2	PD Patients vs Controls: Adj. OR (95% CI)[b]	Adj. p^b	Adj. p^c	Adj. p, Previous Studies: Gao 2009[9]	Dong 2014[16]
rs1805005	p.V60L[e]	G/T	T	0.13	0.15	0.149	1.000	0.77	0.21	0.02	0.734	0.242	0.024	0.83 (0.66–1.05)	0.120	0.840	NA	NA
rs2228479	p.V92M[r]	G/A	A	0.06	0.05	0.417	1.000	0.882	0.114	0.004	0.895	0.102	0.003	1.14 (0.83–1.56)	0.420	1.000	NA	NA
rs1805007	p.R151C[d]	C/T	T	0.03	0.02	0.315	1.000	0.94	0.06	0	0.958	0.039	0.003	1.25 (0.80–1.95)	0.330	1.000	0.060	0.370
rs1110400	p.I155T[d]	T/C	C	0.01	0.01	0.756	1.000	0.97	0.03	0	0.98	0.02	0	1.10 (0.58–2.10)	0.760	1.000	NA	NA
rs1805008	p.R160W[d]	C/T	T	0.02	0.01	0.009	0.063	0.95	0.05	0	0.98	0.02	0	2.10 (1.18–3.73)	0.009	0.063	NA	0.295
rs885479	p.R163Q[r]	G/A	A	0.02	0.02	0.488	1.000	0.97	0.03	0	0.96	0.04	0	0.83 (0.48–1.41)	0.490	1.000	NA	NA
rs1805009	p.D294H[d]	G/C	C	0.01	0.02	0.135	0.945	0.98	0.02	0	0.962	0.037	0.001	0.60 (0.34–1.06)	0.078	0.546	NA	NA

[a]Alleles are denoted as 1 (wild-type allele) or 2 (variant or minor allele).
[b]Adjusted for gender and age.
[c]Adjusted for gender and age and corrected by Bonferroni correction for multiple comparisons.
[d]Red hair color variant [e] non–red hair color variant.
Adj. = adjusted; CI = confidence interval; MAF = minor allele frequency; NA = not assessed in previous studies; OR = odds ratio; PD = Parkinson disease.

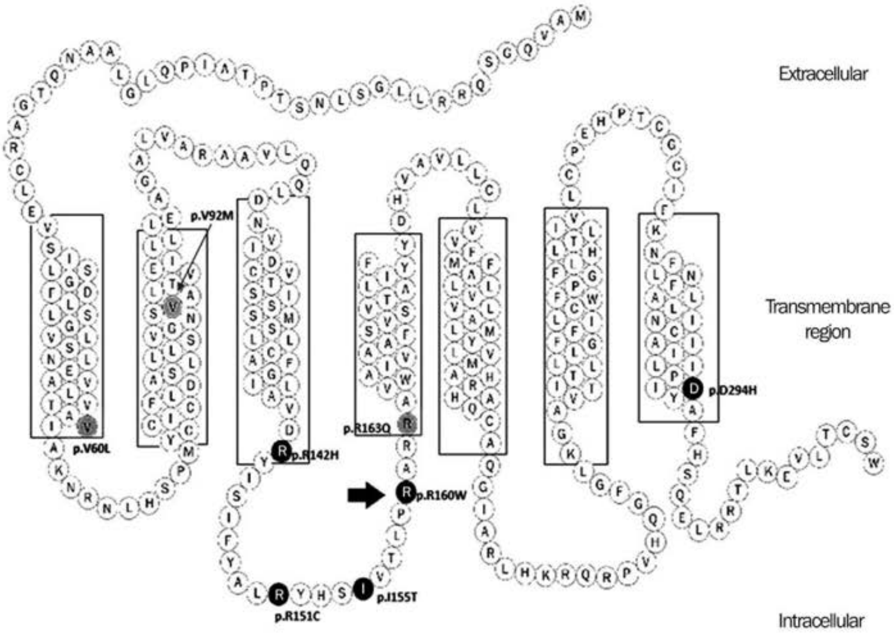


FIGURE 1: Localization of the common nonsynonymous genetic variants with a minor allele frequency ≥ 0.01 depicted in the MC1R protein. Relative positions of the R (red hair color) and r (non–red hair color) variants are shown in black and gray, respectively. The black arrow indicates variant p.R160W, which is associated with an increased Parkinson disease risk in our study.

and controls ascertained in Spain. It is important to note here that both *MC1R* variants associated with PD to date (p.R160W and p.R151C) have previously shown the strongest association with RHC phenotype and CM risk.[11] This is consistent with the observed overrepresentation of RHC individuals among PD and CM patients.[9] Altogether, these data suggest that the genetic variation in the *MC1R* gene may modulate the susceptibility not only for CM but also for PD.

The exact biological mechanism underlying the association of *MC1R* variants with PD is not known. Nonetheless, it should be mentioned that MC1R is expressed not only in melanocytes[18] but also in neurons, where it has a neuroprotective effect.[19] More specifically, *MC1R* has been shown to induce the downstream expression of *NURR1* (also known as *NR4A2*), which is an essential transcription factor for the development and maintenance of the dopaminergic neurons from the substantia nigra.[20–22] Moreover, postmortem studies in PD patients showed that the downregulation of *NURR1* correlates with the presence of α-synuclein containing inclusion bodies in the substantia nigra.[23] One attractive hypothesis is that *MC1R* loss-of-function R variants may be associated with PD through *NURR1* signaling.[20–23] Interestingly, primary human melanocytes carrying homozygous R *MC1R* variants have also shown the downregulation of NURR1.[20] However, future functional studies are needed to further explore this hypothesis in neuronal models of PD.

Our study, although exploratory, has some limitations. First, the sample size, albeit relatively large, is somewhat limited when considering the low frequency of the p.R160W risk allele T in our Spanish population (2% in PD, 1% in controls). Therefore, the risk associated with this variant may be somewhat difficult to estimate precisely. Second, it is to be expected that a small proportion of the subjects used as controls in our study, although neurologically healthy at the time of sample donation (56.69 ± 12.74 years old), will develop symptoms of PD in the future according to the age-dependent increasing incidence of PD (1 in 100 in those aged >60 years).[24] Third, it would have been interesting to evaluate whether environmental factors related to PD, such as caffeine intake, alcohol intake, or smoking status, affect the association of the MC1R variant p.R160W with PD, but unfortunately these environmental data were not available for our PD cohort.

In conclusion, our study indicates that the *MC1R* variant p.R160W is marginally associated with PD in the Spanish population. Further studies should be conducted to validate our findings in other PD populations.

TABLE 2. Frequency of Rare Genetic Variants in the *MC1R* Gene in PD and Controls

Rare *MC1R* Variants	PD Patients, n = 870	Controls, n = 736	Impact Score[a]
Synonymous variants			
A103A (c.309C>T)het	—	1/736	—
I138I (c.414C>T)het[b]	—	1/736	—
A139A (c.417C>T)het[b]	1/870	—	—
A161A (c.483G>A)het[b]	3/870	—	—
I168I (c.504C>T)het[b]	—	1/736	—
Y183Y (c.549C>T)het[b]	—	1/736	—
I221I (c.663C>T)het	—	1/736	—
R229R (c.687C>T)het[b]	1/870	—	—
Q233Q (c.699G>A)het	13/870	9/736	—
Y298Y (c.894C>T)het[b]	1/870	—	—
F300F (c.900C>T)het	—	3/736	—
S316S (c.948C>T)het	1/870	2/736	—
Nonsynonymous variants			
V38M (c.112G>A)het	—	1/736	0.49
N56D (c.166A>G)het[b]	1/870	—	1.00
R67W (c.199C>T)het[b]	1/870	—	1.00
L80V (c.238C>G)het[b]	—	1/736	1.00
S83P (c.246T>C)het	1/870	1/736	0.99
D84E (c.252C>A)het	1/780	1/736	0.99
T95M (c.284C>T)het	—	2/736	0.88
V122M (c.364G>A)het	7/870	1/736	0.12
V140M (c.418G>A)het[b]	1/870	—	0.39
R142C (c.424 C>T)het	1/870	—	1.00
Y152* (c.456C>A)het	1/870	—	1.00
V156A (c.467T>C)het	1/870	—	0.95
A161E (c.482C>A)het[b]	2/870	—	0.99
T272M (c.815C>T)het	1/870	—	0.97
V193M (c.577G>A)het[b]	—	1/736	0.89
R213W (c.637C>T)het	3/870	—	0.02
R223W (c.677C>T)het[b]	1/870	—	0.02
A218T (c.652G>A)het	1/870	—	0.93
P230L (c.689C>T)het	—	1/736	0.00
P268R (c.803C>G)het	—	1/736	1.00
C273W (c.819C>G)het[b]	—	1/736	1.00
N281S (c.842A>G)het	—	1/736	0.25
C289R (c.865T>C)het	1/870	—	0.99
D294N (c.880G>A)het[b]	1/870	—	1.00
Y298H (c.892T>C)het[b]	—	1/736	1.00
A299S (c.895G>T)het[b]	1/870	—	0.99
Total of *MC1R* rare variants	46/870 (0.053%)	32/736 (0.043%)	

[a]Impact score of the amino acid substitution on the structure and function of *MC1R* computed using Polyphen2 software (scores close to 0 indicate a benign mutation, whereas scores close to 1.0 indicate damaging mutation).
[b]Variants that are novel and have not been reported before.
het = change detected in heterozygosis; PD = Parkinson disease.

Acknowledgment

The research at the Melanoma Unit in Barcelona was supported by grants from Fondo de Investigaciones Sanitarias (12/00840), Carlos III Health Institute, Fondo Europeo de Desarrollo Regional of the European Commission, "Una manera de hacer Europa," and the Catalan Government (AGAUR 2014 SGR 603).

We thank all PD patients and their families for participating in this study; and the Center for Biomedical Research in Neurodegenerative Diseases Biobank, integrated into the Spanish National Biobank Network, for sample and data procurement.

This work was carried out at the Esther Koplowitz Center and the CELLEX Center at the August Pi i Sunyer Biomedical Research Institute in Barcelona.

Authorship

G.T.-M. performed all the experimental work, analysis of sequences, and statistical analysis of data. M.P. contributed in the analysis of sequences. J.A.P.-B. designed the project and supervised statistical work. G.T.-M., J.A.P.-B., and R.F.-S. wrote the manuscript. C.B. and M.M (Montserrat Milà) designed specific internal *MC1R* primers and supervised the sequence analysis. R.F.-S, M.E., and M.J.M recruited patients, provided the DNA samples evaluated in the study (PD patients and controls), and contributed to data interpretation. S.P. and J.M. designed the project, obtained funding, took responsibility for the integrity of the data and the accuracy of the data analysis, and supervised the entire project including editing the manuscript. All coauthors reviewed and commented on the manuscript.

Potential Conflicts of Interest

Nothing to report.

References

1. Driver JA. Inverse association between cancer and neurodegenerative disease: review of the epidemiologic and biological evidence. Biogerontology 2014;15:547–557.
2. Bajaj A, Driver JA, Schernhammer ES. Parkinson's disease and cancer risk: a systematic review and meta-analysis. Cancer Causes Control 2010;21:697–707.
3. Liu R, Gao X, Lu Y, Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology 2011;76: 2002–2009.
4. Olsen JH, Tangerud K, Wermuth L, et al. Treatment with levodopa and risk for malignant melanoma. Mov Disord 2007;22:1252–1257.
5. Baade PD, Herrero Hernandez E, Freedman DM, et al. No role for melanoma treatment in the association between melanoma and amyotrophic lateral sclerosis or Parkinson's disease. Neuroepidemiology 2010;35:303–304.
6. Rugbjerg K, Harris MA, Shen H, et al. Pesticide exposure and risk of Parkinson's disease—a population-based case-control study evaluating the potential for recall bias. Scand J Work Environ Health 2011;37:427–436.
7. Wright Willis A, Evanoff BA, Lian M, et al. Geographic and ethnic variation in Parkinson disease: a population-based study of US Medicare beneficiaries. Neuroepidemiology 2010;34:143–151.
8. Duffy DL, Zhao ZZ, Sturm RA, et al. Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. J Invest Dermatol 2010;130:520–528.
9. Gao X, Simon KC, Han J, et al. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol 2009;65:76–82.
10. Puig-Butille JA, Escamez MJ, Garcia-Garcia F, et al. Capturing the biological impact of CDKN2A and MC1R genes as an early predisposing event in melanoma and non melanoma skin cancer. Oncotarget 2014;5:1439–1451.
11. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 2008; 122:2753–2760.
12. Williams PF, Olsen CM, Hayward NK, Whiteman DC. Melanocortin 1 receptor and risk of cutaneous melanoma: a meta-analysis and estimates of population burden. Int J Cancer 2011;129:1730–1740.
13. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181–184.
14. Chaudru V, Laud K, Avril MF, et al. Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev 2005;14:2384–2390.
15. Sole X, Guino E, Valls J, et al. SNPStats: a web tool for the analysis of association studies. Bioinformatics 2006;22:1928–1929.
16. Dong J, Gao J, Nalls M, et al. Susceptibility loci for pigmentation and melanoma in relation to Parkinson's disease. Neurobiol Aging 2014;35:1512.e5–1512.e10.
17. Gerstenblith MR, Goldstein AM, Fargnoli MC, et al. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 2007;28:495–505.
18. Garcia-Borron JC, Abdel-Malek Z, Jimenez-Cervantes C. MC1R, the cAMP pathway, and the response to solar UV: extending the horizon beyond pigmentation. Pigment Cell Melanoma Res 2014; 27:699–720.
19. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci 2008;31:353–360.
20. Smith AG, Luk N, Newton RA, et al. Melanocortin-1 receptor signaling markedly induces the expression of the NR4A nuclear receptor subgroup in melanocytic cells. J Biol Chem 2008;283: 12564–12570.
21. Low D, Chen KS. Genome-wide gene expression profiling of the Angelman syndrome mice with Ube3a mutation. Eur J Hum Genet 2010;18:1228–1235.
22. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, et al. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci U S A 1998;95:4013–4018.
23. Chu Y, Le W, Kompoliti K, et al. Nurr1 in Parkinson's disease and related disorders. J Comp Neurol 2006;494:495–514.
24. Nussbaum RL, Ellis CE. Alzheimer's disease and Parkinson's disease. N Engl J Med 2003;348:1356–1364.

Supplementary Table 1: Analysis of Age of onset in PD patients regarding MC1R genotype

MC1R variants	MC1R genotype^ 1-1 (N)	Age of onset Mean±SD*	MC1R genotype^ 1-2, 2-2 (N)	Age of onset Mean±SD*	p-value
p.V60L	662	57.80±11.50	199	57.10±13.12	0.464
p.V92M	760	57.81±11.98	101	56.35±11.13	0.246
p.R151C	813	57.51±11.93	48	59.83±11.13	0.188
p.I155T	839	57.59±11.84	22	59.23±13.94	0.525
p.R160W	820	57.65±11.86	41	57.46±12.62	0.924
p.R163Q	834	57.80±11.76	27	52.70±14.87	0.089
p.D294H	841	57.64±11.81	20	57.35±15.09	0.913

^Alleles are denoted as '1' (wild type allele) or '2' (variant or minor allele).
*SD, standard deviation.

TREBALL II

Treball realitzat per donar resposta als objectiu 2 i 3 de la present tesi doctoral.

Títol:

A Common Variant in the *MC1R* Gene (p.V92M) is associated with Alzheimer's Disease Risk

Doi: 10.3233/JAD-161113

Resum:

Malgrat en els darrers anys, s’han identificat variants al·lèliques de risc a desenvolupar les formes esporàdiques de la malaltia d’Alzheimer (edat d’inici de la malaltia ≥ 65 anys), la suma del risc atribuït a aquestes variants genètiques juntament amb l’atribuït a l’APOEε4, explica poc més del 50% del risc genètic atribuït al desenvolupament d’aquestes formes tardanes d’Alzheimer. Donat que el MC1R s’expressa en les cèl·lules del SNC i que en les cèl·lules cutànies portadores de variants LOF en *MC1R*, s’ha identificat una desregulació constitutiva de gens implicats en les vies de senyalització de malalties neurodegeneratives; varem hipotetitzar que el gen *MC1R* podria estar implicat en la patogènesi de la malaltia d’Alzheimer.

L’objectiu d’aquest treball era avaluar el gen *MC1R* com a factor genètic de risc a desenvolupar les formes esporàdiques de la malaltia d’Alzheimer i com a factor modificador de l’edat d’aparició d’aquesta malaltia.

Per a donar resposta aquest objectiu, es va dissenyar un estudi cas-control que incloïa 525 pacients d’Alzheimer (edat d’inici de la malaltia ≥ 65 anys) i 160 controls, aparellats proporcionalment per edat i sexe, tots ells d’origen espanyol. El genotip de l’APOE va ser determinat mitjançant sondes Taqman (*Applied Biosystems*) en el conjunt total de les mostres. El gen *MC1R* es va caracteritzar molecularment en el conjunt total de casos i controls, seqüenciant la regió codificant sencera del trànscrit (ENST0000555147) que codifica per la forma canònica del receptor MC1R (NP_002377).

De forma global es van identificar un total de 22 variants al·lèliques (4 sinònimes i 18 no sinònimes) en el gen *MC1R.* Entre aquestes, 16 variants (3 sinònimes i 13 no sinònimes) van ser detectades en pocs o en un únic individu i presentaven una MAF < 0,01. La freqüència global d'aquestes variants rares (MAF < 0,01) es va comprar entre casos (0,049) i controls (0,050) i no es va detectar una diferència estadísticament significativa (p-valor = 0,888).

L'anàlisi d'associació genotípica cas-control es va dur a terme amb el conjunt de variants genètiques que complien els següents criteris: variants no sinònimes, en equilibri Hardy-Weinberg en la població control i amb una MAF > 0,01 en els pacients d'Alzheimer. Se'n van identificar 5: p.V60L (0,16), p.V92M (0,7), p.R151C (0,02), p.R160W (0,02) i p.R163Q (0,02). Tots els anàlisis d'associació genotípica es van ajustar per l'edat, el sexe i el genotip de l'*APOE*, que són els 3 principals factors de risc a desenvolupar les formes esporàdiques d'Alzheimer.

Els resultats dels anàlisis d'associació genotípica van mostrar que els individus portadors de l'al·lel p.V92M (rs2228479), variant 'r' que no s'associa amb el fenotip RHC, presenten un augment del risc a desenvolupar Alzheimer sota un model d'herència additiu (OR: 1,99; 95% IC: 1,08-3,64, p-valor ajustat = 0,026). Es va identificar una associació inversa entre la variant al·lèlica p.V92M de *MC1R* i l'al·lel 4 de l'*APOE*. La resta de variants avaluades, tant 'r' (p.V60L i p.R163Q) com 'R' (p.R151C i p.R160W) no es van associar amb un augment del risc a desenvolupar Alzheimer en aquesta cohort d'origen espanyol. De forma interessant, quan l'anàlisi es va restringir a un subgrup de 69 individus on el diagnòstic d'Alzheimer s'havia confirmat mitjançant l'ús del perfil de biomarcadors en LCR (d'Aβ_{42}, T-tau i P-tau), l'associació entre la variant p.V92M i el risc a desenvolupar Alzheimer augmenta (OR: 3,40; 95% IC: 1,40-8,27, p-valor ajustat = 0,007). Els resultats d'aquest treball indiquen que l'al·lel p.V92M del MC1R augmenta el risc a desenvolupar Alzheimer, especialment en aquells individus on el risc genètic no pot atribuir-se a l'APOEε4, a través de la desregulació de vies de senyalització modulades pel MC1R independents de la via canònica de senyalització. Cap de les variants analitzades en *MC1R* es va associar a una variació en l'edat d'aparició de la malaltia d'Alzheimer, indicant que el gen *MC1R* no és un factor modificador de l'edat d'inici d'aquesta malaltia, almenys en aquesta cohort d'origen espanyol.

Journal of Alzheimer's Disease 56 (2017) 1065–1074
DOI 10.3233/JAD-161113
IOS Press

A Common Variant in the *MC1R* Gene (p.V92M) is associated with Alzheimer's Disease Risk

Gemma Tell-Marti[a,b], Joan Anton Puig-Butille[c,b], Miriam Potrony[a], Estel Plana[d], Celia Badenas[c,b], Anna Antonell[e], Raquel Sanchez-Valle[e], José L. Molinuevo[e], Alberto Lleó[f,g], Daniel Alcolea[f,g], Juan Fortea[f,g], Rubén Fernández-Santiago[h], Jordi Clarimón[f,g], Albert Lladó[e] and Susana Puig[a,b,i,*]

[a]*Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain*
[b]*Centro Investigaciòn Biomèdica en Enfermedades Raras (CIBERER), ISCIII, Barcelona, Spain*
[c]*Biochemical and Molecular Genetics Service, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain*
[d]*RTI Healtlh Solutions, Travesera de Gracia 56 Atic 1era, Barcelona, Spain*
[e]*Alzheimer's Disease and Other Cognitive Disorders Unit, Neurology Service, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[f]*Memory Unit, Neurology Department, Hospital de Sant Pau (Sant Pau Biomedical Research Institute), Universitat Autònoma de Barcelona, Barcelona, Spain*
[g]*CIBERNED, Center of Networker Biomedical Research into Neurodegenerative Diseases, Madrid, Spain*
[h]*Laboratory of Neurodegenerative Disorders, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)-Hospital Clínic de Barcelona-Centro de Investigaciòn sobre Enfermedades Neurodegenerativas (CIBERNED), ISCIII, Barcelona, Spain*
[i]*Medicine Department, Universitat de Barcelona, Barcelona, Spain*

Accepted 30 November 2016

Abstract. Despite the recent identification of some novel risk genes for Alzheimer's disease (AD), the genetic etiology of late-onset Alzheimer's disease (LOAD) remains largely unknown. The inclusion of these novel risk genes to the risk attributable to the *APOE* gene accounts for roughly half of the total genetic variance in LOAD. The evidence indicates that undiscovered genetic factors may contribute to AD susceptibility. In the present study, we sequenced the *MC1R* gene in 525 Spanish LOAD patients and in 160 controls. We observed that a common *MC1R* variant p.V92M (rs2228479), not related to pigmentation traits, was present in 72 (14%) patients and 15 (9%) controls and confers increased risk of developing LOAD (OR: 1.99, 95% CI: 1.08–3.64, $p = 0.026$), especially in those patients whose genetic risk could not be explained by *APOE* genotype. This association remains and even increased in the subset of 69 patients with typical AD cerebrospinal fluid profile (OR: 3.40 95% CI: 1.40–8.27, $p = 0.007$). We did not find an association between p.V92M and age of onset of AD. Further studies are necessary to elucidate the role of MC1R in brain cells through the different MC1R pathways.

Keywords: Cerebrospinal fluid biomarkers, common variant, late-onset Alzheimer's disease, melanocortin 1 receptor (*MC1R*) gene, p.V92M, risk

*Correspondence to: Susana Puig, MD, PhD, Chief Dermatology Service, Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona, Villarroel 170. 08036, Barcelona, Spain. Tel.: +34 93 2275400/Ext. 2422; Fax: +34 93 2275438; E-mail: susipuig@gmail.com.

ISSN 1387-2877/17/$35.00

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized clinically by memory and cognitive dysfunction and represents the most common form of dementia in the elderly [1]. The prevalence of the disease increases after the age of 65 years and the disease onset is usually after the age of 70 years [2]. However, familial AD (FAD) patients carry autosomal dominant mutations in high-risk Alzheimer susceptibility genes (*APP*, *PSEN1*, and *PSEN2*) and present an early age of onset (<65 years). These genes do not play an important role in either Sporadic AD cases or late onset cases (>65 years) indicating that other genomic variants may be involved in the common forms of the disease [3]. To date, the ε4 allele polymorphism in the Apolipoprotein E (*APOE*) gene has been well established as a risk factor for developing late-onset Alzheimer's disease (LOAD) [3]. The disease-attributable risk in LOAD patients related to the ε4 allele in APOE is less than 50% [4]. Previous genome-wide association studies (GWAS) have identified low-risk variants associated with LOAD [5–10], which account for a small proportion of risk. The inclusion of these novel risk genes to the risk attributable to the *APOE* gene accounts for roughly half of the total genetic variance [11], indicating that additional undiscovered genetic factors may contribute to AD susceptibility.

The main pathological hallmarks of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles, and loss of neurons and synapses, resulting in brain atrophy [12]. Moreover, an elevated level of oxidative damage products has been observed in areas of degeneration in AD brains, suggesting that oxidative stress and consequent protein oxidation may be potential mechanisms of neuronal death in AD [13]. The accumulation of intracellular damage determined by reactive oxygen species might produce the progressive loss of control over biological homeostasis and the functional impairment typical of damaged brain cells in AD [14].

The Melanocortin 1 receptor (*MC1R*) gene encodes for a G protein-coupled seven transmembrane receptor for melanocortin peptides (α-MSH, ACTH) and mediates its effects mainly by activating a cAMP-dependent signaling pathway [15]. MC1R expression is observed in several types of neuronal cells suggesting that it may be a key regulator in brain cell functions and survival [16]. The MC1R activation has anti-inflammatory and immunomodulatory effects in brain cells [16] and promotes pigmentation synthesis in melanocytes [17]. It has been established that several *MC1R* polymorphisms constitute a risk factor to develop skin cancer (melanoma and non-melanoma skin cancer) [18, 19], in part, by promoting an increased oxidative stress in skin cells [20]. Notably, co-occurrence of Parkinson's disease (PD) and cutaneous melanoma (CM) has been reported in epidemiological studies [21] and previous evidence indicates that *MC1R* is involved in the bidirectional link between both diseases [22, 23]. Thus, we hypothesized that certain *MC1R* variants may increase the oxidative damage and/or deregulate inflammatory processes in brain cells, which consequently, increase the susceptibility of developing other neurodegenerative disorders beyond PD. In the present study, we analyzed the role of the *MC1R* gene as a putative genetic risk factor in LOAD patients, and we observed that a common *MC1R* variant, not related to pigmentation traits, confers risk of developing LOAD in a Spanish population.

MATERIAL AND METHODS

We performed a case-control study of 525 unrelated LOAD patients (Mean age ± SD, 76.35 ± 5.61 years (the age of onset was after 65 years in all patients studied; male 29.7%/female 70.3%) and 160 controls (Mean age ± SD, 73.81 ± 5.87 years; male 36.3%/female 63.7%). All patients studied were recruited from two hospital-based series from the same geographical area: the Alzheimer's Disease and Other Cognitive Disorders Unit at Clinic Hospital of Barcelona and from the Memory Unit at Sant Pau Hospital of Barcelona. All AD patients were diagnosed using the NINCSDS-ADRDA criteria [24]. Furthermore, 69/525 had a CSF biomarker profile typical of AD ($A\beta_{42}$/p-tau ratio <6.43) [25].

The control group included healthy individuals without signs of neurodegenerative or psychiatric disorders obtained from three independent control series from Spain: Hospital Clinic of Barcelona (N = 13), Sant Pau Hospital of Barcelona (N = 85), and the Spanish National Bank of DNA (N = 62) (Table 1).

All individuals included in the study gave their written informed consent according to the Declaration of Helsinki. The Ethical Committee of Clinical Investigation at the Hospital Clinic of Barcelona approved the study.

APOE genotype analysis

DNA was isolated from blood samples using the Wizard® Genomic DNA Purification Kit (Promega,

Table 1
Demographic data and APOE genotype of the samples analyzed

			LOAD (N = 525)	Controls (N = 160)
AOO		Mean ± SD*	76.35 ± 5.61	73.81 ± 5.87
Gender	Male	N (%)	156 (29.7)	58 (36.3)
	Female	N (%)	369 (70.3)	102 (63.7)
[a]APOE	APOE –/–	N (%)	283 (53.9)	128 (80)
	APOE –/+	N (%)	218 (41.5)	32 (20)
	APOE +/+	N (%)	24 (4.6)	0 (0)
Hospital based-series	Hospital Clinic of Barcelona	N (%)	110 (21)	13 (8)
	Sant Pau Hospital of Barcelona	N (%)	415 (79)	85 (53)
	National Bank of DNA[b]	N (%)	0 (0)	62 (39)

[a] –/– individuals who carry no ε4 allele. –/+individuals who carry one ε4 allele. +/+individuals who carry two ε4 alleles [b]from The University of Salamanca. *SD standard deviation.

Fitchburg, Wisconsin, USA). *APOE* genotype was determined through the analysis of rs429358 and rs7412 using TaqMan (Applied Biosystems) genotyping technologies.

CSF biomarkers determination

69 subjects underwent a spinal tap during the morning. The samples were centrifuged and stored in polypropylene tubes at –80°C within 2 h. Levels of $A\beta_{42}$, t-tau, and p-tau were measured by experienced laboratory personnel using commercial sandwich ELISA kits (Innogenetics, Ghent, Belgium) [26]. We are participants of the QC program, and $A\beta_{42}$, t-tau, and p-tau levels obtained in our lab for the Alzheimer's Association QC samples were within mean ± 2 SD.

MC1R molecular screening

The *MC1R* gene, which consists of one single exon encoding a 317 amino-acid protein (ENST00000555147), was sequenced using 50–100 ng of total DNA per sample. PCR amplification was carried out as previously described [27] using an initial denaturizing step at 95°C 5 min, followed by 35 PCR cycles (94°C 1 min, 55°C 1 min, 72°C 3 min), and a final extension at 72°C 10 min. PCR products were purified using Multiscreen Filter plates (Millipore). We sequenced the entire coding region of *MC1R* (a 1,107 bp fragment) using the following internal primers (TM-F: 5'AACCTGCACTCACCCATGTA3' and TM-R: 5'TTTAAGGCCAAAGCCCTGGT3') and the BigDye Terminator v3.1 Cycle Sequencing kit, according to manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequences were run on an ABI3100 automatic sequencer (Applied Biosystems) and analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems). The entire coding region was sequenced in 110 LOAD patients and in all controls. In 415 LOAD patients, the *MC1R* gene was exclusively sequenced using TM-R primer. This strategy allows us to detect all *MC1R* variants, except for the presence of p.D294H and p.T314T variants.

Statistical analysis

We focused the analysis on the non-synonymous *MC1R* variants with an observed minor allele frequency (MAF) in at least 1% of cases. Synonymous variants were considered as wild-type *MC1R* alleles. Public databases such as dbSNP (http://www.ncbi.nlm.nih.gov/), MelGene DB (http://www.melgene.org/), and Ensembl genome browser (http://www.ensembl.org/) were used to determine whether the detected non-synonymous variants have been previously described. *In-silico* analysis of each rare non-synonymous variant was carried out using software Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) [28].

The genotypic association analysis was performed using multiple logistic regression models (co-dominant, dominant, recessive, over-dominant, and log-additive) in the whole set of patients (N = 525) and in the subset of patients with CSF biomarkers data (N = 69). The selection of the most suitable model of inheritance was performed based on both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). All reported odds ratios (ORs), 95% confidence intervals (CI), and *p*-values were adjusted for age, gender, and *APOE* genotype.

The goodness-of-fit of our logistic regression model was evaluated by the Hosmer-Lemeshow test. In the genotypic association analysis with the whole set of samples (525 patients and 160 controls), we obtained the best fit of our model by categorical transformation of the age. We converted the numerical

Table 2
Genetic variants detected in the *MC1R* gene

MC1R variants				Subjects		
				LOAD (N = 525)	Controls (N = 160)	Polyphen
rs	MAF (%) (EA/AA/All) [#]	AA change	cDNAchange	N (%)	N (%)	Score 2
Synonymous						
rs146544450	0.2151/0.0969/0.1761	p.Q233Q	(c.699G>A)Het	4 (0.7)	–	–
rs181269865	0.1064/0.0712/0.0947	p.I264I	(c.792C>T)Het	2 (0.4)	–	–
UN	UN	**p.L309L**	**(c.927C>G)Het**	1 (0.2)	–	–
rs2228478	10.7936/40.8062/20.8104	^p.T314T[a]	(c.942A>G)Het	21 (19)[a]	24 (15)[a]	–
			(c.942A>G)Homo	2 (2)[a]	–	
Non-synonymous						
rs1805005	13.3419/2.2612/9.6064	^p.V60L	(c.178T>G)Het	137 (26)	40 (25)	0.988
			(c.178T>G)Homo	18 (3)	5 (3)	
rs372590533	0.0117/0.0/0.0078	p.R67Q	(c.200G>A)Het	–	3 (2)	0.744
rs34474212	0.1051/0.0228/0.0773	p.S83P	(c.247T>C)Het	1 (0.2)	–	0.999
rs1805006	1.0028/0.1826/0.7254	^p.D84E	(c.252C>A)Het	1 (0.2)	–	1.000
rs2228479	8.5784/1.9389/6.3311	^p.V92M	(c.274G>A)Het	72 (14)	15 (9)	0.015
			(c.274G>A)Homo	2 (0.4)	–	
rs201192930	0.0116/0.0228/0.0154	p.V122M	(c.364G>A)Het	1 (0.2)	–	0.126
rs374235260	0.0116/0.0/0.0077	p.M128T	(c.383T>C)Het	–	1 (0.6)	0.235
rs11547464	0.7442/0.0682/0.5155	^p.R142H	(c.425G>A)Het	6 (1)	1 (0.6)	1.000
rs1805007	7.6163/1.7061/5.6171	^p.R151C	(c.451C>T)Het	26 (5)	9 (6)	1.000
rs201326893	0.0349/0.0/0.0231	p.Y152*	(c.456C>A)Het	1 (0.2)	–	1.000
rs1110400	1.0468/0.2959/0.7928	^p.I155T	(c.464T>C)Het	6 (1)	5 (3)	0.986
UN	UN	**p.V156E**	**(c.467T>A)Het**	1 (0.2)	–	0.981
rs1805008	7.7147/1.3452/5.5624	^p.R160W	(c.478C>T)Het	18 (3)	4 (2)	0.861
rs885479	4.8207/1.5269/3.7068	^p.R163Q	(c.488G>A)Het	24 (5)	3 (2)	0.004
UN	UN	**p.V174I**	**(c.520G>A)Het**	–	1 (0.6)	0.002
rs200000734	0.0594/0.0239/0.0476	p.R213W	(c.637C>T)Het	1 (0.2)	–	0.019
rs1805009	2.0716/0.6859/1.6111	^p.D294H[a]	(c.880G>C)Het	5 (4)[a]	6 (4)[a]	1.000
			(c.880G>C)Homo	1 (0.9)[a]	–	
UN	UN	**p.Y298H**	**(c.892T>C)Het**	–	2 (1.2)	1.000

[#]The minor-allele frequency in percent listed in the order of European American (EA), African American (AA) and all populations (All) (delimited by /). Variants in bold have not been reported before. UN = Unknown. Het: variant in heterozygosis. Homo: variant in homozygosis.^ *MC1R* common variants.Score Polyphen2: predicts possible impact of an amino acid substitution on the structure and function of a human protein (scores close to 0.0, indicate a benign mutation; score close to 1.0, damaging mutation) [a] *MC1R* variants evaluated in 110 LOAD patients and 160 controls.

variable (age) into a categorical variable, dividing the age into different categories. In this analysis, the logistic regression model was adjusted for age (as categorical variable), gender, and APOE genotype. Otherwise, in the genotypic association analysis with the subset of patients with typical AD CSF biomarkers (69 patients and 160 controls), we obtained the best fit of our model, including the age as a numerical variable. In this second analysis, the logistic regression model was adjusted for age (as numerical variable), gender, and APOE genotype.

We evaluated whether *MC1R* and *APOE* genotypes modify age of onset (AOO) among the Spanish LOAD group using the Student's t-test. We evaluated whether interaction between *MC1R* variants and *APOE* alleles exists using the two-tail χ^2 test.

p-values less than 0.05 were considered statistically significant. All tests were two sided, and Bonferroni correction for multiple comparisons was applied to all p-values <0.05. We performed all the analyses using STATA v.11 software.

RESULTS

MC1R genotyping was carried out in 525 LOAD patients and in 160 control subjects. Non-significant difference in the gender distribution was detected between patients and controls ($p = 0.118$). The study identified 22 *MC1R* variants (4 synonymous and 18 non-synonymous), four rare variants (p.V156E, p.V174E, p.Y298H, and p.L309L) had not been previously identified (Table 2). Five recurrent non-synonymous variants showed a frequency $\geq 1\%$ in LOAD patients: p.V60L (16%), p.V92M (7%), p.R151C (2%), p.R160W (2%), and p.R163Q (2%). All variants were in Hardy-Weinberg equilibrium within both the control population and LOAD

Table 3
Results of association analysis of *MC1R* variants and LOAD

AA change	Controls (N = 160)				LOAD patients (N = 525)				CONTROL vs LOAD patients	
	[a]MAF	[a]Genotype frequency			MAF	Genotype frequency				
	2	1–1	1–2	2–2	2	1–1	1–2	2–2	OR (95% CI)[b]	*p-value*[b]
p.V60L	0.16	0.72	0.25	0.03	0.16	0.71	0.26	0.03	0.93 (0.69–1.33)	0.695
p.V92M	0.05	0.9	0.1	0	0.07	0.859	0.137	0.004	**1.99 (1.08–3.64)**	**0.026**
p.R163Q	0.01	0.98	0.02	0	0.02	0.95	0.05	0	2.47 (0.69–8.81)	0.162
p.R151C	0.03	0.94	0.06	0	0.025	0.95	0.05	0	1.01 (0.44–2.30)	0.979
p.R160W	0.01	0.975	0.025	0	0.02	0.966	0.034	0	0.92 (0.29–2.91)	0.882

MAF: Minor allele frequency. [a]Allele described as 1 (wild type allele) or 2 (variant allele). [b]Adjusted for gender, age (converted in a categorical variable) and APOE genotype. The genetic model used was log-additive, the homozygous for 'variant allele' (a/a) has double the risk of the heterozygous (A/a). The statistically significant result is highlighted in bold.

patients (allelic and genotypic frequencies are listed in Table 3). Moreover, frequencies of *MC1R* variants observed in control subjects were compared with those observed in other set of controls (N = 736) free from neurodegenerative disorders used in other study [29]. No statistical significant differences were observed between both groups.

The *APOE* genotype was obtained for all cases and controls. The frequency of heterozygous *APOE* $\varepsilon 4$ carriers was 41.5% (218/525) in LOAD patients and 20% (32/160) in controls. The *APOE* $\varepsilon 4/\varepsilon 4$ genotype was only detected in 4.6% (24/525) of LOAD patients. The carriers of at least one APOE $\varepsilon 4$ allele had a higher than three-fold increased risk of developing LOAD (OR: 3.47, 95% CI: 2.24–5.39, $p < 0.0001$).

We evaluated the 5 most common *MC1R* variants detected in the study with the risk of developing LOAD (Table 3). We detected that variant p.V92M was enriched in LOAD patients compared to controls, being associated with an increased risk of developing LOAD under the log-additive genetic model (OR: 1.99, 95% CI: 1.08–3.64, $p = 0.026$), after Bonferroni correction this association did not reach statistical significance (Bonferroni corrected $p = 0.13$).

We re-evaluated the association between *MC1R* variant p.V92M and LOAD risk within the subset of 69 patients with typical AD CSF profile (Table 4) and we found it was statistically significant and we evidenced an even higher OR (OR: 3.40 95% CI: 1.40–8.27, $p = 0.007$). We did not detect statistically significant differences in the different CSF biomarkers levels between p.V92M carriers and non-carriers (data not shown).

In order to identify whether the p.V92M association with LOAD risk was modulated by the presence of the APOE $\varepsilon 4$ allele, we evaluated the interaction between both alleles within the whole set of LOAD patients (Table 5). The frequency of variant p.V92M

Table 4
Demographic data and CSF biomarkers levels of LOAD patients subset confirmed by CSF analysis

			LOAD (N = 69)
AOO		Mean ± SD*	71.46 ± 4.74
Gender	Male	N (%)	26 (37.7%)
	Female	N (%)	43 (62.3%)
[a]APOE	APOE –/–	N (%)	30 (43.5%)
	APOE –/+	N (%)	31 (44.9%)
	APOE +/+	N (%)	8 (11.6%)
CSF protein levels	$A\beta_{42}$	Mean ± SD	348.64 ± 143.14
	t-tau	Mean ± SD	711.25 ± 385.51
	p-tau	Mean ± SD	100.46 ± 42.002

[a] –/– patients who carry no $\varepsilon 4$ allele. –/+patients who carry one $\varepsilon 4$ allele. +/+patients who carry two $\varepsilon 4$ alleles. *SD standard deviation.

in the *MC1R* gene was significantly lower ($p = 0.041$) in carriers of at least one APOE $\varepsilon 4$ allele compared to non-carriers (35.1% and 64.9%, respectively). This result suggests an inverse correlation between both alleles.

Finally, we observed a significantly lower AOO associated with APOE $\varepsilon 4$ allele ($p < 0.0001$), in contrast, no significant effect on AOO was observed for *MC1R* variant p.V92M (Table 6).

DISCUSSION

LOAD form accounts for more than 90% of AD cases [30]. To date, the *APOE* gene is the major genetic factor in LOAD susceptibility [31], while other genetic factors related with LOAD susceptibility remain largely unknown. Thus, identification of novel genetic factors may be crucial to detect individuals with an inherited AD risk.

In this study, we report a novel association between the p.V92M variant in the *MC1R* gene and the risk of developing LOAD. After Bonferroni correction, the association detected in the overall set of patients did not reach statistical significance. However, the

Table 5
Analysis of p.V92M distribution regarding APOE genotype within LOAD patients (N = 525)

	^p.V92M				p-value
	0		≥1		
APOE genotype	N	%	N	%	
APOE ε4 (–)	235	52.1	48	64.9	0.041
APOE ε4 (+)	216	47.9	26	35.1	

APOE ε4 (–): non carriers of APOE ε4 allele. (+): carriers of at least one APOE ε4 allele. ^ p.V92M (0): non carriers of variant p.V92M. (≥1): carriers of at least one p.V92M allele.

Table 6
Analysis of AOO regarding APOE genotype and p.V92M variant genotype

	Age of onset		p-value
[a]APOE genotype	N	Mean ± SD*	
APOE ε4 –	283	77.36 ± 5.33	<0.0001
APOE ε4 +	242	75.17 ± 5.70	
^MC1R genotype			
p.V92M (–)	451	76.38 ± 5.64	0.758
p.V92M (+)	74	76.16 ± 5.43	

*SD, standard deviation.[a]–/– patients who carry no ε4 allele. –/+ patients who carry one ε4 allele. +/+ patients who carry two ε4 alleles. ^ p.V92M (–): non carriers of p.V92M variant. (+): carriers of at least one p.V92M allele.

association in the subset of biochemically confirmed AD patients was even stronger than in the overall set of patients, suggesting that the *MC1R* variant plays a role in the etiology of AD. This gene is highly polymorphic, and p.V92M and other variants are common in the Caucasian population. During the last few years, several GWAS have been conducted to identify common LOAD risk variants [5–10], and none of these studies reported an association between *MC1R* and the disease. This could be caused by methodological issues (coverage level of *MC1R* in SNP-array platforms or conservative statistical correction procedures) or by the molecular or clinical heterogeneity of patients included. Interestingly, we observed an inverse tendency between the *APOE* genotype and p.V92M suggesting that the presence of the *MC1R* variant could contribute to AD susceptibility, especially in those patients whose genetic risk could be not attributable to the *APOE* genotype. Further studies restricted to *MC1R* variants and *APOE* alleles should be conducted using previous GWAS data to elucidate such an inverse correlation.

Clinicopathological studies have shown that the sensitivity and specificity of the classical clinical criteria for AD diagnosis are roughly 85% and 70%, respectively [32]. CSF studies measuring $A\beta_{42}$ and tau protein levels in AD patients with confirmed pathology have demonstrated that abnormal levels of both biomarkers constitute a specific signature of the underlying AD-pathology (senile plaques and neurofibrillary tangles, respectively). Furthermore, multiple studies have shown that the sensitivity and specificity with the inclusion of specific CSF biomarkers profiles are roughly 90% and 85%, respectively [33, 34]. In accordance with this evidence, a recent study has found after inclusion of CSF results, 90% of amnestic and 82% of the non-amnestic AD presentation could be categorized as "high probability of AD etiology", while 3% of AD patients fit into the category "dementia probably not due to AD" [35]. Thus, we included a subset of patients with typical CSF AD biomarkers, demonstrating evidence of AD pathophysiological process and increased probability of AD etiology as a cause of symptomatology of the patient according to NIA-AA criteria [36]. This fact is relevant, because different studies have demonstrated neuropathological changes that sometimes do not correlate with clinical diagnosis [37]. Therefore, the role of the *MC1R* variant p.V92M in the AD risk was re-analyzed in these well-characterized AD patients. Notably, in spite of the sample size reduction, the association remains statistically significant and the LOAD risk in p.V92M carriers increased more than three fold. Thereby, the risk of p.V92M is more evident within more accurately diagnosed AD patients.

In this study, decreasing age of onset was restricted to the APOE ε4 allele. Thus, the p.V92M variant should be considered like those prior variants which increase the risk of developing AD but do not modulate AOO [38].

The *MC1R* gene encodes a membrane receptor, which is expressed, in neurons of the periaqueductal gray matter, astrocytes, and Schwann cells activated by melanocortin peptides [16, 39]. This receptor may have an important role in the anti-inflammatory brain response [16] and in female specific mediation mechanisms of analgesia [40]. MC1R is also expressed in melanocytes, a cell type with a common embryonic origin with brain cells [41], which determine hair and skin color [42], and certain variants increase the risk for skin cancer (melanoma and non-melanoma skin cancer) [18, 19]. Functional studies of *MC1R* variants conducted in melanocytes, reveal that certain variants reduce cell surface protein expression and diminished capacity to stimulate cAMP, resulting in the red hair color phenotype [17]. Interestingly, two *MC1R* variants related to red hair color phenotype modulate the risk to develop PD [22, 29]. These findings partially explain the previous epidemiological evidence describing a bidirectional link between PD and CM [21]. In contrast to PD, an increased incidence of CM among AD patients and overrepresentation of individuals with natural red hair within AD patients compared to control population has not been reported. This can be explained as the p.V92M variant does not confer a risk to develop CM [18] and by the fact that variant p.V92M promotes a decrease in the affinity of the receptor for its ligand α-MSH, but showed normal cell surface expression and normal capacity to stimulate cAMP, consequently it does not impact on the phenotype [17, 39]. Notably, a functional deficiency of α-MSH in the brain cells of LOAD patients had been previously reported, suggesting that α-MSH may be critical in the development of LOAD [43].

To date, functional evaluation of *MC1R* variants in other cell types such as the nervous system cells is limited. However, there is evidence that certain variants may also impact physiological conditions beyond skin and hair pigmentation, such as risk of depression disorders [44], pain response [40], and anesthetic requirement [45]. Interestingly, a case-control study indicates that variant p.V92M is associated with the response of desipramine treatment in depression disorder [44].

Our study, although exploratory, has some limitations. The major one being sample size, which is not large enough to provide reliable evidence for a genetic AD risk factor, especially if we focus on the controls size (N = 160) which is small. However, our work provides positive results from our hypothesis and highlights a putative role of the *MC1R* gene in the genetic susceptibility to developing neurodegenerative diseases, which is in the same line as previously published works [22, 23].

Another minor limitation is the exclusion of the p.D294H *MC1R* variant from the genetic association analysis. As we explained in the Material and Methods section, the entire coding region was sequenced in 110 LOAD patients, but the other 415 LOAD patients were exclusively sequenced using TM-R primer. This strategy allowed us to detect all *MC1R* variants except one common non-synonymous variant (p.D294H). However, we compared the frequency of the p.D294H variant observed in 110 LOAD patients (minor allele frequency was 3%) with the frequency observed in 160 controls (minor allele frequency was 2%), and we did not detect any statistically significant difference.

In conclusion, the present study suggests that *MC1R* variant p.V92M may increase the risk to develop LOAD. Although, the molecular mechanisms underlying the increased risk of LOAD associated with p.V92M variant are not known, this variant may have biological relevance through non-pigmentation pathways involved in inflammatory or immunomodulatory processes.

Larger genetic studies are necessary to confirm the association of p.V92M with AD. Additionally, further functional studies should be carried out to elucidate the role of the MC1R receptor in brain cells.

ACKNOWLEDGMENTS

We thank the National Bank of DNA from the University of Salamanca (Spain) for kindly providing DNAs from control healthy subjects. The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (12/00840) Spain, co-funded by "Fondo Europeo de Desarrollo Regional (FEDER). Uniòn Europea. Una manera de hacer Europa", by the CERCA Programme/Generalitat de Catalunya, AGAUR 2014_SGR_603 of the Catalan Government, Spain. The work was carried out at the Esther Koplowitz Centre, Barcelona, Spain. This work was partially supported by grant to Dr. Josè Luis Molinuevo (CONSOLIDER-Ingenio program 2010: CSD2010-00045). The research at the Memory Unit at Sant Pau Hospital of Barcelona is partially funded by the AGAUR_2014_SGR_235 grant of the Catalan Government, Spain. Miriam Potrony is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain.

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-1113r1).

REFERENCES

[1] Gilbert BJ (2013) The role of amyloid β in the pathogenesis of Alzheimer's disease. *J Clin Pathol* **66**, 362-366.

[2] Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, Andreassen OA, Engedal K, Ulstein I, Djurovic S, Ibrahim-Verbaas C, Hofman A, Ikram MA, van Duijn CM, Thorsteinsdottir U, Kong A, Stefansson K (2013) Variant of TREM2 associated with the risk of Alzheimer's disease. *N Engl J Med* **368**, 107-116.

[3] Bettens K, Sleegers K, Van Broeckhoven C (2013) Genetic insights in Alzheimer's disease. *Lancet Neurol* **12**, 92-104.

[4] Ashford JW, Mortimer JA (2002) Non-familial Alzheimer's disease is mainly due to genetic factors. *J Alzheimers Dis* **4**, 169-177.

[5] Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, Pasquier F, Fievet N, Barberger-Gateau P, Engelborghs S, De Deyn P, Mateo

I, Franck A, Helisalmi S, Porcellini E, Hanon O, European Alzheimer's Disease Initiative Investigators, de Pancorbo MM, Lendon C, Dufouil C, Jaillard C, Leveillard T, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossu P, Piccardi P, Annoni G, Seripa D, Galimberti D, Hannequin D, Licastro F, Soininen H, Ritchie K, Blanche H, Dartigues JF, Tzourio C, Gut I, Van Broeckhoven C, Alperovitch A, Lathrop M, Amouyel P (2009) Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat Genet* **41**, 1094-1099.

[6] Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Morgan K, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Love S, Kehoe PG, Hardy J, Mead S, Fox N, Rossor M, Collinge J, Maier W, Jessen F, Schürmann B, Heun R, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frolich L, Hampel H, Hüll M, Rujescu D, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Mühleisen TW, Nöthen MM, Moebus S, Jöckel KH, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet* **41**, 1088-1093.

[7] Seshadri S, Fitzpatrick AL, Ikram MA, DeStefano AL, Gudnason V, Boada M, Bis JC, Smith AV, Carassquillo MM, Lambert JC, Harold D, Schrijvers EM, Ramirez-Lorca R, Debette S, Longstreth WT Jr, Janssens AC, Pankratz VS, Dartigues JF, Hollingworth P, Aspelund T, Hernandez I, Beiser A, Kuller LH, Koudstaal PJ, Dickson DW, Tzourio C, Abraham R, Antunez C, Du Y, Rotter JI, Aulchenko YS, Harris TB, Petersen RC, Berr C, Owen MJ, Lopez-Arrieta J, Varadarajan BN, Becker JT, Rivadeneira F, Nalls MA, Graff-Radford NR, Campion D, Auerbach S, Rice K, Hofman A, Jonsson PV, Schmidt H, Lathrop M, Mosley TH, Au R, Psaty BM, Uitterlinden AG, Farrer LA, Lumley T, Ruiz A, Williams J, Amouyel P, Younkin SG, Wolf PA, Launer LJ, Lopez OL, van Duijn CM, Breteler MM, Consortium CHARGE, GERAD1 Consortium, EADI1, Consortium (2010) Genome-wide analysis of genetic loci associated with Alzheimer disease. *JAMA* **303**, 1832-1840.

[8] Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, Gallins PJ, Buxbaum JD, Jarvik GP, Crane PK, Larson EB, Bird TD, Boeve BF, Graff-Radford NR, De Jager PL, Evans D, Schneider JA, Carrasquillo MM, Ertekin-Taner N, Younkin SG, Cruchaga C, Kauwe JS, Nowotny P, Kramer P, Hardy J, Huentelman MJ, Myers AJ, Barmada MM, Demirci FY, Baldwin CT, Green RC, Rogaeva E, St George-Hyslop P, Arnold SE, Barber R, Beach T, Bigio EH, Bowen JD, Boxer A, Burke JR, Cairns NJ, Carlson CS, Carney RM, Carroll SL, Chui HC, Clark DG, Corneveaux J, Cotman CW, Cummings JL, DeCarli C, DeKosky ST, Diaz-Arrastia R, Dick M, Dickson DW, Ellis WG, Faber KM, Fallon KB, Farlow MR, Ferris S, Frosch MP, Galasko DR, Ganguli M, Gearing M, Geschwind DH, Ghetti B, Gilbert JR, Gilman S, Giordani B, Glass JD, Growdon JH, Hamilton RL, Harrell LE, Head E, Honig LS, Hulette CM, Hyman BT, Jicha GA, Jin LW, Johnson N, Karlawish J, Karydas A, Kaye JA, Kim R, Koo EH, Kowall NW, Lah JJ, Levey AI, Lieberman AP, Lopez OL, Mack WJ, Marson DC, Martiniuk F, Mash DC, Masliah E, McCormick WC, McCurry SM, McDavid AN, McKee AC, Mesulam M, Miller BL, Miller CA, Miller JW, Parisi JE, Perl DP, Peskind E, Petersen RC, Poon WW, Quinn JF, Rajbhandary RA, Raskind M, Reisberg B, Ringman JM, Roberson ED, Rosenberg RN, Sano M, Schneider LS, Seeley W, Shelanski ML, Slifer MA, Smith CD, Sonnen JA, Spina S, Stern RA, Tanzi RE, Trojanowski JQ, Troncoso JC, Van Deerlin VM, Vinters HV, Vonsattel JP, Weintraub S, Welsh-Bohmer KA, Williamson J, Woltjer RL, Cantwell LB, Dombroski BA, Beekly D, Lunetta KL, Martin ER, Kamboh MI, Saykin AJ, Reiman EM, Bennett DA, Morris JC, Montine TJ, Goate AM, Blacker D, Tsuang DW, Hakonarson H, Kukull WA, Foroud TM, Haines JL, Mayeux R, Pericak-Vance MA, Farrer LA, Schellenberg GD (2011) Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat Genet* **43**, 436-441.

[9] Hollingworth P, Harold D, Sims R, Gerrish A, Lambert JC, Carrasquillo MM, Abraham R, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Jones N, Stretton A, Thomas C, Richards A, Ivanov D, Widdowson C, Chapman J, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Beaumont H, Warden D, Wilcock G, Love S, Kehoe PG, Hooper NM, Vardy ER, Hardy J, Mead S, Fox NC, Rossor M, Collinge J, Maier W, Jessen F, Rüther E, Schürmann B, Heun R, Kölsch H, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frölich L, Hampel H, Gallacher J, Hull M, Rujescu D, Giegling I, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Mühleisen TW, Nöthen MM, Moebus S, Jockel KH, Klopp N, Wichmann HE, Pankratz VS, Sando SB, Aasly JO, Barcikowska M, Wszolek ZK, Dickson DW, Graff-Radford NR, Petersen RC, Alzheimer's Disease Neuroimaging Initiative, van Duijn CM, Breteler MM, Ikram MA, DeStefano AL, Fitzpatrick AL, Lopez O, Launer LJ, Seshadri S, consortium CHARGE, Berr C, Campion D, Epelbaum J, Dartigues JF, Tzourio C, Alpèrovitch A, Lathrop M, EADI1 consortium, Feulner TM, Friedrich P, Riehle C, Krawczak M, Schreiber S, Mayhaus M, Nicolhaus S, Wagenpfeil S, Steinberg S, Stefansson H, Stefansson K, Snaedal J, Björnsson S, Jonsson PV, Chouraki V, Genier-Boley B, Hiltunen M, Soininen H, Combarros O, Zelenika D, Delepine M, Bullido MJ, Pasquier F, Mateo I, Frank-Garcia A, Porcellini E, Hanon O, Coto E, Alvarez V, Bosco P, Siciliano G, Mancuso M, Panza F, Solfrizzi V, Nacmias B, Sorbi S, Bossu P, Piccardi P, Arosio B, Annoni G, Seripa D, Pilotto A, Scarpini E, Galimberti D, Brice A, Hannequin D, Licastro F, Jones L, Holmans PA, Jonsson T, Riemenschneider M, Morgan K, Younkin SG, Owen MJ, O'Donovan M, Amouyel P, Williams J (2011) Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nat Genet* **43**, 429-435.

[10] Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, DeStafano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thorton-Wells TA, Jones N,

Smith AV, Chouraki V, Thomas C, Ikram MA, Zelenika D, Vardarajan BN, Kamatani Y, Lin CF, Gerrish A, Schmidt H, Kunkle B, Dunstan ML, Ruiz A, Bihoreau MT, Choi SH, Reitz C, Pasquier F, Cruchaga C, Craig D, Amin N, Berr C, Lopez OL, De Jager PL, Deramecourt V, Johnston JA, Evans D, Lovestone S, Letenneur L, Moròn FJ, Rubinsztein DC, Eiriksdottir G, Sleegers K, Goate AM, Fièvet N, Huentelman MW, Gill M, Brown K, Kamboh MI, Keller L, Barberger-Gateau P, McGuiness B, Larson EB, Green R, Myers AJ, Dufouil C, Todd S, Wallon D, Love S, Rogaeva E, Gallacher J, St George-Hyslop P, Clarimon J, Lleo A, Bayer A, Tsuang DW, Yu L, Tsolaki M, Bossú P, Spalletta G, Proitsi P, Collinge J, Sorbi S, Sanchez-Garcia F, Fox NC, Hardy J, Deniz Naranjo MC, Bosco P, Clarke R, Brayne C, Galimberti D, Mancuso M, Matthews F, European Alzheimer's Disease Initiative (EADI), Genetic, Environmental Risk in Alzheimer's Disease, Alzheimer's Disease Genetic Consortium, Cohorts for Heart, Aging Research in Genomic Epidemiology, Moebus S, Mecocci P, Del Zompo M, Maier W, Hampel H, Pilotto A, Bullido M, Panza F, Caffarra P, Nacmias B, Gilbert JR, Mayhaus M, Lannefelt L, Hakonarson H, Pichler S, Carrasquillo MM, Ingelsson M, Beekly D, Alvarez V, Zou F, Valladares O, Younkin SG, Coto E, Hamilton-Nelson KL, Gu W, Razquin C, Pastor P, Mateo I, Owen MJ, Faber KM, Jonsson PV, Combarros O, O'Donovan MC, Cantwell LB, Soininen H, Blacker D, Mead S, Mosley TH Jr, Bennett DA, Harris TB, Fratiglioni L, Holmes C, de Bruijn RF, Passmore P, Montine TJ, Bettens K, Rotter JI, Brice A, Morgan K, Foroud TM, Kukull WA, Hannequin D, Powell JF, Nalls MA, Ritchie K, Lunetta KL, Kauwe JS, Boerwinkle E, Riemenschneider M, Boada M, Hiltuenen M, Martin ER, Schmidt R, Rujescu D, Wang LS, Dartigues JF, Mayeux R, Tzourio C, Hofman A, Nothen MM, Graff C, Psaty BM, Jones L, Haines JL, Holmans PA, Lathrop M, Pericak-Vance MA, Launer LJ, Farrer LA, van Duijn CM, Van Broeckhoven C, Moskvina V, Seshadri S, Williams J, Schellenberg GD, Amouyel P (2013) Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat Genet* **45**, 1452-1458.

[11] Bertram L (2011) Alzheimer's genetics in the GWAS era: A continuing story of 'replications and refutations'. *Curr Neurol Neurosci Rep* **11**, 246-253.

[12] Castellani RJ, Lee HG, Zhu X, Perry G, Smith MA (2008) Alzheimer disease pathology as a host response. *J Neuropathol Exp Neurol* **67**, 523-531.

[13] Roberts PJ (1990) Development, characterization, and subcellular location of DNAse activity in HL-60 cells and monocytes. *Blood* **75**, 976-983.

[14] Cencioni C, Spallotta F, Martelli F, Valente S, Mai A, Zeiher AM, Gaetano C (2013) Oxidative stress and epigenetic regulation in ageing and age-related diseases. *Int J Mol Sci* **14**, 17643-17663.

[15] Gerstenblith MR, Goldstein AM, Fargnoli MC, Peris K, Landi MT (2007) Comprehensive evaluation of allele frequency differences of MC1R variants across populations. *Hum Mutat* **28**, 495-505.

[16] Catania A (2008) Neuroprotective actions of melanocortins: A therapeutic opportunity. *Trends Neurosci* **31**, 353-360.

[17] Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ (2011) Melanocortin 1 receptor variants: Functional role and pigmentary associations. *Photochem Photobiol* **87**, 978-987.

[18] Raimondi S, Sera F, Gandini S, Iodice S, Caini S, Maisonneuve P, Fargnoli MC (2008) MC1R variants, melanoma and red hair color phenotype: A meta-analysis. *Int J Cancer* **122**, 2753-2760.

[19] Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, Bavinck JN, Leiden Skin Cancer Study Team (2001) Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. *Am J Hum Genet* **68**, 884-894.

[20] Kadekaro AL, Chen J, Yang J, Chen S, Jameson J, Swope VB, Cheng T, Kadakia M, Abdel-Malek Z (2012) Alpha-melanocyte-stimulating hormone suppresses oxidative stress through a p53-mediated signaling pathway in human melanocytes. *Mol Cancer Res* **10**, 778-786.

[21] Liu R, Gao X, Lu Y, Chen H (2011) Meta-analysis of the relationship between Parkinson disease and melanoma. *Neurology* **76**, 2002-2009.

[22] Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A (2009) Genetic determinants of hair color and Parkinson's disease risk. *Ann Neurol* **65**, 76-82.

[23] Tell-Marti G, Puig-Butille JA, Potrony M, Badenas C, Milà M, Malvehy J, Marti MJ, Ezquerra M, Fernandez-Santiago R, Puig S (2015) The MC1R melanoma risk variant p. R160W is associated with Parkinson disease. *Ann Neurol* **77**, 889-894.

[24] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-944.

[25] Welge V, Fiege O, Lewczuk P, Mollenhauer B, Esselmann H, Klafki HW, Wolf S, Trenkwalder C, Otto M, Kornhuber J, Wiltfang J, Bibl M (2009) Combined CSF tau, p-tau181 and amyloid-beta 38/40/42 for diagnosing Alzheimer's disease. *J Neural Transm (Vienna)* **116**, 203-212.

[26] Antonell A, Fortea J, Rami L, Bosch B, Balasa M, Sánchez-Valle R, Iranzo A, Molinuevo JL, Lladó A (2011) Different profiles of Alzheimer's disease cerebrospinal fluid biomarkers in controls and subjects with subjective memory complaints. *J Neural Transm (Vienna)* **118**, 259-262.

[27] Chaudru V, Laud K, Avril MF, Miniére A, Chompret A, Bressac-de Paillerets B, Demenais F (2005) Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. *Cancer Epidemiol Biomarkers Prev* **14**, 2384-2390.

[28] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR (2010) A method and server for predicting damaging missense mutations. *Nat Methods* **7**, 248-249.

[29] Tell-Marti G, Puig-Butille JA, Potrony M, Badenas C, Milà M, Malvehy J, Marti MJ, Ezquerra M, Fernández-Santiago R, Puig S (2015) The MC1R melanoma risk variant p. R160W is associated with Parkinson disease. *Ann Neurol* **77**, 889-894.

[30] Bosco P, Ferri R, Salluzzo MG, Castellano S, Signorelli M, Nicoletti F, Nuovo SD, Drago F, Caraci F (2013) Role of the transforming-growth-factor-β1 gene in late-onset Alzheimer's disease: Implications for the treatment. *Curr Genomics* **14**, 147-156.

[31] Li Y, Grupe A (2007) Genetics of late-onset Alzheimer's disease: Progress and prospect. *Pharmacogenomics* **8**, 1747-1755.

[32] Beach TG, Monsell SE, Phillips LE, Kukull W (2012) Accuracy of the clinical diagnosis of Alzheimer disease at National Institute on Aging Alzheimer Disease Centers, 2005-2010. *J Neuropathol Exp Neurol* **71**, 266-273.

[33] Molinuevo JL, Gispert JD, Dubois B, Heneka MT, Lleo A, Engelborghs S, Pujol J, de Souza LC, Alcolea D, Jessen

F, Sarazin M, Lamari F, Balasa M, Antonell A, Rami L (2013) The AD-CSF-index discriminates Alzheimer's disease patients from healthy controls: A validation study. *J Alzheimers Dis* **36**, 67-77.

[34] Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, Kimmel LH, Bergeson J, Manetti GJ, Zimmermann M, Tang B, Bartko JJ, Cohen RM (2003) Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. *JAMA* **289**, 2094-2103.

[35] Balasa M, Sánchez-Valle R, Antonell A, Bosch B, Olives J, Rami L, Castellvi M, Molinuevo JL, Lladó A (2014) Usefulness of biomarkers in the diagnosis and prognosis of early-onset cognitive impairment. *J Alzheimers Dis* **40**, 919-927.

[36] McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR Jr, Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**, 263-269.

[37] Balasa M, Gelpi E, Antonell A, Rey MJ, Sanchez-Valle R, Molinuevo JL, Llado A, Neurological Tissue Bank/University of Barcelona/Hospital Clínic NTB/UB/HC Collaborative Group (2011) Clinical features and APOE genotype of pathologically proven early-onset Alzheimer disease. *Neurology* **76**, 1720-1725.

[38] Thambisetty M, An Y, Tanaka T (2013) Alzheimer's disease risk genes and the age-at-onset phenotype. *Neurobiol Aging* **34**, 2696 e2691-2695.

[39] García-Borrón JC, Sánchez-Laorden BL, Jiménez-Cervantes C (2005) Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res* **18**, 393-410.

[40] Liem EB, Joiner TV, Tsueda K, Sessler DI (2005) Increased sensitivity to thermal pain and reduced subcutaneous lidocaine efficacy in redheads. *Anesthesiology* **102**, 509-514.

[41] Christiansen JH, Coles EG, Wilkinson DG (2000) Molecular control of neural crest formation, migration and differentiation. *Curr Opin Cell Biol* **12**, 719-724.

[42] Valverde P, Healy E, Jackson I, Rees JL, Thody AJ (1995) Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet* **11**, 328-330.

[43] Rainero I, May C, Kaye JA, Friedland RP, Rapoport SI (1988) CSF alpha-MSH in dementia of the Alzheimer type. *Neurology* **38**, 1281-1284.

[44] Wu GS, Luo HR, Dong C, Mastronardi C, Licinio J, Wong ML (2011) Sequence polymorphisms of MC1R gene and their association with depression and antidepressant response. *Psychiatr Genet* **21**, 14-18.

[45] Liem EB, Lin CM, Suleman MI, Doufas AG, Gregg RG, Veauthier JM, Loyd G, Sessler DI (2004) Anesthetic requirement is increased in redheads. *Anesthesiology* **101**, 279-283.

Supplementary Table 1: Results of association analysis of MC1R variants and typical AD CSF profile patients

AA change	Controls (N=160)				LOAD patients (N=69)					
	[a]MAF	[a]Genotype frequency			MAF	Genotype frequency			CONTROL vs LOAD patients	
	2	1-1	1-2	2-2	2	1-1	1-2	2-2	OR (95% CI)[b]	P-value[b]
p.V60L	0.16	0.72	0.25	0.03	0.17	0.71	0.25	0.04	1.14 (0.64-2.02)	0.66
p.V92M	0.05	0.9	0.1	0	0.09	0.83	0.16	0.01	**3.40 (1.40-8.27)**	**0.007**
p.R163Q	0.01	0.98	0.02	0	0.03	0.94	0.06	0	1.57 (0.30-8.22)	0.59
p.R151C	0.03	0.94	0.06	0	0.01	0.97	0.03	0	0.77 (0.15-4.04)	0.77
p.R160W	0.01	0.975	0.025	0	0.01	0.99	0.01	0	0.54 (0.05-6.47)	0.62

MAF: Minor allele frequency

[a]Allele described as 1 (wild type allele) or 2 (variant allele)

[b] Adjusted for gender, age and APOE genotype.

The genetic model used was the log-additive, the homozygous for 'variant allele' (a/a) has double the risk of the heterozygous (A/a).

The statistically significant result is highlighted in bold.

Supplementary Table 2: Analysis of Age of onset in AD patients regarding MC1R genotype

MC1R variants	MC1R genotype^ 1-1 (N)	Age of onset Mean±SD*	MC1R genotype^ 1-2, 2-2 (N)	Age of onset Mean±SD*	p-value
p.V60L	370	76.18±5.50	155	76.75±5.85	0.281
p.V92M	451	76.38±5.64	74	76.16±5.43	0.758
p.R163Q	501	76.41±5.59	24	75.04±5.83	0.272
p.R151C	499	76.33±5.59	26	76.77±6.02	0.716
p.R160W	507	76.30±5.64	18	77.56±4.63	0.353

^Alleles are denoted as '1' (wild type allele) or '2' (variant or minor allele).
*SD, standard deviation.

TREBALL III

Treball realitzat per donar resposta a l'objectiu 3 de la present tesi doctoral.

Títol:

The p.R151C Polymorphism in *MC1R* Gene Modifies the Age of Onset in Spanish Huntington's Disease Patients

Doi: 10.1007/s12035-016-0305-5

Resum:

La longitud de l'expansió del trinucleòtid CAG en l'exó 1 del gen *HTT*, correlaciona inversament amb l'edat d'aparició de la malaltia de Huntington, però no la determina per complet. Part de la variació restant s'ha atribuït a altres factors genètics i es coneix que en queden d'altres pendents d'identificar. Donat que el MC1R s'expressa en les cèl·lules del SNC i que en les cèl·lules cutànies portadores de variants LOF en *MC1R*, s'ha identificat una desregulació constitutiva de gens implicats en les vies de senyalització de malalties neurodegeneratives; varem hipotetitzar que el gen *MC1R* podria ser un factor modificador de l'edat d'aparició de la malaltia de Huntington.

L'objectiu d'aquest treball era avaluar el gen *MC1R* com a factor modificador de l'edat d'inici de la malaltia de Huntington.

El gen *MC1R* es va caracteritzar molecularment en 600 casos de Huntington d'origen espanyol, seqüenciant la regió codificant sencera del trànscrit (ENST0000555147) que codifica per la forma canònica del receptor MC1R (NP_002377). Mitjançant un model de regressió lineal es va avaluar el percentatge de variació en l'edat d'inici de la malaltia atribuït a l'expansió del trinucleòtid CAG. L'efecte de les variants al·lèliques de *MC1R* es va avaluar mitjançant un model de regressió lineal múltiple, en el qual l'edat d'inici es definia com a variable dependent i l'expansió del triplet CAG i les variants al·lèliques de *MC1R* com a variables independents.

De forma global es van identificar 34 variants al·lèliques (10 sinònimes i 24 no sinònimes). Per a l'anàlisi, es van seleccionar el conjunt de variants no sinònimes que presentaven una MAF >0,01. Se'n van identificar 8: p.V60L (0,14), p.V92M (0,05), p.R142H (0,02), p.R151C (0,02), p.I155T (0,02), p.R160W(0,01), p.R163Q (0,02) i p.D294H (0,01). Les freqüències al·lèliques de les variants de *MC1R* identificades en els pacients de Huntington no van mostrar diferencies significatives respecte a les freqüències identificades en població control espanyola.

A través del model de regressió lineal varem identificar que un 58% de la variació en l'edat d'aparició de la malaltia esta determinat pel número de repeticions CAG en la cohort espanyola analitzada. La inclusió de la variant p.R151C (rs1805007) en el model de regressió lineal múltiple va mostrar un efecte significatiu (R^2 = 0,583, p-valor = 0,004, p-valor corregit per Bonferroni = 0,032). L'al·lel p.R151C de *MC1R* contribueix a explicar un 1,42% de la variació restant que no s'explica per l'expansió del triplet CAG. Aquest percentatge de variació és similar al prèviament atribuït a altres variants genètiques identificades en els gens *UCHL1* (1,7%), *PGC-1α* (1.5%) i *ATG7* (1%). Els pacients de Huntington portadors de l'al·lel p.R151C en heterozigosi presenten una edat mitjana d'inici = 43,95 anys, que representa una disminució de 5,13 anys respecte als individus no portadors d'aquest l'al·lel.

Aquests resultats suggereixen per primera vegada un paper modificador del gen *MC1R* en l'edat d'inici de la malaltia de Huntington i indiquen que la pèrdua de funció del MC1R, que s'associa a l'al·lel p.R151C, pot contribuir a la fisiopatologia del Huntington.

Mol Neurobiol
DOI 10.1007/s12035-016-0305-5

The p. R151C Polymorphism in *MC1R* Gene Modifies the Age of Onset in Spanish Huntington's Disease Patients

Gemma Tell-Marti[1,2] · Joan Anton Puig-Butille[2,3] · Pol Gimenez-Xavier[1,2] · Ariadna Segu-Roig[1] · Miriam Potrony[1] · Celia Badenas[2,3] · Victoria Alvarez[4] · José M. Millán[2,5] · María José Trujillo-Tiebas[2,6] · María A Ramos-Arroyo[7] · Montserrat Milà[2,3] · Susana Puig[1,2,8]

Received: 20 June 2016 / Accepted: 16 November 2016

Abstract The expansion of CAG repeats (≥36 CAG) in the *HTT* gene is the only known genetic cause of Huntington's disease (HD) and the main determinant of the course of the disease. The length of the expanded CAG repeats correlates inversely with the age of onset (AOO) but does not completely determine it. We investigated the role of the melanocortin 1 receptor (*MC1R*) gene as a modifier factor of AOO in 600 HD patients from Spain. We sequenced the entire region of the *MC1R* gene and analyzed all the nonsynonymous *MC1R* genetic variants with a minor allele frequency of at least 0.01 in HD patients. The variability in AOO attributable to the CAG repeats and *MC1R* polymorphisms was evaluated using a multiple linear regression model. We found that the loss-of-function p. R151C *MC1R* polymorphism has a significant influence on the AOO (P = 0.004; Bonferroni-corrected P = 0.032) which explains 1.42% of the variance in AOO that cannot be accounted for by the expanded CAG repeat. Our results suggest that the *MC1R* gene could modify the AOO in Spanish HD patients and encourage the evaluation of loss-of-function *MC1R* polymorphisms in other HD populations with a higher frequency of these *MC1R* polymorphisms.

Keywords Huntington's disease · Age of onset · Melanocortin 1 receptor (*MC1R*) gene · p. R151C · Neurodegenerative diseases

Introduction

The expansion of CAG repeats in the *HTT* gene (≥36 CAG) is the only known genetic cause of Huntington's disease (HD) [1]. The length of the expanded CAG repeats correlates inversely with the age of onset (AOO), explaining 42–73% of its variance, but does not completely determine it. The existence of additional genetic modifying factors have been reported, indicating that AOO is modulated by multiple genes involved in a wide range of physiological functions such as oxidative stress (*ASK1*, *MAP2K6*, and *PGC-1α)* [2, 3], synaptic transmission (*HAP1*

Montserrat Milà and Susana Puig contributed equally to this work.

✉ Susana Puig
susipuig@gmail.com; spuig@clinic.ub.es

1 Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain

2 Centro de Investigación Biomédica en Red Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain

3 Biochemical and Molecular Genetics Service, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain

4 Laboratorio de Genética. AGC Laboratorio de Medicina, Hospital Universitario Central de Asturias, Oviedo, Spain

5 Instituto de Investigación Sanitaria Hospital La Fe, IIS-La Fe, Valencia, Spain

6 Department of Genetics, Health Research Institute–Jimenez Diaz Foundation University Hospital (IIS-FJD), Madrid, Spain

7 Medical Genetics Department, Complejo Hospitalario de Navarra, IdiSNA, Navarra Institute for Health Research, Irunlarrea 4, 31008 Pamplona, Navarra, Spain

8 Medicine Department, Universitat de Barcelona, Barcelona, Spain

Published online: 06 December 2016

and *ADORA2*) [4, 5], glutamatergic response (*GRIN2A* and *GRIN2B*) [6], autophagy (*ATG7*) [7], proteolysis (*UCHL*) [8] or DNA handling and repair mechanisms (*MLH1, MLH3*) [9].

The melanocortin 1 receptor (*MC1R*) gene encodes for a protein that activates pigmentation, antioxidant, DNA repair, and survival pathways [10]. Although the MC1R protein is mainly expressed in melanocytes, it is also detected in neurons where it has a neuroprotective effect [11]. We have observed that skin cells harboring loss-of-function *MC1R* polymorphisms show a deregulation of genes involved in neurodegenerative diseases pathways, including Huntington's disease [12]. It has also been shown that skin cells with loss-of-function *MC1R* polymorphisms showed a significantly greater oxidative DNA and a lipid damage [13]. Elevated levels of oxidative damage are a physiological condition found in areas of degeneration in HD brains suggesting that oxidative stress and consequent protein oxidation is a potential mechanism of neuronal death in HD patients [14]. Thus, we hypothesize that the loss-of-function *MC1R* polymorphisms may impact on the onset of HD by increasing the oxidative stress in the brain cells. To explore the role of the *MC1R* gene as a modifying factor of the AOO, we sequenced the entire coding region of *MC1R* in 600 HD Spanish patients and analyzed all the nonsynonymous genetic variants with an observed minor allele frequency (MAF) of at least 0.01.

Subjects and Methods

Subjects

The study includes 600 unrelated HD patients (265 (44.2%) males and 335 (55.8%) females) from five hospital-based series from Spain: the Biochemical and Molecular Genetics Service from Hospital Clínic of Barcelona ($N = 213$), the Medical Genetics Department of the Complejo Hospitalario de Navarra ($N = 141$), the Genetics Service from Hospital Universitario Central of Oviedo ($N = 112$), the Health Research Institute Hospital La Fe of Valencia ($N = 76$), and the Department of Genetics from Health Research Institute–Jimenez Diaz Foundation University Hospital (IIS-FJD) of Madrid ($N = 58$).

The mean age of 600 studied patients was 49 ± 14 (SD) years and the expanded (CAG)n sequence ranged from 36 to 90 repeats (4.8% of patients had <40 CAGs, 89.7% had 40–50 CAGs, and 5.5% had >50 CAGs), with an average of 44 ± 4.8 (SD) CAG repeats. All HD patients were clinically diagnosed by neurologists, and AOO was estimated as the age at which motor symptoms first occurred.

All study participants gave their written informed consent, and the study was approved by the ethics committee of the Hospital Clínic of Barcelona.

HTT (CAG)n Analysis

DNA was obtained from blood samples, and the (CAG) repeat number in the *HTT* gene (NM_002111.6) was determined by PCR using the primers HD1/HD2 and nested HD1/HD3 [15] FAM labeled and sizing by capillary electrophoresis in an ABI 3100 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

MC1R Molecular Screening

The *MC1R* gene, which consists of one single exon encoding a 317 amino-acid protein (ENST00000555147), was sequenced using 50–100 ng of the total DNA per sample and carrying out the methodology previously described [16].

Statistical Analysis

Allelic and genotypic frequencies of *MC1R* variants were determined by SNPStats software (http://bioinfo.iconcologia.net/SNPstats). *In-silico* analysis of nonsynonymous variants was carried out using Polyphen2 software (http://genetics.bwh.harvard.edu/pph2/).

We analyzed nonsynonymous *MC1R* variants with an observed MAF of at least 0.01 in HD patients. The association of *MC1R* polymorphisms with AOO was evaluated by encoding the genotype of *MC1R* polymorphisms as a binary independent variable based on the absence/presence of the polymorphism (major allele homozygotes = 0 and heterozygotes or minor allele homozygotes = 1). Variability in AOO attributable to the expanded HD allele was assessed by a linear regression using AOO as the dependent variable and expanded HD allele as the independent variable. The goodness-of-fit was evaluated by the proportion of variation in the AAO explained by the coefficient of determination (R^2). We obtained the best fit of our data by logarithmic transformation of the AAO and the CAG repeat number. The association of *MC1R* polymorphisms with AOO was assessed by multiple linear regressions, correcting for the effect of expanded HD allele, and using AOO as the dependent variable and the polymorphisms and the expanded HD allele as the independent variables. A change of R2 after adding the effect of the polymorphisms indicated a relative improvement of the model and thus identified the percentage of variance attributable to the *MC1R* polymorphisms when there was a significant P value ($P < 0.05$). Bonferroni correction for multiple comparisons was applied to all $P < 0.05$. All calculations were carried out using R version 2.15.2.

Results

MC1R genotyping was carried out in 600 HD Spanish patients with similar CAG repeat distribution as previously observed

Table 1. Genetic variants in the *MC1R* gene detected in HD patients

Huntington disease (HD) patients				
MC1R variants		HD patients minor alleles (N = 1200 total alleles)	MAF^	Impact score[a]
AA change	cDNA change			
Synonymous variants				
p. A110A	c.330 T > C	2/1200	_	_
p. A111A	c.333G > A	1/1200	_	_
p. C133C	c.399C > T	1/1200	_	_
p. R151R	c.453C > T	1/1200	_	_
p. A161A	c.483G > A	1/1200	_	_
p. Y182Y	(c.546C > T)	2/1200	_	_
p. Q233Q	c.699G > A	8/1200	_	_
p. I264I	c.792C > T	1/1200	_	_
p. T314 T	c.942A > G	93/1200	0.08	_
p. S316S	c.948C > T	1/1200	_	_
Nonsynonymous variants				
p. V60 L	c.178G > T	163/1200	0.14	0.988
p. A81T	c.241G > A	1/1200	_	1000
p. D84E	c.252C > A	1/1200	_	0.94
p. V92 M	c.274G > A	65/1200	0.05	0.015
p. V122 M	c.364G > A	2/1200	_	0.120
p. R142C	c.424 C > T	1/1200	_	1.000
p. R142H	c.425G > A	18/1200	0.02	1.000
p. R151C	c.451C > T	21/1200	0.02	1.000
p. Y152[a]	c.456C > A	1/1200	_	1.000
p. I155T	c.464 T > C	19/1200	0.02	0.985
p. V156A	c.467 T > C	1/1200	_	0.951
p. P159L	c.476C > T	1/1200	_	0.084
p. R160W	c.478C > T	17/1200	0.01	0.934
p. R163Q	c.488G > A	22/1200	0.02	0.032
p. V170 M	c.508G > A	1/1200	_	0.292
p. V193 M	c.577G > A	1/1200	_	0.532
p. R213W	c.637C > T	1/1200	_	0.079
p. I245V	c.733A > G	1/1200	_	0.205
p. G248D	c.743G > A	1/1200	_	1.000
p. H270Y	c.808C > T	1/1200	_	0.998
p. A285T	c.853G > A	1/1200	_	0.004
p. D294H	c.880G > C	15/1200	0.01	1.000
p. R306C	c.916C > T	1/1200	_	0.067
p. R307K	c.920G > A	1/1200	_	0.001

^*MAF* minor allele frequency of all the variants with an observed minor allele frequency (MAF) of at least 0.01

[a] Impact score of the amino-acid substitution on the structure and function of *MC1R* computed using the Polyphen2 software (scores close to 0 indicate a benign mutation whereas scores close to 1.0 indicate a harmful mutation)

in other Spanish studies [17]. We detected 34 variants within the coding region of the *MC1R* gene (10 synonymous and 24 nonsynonymous) (Table 1), eight of which were nonsynonymous *MC1R* variants with a MAF ≥ 0.01: p. V60 L (0.14), p. V92 M (0.05), p. R142H (0.02), p. R151C (0.02), p. I155T (0.02), p. R160W (0.01), p. R163Q (0.02), and p. D294H (0.01) (Table 1). The allelic frequencies observed in HD Spanish patients were not statistically different from those observed in a set of 736 controls free from neurodegenerative disorders used in a previous study [16] and from those reported in other Spanish controls sets [18, 19].

Table 2. Effect of *MC1R* SNPs on the age of onset in 600 HD patients

Variable	R^2	ΔR^2	Additional explained variance (%)	*P value*
HD CAG (36–90)	0.577	–	–	<0.0001
HD CAG + MC1R p. V60 L	0.577	0.000	–	n.s
HD CAG + MC1R p. V92 M	0.577	0.000	–	n.s
HD CAG + MC1R p. R142H	0.578	0.001	–	n.s
HD CAG + MC1R p. R151C	0.583	0.006	1.42	0.004
HD CAG + MC1R p. I155T	0.578	0.001	–	n.s
HD CAG + MC1R p. R160W	0.577	0.000	–	n.s
HD CAG + MC1R p. R163Q	0.578	0.001	–	n.s
HD CAG + MC1R p. D294H	0.578	0.001	–	n.s

HD CAG expanded CAG allele in *HTT* gene, R^2 coefficient of determination, illustrates the relative improvement of the regression model when the genotypes were considered in addition to the CAG repeats, ΔR^2 value quantify these differences

n.s not statistically significant

The level of significance was set to *P* value < 0.05

Linear regression analysis of the effect of the expanded HD allele in AOO indicated that the HD mutation explained ~58% of the variation in AOO ($R^2 = 0.577$, $P < 0.0001$) (Table 2). We then assessed *MC1R* as a modifying factor of AOO using a multiple linear regression model, including the *MC1R* variants with a MAF ≥ 0.01 as covariants (Table 2). The inclusion of p. R151C polymorphism revealed a significant effect on the AOO ($R^2 = 0.583$, $P = 0.004$, Bonferroni-corrected $P = 0.032$). The p. R151C polymorphism constituted 1.42% of the variance that could not be explained by the expanded HD allele (Table 2). HD patients with a heterozygous p. R151C genotype developed their first symptoms at an average age of 43.95 years, which was 5.13 years earlier than homozygotes for the major allele (Table 3).

Discussion

We have investigated the role of the *MC1R* gene as a modifier of the AOO in a set of Spanish HD patients; we found evidence that the p. R151C polymorphism, which impairs MC1R function, modifies the AOO in HD patients. The percentage of the variance attributed to the p. R151C polymorphism (1.42%) is similar to that reported for other genetic variants located in the *UCHL1* (1.7%) [8], *PGC-1α* (1.5%) [3], and *ATG7* (1%) [7] genes.

Table 3. Mean age of onset by the presence of the p. R151C *MC1R* polymorphism in HD patients

Genotype	Genotypic frequency (%)	Mean CAGexp (SD)	Mean AOO (SD)
CC	96	43.97 (4.88)	49.08 (14.12)
CT	4	43.38 (2.16)	43.95 (11.81)
TT	–	–	–

CAGexp expanded CAG repeat number, *SD* standard deviation, *AOO* age of onset, *C* major allele, *T* minor allele

Previous studies indicate that the loss-of-function polymorphisms, such as p. R151C and p. R160W, increase the risk of developing Parkinson's disease [16, 20]. Thus, our findings reinforce the hypothesis that *MC1R* could play a role in the etiology of multiple neurodegenerative diseases [12].

Few studies have assessed the role of *MC1R* in neurons; in contrast, the gene has been widely studied in the field of skin cancer, functional studies in melanocytes indicate that p. R151C reduces MC1R expression on the cell surface and impairs protein function [21, 22]. Melanocytes with a non-functional MC1R protein have a compromised DNA repair capacity and sustain high levels of reactive oxygen species [10, 13]. Although functional studies of *MC1R* variants should be carried out in brain cells, any modifier effect on the AOO in HD attributable to the p. R151C polymorphism could be explained by a role under similar physiological conditions to those observed in skin cells. Brain cells of HD patients present high levels of oxygen species [14]. In addition, other AOO modifier genes in HD, such as *PGC-1α* [3], *ASK*, [2], and *MAP2K6* [2] are involved in this biological process. Additional functional studies are needed to further explore and clarify the effect of *MC1R* polymorphisms on cells of the nervous system.

In conclusion, we report for the first time the possible role of the p. R151C *MC1R* polymorphism as a genetic modifier of the AOO in Spanish HD patients. Further studies should be conducted to validate our findings in other HD populations with a higher frequency of these *MC1R* polymorphisms.

Acknowledgements We are grateful to the patients for participating in the study, to the geneticists for providing DNA samples, and to Pablo Gonzalez Navarro, technician from the Department of Genetics-Jimenez

Diaz Foundation University Hospital (IIS-FJD), for his work in relation to this project.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Funding The research at the Melanoma Unit in Barcelona is partially funded by the Grants from Fondo de Investigaciones Sanitarias (12/00840) Spain, by the AGAUR 2014_SGR_603 of the Catalan Government, Spain, by the European Commission under the 6th Framework Programme, Contract nr: LSHC-CT-2006-018702 (GenoMEL), and by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115). Miriam Potrony is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain.

References

1. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group (1993). Cell 72 (6):971–983
2. Arning L, Monte D, Hansen W, Wieczorek S, Jagiello P, Akkad DA, Andrich J, Kraus PH et al (2008) ASK1 and MAP2K6 as modifiers of age at onset in Huntington's disease. J Mol Med (Berl) 86(4):485–490. doi:10.1007/s00109-007-0299-6
3. Taherzadeh-Fard E, Saft C, Andrich J, Wieczorek S, Arning L (2009) PGC-1alpha as modifier of onset age in Huntington disease. Mol Neurodegener 4:10. doi:10.1186/1750-1326-4-10
4. Metzger S, Rong J, Nguyen HP, Cape A, Tomiuk J, Soehn AS, Propping P, Freudenberg-Hua Y et al (2008) Huntingtin-associated protein-1 is a modifier of the age-at-onset of Huntington's disease. Hum Mol Genet 17(8):1137–1146. doi:10.1093/hmg/ddn003
5. Dhaenens CM, Burnouf S, Simonin C, Van Brussel E, Duhamel A, Defebvre L, Duru C, Vuillaume I et al (2009) A genetic variation in the ADORA2A gene modifies age at onset in Huntington's disease. Neurobiol Dis 35(3):474–476. doi:10.1016/j.nbd.2009.06.009
6. Arning L, Saft C, Wieczorek S, Andrich J, Kraus PH, Epplen JT (2007) NR2A and NR2B receptor gene variations modify age at onset in Huntington disease in a sex-specific manner. Hum Genet 122(2):175–182. doi:10.1007/s00439-007-0393-4
7. Metzger S, Saukko M, Van Che H, Tong L, Puder Y, Riess O, Nguyen HP (2010) Age at onset in Huntington's disease is modified by the autophagy pathway: implication of the V471A polymorphism in Atg7. Hum Genet 128(4):453–459. doi:10.1007/s00439-010-0873-9
8. Metzger S, Bauer P, Tomiuk J, Laccone F, Didonato S, Gellera C, Soliveri P, Lange HW et al (2006) The S18Y polymorphism in the UCHL1 gene is a genetic modifier in Huntington's disease. Neurogenetics 7(1):27–30. doi:10.1007/s10048-005-0023-z
9. Pinto RM, Dragileva E, Kirby A, Lloret A, Lopez E, St Claire J, Panigrahi GB, Hou C et al (2013) Mismatch repair genes Mlh1 and Mlh3 modify CAG instability in Huntington's disease mice: genome-wide and candidate approaches. PLoS Genet 9(10): e1003930. doi:10.1371/journal.pgen.1003930
10. Abdel-Malek ZA, Swope VB, Starner RJ, Koikov L, Cassidy P, Leachman S (2014) Melanocortins and the melanocortin 1 receptor, moving translationally towards melanoma prevention. Arch Biochem Biophys. doi:10.1016/j.abb.2014.07.002
11. Catania A (2008) Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci 31(7):353–360. doi:10.1016/j.tins.2008.04.002
12. Puig-Butille JA, Escamez MJ, Garcia-Garcia F, Tell-Marti G, Fabra A, Martinez-Santamaria L, Badenas C, Aguilera P et al (2014) Capturing the biological impact of CDKN2A and MC1R genes as an early predisposing event in melanoma and non melanoma skin cancer. Oncotarget 5(6):1439–1451
13. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK et al (2012) An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature 491(7424):449–453. doi:10.1038/nature11624
14. Browne SE, Ferrante RJ, Beal MF (1999) Oxidative stress in Huntington's disease. Brain Pathol 9(1):147–163
15. Warner JP, Barron LH, Brock DJ (1993) A new polymerase chain reaction (PCR) assay for the trinucleotide repeat that is unstable and expanded on Huntington's disease chromosomes. Mol Cell Probes 7(3):235–239. doi:10.1006/mcpr.1993.1034
16. Tell-Marti G, Puig-Butille JA, Potrony M, Badenas C, Mila M, Malvehy J, Marti MJ, Ezquerra M et al (2015) The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol. doi:10.1002/ana.24373
17. Ramos-Arroyo MA, Moreno S, Valiente A (2005) Incidence and mutation rates of Huntington's disease in Spain: experience of 9 years of direct genetic testing. J Neurol Neurosurg Psychiatry 76(3):337–342. doi:10.1136/jnnp.2004.036806
18. Scherer D, Nagore E, Bermejo JL, Figl A, Botella-Estrada R, Thirumaran RK, Angelini S, Hemminki K et al (2009) Melanocortin receptor 1 variants and melanoma risk: a study of 2 European populations. Int J Cancer 125(8):1868–1875. doi:10.1002/ijc.24548
19. Cordoba-Lanus E, Hernandez-Jimenez JG, Medina-Coello C, Espinoza-Jimenez A, Gonzalez A, Rodriguez-Perez MD, Carretero-Hernandez G, Almeida P et al (2014) MC1R gene variants and sporadic malignant melanoma susceptibility in the Canary Islands population. Arch Dermatol Res 306(1):51–58. doi:10.1007/s00403-013-1420-z
20. Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A (2009) Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol 65(1):76–82. doi:10.1002/ana.21535
21. Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ (2011) Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol 87(5):978–987. doi:10.1111/j.1751-1097.2011.00970.x
22. Garcia-Borron JC, Abdel-Malek Z, Jimenez-Cervantes C (2014) MC1R, the cAMP pathway, and the response to solar UV: extending the horizon beyond pigmentation. Pigment Cell Melanoma Res 27(5):699–720. doi:10.1111/pcmr.12257

TREBALL IV

Treball realitzat per donar resposta a l'objectiu 4 de la present tesi doctoral.

Títol:

Genomic expression differences between cutaneous cells from red hair color individuals and black hair color individuals based on bioinformatic analysis

Doi: 10.18632/oncotarget.14140.

Resum:

Nombrosos treballs han identificat una associació entre les variants al·lèliques LOF de *MC1R* i un augment del risc a desenvolupar càncer cutani, tan melanoma com no melanoma. Els co-cultius de melanòcits i queratinòcits on la funció del MC1R és nul·la, mostraven 3570 gens diferencialment expressats respecte als co-cultius MC1R WT. Entre aquests, 1954 gens estaven diferencialment sobreexpressats i 1616 infraexpressats.

Amb l'objectiu de posar en context biològic aquesta signatura gènica associada a la pèrdua de funció del MC1R, es va realitzar un anàlisi de xarxes d'interacció proteïna-proteïna (PPI). Es tracta d'un anàlisi computacional, basat en la cerca de mòduls funcionals en llistats de gens (trànscrits), segons la interacció existent entre les proteïnes per les quals codifiquen aquests gens.

Aquest anàlisi ens va permetre identificar dues xarxes estadísticament significatives (p-valor < 0,001). Una composada per 557 gens identificada a partir dels 1954 gens sobreexpressats i una altre constituïda per 450 gens identificada a partir dels 1616 gens infraexpressats. En base a dos paràmetres topològics de les xarxes, com el nombre de connexions d'un node dins la xarxa i el grau de centralitat d'aquest node, es van identificar 23 nodes (*hub genes*) com a gens més rellevants en aquestes xarxes. Entre aquests, 12 connecten el 48% (270 gens de 557) de la xarxa identificada a partir dels gens sobreexpressats i 11 connecten el 39% (177 gens de 450) de la xarxa identificada a partir dels gens infraexpressats.

D'aquests 23 gens, 12 estan implicats en la regulació de l'autofàgia (*PIK3C3*, *ATG4C*, *ATG10*, *SNX2*, *TRAF2*, *SQSTM1*, *CLN3*, *WIPI2*, *GABARAPL1*, *GABARAPL2*, *MAP1LC3B* i *MAP1LC3A*), 6 implicats en la reparació del dany en l'ADN i el manteniment de l'homeòstasi cel·lular (*PRKAA1*, *CDK1*, *BUB1B*, *PCNA*, *RPA1* i *BRCA1*) i dos en la regulació de la fosforilació oxidativa i la funció mitocondrial (*GBAS*, *ICT1*).

L'expressió diferencial d'aquests 23 gens es va avaluar en dos *sets* de dades independents: un que contenia l'expressió gènica global de 14 biòpsies de pell sana d'individus pèl-roigs i de 7 biòpsies de pell sana d'individus amb cabell negre (model *in vivo*), i un altre que contenia l'expressió gènica global de 20 melanomes de pacients pèl-roigs i 6 melanomes de pacients amb el cabell negre.

L'anàlisi d'expressió diferencial realitzat amb les biòpsies de pell sana va identificar 1952 gens expressats diferencialment al comparar les biòpsies d'individus pèl-roigs *vs* les biòpsies d'individus amb el cabell negre, entre aquests, 378 gens formen part de la signatura gènica identificada en els co-cultius portadors d'un MC1R no funcional. L' expressió gènica de 18 dels 23 *hub genes* identificats en el model *in vitro*, es va poder avaluar en el *set* de dades del model *in vivo*. L'anàlisi de revalidació, va identificar que 8 d'aquests gens es troben expressats de forma diferencial (*CLN3*, *ATG10*, *WIPI2*, *SNX2*, *GABARAPL2*, *YWHA*, *PCNA i GBAS*) en les biòpsies de pell dels individus pèl-roigs. Per contra, cap d'aquests 23 gens es troba expressat diferencialment al comprar l'expressió detectada en els tumors de pèl-roigs *vs* la detectada en els tumors d'individus amb el cabell negre.

Així doncs, aquests resultats confirmen que la pèrdua de funció del MC1R promou una desregulació gènica a nivell constitutiu associada a la síntesi de feomelanina. A més, destaquen que part de la signatura gènica identificada en les cèl·lules sanes *in vitro*, es revalida en les cèl·lules sanes *in vivo* i no en les cèl·lules tumorals. Per tant, la signatura gènica identificada s'associa a l'alteració funcional que predisposa als melanòcits a transformar-se en els estadis inicials de la carcinogènesi i no a la pròpia progressió del tumor.

De forma interessant, el treball confirma un augment de l'estrès oxidatiu en les cèl·lules portadores d'un MC1R no funcional i destaca l'autofàgia com un dels processos biològics que pot estar desregulat a nivell basal en aquestes cèl·lules.

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 7), pp: 11589-11599

Research Paper

Genomic expression differences between cutaneous cells from red hair color individuals and black hair color individuals based on bioinformatic analysis

Joan Anton Puig-Butille[1], Pol Gimenez-Xavier[2], Alessia Visconti[3], Jérémie Nsengimana[4], Francisco Garcia-García[5], Gemma Tell-Marti[2], Maria José Escamez[6], Julia Newton-Bishop[4], Veronique Bataille[3], Marcela del Río[6], Joaquín Dopazo[5,7,8], Mario Falchi[3], Susana Puig[2]

[1]Biochemistry and Molecular Genetics Department, Melanoma Unit, Hospital Clinic & IDIBAPS, CIBER de Enfermedades Raras (CIBERER), Barcelona, Spain

[2]Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS, CIBER de Enfermedades Raras (CIBERER), Barcelona, Spain

[3]Department of Twin Research and Genetic Epidemiology, King's College London, London, UK

[4]Section of Epidemiology and Biostatistics, Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, UK

[5]Computational Genomics Department, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain

[6]Departamento de Bioingeniería, Universidad Carlos III de Madrid, CIEMAT, IIS-Fundación Jiménez Díaz, CIBER de Enfermedades Raras (CIBERER), Madrid, Spain

[7]Functional Genomics Node, (INB) at CIPF, Valencia, Spain

[8]CIBER de Enfermedades Raras (CIBERER), Valencia, Spain

Correspondence to: *Joan Anton Puig-Butille,* ***email:*** *jantonpuig@gmail.com; japuig@clinic.ub.es*

Keywords: *MC1R, red hair phenotype, melanocyte, melanoma, autophagy*

Received: *July 28, 2016* ***Accepted:*** *November 21, 2016* ***Published:*** *December 24, 2016*

ABSTRACT

The *MC1R* gene plays a crucial role in pigmentation synthesis. Loss-of-function *MC1R* variants, which impair protein function, are associated with red hair color (RHC) phenotype and increased skin cancer risk. Cultured cutaneous cells bearing loss-of-function *MC1R* variants show a distinct gene expression profile compared to wild-type *MC1R* cultured cutaneous cells. We analysed the gene signature associated with RHC co-cultured melanocytes and keratinocytes by Protein-Protein interaction (PPI) network analysis to identify genes related with non-functional *MC1R* variants. From two detected networks, we selected 23 nodes as hub genes based on topological parameters. Differential expression of hub genes was then evaluated in healthy skin biopsies from RHC and black hair color (BHC) individuals. We also compared gene expression in melanoma tumors from individuals with RHC versus BHC. Gene expression in normal skin from RHC cutaneous cells showed dysregulation in 8 out of 23 hub genes (*CLN3, ATG10, WIPI2, SNX2, GABARAPL2, YWHA, PCNA* and *GBAS*). Hub genes did not differ between melanoma tumors in RHC versus BHC individuals. The study suggests that healthy skin cells from RHC individuals present a constitutive genomic deregulation associated with the red hair phenotype and identify novel genes involved in melanocyte biology.

INTRODUCTION

Human cutaneous pigmentation is dependent on melanin pigment production (eumelanin and pheomelanin) by epidermal and follicular melanocytes. Melanin synthesis is controlled by the melanocortin receptor type 1 (*MC1R*) which encodes a 7-pass transmembrane G-protein-coupled receptor. In wild-type *MC1R* melanocytes, activation of the receptor by the α-melanocyte stimulating hormone (α-MSH) promotes the synthesis of eumelanin pigment (dark pigment), reduces UV-induced oxidative stress and enhances DNA repair through base-excision repair and NER mechanisms which repairs UV-photoproducts [1]. *MC1R* is a highly polymorphic gene and loss-of-function variants (p.R151C, p.R142H, p.R161W, p.D294H p.D84E and p.I155T) result in a minimal receptor activity and mainly produces pheomelanin (red/yellow) pigment [2].

Epidemiological studies have found that loss-of-function variants in *MC1R* can, in part, predict the red-hair color (RHC) phenotype (red hair, fair skin, low tan capacity and high UV sensitivity) as well as melanoma [3] and non-melanoma skin cancer risk [4]. The decreased eumelanin production compared to pheomelanin in RHC individuals increases skin cancer risk due to the weak UV shielding capacity of pheomelanin, increase in UVA-induced reactive oxygen species [5, 6] and altered NER pathway. However, recent data indicate that RHC *MC1R* variants also contribute to carcinogenesis by UV-independent mechanisms. Mitra D et al. observed that the absence of pheomelanin is protective against melanoma development in mice models [7]. They detected high levels of oxidative DNA and lipid damage in RHC mice in a UV-independent model which still leads to oxidative damage. In addition, we h[2]ave previously reported that co-cultured melanocytes and keratinocytes harbouring loss-of-function *MC1R* variants show a constitutive overexpression of genes involved in oxidative phosphorylation pathway and DNA repair mechanisms [8].

The aim of this study was to further identify genes related to the RHC phenotype by analysing the previously reported expression signature pattern associated with RHC MC1R variants [8] using protein-protein interaction (PPI) network analyses. Gene co-expression networks have the potential to highlight specific molecular mechanisms and genes related to a specific trait or disease [9–11]. Expression of candidate genes from the PPI network analyses were further analysed in independent gene expression data generated from normal skin biopsy from RHC individuals and Black hair color (BHC) using the UK MuTHER dataset and in a subset of melanomas from RHC and BHC patients.

RESULTS

We identified 3,570 differentially expressed genes (DEGs) in co-cultured melanocytes and keratinocytes from a pair of RHC siblings carrying non-functional *MC1R* variants versus a pair of siblings with wild-type *MC1R* alleles as previously described [8]. The expression signature pattern associated with RHC *MC1R* variants was evaluated by PPI network analysis. Networks from up-regulated and down-regulated DEGs were constructed separately as, from a systems biology perspective, functionally-related genes are frequently co-expressed across a set of samples [12] and up-regulated and down-regulated transcripts tend to sub-connect [13, 14]. A statistically significant network composed of 557 nodes was detected among the set of 1954 up-regulated genes ($p< 0.001$; Figure 1). Also, we found a statistically significant network composed of 450 nodes among the set of 1616 down-regulated genes ($p<0.001$, Figure 2). Topological information of networks and name of nodes (genes) are indicated in Supplementary Data Table 1 and Supplementary Data Table 2.

We evaluated degree and betweenness centrality in both networks to investigate relationship between nodes and select the hub genes in each network. Twenty-three nodes were selected as hub genes (Table 1). In both networks, highly connected nodes tend to also show high betweenness centrality values. However, two nodes with a low degree centrality value (*BUB1B* and *SNX2* gene) were selected based on their high Betweenness centrality value.

In the network of up-regulated genes, 13 nodes were classified as hub genes (Figure 3). These genes play a role in DNA repair and cell-cycle homeostasis (*PRKAA1, CDK1, BUB1B, PCNA, RPA1* and *BRCA1*), oxidative phosphorylation (*GBAS, ICT1*) and autophagy (*PIK3C3, ATG4C, ATG10* and *SNX2*). The neighbouring genes of each hub gene are listed in Supplementary Tables 3-14.

In the network of down-regulated genes, 11 nodes were classified as hub genes (Figure 4). These genes are involved in apoptosis (*SMAD3, YWHAG*), mRNA metabolism (*PABPC1*), and autophagy (*TRAF2, SQSTM1, CLN3, WIPI2, GABARAPL1, GABARAPL2, MAP1LC3B, MAP1LC3A*). The neighbouring genes of each hub gene are listed in Supplementary Tables 15-25.

Next, we assessed the differential expression of those 23 genes in an additional whole genome expression dataset of healthy skin biopsies from the TwinsUK MuTHER dataset in 14 RHC individuals and 7 BHC individuals. At 10% FDR, genome wide differential expression analyses identified 1,952 DEGs between RHC and BHC individuals, consisting of 1,074 up-regulated and 878 down-regulated genes in RHC (Supplementary Table 26). Overall, 378 DEGs were common between the healthy skin and the co-cultured melanocyte-keratinocyte datasets. No statistically significant PPI networks were detected among DEGs sets (data not shown). Expression data from 18 out of 23 hub genes identified in the melanocyte-keratinocyte network analysis was available in the MuTHER dataset (*MAPL1C3B* was not included in the array and *PABPC1, ATG4C, CDK1* and *PIK3C3* genes failed the quality control). Five out of 18 genes classified as hub genes in the melanocyte-keratinocyte network analysis were differentially expressed in the MuTHER dataset with RHC skin showing down-regulation of *CLN3* and *ATG10* genes and up-regulation of *SNX2, PCNA* and *GBAS* genes. When the differential expression analysis was restricted to these 18 hub genes, up-regulation of

GABARAPL2 and *YWHA* genes and down-regulation of *WIPI2* gene also reached statistically significant values (Table 2).

Finally, expression of hub genes was evaluated in a whole genome expression dataset of 26 melanomas from 6 BHC and 20 RHC patients. None of these genes were found to be differentially expressed between tumors from RHC patients compared with BHC patients. Interestingly, the top two upregulated genes in melanomas from RHC were *PRKAA1* (fold change=1.37, p=0.08) and *PIK3C3* (fold change=1.23, p=0.09) while the top two downregulated were *YWHAG* (fold change=0.62, p=0.29) and *CLN3* (fold change=0.77, p=0.3). These p-values were unadjusted for multiple testing.

DISCUSSION

In this study, we aimed to uncover genes associated with the RHC phenotype by analysing a previously reported gene expression pattern [8]. By PPI network analysis, we identified two gene co-expression networks that reached statistically significant values in loss-of-function *MC1R* variants cutaneous cells. Since highly connected nodes are central to the network's architecture [15], the study was focused on those nodes with higher degree and betweenness centrality values. Overall, 23 nodes were selected as hub genes and their expression was analysed in an additional dataset from non-lesional skin tissue from RHC individuals or BHC individuals.

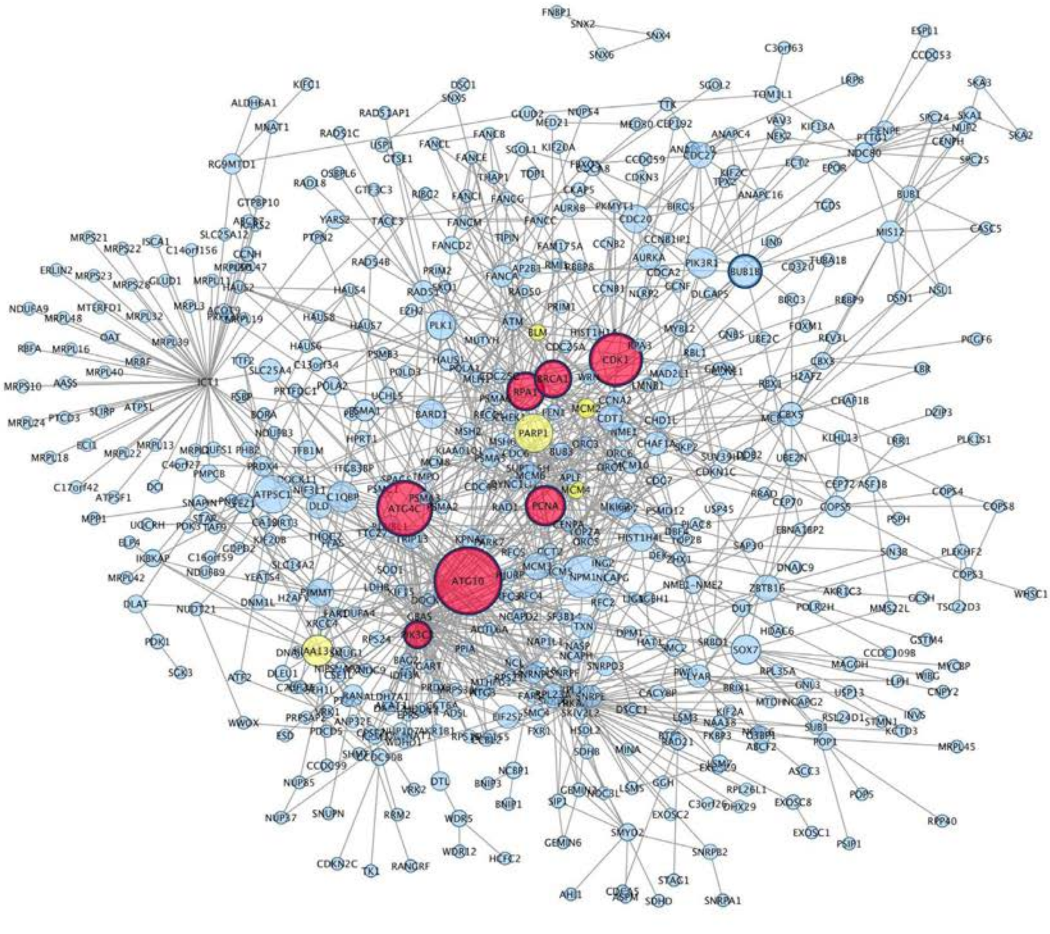


Figure 1: Protein Protein interaction network among up–regulated genes detected in co-cultured keratinocytes and melanocytes from individuals harbouring Red hair *color MC1R* variants (GSE44805 dataset). Nodes represent genes and edges indicate connections between proteins. Nodes are coloured based on number of degree in: low connected node (blue), medium connected node (yellow) and high connected node (red). Node size is proportional to the betweenness centrality value: the higher the value, the larger the node. Hub genes are represented with a thicker black border. The *GBAS*, *PRKAA1*, *ICT1* and *SNX2* hub genes do not follow the node size and border criteria to improve graphical representation of the network.

We detected a gene expression pattern composed of 1,952 transcripts in cutaneous cells from RHC individuals indicating that skin from those individuals presents a distinct gene expression pattern compared to BCH individuals independent of the UV radiation effect. Altogether, these results confirm that loss-of-function *MC1R* variants promote a constitutive genomic deregulation associated with pheomelanin synthesis as observed *in vitro* [8] and in mice models [7]. Three hundred seventy-eight genes were in common in the set of deregulated transcripts both *in vitro* and *in vivo* cutaneous cells from RHC individuals, including a set of the network's hub genes such as *CLN3*, *ATG10*, *WIPI2*, *SNX2*, *PCNA*, *GBAS*, *GABARAPL2* and *YWHAG*.

Notably, *CLN3*, *ATG10*, *WIPI2*, *SNX2* and *GABARAPL2* are members of the autophagy interaction network [16]. Autophagy is a highly conserved lysosomal pathway involved in tissue homeostasis, adaptation to starvation and removal of dysfunctional organelles or pathogens [17]. Additional autophagy genes (*ATG12, ATG2A*, *ATG9A, ATG7)* are also down-deregulated in healthy skin from RHC individuals (Supplementary Table 26). Previous data suggest that regulators of autophagosome formation play a role in melanosome formation and destruction of abnormal melanosomes [18]. Autophagosome formation is a multistep process in which the expansion and closure of the vesicle membrane is controlled by the UBL complex [19]. Notably, most of deregulated genes (*ATG7, ATG12, ATG10, ATG5* and *WIP*I) are related to the UBL complex [20, 21]. Moreover, cutaneous cells from RHC individuals show over-expression of *GABARAPL2* which is essential for

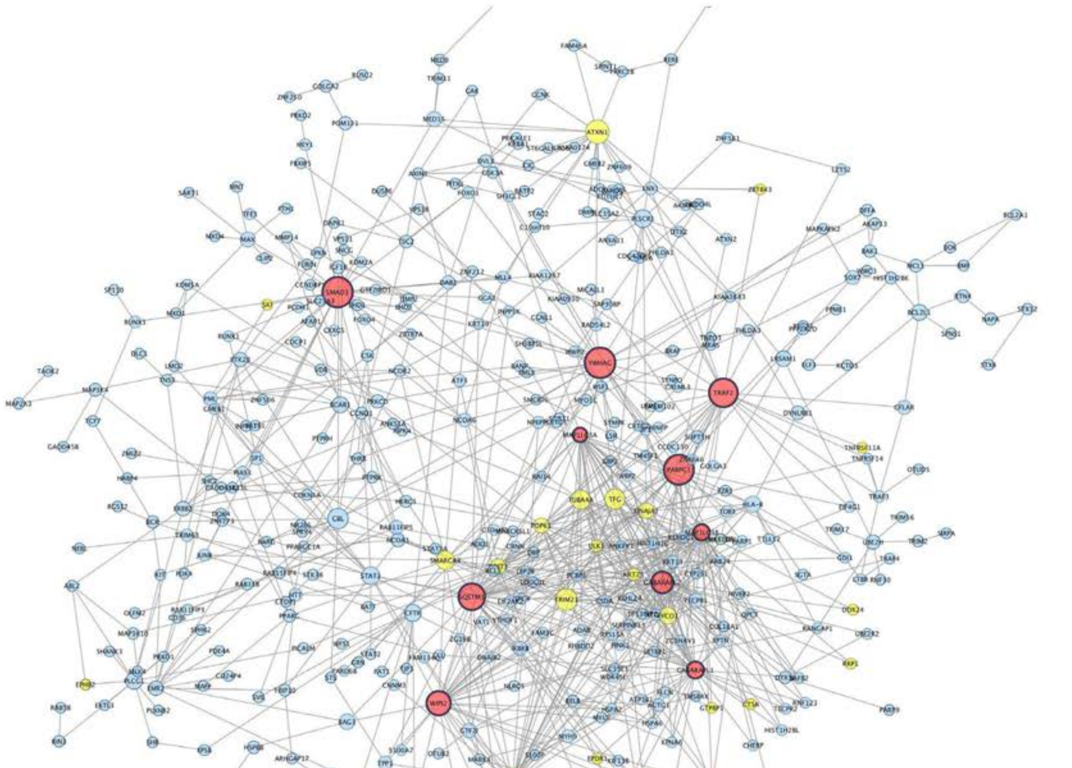


Figure 2: Protein Protein interaction network among down–regulated genes detected in co-cultured keratinocytes and melanocytes from individuals harbouring Red hair color *MC1R variants* (GSE44805 dataset). Nodes represent genes and edges indicate interaction between proteins. Nodes are coloured based on number of degree in: low connected node (blue), medium connected node (yellow) and high connected node (red). Node size indicate Betweenness centrality values. Hub genes are represented with a thicker black border.

Table 1: Topological information of networks detected among deregulated genes detected co-cultured keratinocytes and melanocytes from individuals harbouring Red hair color MC1R variants (GSE44805 dataset)

Degree Centrality						
Network	N. of nodes	N. of edges	Highest Value	Lowest Value	Mean ± SD	*Hub genes**
Up regulated genes	514	1144	77	1	4.45±6.78	*GBAS (77), PRKAA1 (54), ICT1* (54), *ATG4C (42), ATG10 (50) PIK3C3 (30), PCNA (28), CDK1 (27), BRCA1(27) RPA1 (25), BUB1B (12)[a], SNX2(3)[a]*
Down regulated genes	411	818	42	1	3.98±6.07	*GABARAPL2 (42) SQSTM1 (39) GABARAPL1 (38), MAP1LC3B (38), WIPI2 (34), MAP1LC3A(31) CLN3(30), YWHAG(29), SMAD3(27), TRAF2(27), PABPC1(24)*
Betweenness Centrality						
Network	N. of nodes	N. of edges	Highest Value	Lowest Value	Mean ± SD	*Hub genes**
Up regulated genes	514	1144	0.66	0.00	0.01±0.03	*SNX2(0.66), GBAS (0.29), ICT1* (0.18), *PRKAA1 (0.16), ATG10 (0.11), ATG4C (0.09), CDK1 (0.08), PCNA (0.06), RPA1 (0.05), BRCA1(0.05) BUB1B (0.04), PIK3C3 (0.03)*
Down regulated genes	411	818	0.15	0.00	0.01±0.02	*SMAD3(0.15), YWHAG (0.15), PABPC1(0.14) TRAF2(0.13), SQSTM1 (0.12), WIPI2 (0.10) CLN3(0.06), GABARAPL1 (0.05), MAP1LC3B (0.05) GABARAPL2(0.04), MAP1LC3A(0.04)*

Numberof nodes, number of edges, Betweenness Centrality and Degree Centrality from both detected networks, *Name of hub genes and its Betweenness centrality and degree values. [a]: *BUB1B* and *SNX2* were selected based on its Betweenness Centrality values.

autophagosome maturation [19] and down-regulation of *CLN3* which play a role in autophagy, endocytosis and vesicular trafficking [16]. Previous comparison of black and red human hair melanosomes reveals differences between eumelanosomes and pheomelanosomes in physical terms such as shape or structural integrity [22]. Our findings support that autophagy genes are closely related to melanosome biogenesis and further suggest that these genes could underlie part of the physical differences between types of melanosomes.

Findings from healthy skins could not be replicated in melanomas. This could be because if the network's hub genes play a role in melanoma development, it may be in early tumor initiation as opposed to progression. In addition, gene expression is under a tighter regulatory control in tumors than in healthy tissues and differences are often subtler, requiring a larger sample to be detected. The largest fold change between RHC and BHC hub gene expression was 1.37 and the power to detect a difference of this magnitude at α=0.05 with 20 RHC and

6 BHC tumors was only 37% without multiple testing adjustments, reducing to 5% with adjustment. This is in contrast with the gene showing the largest difference in healthy skins (fold change=2.7, power=0.97 after multiple-tests correction). Nonetheless, the tumor data reinforce the possible involvement of cell cycle homeostasis and autophagy as *PRKAA1* and *PIK3C3* were the top 2 ranked genes. Additional studies of genes identified in this study should be conducted in skin cancer tumors to elucidate their role in malignant transformation.

A previous PPI network analyses conducted in a whole gene expression dataset of 31 primary melanomas and 52 metastases reported a PPI network in which *PCNA, CDK1, MAD2L1, RFC4* and *BRCA1* genes showed highest degree centrality values among upregulated genes in metastases [14]. We observed a similar behaviour in *in vitro* RHC cutaneous cells since *PCNA*, *CDK1* and *BRCA1* were hub genes in the network of up-regulated genes. However, only *PCNA* reaches statistically significant values in *in vivo* RHC cutaneous cells. Since these genes are essential for cell cycle progression and DNA repair [23–24], these findings suggest that up-regulation of these genes may be caused by an increased and continuous DNA damage. The intrinsic pheomelanin pathway represents an additional contributor to DNA damage by increasing oxidation [7, 8]. However, the molecular process underlying the increased DNA damage is poorly understood. Both *in vitro* and *in vivo* RHC cutaneous cells show an overexpression of *GBAS* which encodes a mitochondrial protein involved in oxidative

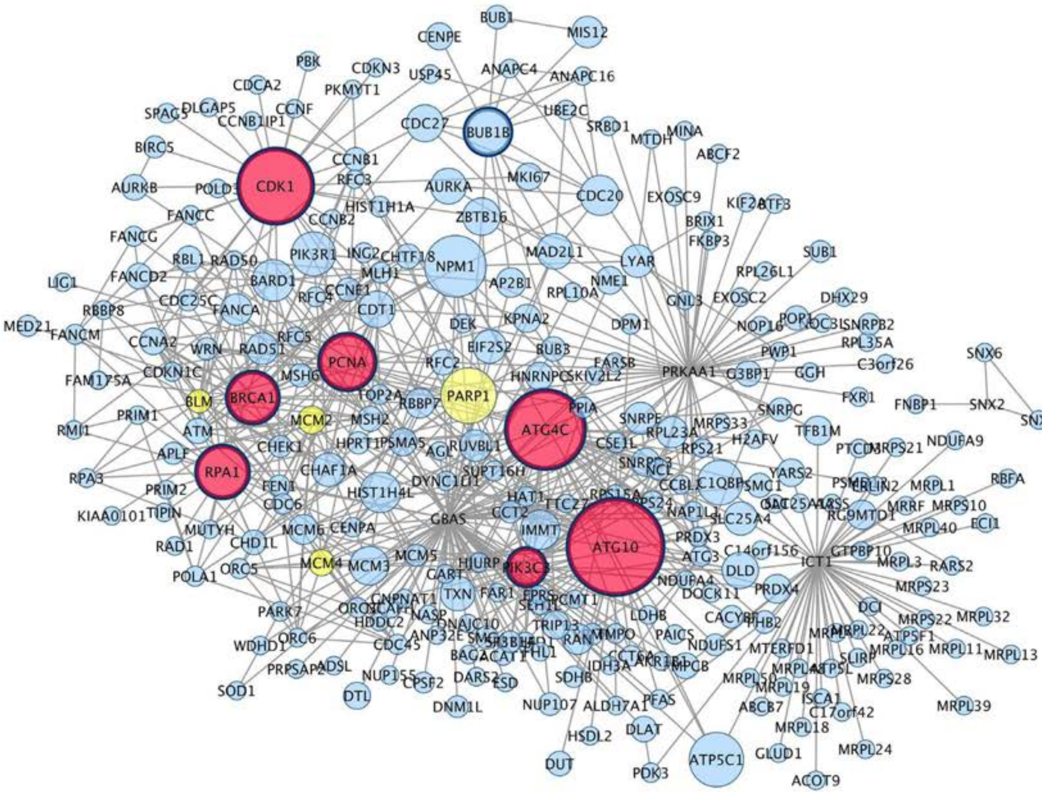


Figure 3: Hub genes and their first connected genes from network detected among up-regulated genes in co-cultured keratinocytes and melanocytes from individuals harbouring Red hair color *MC1R* variants (GSE44805 dataset). Only the first connection of hub genes is indicated. Nodes represent proteins and edges indicate connections between proteins. Nodes are coloured based on number of degree: Red (degrees >μ+ 3σ; x>24.8), yellow (μ+ 3σ < degree>μ+ 2σ; 24.8<degree>18.6), blue (degrees<μ+2σ, degree<18.6). Node size indicate betweenness centrality values. Hub genes are represented with a thicker black border. The hub genes (N=13 nodes) are directly connected with 270 out of 557 nodes within network. The *GBAS*, *PRKAA1*, *ICT1* and *SNX2* hub genes do not follow the node size and border criteria to improve graphical representation of the network.

phosphorylation [25, 26]. Further studies are required to clarify the biological role of mitochondrial related gene in melanocyte and melanogenesis.

The main limitations of present study are the disparity between platforms used for gene expression capture (resulting in presence/absence of gene probes and probe design), the low frequency of phenotypically black hair individuals included in the datasets and the differences in type of samples analyzed (cultured skin cells vs non-cultured skin cells) Establishment of cultured cutaneous cells might influence the expression of certain genes resulting in distinct expression such as *ATG10* gene expression which is found up-regulated in cultured cells and down-regulated in non-cultured cells.

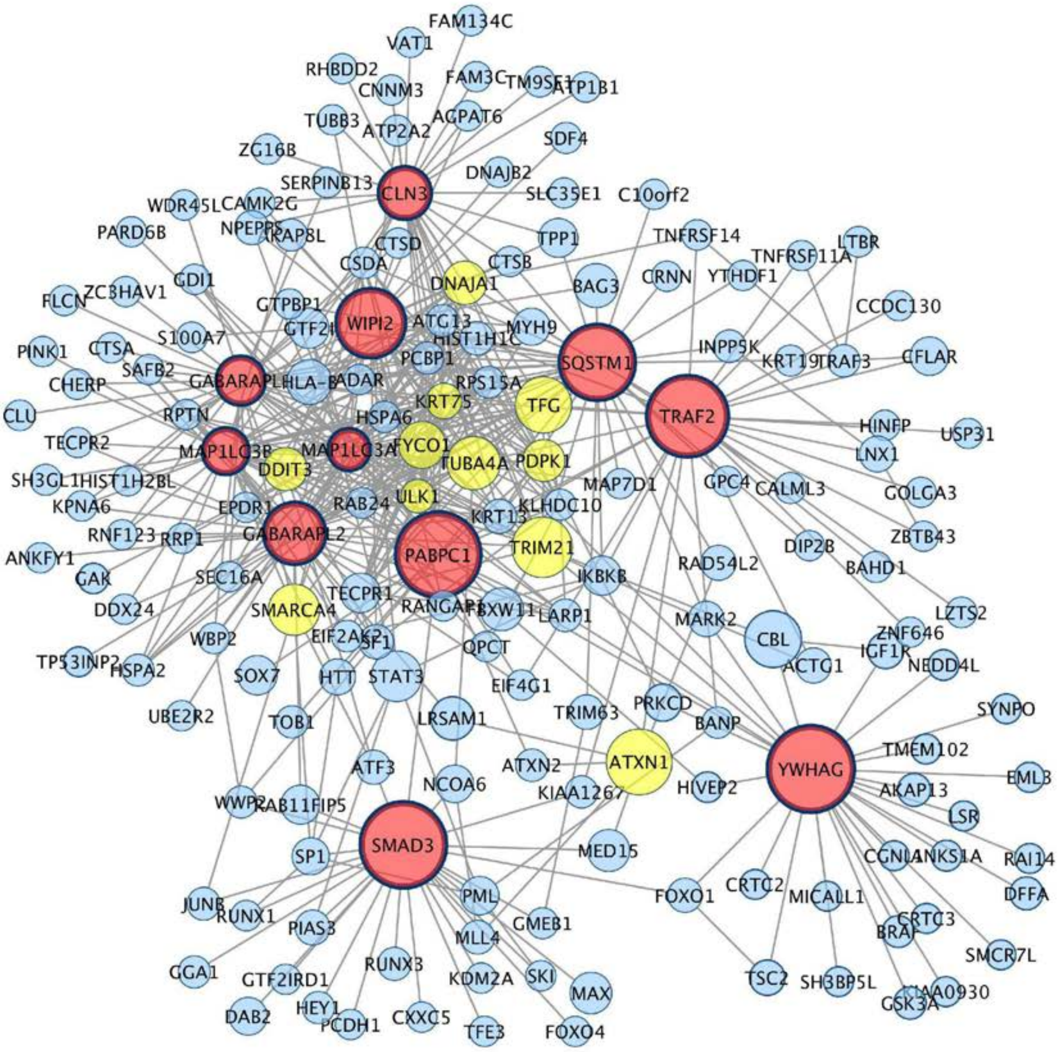


Figure 4: Hub genes and their first connected genes from network detected among down-regulated genes in co-cultured keratinocytes and melanocytes from individuals harbouring Red hair color *MC1R* variants (GSE44805 dataset). Only the first connection of hub genes is indicated. Nodes represent genes and edges indicate direct interaction between proteins. Nodes are coloured based on number of degree in Red (degrees $>\mu+3\sigma$; $x>22.2$), yellow ($\mu+3\sigma <$ degree$>\mu+2\sigma$; 22.2<degree>16.3), blue (degrees< $\mu+2\sigma$, degree<16.3 Node size indicate Betweenness centrality values. Hub genes are those nodes with the widest border. The hub genes (N=11 nodes) connects 177 out of 411 nodes.

Table 2: Differential Expression of hub genes in healthy skin from Red hair color individuals vs Black hair color individuals

ID	Results for whole list of genes(A) (N=1952)			Results from list of hub genes(B) (N=18)		
	logFC	**p.value**	**adj. p.value**	**logFC**	**p.value**	**adj. p.value**
ATG10	-0.431	0.001	0.032	-0.432	> 0.001	0.007
CLN3	-0.339	> 0.001	0.024	-0.339	> 0.001	0.007
GABARAPL2	ns	ns	ns	0.254	0.0188	0.056
GBAS	0.374	0.005	0.067	0.373	0.005	0.017
PCNA	0.209	> 0.001	0.026	0.209	0.001	0.007
SNX2	0.506	0.002	0.041	0.508	0.001	0.007
WIPI2	ns	ns	ns	-0.156	0.034	0.076
YWHAG	ns	ns	ns	0.273	0.023	0.060

Differential expression of hub genes was assessed (A) by analyzing the whole gene expression dataset and (B) by analyzing the differential expression of only hub genes. Table shows hub genes with statistically significant p-values and adjusted P-values (adj.p.value). logFC= logarithm of Fold Change; ns=not significant P-value

In summary, the genes identified may help to reveal underlying molecular mechanisms associated with red hair color phenotype and future studies of these genes may provide insight to better understand the increased skin cancer risk observed in individuals harbouring loss-of-function MC1R variants.

MATERIALS AND METHODS

Whole genome expression data from three different datasets were included in the study. An initial dataset included whole genome expression from four co-cultured keratinocytes and melanocytes from two pair of siblings. Phenotypical data and genetic variants in melanoma susceptibility genes *MC1R* and *CDKN2A* were obtained in all individuals. A pair of siblings, phenotypically classified as RHC individuals were double heterozygous for the p.R160W, p.R151C variants in *MC1R* (individual 1 and individual 2 were 38 and 33 years old, respectively). In contrast, a pair of siblings with brown hair color carried wild-type *MC1R* alleles (individual 1 and individual 2 were 50 and 51 years old, respectively). Moreover, the germline p.G101W *CDKN2A* mutation was detected in one of each pair of siblings. Extraction of the expression data was carried out using the Whole Human Genome Microarray 4x44K (Agilent). Generation, pre-processing and differential expression and pathway based analyses from this dataset (GSE44805) has been previously reported [8].

Expression profiling in healthy skin tissue was obtained using Illumina Human HT-12 V3 BeadChips (Illumina) in 21 of 705 female individuals from the TwinsUK Cohort (http://www.muther.ac.uk/public.html). The only eligibility criterion for the TwinsUK Cohort was twin status and therefore the sample is held to be representative of the general population [27, 28]. For the present study, individuals were selected based on phenotypical data since the genetic status of the *MC1R* gene was not available. Fourteen red hair individuals (two monozygotic twin pairs, one dizygotic twin pair, and 8 singletons) and 7 with black hair (all singletons) of Caucasian ancestry were selected. The mean age for the RHC individuals was 50.88 years old (range=43.33-59.84), while for the BHC individuals was 62.04 years old (range=49.23-74.54). All red hair individuals but two (missing the *MC1R* genotype) carry at least one RHC allele. For the pair without genetic data, multiple photographs taken in different years were used in order to confirm that they actually had red hair. Non RHC alleles were detected in black hair individuals. Probe expression levels were log transformed and quantile normalized, and only probes mapping uniquely to genes of known function and not containing common SNPs were included in the analysis, as detailed elsewhere [28], resulting in 16,646 genes. Mean expression profile was used when the same gene was targeted by multiple probes. Written informed consent was provided by all the twins, and the Guy's and St Thomas' Hospital NHS Trust Research Ethics Committee approved the study.

Generation, pre-processing and analysis of whole genome gene expression in melanoma tumors from the Leeds Melanoma Cohort (LMC, ethical approval MREC 1/3/57, PIAG 3-09(d)/2003) has been described elsewhere [29]. This data contains 204 melanoma primaries expression-profiled using DASL array HT12 v4. Gene expression was compared between 20 melanoma tumors from RHC and 6 melanoma tumors from BHC patients. All RHC melanoma patients carried at least one RHC allele). One patient has missing MC1R genotype. We observed that among black haired individuals, one patient carried two RHC variants. One patient was missing their MC1R genotype.

Statistical analyses

Standardization of gene expression data across arrays from co-cultured keratinocytes and melanocytes was performed with Agilent Processed Signal (Agilent Feature Extraction Software) using quantile normalization [30]. Differential gene expression analysis was carried out using the *limma* [31] package from Bioconductor. Multiple testing adjustment of p-values was performed according to Benjamini and Hochberg (Benjamini & Hochberg, 1995; Benjamini and Yekutieli, 2001) controlling False Discovery Rate (FDR = 5%).

Protein-protein interaction (PPI) networks were built based on differentially expressed genes. Identification of statistically significant PPI networks within the set of differentially expressed genes was carried out by SNOW [32] as implemented in Babelomics 5 suite [33], where approaches for protein-protein interaction data from 5 databases such as IntAct Molecular Interaction Database [34], Molecular INTeraction database [35], Biomolecular Interaction Network Database [36], Database of Interacting Proteins [37] and Human Protein Reference database [38] are included for functional profiling of genomic data, evaluating the cooperative behavior of a list of genes as a functional module. SNOW calculates the Minimal connected network (MCN) for the proteins/genes in the list. The topology of this MCN is then compared against 10,000 random MCNs with same size range to obtain a p-value. Statistically significant connected networks of differentially expressed genes were obtained for up and down regulated transcripts in RHC co-cultured melanocytes and keratinocytes. Further visualization and evaluation of networks was conducted using CytoscapeWeb [39]. Networks were evaluated using two topological parameters, namely degree and betweenness centrality. Degree centrality counts the number of edges connected to each node (protein/gene). Betweenness centrality counts the number of times each node is included in the shortest path between any two other nodes, indicating which node(s) have the larger influence on the communication between proteins/genes included in the network. Nodes were classified based on the mean (μ) and standard deviation (σ) of degree centrality value calculated over all proteins/genes in: lower connected node (degree$<\mu+2\sigma$), medium connected node ($\mu+2\sigma>$degree$<\mu+3\sigma$), and highly connected node (degree$>\mu+3\sigma$). Highly connected nodes or those not highly connected nodes but with high betweenness centrality values were classify as top nodes (hub genes).

Differential gene expression analysis in healthy skin tissue was carried out using the limma R package controlling for family structure and batch effects. Benjamini and Hochberg's method, as implemented in *limma*, was used to control the false discovery rate at 10%. Differential expression of the hub genes identified in the gene expression data from co-cultured melanocytes and keratinocytes was assessed firstly by analysing the whole gene expression dataset and then by restricting it to hub genes only.

Whole differential gene expression was evaluated by SNOW following the same analyses as in co-cultured melanocytes-keratinocytes data.

Differential expression analysis in tumors was restricted to the genes that were identified as hub genes in the melanocytes-keratinocytes dataset (N=23). Examination of the probe list in the Illumina DASL whole genome array confirmed that 33 probes were arrayed covering all 23 genes selected as hub genes in networks. Differential expression in tumors from 20 RHC vs 6 BHC patients from LMC was performed using linear regression and adjusting for potential batch effect. Analysis was conducted using STATA v12 software.

CONFLICTS OF INTEREST

There is no conflicts of interest to disclose.

GRANT SUPPORT

This work was mainly supported by intramural grants INTRA/07/726.2 and ACCI2014 (U726 and U714) from the Biomedical Network Research on Rare Diseases (CIBERER) of the Instituto de Salud Carlos III, Spain. The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias (PI15/00716 and grants PI15/00956); AGAUR 2014 SGR 603 of the Catalan Government, Spain; European Commission under the 6th Framework Programme, Contract No.LSHC-CT-2006-018702 (GenoMEL) and by the European Commission under the 7th Framework Programme, Diagnoptics; The National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115) and a grant from "Fundació La Marató de TV3, 201331-30", Catalonia, Spain. The Department of Bioengineering UC3M-CIEMAT-IISFJD in Madrid is partially funded from the Spanish Ministerio de Economía y Competitividad (SKINNOVA; SAF2013-43475-R) and (CELLCAM; S2010/BMD-2420) from Comunidad de Madrid. The TwinsUK study was funded by the Wellcome Trust; European Community's Seventh Framework Programme (FP7/2007-2013). The study also received support from the National Institute for Health Research (NIHR)- funded BioResource, Clinical Research Facility and Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London. MF, VB and AV are supported by BSF grant n. 5044i. The Leeds contribution (tumour gene expressions) was funded by Cancer Research UK Programme grant C588/ A19167 and Project GrantC8216/A6129 and NIH Award CA83115.

REFERENCES

1. Abdel-Malek ZA, Swope VB, Starner RJ, Koikov L, Cassidy P, Leachman S. Melanocortins and the melanocortin 1 receptor, moving translationally towards melanoma prevention. Arch Biochem Biophys. 2014; 563:4-12.
2. Garcia-Borron JC, Abdel-Malek Z, Jimenez-Cervantes C. MC1R, the cAMP pathway, and the response to solar UV: extending the horizon beyond pigmentation. Pigment Cell Melanoma Res. 2014; 27:699-720.
3. Pasquali E, Garcia-Borron JC, Fargnoli MC, Gandini S, Maisonneuve P, Bagnardi V, Specchia C, Liu F, Kayser M, Nijsten T, Nagore E, Kumar R, Hansson J, Kanetsky PA, Ghiorzo P, Debniak T, et al. MC1R variants increased the risk of sporadic cutaneous melanoma in darker-pigmented Caucasians: a pooled-analysis from the M-SKIP project. Int J Cancer. 2015; 136:618-631.
4. Tagliabue E, Fargnoli MC, Gandini S, Maisonneuve P, Liu F, Kayser M, Nijsten T, Han J, Kumar R, Gruis NA, Ferrucci L, Branicki W, Dwyer T, Blizzard L, Helsing P, Autier P, et al. MC1R gene variants and non-melanoma skin cancer: a pooled-analysis from the M-SKIP project. Br J Cancer. 2015; 113:354-363.
5. Rouzaud F, Kadekaro AL, Abdel-Malek ZA, Hearing VJ. MC1R and the response of melanocytes to ultraviolet radiation. Mutat Res. 2005; 571:133-152.
6. Hill HZ, Hill GJ. UVA, pheomelanin and the carcinogenesis of melanoma. Pigment Cell Res. 2000; 13:140-144.
7. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491:449-453.
8. Puig-Butille JA, Escamez MJ, Garcia-Garcia F, Tell-Marti G, Fabra A, Martinez-Santamaria L, Badenas C, Aguilera P, Pevida M, Dopazo J, del Rio M, Puig S. Capturing the biological impact of CDKN2A and MC1R genes as an early predisposing event in melanoma and non melanoma skin cancer. Oncotarget. 2014; 5:1439-1451. doi: 10.18632/ oncotarget.1444.
9. Yue Z, Li HT, Yang Y, Hussain S, Zheng CH, Xia J, Chen Y. Identification of breast cancer candidate genes using gene co-expression and protein-protein interaction information. Oncotarget. 2016; 7:36092-36100. doi: 10.18632/ oncotarget.9132.
10. Keller MP, Choi Y, Wang P, Davis DB, Rabaglia ME, Oler AT, Stapleton DS, Argmann C, Schueler KL, Edwards S, Steinberg HA, Chaibub Neto E, Kleinhanz R, Turner S, Hellerstein MK, Schadt EE, et al. A gene expression network model of type 2 diabetes links cell cycle regulation in islets with diabetes susceptibility. Genome Res. 2008; 18:706-716.
11. Deng SP, Zhu L, Huang DS. Predicting Hub Genes Associated with Cervical Cancer through Gene Co-Expression Networks. IEEE/ACM Trans Comput Biol Bioinform. 2016; 13:27-35.
12. Diao H, Li X, Hu S, Liu Y. Gene expression profiling combined with bioinformatics analysis identify biomarkers for Parkinson disease. PLoS One. 2012; 7:e52319.
13. Sun H. Identification of key genes associated with gastric cancer based on DNA microarray data. Oncol Lett. 2016; 11:525-530.
14. Qiu T, Wang H, Wang Y, Zhang Y, Hui Q, Tao K. Identification of genes associated with melanoma metastasis. Kaohsiung J Med Sci. 2015; 31:553-561.
15. Almaas E. Biological impacts and context of network theory. J Exp Biol. 2007; 210:1548-1558.
16. Behrends C, Sowa ME, Gygi SP, Harper JW. Network organization of the human autophagy system. Nature. 2010; 466:68-76.
17. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132:27-42.
18. Ho H, Ganesan AK. The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res. 2011; 24:595-604.
19. Tanida I. Autophagy basics. Microbiol Immunol. 2011; 55:1-11.
20. Nemoto T, Tanida I, Tanida-Miyake E, Minematsu-Ikeguchi N, Yokota M, Ohsumi M, Ueno T, Kominami E. The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. J Biol Chem. 2003; 278:39517-39526.
21. Dooley HC, Razi M, Polson HE, Girardin SE, Wilson MI, Tooze SA. WIPI2 links LC3 conjugation with PI3P, autophagosome formation, and pathogen clearance by recruiting Atg12-5-16L1. Mol Cell. 2014; 55:238-252.
22. Liu Y, Hong L, Wakamatsu K, Ito S, Adhyaru B, Cheng CY, Bowers CR, Simon JD. Comparison of structural and chemical properties of black and red human hair melanosomes. Photochem Photobiol. 2005; 81:135-144.
23. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell. 2007; 129:665-679.
24. Tanaka S, Tak YS, Araki H. The role of CDK in the initiation step of DNA replication in eukaryotes. Cell Div. 2007; 2:16.
25. Smits P, Rodenburg RJ, Smeitink JA, van den Heuvel LP. Sequence variants in four candidate genes (NIPSNAP1,

GBAS, CHCHD1 and METT11D1) in patients with combined oxidative phosphorylation system deficiencies. J Inherit Metab Dis. 2010; 33:S13-19.

26. Martherus RS, Sluiter W, Timmer ED, VanHerle SJ, Smeets HJ, Ayoubi TA. Functional annotation of heart enriched mitochondrial genes GBAS and CHCHD10 through guilt by association. Biochem Biophys Res Commun. 2010; 402:203-208.

27. Andrew T, Hart DJ, Snieder H, de Lange M, Spector TD, MacGregor AJ. Are twins and singletons comparable? A study of disease-related and lifestyle characteristics in adult women. Twin Res. 2001; 4:464-477.

28. Grundberg E, Small KS, Hedman AK, Nica AC, Buil A, Keildson S, Bell JT, Yang TP, Meduri E, Barrett A, Nisbett J, Sekowska M, Wilk A, Shin SY, Glass D, Travers M, et al. Mapping cis- and trans-regulatory effects across multiple tissues in twins. Nat Genet. 2012; 44:1084-1089.

29. Nsengimana J, Laye J, Filia A, Walker C, Jewell R, Van den Oord JJ, Wolter P, Patel P, Sucker A, Schadendorf D, Jonsson GB, Bishop DT, Newton-Bishop J. Independent replication of a melanoma subtype gene signature and evaluation of its prognostic value and biological correlates in a population cohort. Oncotarget. 2015; 6:11683-11693. doi: 10.18632/oncotarget.3549.

30. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19:185-193.

31. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004; 3:Article3.

32. Minguez P, Gotz S, Montaner D, Al-Shahrour F, Dopazo J. SNOW, a web-based tool for the statistical analysis of protein-protein interaction networks. Nucleic Acids Res. 2009; 37:W109-114.

33. Alonso R, Salavert F, Garcia-Garcia F, Carbonell-Caballero J, Bleda M, Garcia-Alonso L, Sanchis-Juan A, Perez-Gil D, Marin-Garcia P, Sanchez R, Cubuk C, Hidalgo MR, Amadoz A, Hernansaiz-Ballesteros RD, Aleman A, Tarraga J, et al. Babelomics 5.0: functional interpretation for new generations of genomic data. Nucleic Acids Res. 2015; 43:W117-121.

34. Kerrien S, Aranda B, Breuza L, Bridge A, Broackes-Carter F, Chen C, Duesbury M, Dumousseau M, Feuermann M, Hinz U, Jandrasits C, Jimenez RC, Khadake J, Mahadevan U, Masson P, Pedruzzi I, et al. The IntAct molecular interaction database in 2012. Nucleic Acids Res. 2012; 40:D841-846.

35. Licata L, Briganti L, Peluso D, Perfetto L, Iannuccelli M, Galeota E, Sacco F, Palma A, Nardozza AP, Santonico E, Castagnoli L, Cesareni G. MINT, the molecular interaction database: 2012 update. Nucleic Acids Res. 2012; 40:D857-861.

36. Bader GD, Betel D, Hogue CW. BIND: the Biomolecular Interaction Network Database. Nucleic Acids Res. 2003; 31:248-250.

37. Salwinski L, Miller CS, Smith AJ, Pettit FK, Bowie JU, Eisenberg D. The Database of Interacting Proteins: 2004 update. Nucleic Acids Res. 2004; 32:D449-451.

38. Keshava Prasad TS, Goel R, Kandasamy K, Keerthikumar S, Kumar S, Mathivanan S, Telikicherla D, Raju R, Shafreen B, Venugopal A, Balakrishnan L, Marimuthu A, Banerjee S, Somanathan DS, Sebastian A, Rani S, et al. Human Protein Reference Database--2009 update. Nucleic Acids Res. 2009; 37:D767-772.

39. Lopes CT, Franz M, Kazi F, Donaldson SL, Morris Q, Bader GD. Cytoscape Web: an interactive web-based network browser. Bioinformatics. 2010; 26:2347-2348.

www.impactjournals.com/oncotarget/ Oncotarget, Supplementary Materials 2016

Genomic expression differences between cutaneous cells from red hair color individuals and black hair color individuals based on bioinformatic analysis

SUPPLEMENTARY TABLES

Supplementary Table 1: Atributes of nodes from networks of up-regulated genes (N=557)

See Supplementary File 1

Supplementary Table 2: Atributes of nodes from networks of down-regulated genes (N=450)

See Supplementary File 1

Supplementary Table 3: First connections of the *ATG4C* gene: Table shows the first connections of the *ATG4C* gene within the network of up-regulated transcripts. Name gene and KEGGKEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGGKEGG pathway.

See Supplementary File 1

Supplementary Table 4: First connections of the *ATG10*: Table shows the first connections of the *ATG10* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 5: First connection of *BRCA1*: Table shows the first connections of *BRCA1* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

www.impactjournals.com/oncotarget/ Oncotarget, Supplementary Materials 2016

Supplementary Table 6: First connections of the *BUB1B*

GEN	KEGG
ANAPC16	NA
ANAPC4	HTLV-I infection (hsa05166), Progesterone-mediated oocyte maturation (hsa04914), Oocyte meiosis (hsa04114), Ubiquitin mediated proteolysis (hsa04120), Cell cycle (hsa04110)
AP2B1	Endocytosis (hsa04144), Synaptic vesicle cycle (hsa04721), Endocrine and other factor-regulated calcium reabsorption (hsa04961), Huntington's disease (hsa05016)
BUB1	Progesterone-mediated oocyte maturation (hsa04914), Cell cycle (hsa04110), Oocyte meiosis (hsa04114)
BUB3	Cell cycle (hsa04110), HTLV-I infection (hsa05166)
CDC20	HTLV-I infection (hsa05166), Pyrimidine metabolism (hsa00240), Metabolic pathways (hsa01100), Oocyte meiosis (hsa04114), Viral carcinogenesis (Viral carcinogenesis (hsa05203)), Cell cycle (hsa04110), Ubiquitin mediated proteolysis (hsa04120)
CDC27	Oocyte meiosis (hsa04114), Ubiquitin mediated proteolysis (hsa04120), Cell cycle (hsa04110), Ubiquinone and other terpenoid-quinone biosynthesis (hsa00130), HTLV-I infection (hsa05166), Progesterone-mediated oocyte maturation (hsa04914)
CENPE	Pathways in cancer (hsa05200), HTLV-I infection (hsa05166), Signaling pathways regulating pluripotency of stem cells (hsa04550), Hippo signaling pathway (hsa04390), Wnt signaling pathway (hsa04310), Basal cell carcinoma (hsa05217), Melanogenesis (hsa04916), Notch signaling pathway (hsa04330)
MAD2L1	HTLV-I infection (hsa05166), Progesterone-mediated oocyte maturation (hsa04914), Cell cycle (hsa04110), Oocyte meiosis (hsa04114)
MIS12	NA
PARP1	NF-kappa B signaling pathway (hsa04064), Purine metabolism (hsa00230), Metabolic pathways (hsa01100), Base excision repair (hsa03410)
UBE2C	Ubiquitin mediated proteolysis (hsa04120)

Table shows the first connections of the *BUB1B* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

www.impactjournals.com/oncotarget/ Oncotarget, Supplementary Materials 2016

Supplementary Table 7: First connection of *CDK1*. Table shows the first connections of the *CDK1* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 8: First connections of the *GBAS* gene. Table shows the first connections of the *GBAS* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated.

See Supplementary File 1

Supplementary Table 9: First connections of the *ICT1* gene. Table shows the first connections of the *ICT1* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 10: First connectios of *PCNA*. Table shows the first connections of the *PCNA* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 11: First connections of the *PIK3C3*. Table shows the first connections of the *PIK3C3* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 12: First connections of the *PRKAA1*. Table shows the first connections of the *PRKAA1* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 13: First connections of the SNX2

GEN	KEGG
FNBP1	NA
SNX4	Endocytosis (hsa04144)
SNX6	Endocytosis (hsa04144)

Table shows the first connections of the *SNX2* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

www.impactjournals.com/oncotarget/ Oncotarget, Supplementary Materials 2016

Supplementary Table 14: First connections of the *RPA1*. Table shows the first connections of the *RPA1* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 15: First connections of the *GABARPAL1*. Table shows the first connections of the *GABARPAL1* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 16: First connection of *CLN3*. Table shows the first connections of the *CLN3* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 17: First connections of the *GABARAPL2*. Table shows the first connections of the *GABARAPL2* gene within the network of up-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 18: First connections of the *MAP1LC3A*. Table shows the first connections of the *MAP1LC3A* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

www.impactjournals.com/oncotarget/ Oncotarget, Supplementary Materials 2016

Supplementary Table 19: First connections of the *MAPL1C3B*. Table shows the first connections of the *MAPL1C3B* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 20: First connections of the *PABPC1*. Table shows the first connections of the *PABPC1* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 21: First connections of the *SMAD3*. Table shows the first connections of the *SMAD3* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 22: First connections of the *SQSTM1*. Table shows the first connections of the *SQSTM1* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 23: First connections of the *TRAF2*. Table shows the first connections of the *TRAF2* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 24: First connections of the *WIPI2*. Table shows the first connections of the *WIPI2* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 25: First connections of the *YWHAG*. Table shows the first connections of the *YWHAG* gene within the network of down-regulated transcripts. Name gene and KEGG pathway in which gene is associated with, is indicated. NA= Not associated KEGG pathway.

See Supplementary File 1

Supplementary Table 26: List of differentially expressed genes in healthy skin from Red hair color individuals vs Black hair color individuals. Table contains Log2 Fold Change (logFc), P-Value and adjusted P-value (adj.P-Val) for each gene.

See Supplementary File 2

DISCUSSIÓ

1. EL GEN *MC1R* COM A FACTOR DE RISC A DESENVOLUPAR LA MALALTIA DE PARKINSON

En el treball I, l'anàlisi d'associació genotípica cas-control indica que els individus portadors de la variant 'R' p.R160W (rs1805008) presenten un augment del risc a desenvolupar Parkinson (OR=2,10; 95% IC: 1,18-3,73; p-valor ajustat = 0,009) (Taula 1, Treball I). Aquest resultat indica que el gen *MC1R* pot estar implicat en la etiologia de la malaltia de Parkinson.

Dos estudis previs han avaluat determinades variants de *MC1R* en pacients de Parkinson en altres poblacions. Gao *et al*. [215] van genotipar la variant p.R151C (rs1805007) en 272 pacients de Parkinson i 1185 controls nord-americans i Dong *et al*. [217] van analitzar la variant p.R160W en dos *sets* de dades GWAS independents: un primer *set* que inclou les dades genotípiques de 808 casos de Parkinson i 1.623 controls de població nord-americana no hispànica (Estudi PAGE); i un segon *set*, que inclou les dades genotípiques de 5.333 casos i 12.019 controls provinents de múltiples poblacions caucàsiques d'ètnia blanca, reclutats per membres del IPDGC (International Parkinson's Disease Genomics Consortium).

L'estudi de Gao *et al*. [215] va identificar que els individus portadors de l'al·lel p.R151C en homozigosi presenten un augment del risc a desenvolupar la malaltia de Parkinson (OR=3,10; 95% IC: 1,06-9,37; p-valor ajustat = 0,06) en població nord-americana. A causa de la baixa freqüència al·lèlica de les variants LOF de *MC1R* en la població espanyola [218, 219], no es va identificar cap cas homozigot per a aquestes variants al·lèliques en la cohort espanyola (Taula 1, Treball I). Per tant, l'associació amb el Parkinson identificada en els individus homozigots per l'al·lel p.R151C no es va poder revalidar en el treball I. No obstant, el grau d'associació amb el Parkinson que s'observa en els individus heterozigots per l'al·lel p.R151C en la cohort espanyola (OR=1,25; 95% IC: 0,80-1,95) (Taula 1, Treball I), és similar al observat per Gao *et al*. en població nord-americana (OR=1,23; 95% IC: 0,84-1,82).

En conjunt, els resultats del treball I i els identificats per Gao *et al.* indiquen una associació entre les variants LOF en *MC1R* i un augment del risc a desenvolupar Parkinson.

Concretament, la susceptibilitat genètica a desenvolupar Parkinson, s'atribueix a les dues variants al·lèliques (p.R151C i p.R160W) que presenten una major associació amb el fenotip RHC [28] i un major risc atribuïble a desenvolupar melanoma [64]. Aquest fet, explica la sobrerepresentació d'individus pèl-roigs que s'ha identificat en els pacients de Parkinson, i explica, en part, la relació epidemiològica bidireccional entre el risc a desenvolupar Parkinson i el risc a desenvolupar melanoma, ja que el gen *MC1R* és un factor de risc per ambdues malalties.

En el treball presentat per Dong *et al*. [217], els autors van avaluar una associació entre 11 SNPs identificats en 9 gens (*IRF4*, *TYR*, *TPCN2*, *KITLG*, *SLC24A4*, *MC1R*, *CDK10*, *ASIP* i *PIGU*) associats amb la pigmentació i/o la susceptibilitat a melanoma i el risc a Parkinson. L'associació entre l'al·lel p.R160W de *MC1R* (rs1805008) i el risc a desenvolupar Parkinson es va avaluar en els dos *sets* de dades prèviament esmentats (PAGE i IPDGC). L'estudi no va identificar una associació significativa ni en la cohort PAGE (OR=1,14; 95% IC: 0,89-1,44) ni en la del IPDGC (OR=0,98; 95% IC: 0,89-1,07). Tampoc es va revalidar l'associació entre la variant p.R151C (rs1805007) i el risc a desenvolupar Parkinson (OR=1,06; 95% IC: 0.94-1.19), que es va analitzar únicament en la cohort IPDGC [217]. Cap dels SNPs avaluats en aquest treball es va associar amb un increment del risc a desenvolupar la malaltia de Parkinson. En el mateix treball, es va avaluar la relació entre característiques fenotípiques que augmenten el risc a desenvolupar melanoma (color de cabell pèl-roig, color clar d'ulls i fototip cutani clar) i el risc a desenvolupar Parkinson. Tampoc es va identificar una associació entre cap d'aquestes característiques fenotípiques i el risc a Parkinson. Per tant, els resultats d'aquest treball no confirmen un increment del risc a Parkinson en els individus pèl-roigs i tampoc van identificar el gen *MC1R*, ni la resta de gens de pigmentació, com a factors genètics de risc a desenvolupar la malaltia.

Posteriorment a la publicació del treball I, diversos treballs han analitzat el paper de *MC1R* en el risc a desenvolupar Parkinson en varies poblacions mitjançant diferents estratègies metodològiques (Taula 2).

Taula 2: Avaluació del gen *MC1R* com a factor de risc a desenvolupar la malaltia de Parkinson en diferents cohorts.

Estudi	Població	Estratègia metodològica	Casos/Controls	Resultats Globals	p.R160W OR (95% IC)	p.R151C OR (95% IC)
Foo *et al*. [220]	Xinesa	Genotipació de p.R160W	778/517	La variant p.R160W no s'identifica en cap individu	NI	NI
		Seqüenciació de *MC1R*	99/99	Cap de les variants al·lèliques identificades s'associa a Parkinson		
Jiang & Liu [221]	Asiàtics amb ascendència europea	Metanàlisi de variants *MC1R* a partir de dades de 15 estudis GWAS	13.798/95.282	Cap de les variants al·lèliques identificades s'associa a Parkinson	NI	NI
Lubbe *et al*. (IPDGC) [222]	Caucàsics d'ètnia blanca#	Metanàlisi de la variant p.R160W genotipada mitjançant *Neuro X chip*	5.944/4.642	La variant p.R160W no s'associa a Parkinson	1,01 (0,90-1,13)	NA
Lorenzo-Betancor *et al*.[223]	Caucàsics no hispànics de Florida	Seqüenciació de *MC1R*	889/940	Cap de les variants al·lèliques identificades s'associa a Parkinson	1,06 (P = 0.62)	1,27 (P = 0.068)
Gan-Or *et al*. (EA) [224]	Nord-americans amb ascendència europea	Seqüenciació de *MC1R*	551/956	Cap de les variants al·lèliques identificades s'associa a Parkinson	1,13 (0,68-1,90)	0,92 (0,57-1,47)
Gan-Or *et al*. (NY) [224]	Nord-americans de Nova York	Seqüenciació de *MC1R*	539/265	Cap de les variants al·lèliques identificades s'associa a Parkinson	0,92 (0,53-1,57)	0,77(0,48-1,23)

NI: variant no identificada en la població analitzada.

NA: variant no avaluada en la població analitzada.

#Població caucàsica d'ètnia blanca. Aquesta cohort inclou diferents poblacions: holandesa, alemanya, britànica, nord-americana, francesa i grega, cohorts estudiades dins del IPDGC (International Parkinson's Disease Genomics Consortium).

P: p-valor

Cap d'aquests treballs va identificar el gen *MC1R* com a factor de risc a desenvolupar la malaltia de Parkinson [220-224]. La discrepància de resultats entre els diferents estudis pot ser deguda a diferents factors metodològics. Alguns d'aquests treballs [217, 220-222] van analitzar el gen *MC1R* com a factor de risc a Parkinson, avaluant únicament variants genètiques concretes (p.R160W i/o p.R151C). Donat que la freqüència de les variants al·lèliques de *MC1R* varia molt entre les diferents poblacions [9, 225, 226] i que pot existir un efecte poblacional específic associat a una/unes determinades variants al·lèliques [64, 226], és clau caracteritzar molecularment el gen *MC1R* en cada població, seqüenciant tota la regió codificant.

D'altra banda, cap d'aquests estudis [215, 217, 220-224] indica l'exclusió d'individus que hagin desenvolupat melanoma o que presentin antecedents familiars de melanoma com a població control. Aquest hauria de ser un criteri d'elegibilitat a tenir en compte, ja que s'ha identificat una major freqüència de variants al·lèliques de *MC1R* en els individus susceptibles a desenvolupar melanoma [28, 64]. La inclusió d'aquests individus com a controls, pot introduir un biaix que modifiqui la freqüència amb la que es produeix l'esdeveniment que s'avalua i donar lloc a una conclusió equivocada (Annex II).

A més, algunes de les cohorts analitzades en aquests treballs [217, 221-224] agrupen diferents poblacions. Com es demostra en el treball de Lubbe *et al.*[222], la freqüència de l'al·lel p.R160W varia molt entre les diferents subpoblacions (holandesa, alemanya, britànica, nord-americana, francesa i grega) que es van analitzar conjuntament com a població caucàsica d'ètnia blanca (Taula 3). Aquest fet es deu a l'alt grau de variabilitat interpoblacional de les variants de *MC1R* [9]. Per tant, si degut a la heterogeneïtat interpoblacional, la homogeneïtat genètica en les cohorts avaluades és baixa [217, 221-224], es pot introduir un biaix que alteri els resultats dels anàlisis d'associació i generi falsos negatius.

Taula 3: Anàlisi d'associació entre la variant p.R160W i el Parkinson en les diferents poblacions estudiades. Adaptada de Lubbe *et al.* [222].

	OR (95% CI)	*p*	Controls		Cases	
			MAF	N	MAF	N
NeuroX Genotyped samples[a]	1.01 (0.90–1.13)	0.853	0.062	4,642	0.064	5,944
Dutch	0.86 (0.57–1.30)	0.469	0.075	409	0.066	312
French	1.00 (0.61–1.66)	0.989	0.040	451	0.036	508
German	1.10 (0.87–1.41)	0.425	0.068	880	0.075	1,296
Greek	1.34 (0.92–1.95)	0.131	0.026	882	0.034	965
UK	0.95 (0.72–1.24)	0.624	0.080	583	0.073	1,107
US	0.92 (0.76–1.12)	0.385	0.077	1,437	0.074	1,756
Tell-Marti et al[b]	2.10 (1.18–3.73)	0.009	0.010	736	0.025	870
Dong et al[c]	0.98 (0.89–1.07)	0.624	—	13,642	—	6,141

[a]Logistic regression correcting for sex and population stratification.
[b]Logistic regression correcting for gender and age.
[c]Meta-analysis of 5,333 cases and 12,019 controls from the International Parkinson's Disease Genomics Consortium; and 808 cases and 1,623 controls from the Parkinson's Genes and Environment Study.
CI = confidence interval; MAF = minor allele frequency; N = number of samples; OR = odds ratio; UK = United Kingdom; US = United States.

En base a aquesta evidència, Lubbe *et al.*[222] van analitzar una possible associació de l'al·lel p.R160W i el risc a desenvolupar Parkinson estratificant per poblacions (Taula 3). No obstant, aquesta associació tampoc es va revalidar en cap dels subanàlisis poblacionals. Cal indicar però, que la freqüència de l'al·lel p.R160W prèviament identificada en altres cohorts control d'origen grec (MAF = 0,010 o 0,012) (Taula 4) (Annex II) és: 1) notablement inferior a la MAF observada per Lubbe *et al*. en el *set* de controls grecs (MAF = 0,026), 2) notablement inferior a la MAF identificada per Lubbe *et al*. en els pacients de Parkinson grecs (MAF = 0,034) i 3) és la mateixa freqüència que s'ha identificat en la cohort de controls d'origen espanyol (MAF = 0,012) (Taula 4). Donat que diversos estudis han mostrat una elevada heterogeneïtat genètica entre les poblacions de l'Europa del Nord i del Sud, i han confirmat una major homogeneïtat genètica entre les poblacions de cada subgrup [227], els resultats identificats per Lubbe *et al*. en la població grega (Europa del Sud) han de ser interpretats amb precaució, ja que s'esperaria que poguessin ser similars als identificats en població espanyola (Annex II).

Taula 4: Freqüència al·lèlica de la variant p.R160W identificada en diferents estudis cas-control. Adaptada de Tell-Marti *et al.* [228].

	p.R160W in PD Studies				p.R160W in Different Control Populations	
Population	**MAF, Controls (N)**	**MAF, PD (N)**	**OR [95% CI]**	***p***	**Population**	**MAF, Controls (N)**
Spain[2]	0.012 (736)	0.024 (870)	2.10 [1.18–3.73]	0.009[a]	Spain[4] Spain[5]	0.014 (544) 0.010 (491)
Letter IPDGC						
Greece	0.026 (882)	0.034 (965)	1.34 [0.92–1.95]	0.131	Greece[8] Greece[9]	0.010 (155) 0.012 (166)
France	0.040 (451)	0.036 (508)	1.00 [0.61–1.66]	0.989	France[10] France[11]	0.024 (210) 0.030 (869)
Germany	0.068 (880)	0.075 (1,296)	1.10 [0.87–1.41]	0.425	Germany[4]	0.060 (1038)
The Netherlands	0.075 (409)	0.066 (312)	0.86 [0.57–1.30]	0.469	The Netherlands[12]	0.105 (385)
United States	0.077 (1,437)	0.074 (1,756)	0.92 [0.76–1.12]	0.385	United States[13] United States[14]	0.060 (166) 0.062 (179)
United Kingdom	0.080 (583)	0.073 (1,107)	0.95 [0.72–1.24]	0.624	United Kingdom[15] United Kingdom[16]	0.091 (93) 0.066 (332)
Pooled population[b]	0.062 (4,642)	0.064 (5,944)	1.01 [0.90–1.13]	0.853	Pooled population[c]	0.053 (3,593)

[a]Statistically significant.
[b]Includes all populations assessed in the IPDGC study.
[c]Includes all control populations listed in this table, except those from Spain.
CI = confidence interval; IPDGC = International PD Genetic Consortium; MAF = minor allele frequency; N = number of individuals included in each study; OR = odds ratio; PD = Parkinson disease.

No obstant, Lubbe *et al*. [222] van concloure que la variant p.R160W no s'associa a un augment del risc a desenvolupar Parkinson i atribuïen els resultats presentats en el treball I a un fals positiu. Els autors van indicar un possible biaix en les freqüències al·lèliques detectades en el treball I, ocasionat per un origen poblacional diferent en la selecció de casos i controls, ja que els pacients de Parkinson provenien de Barcelona i els controls del conjunt del territori espanyol.

Posteriorment a aquesta suggerència, varem reavaluar la MAF de la variant p.R160W estratificant la cohort control (N= 736), segons si els individus havien estat reclutats al Hospital Clínic de Barcelona (N= 131, MAF = 0,015) o en altres hospitals d'Espanya (N= 605, MAF= 0,011). La diferència no era estadísticament significativa i a més, la freqüència de l'al·lel p.R160W identificada en la cohort control d'origen espanyol (MAF = 0,012) coincidia amb la freqüència al·lèlica que s'ha identificat en altres cohorts control d'origen espanyol (Taula 4) (Annex II).

En base aquests resultats, i tenint en compte, que estudis previs han demostrat que la població espanyola és suficientment homogènia com per realitzar estudis d'associació cas-control [229, 230], sense que aquests es vegin alterats per l'estratificació intraterritorial, descartem que els resultats presentats en el treball I es deguin a un biaix ocasionat per una baixa homogeneïtat genètica poblacional entre la cohort de casos i controls.

D'altra banda, un factor poblacional pot explicar la discrepància entre els resultats del treball I i els resultats identificats per Foo *et al*. [220] i Jiang & Liu [221] en població asiàtica. En aquests treballs no es va identificar cap de les variants LOF de *MC1R* identificades en població caucàsica d'ètnia blanca, i únicament 2 de les variants (p.V92M i p.R163Q) comunament identificades en població caucàsica d'ètnia blanca, es van identificar en aquestes poblacions asiàtiques. Els autors van concloure que les variants LOF de *MC1R* són rares en la població asiàtica i que el gen *MC1R* no és un factor de risc a Parkinson en aquesta població [220, 221].

Aquests resultats, concorden amb el fet que la incidència del melanoma en la població asiàtica sigui molt més baixa que en la població caucàsica i amb que no s'hagi reportat una comorbiditat entre el melanoma i el Parkinson en aquesta població. A més, els resultats coincideixen en que els al·lels LOF de *MC1R* són inexistents o extremadament rars en la població asiàtica [226], i per tant, és poc probable que modulin el risc a desenvolupar Parkinson en aquesta població. D'altra banda, la variant p.R163Q, la més freqüent en la població asiàtica [231, 232], disminueix lleugerament la senyalització a través de la via canònica de MC1R, però l'activitat residual del MC1R associada a aquesta variant, és molt superior a l'activitat residual associada a les variants LOF [7, 233]. La p.R163Q augmenta el risc a desenvolupar melanomes que apareixen en zones crònicament exposades a la RUV, a través però, de mecanismes independents a la pigmentació i la síntesi de feomelanina [234] (Annex III). A més, altres variants prèviament identificades en gens de risc a desenvolupar Parkinson, no s'han identificat o han resultat ser molt infreqüents en la població asiàtica [235] (Annex III).

Els resultats d'aquests treballs, reforcen la hipòtesi de que únicament les variants LOF de *MC1R* són variants genètiques de risc a Parkinson i indiquen un efecte poblacional, ja que aquestes serien variants de risc en població caucàsica d'ètnia blanca, però és poc probable que ho siguin en població asiàtica (Annex III i Annex IV).

En aquesta mateixa línia, un metanàlisi va analitzar els resultats de les 6 publicacions ([215, 217, 222-224] i Treball I), que fins a la data, han analitzat el gen *MC1R* com a factor de risc a Parkinson en poblacions caucàsiques d'ètnia blanca [236]. En concret, el metanàlisi va avaluar el risc a desenvolupar Parkinson en base a: tenir el cabell pèl-roig (Figura 17A), ser portador de l'al·lel p.R151C de *MC1R* (Figura 17B) o ser portador de l'al·lel p.R160W de *MC1R* (Figura 17C) [236].

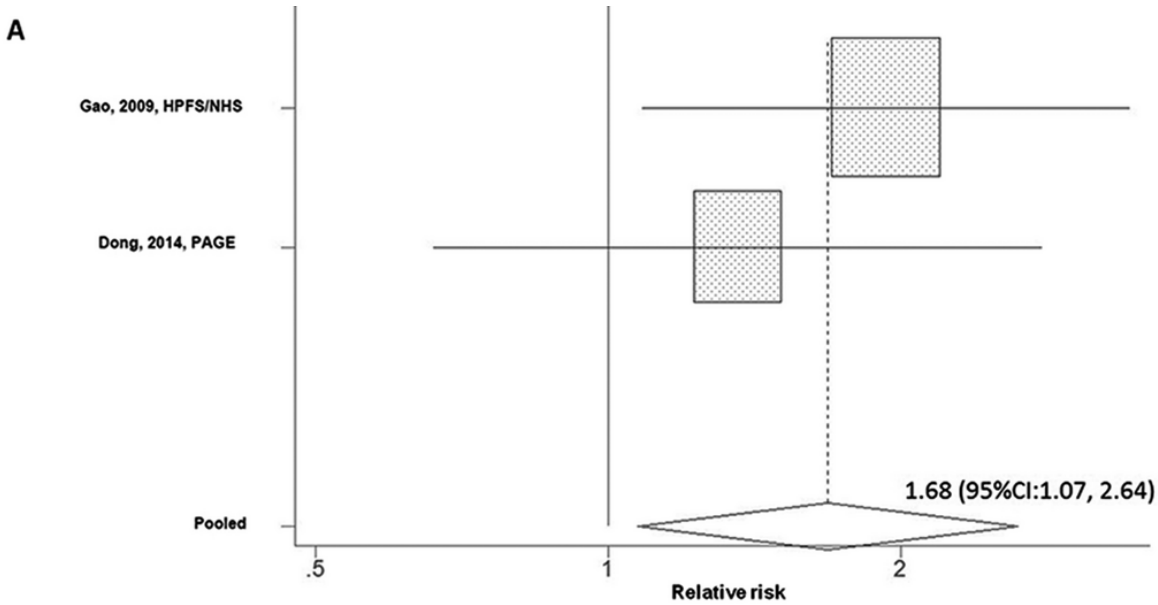


Figura 17A. Avaluació del risc a desenvolupar Parkinson en els individus pèl-rojos. Inclou 1.347 casos de Parkinson. Adaptada de Chen *et al.* [236].

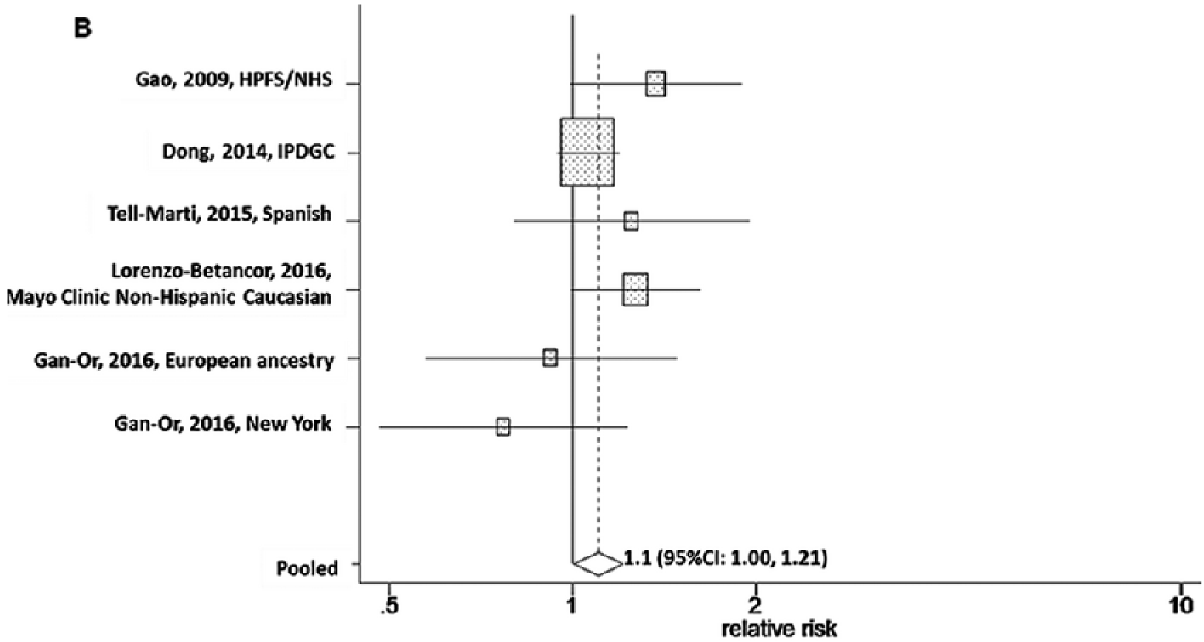


Figura 17B. Avaluació del risc a desenvolupar Parkinson en els individus portadors de l'al·lel p.R151C de *MC1R*. Inclou 8.454 casos de Parkinson. Adaptada de Chen *et al.* [236].

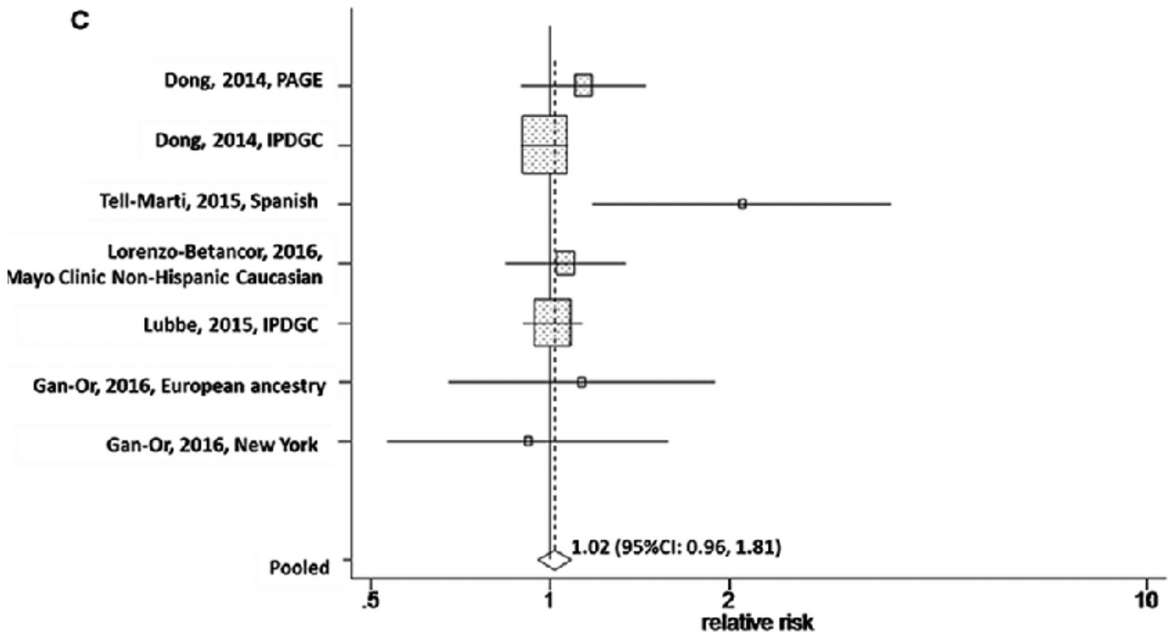


Figura 17C. Avaluació del risc a desenvolupar Parkinson en els individus portadors de l'al·lel p.R160W de *MC1R*. Inclou 14.934 casos de Parkinson. Adaptada de Chen *et al.* [236].

Els resultats d'aquest metanàlisi confirmen un augment del risc a Parkinson en els individus pèl-rojos i en els portadors de l'al·lel p.R151C. Ara bé, calen més estudis d'associació cas-control que confirmin si l'al·lel p.R160W i la resta d'al·lels 'R' de *MC1R* augmenten el risc a Parkinson.

Per tal d'evitar falsos negatius i identificar possibles efectes poblacionals específics, és clau que futurs estudis d'associació cas-control caracteritzin molecularment tot el gen *MC1R* en poblacions genèticament homogènies. Diversos treballs han reportat la necessitat de considerar l'estratificació per poblacions quan es realitzen estudis genètics en població Europea o amb ascendència Europea. La heterogeneïtat genètica que existeix entre la població de l'Europa del Sud, l'Europa del Nord o la població americana d'ascendència Europea, impedeix analitzar-les conjuntament, sense assumir un important biaix causat per aquesta heterogeneïtat genètica [227, 230].

D'altra banda, el fet que les variants de *MC1R* presentin una baixa freqüència i una baixa penetrància [236] en comparació a altres variants al·lèliques identificades en altres gens de risc a Parkinson [116], dificulta la identificació d'aquestes possibles associacions.

Ara bé, de manera molt rellevant, Chen *et al*. [237] han confirmat, mitjançant un estudi funcional, que la pèrdua de funció del MC1R augmenta la susceptibilitat a desenvolupar Parkinson. El treball va avaluar el paper del MC1R en el SNC comparant ratolins negres ($Mc1r^{+/+}$) *vs* ratolins pèl-roigs ($Mc1r^{e/e}$) [237]. Els models murins utilitzats en aquest treball són aquells en els quals s'ha identificat una major susceptibilitat a desenvolupar melanoma (Figura 10) [70].

Els autors van confirmar l'expressió del MC1R en la substància negra *pars compacta* del mesencèfal, regió rica en neurones dopaminèrgiques i que es veu compromesa en els pacients de Parkinson. Per tal d'identificar un possible impacte del MC1R en el sistema nigroestriatal dopaminèrgic, els autors van comparar la integritat de les neurones dopaminèrgiques en els ratolins $Mc1r^{+/+}$ *vs* els $Mc1r^{e/e}$ i van monotoritzar l'activitat locomotora d'aquests ratolins. A nivell constitutiu, els ratolins pèl-roigs ($Mc1r^{e/e}$) presentaven una pèrdua significativa de neurones dopaminèrgiques en la substància negra *pars com-*

pacta i en conseqüència una disminució dels nivells de dopamina en el nucli estriat. Malgrat de forma general, els ratolins $Mc1r^{e/e}$ eren sans, aquests presentaven una disminució de l'activitat locomotora respecte als ratolins $Mc1r^{+/+}$. Aquestes alteracions, eren significatives en els ratolins $Mc1r^{e/e}$ que tenien 8 i 14 mesos, i en canvi, no es van identificar en els ratolins més joves (2 mesos de vida). Aquests resultats indiquen que la pèrdua de funció del MC1R impacta en el manteniment i funcionament de les neurones dopaminèrgiques, i que amb l'envelliment, factor principal de risc a Parkinson, aquests ratolins presenten alteracions fisiològiques que els predisposen a desenvolupar aquesta malaltia [237].

Donat que en les cèl·lules cutànies d'aquests ratolins ($Mc1r^{e/e}$) s'ha identificat un augment de les ROS i del dany oxidatiu [70] i que l'estrès oxidatiu està implicat en la patogènesi del Parkinson [238], els autors van avaluar si aquests ratolins ($Mc1r^{e/e}$) presenten un major dany oxidatiu en les cèl·lules del SNC. Concretament en la regió ventral del mesencèfal, rica en neurones dopaminèrgiques, i en el nucli estriat, regió cap a on és projecten aquestes neurones dopaminèrgiques. De forma interessant, en els ratolins $Mc1r^{e/e}$ es va identificar un augment del dany oxidatiu en la regió ventral el mesencèfal [237]. Aquest resultat concorda amb que l'activació del MC1R comporta una resposta antioxidant [67] i indica que ens els ratolins on l'activitat del MC1R és nul·la ($Mc1r^{e/e}$), s'observa un augment de l'estrès oxidatiu, tant en les cèl·lules cutànies com en les cèl·lules del SNC.

Per a confirmar si els ratolins $Mc1r^{e/e}$ són més susceptibles a desenvolupar Parkinson que els ratolins $Mc1r^{+/+}$, els autors els van subministrar les neurotoxines (6-OHDA i MPTP), comunament utilitzades per a generar models experimentals que reprodueixin la malaltia de Parkinson. Posteriorment a l'administració d'aquestes neurotoxines, els ratolins $Mc1r^{e/e}$ presentaven una disminució significativa dels nivells de dopamina en el nucli estriat i una reducció notable de neurones dopaminèrgiques. Els resultats d'aquest treball indiquen que la susceptibilitat d'aquests ratolins ($Mc1r^{e/e}$) a desenvolupar Parkinson, es pot explicar per l'augment del dany oxidatiu i la pèrdua de neurones dopaminèrgiques que s'observa constitutivament en aquests ratolins [237].

En base a aquests resultats, els autors van voler avaluar el paper del MC1R com a possible diana farmacològica. L'activació del MC1R mitjançant un agonista selectiu (BMS-470539) [239], va mostrar un efecte protector, disminuint la mortalitat de les neurones dopaminèrgiques induïda per l'efecte neurotòxic del MPTP [237]. Aquesta acció neuroprotectora, proporciona una nova evidencia per pensar que la pèrdua de neurones dopaminèrgiques que s'identifica en els ratolins Mc1r$^{e/e}$ i la neurotoxicitat dopaminèrgica que aquests mostren en resposta a les neurotoxines, depenen directament de la funcionalitat del MC1R.

El fet que els ratolins portadors d'un MC1R no funcional presentin una major susceptibilitat a desenvolupar tant melanoma com Parkinson, sumat a l'associació epidemiològica entre les variants 'R' i l'augment del risc a desenvolupar les dues malalties, indica que el gen *MC1R* és un factor de risc tant a melanoma com a Parkinson.

2. EL GEN *MC1R* COM A FACTOR DE RISC A DESENVOLUPAR LA MALALTIA D'ALZHEIMER

En el treball II, l'anàlisi d'associació genotípica cas-control indica que els individus portadors de l'al·lel p.V92M de *MC1R*, presenten un augment del risc a desenvolupar la malaltia d'Alzheimer sota un model d'herència additiu (OR=1,99; 95% IC: 1,08-3,64; p-valor ajustat = 0,026) (Taula 3, Treball II). Aquest resultat indica que el gen *MC1R* pot estar implicat en la etiologia de la malaltia d'Alzheimer.

En aquest treball es va identificar una associació inversa entre l'al·lel p.V92M de *MC1R* i l'al·lel 4 de l'*APOE* (Taula 5, Treball II). Aquest resultat indica que la presència d'aquesta variant en *MC1R* augmenta la susceptibilitat a desenvolupar Alzheimer, especialment en aquells individus on el risc a desenvolupar la malaltia no pot atribuir-se al principal factor genètic de risc identificat en les formes esporàdiques d'Alzheimer [143].

Donat que el diagnòstic de la malaltia d'Alzheimer, en base als criteris clínics utilitzats avui en dia [131], té una sensibilitat i especificitat del 85% i 70%, respectivament [135], és molt probable que alguns dels individus diagnosticats com a pacients d'Alzheimer en el treball II i en d'altres treballs, presentin una malaltia neurodegenerativa que no sigui

Alzheimer [240]. Aquest error en el diagnòstic pot introduir un biaix en la selecció dels casos, que modifiqui la freqüència amb la que es produeix l'esdeveniment que s'avalua i donar lloc a una conclusió equivocada. L'ús del perfil de biomarcadors de LCR (d' $A\beta_{42}$, T-tau i P-tau) en el diagnòstic de la malaltia d'Alzheimer augmenta la sensibilitat i especificitat diagnòstica en un 90% i 85%, respectivament [136, 137]. En base aquesta evidència, en el treball II es va repetir l'anàlisi d'associació genotípica cas-control utilitzant el mateix grup de controls i restringint els casos a un subgrup de pacients d'Alzheimer on el diagnòstic clínic havia estat confirmat pel perfil de biomarcadors en LCR. Malgrat la reducció de la mida mostral, es va revalidar l'associació entre l'al·lel p.V92M i el risc a desenvolupar Alzheimer. De fet, aquesta associació augmentava més de 3 vegades en aquest subgrug de pacients (Taula suplementària 1, Treball II).

L'al·lel p.V92M és comú en les poblacions caucàsiques d'ètnia blanca [9] i en la nostre cohort control presenta una freqüència al·lèlica de 0,05 (Taula 3, Treball II). Malgrat no ser una variant rara, cap dels estudis GWAS realitzats en la darrera dècada [146-151] ha identificat una associació entre el gen *MC1R* i la malaltia d'Alzheimer. Aquest fet podria justificar-se per les pròpies limitacions que presenten aquests estudis GWAS. En primer lloc, el gen *MC1R* presenta un baix nivell de cobertura en les plataformes de genotipat d'SNPs que s'han utilitzat en aquests estudis. D'altra banda els estudis GWAS presenten una limitació deguda a l'estratificació poblacional [241, 242]. Per tal d'augmentar el poder estadístic per a detectar diferències entre casos i controls, s'analitzen cohorts d'una gran mida mostral que agrupen diferents poblacions en una única cohort. Tenint en compte l'alt grau de variabilitat interpoblacional, tant de l'al·lel p.V92M (MAF de 0,3-0,12 en les poblacions caucàsiques) com de les altres variants al·lèliques de *MC1R* [9], és clau que els estudis d'associació es facin en cohorts genèticament homogènies, ja que el biaix que introdueix l'estratificació poblacional pot donar lloc a conclusions incorrectes. A més, els tests de correcció múltiple que s'utilitzen en els estudis GWAS són estadísticament molt conservadors i dificulten la identificació de variants genètiques que expliquen un petit percentatge del risc a desenvolupar una malaltia.

El treball II presenta una limitació pel que fa a la cohort control utilitzada. Malgrat està ben caracteritzada i permet corregir l'anàlisi d'associació genotípica per els principals factors de risc a desenvolupar Alzheimer (sexe, edat i genotip *APOE*), aquesta presenta una mida mostral limitada (N=160). Aquest fet disminueix el poder estadístic del treball II per a detectar diferències entre casos i controls. Malgrat això, la freqüència de l'al·lel p.V92M que s'identifica en el *set* de controls coincideix amb la prèviament identificada en població control espanyola (MAF= 0,05) [218]. A més, les freqüències al·lèliques de les variants de *MC1R* identificades en aquesta cohort control (Taula 3, Treball II) es van comparar amb les identificades a la cohort control (N= 736) d'origen espanyol analitzada en el treball I (Taula 2, Treball I), i no es van detectar diferencies significatives (Annex V). Per tant, la cohort control utilitzada en el treball II reflecteix les freqüències al·lèliques de les variants de *MC1R* a la població espanyola.

L'al·lel p.V92M disminueix a la meitat l'afinitat del MC1R amb el seu lligand (α-MSH), en canvi, els nivells d'expressió del MC1R en la membrana de les cèl·lules portadores d'aquest al·lel són normals [8, 233]. Les cèl·lules portadores de la variant p.V92M mostren una lleugera disminució de la senyalització a través de la via canònica del MC1R. No obstant, aquestes cèl·lules presenten una activitat residual molt superior a l'activitat residual identificada en les cèl·lules portadores de variants LOF de *MC1R* [7, 233]. Això explica perquè l'alteració funcional associada a la variant p.V92M, no es tradueix en una síntesi notable de feomelanina i l'al·lel p.V92M no s'associa amb el fenotip RHC [7, 8, 28]. Els estudis epidemiològics presenten una associació controvertida entre la presència de l'al·lel p.V92M i l'augment dels risc a desenvolupar melanoma, mentre alguns treballs afirmen que no s'associa amb un augment del risc a melanoma [28, 218, 243, 244], altres indiquen que si l'incrementa lleugerament [245, 246]. Aquestes evidències expliquen perquè a diferència del que succeeix amb la malaltia de Parkinson, no s'ha descrit una sobrerepresentació d'individus pèl-roigs entre els pacients d'Alzheimer, ni tampoc un augment del risc a desenvolupar melanoma en aquests pacients.

Fins a la data, s'han realitzat pocs estudis que avaluïn l'impacte de les variants al·lèliques de *MC1R* més enllà de l'efecte sobre el càncer cutani. No obstant, s'ha descrit que els individus portadors de la variant p.R163Q, que tampoc s'associa amb el fenotip RHC, presenten un augment del risc a patir una depressió i que els individus portadors de la variant p.V92M presenten una remissió de la depressió en resposta al tractament amb desipramina [247]. La prevalença de la depressió és duplica en les dones, indicant que el sexe és un factor de risc a desenvolupar aquesta malaltia [248]. Tenint en compte que la disepramina s'ha utilitzat com a analgèsic i que el MC1R modula la resposta al dolor en les dones a traves del receptors κ-opioides [83], s'ha suggerit que la remissió de la depressió en resposta a la desipramina podria estar regulada pel MC1R a través de la via del dolor [247]. Malgrat cap treball ha avaluat a nivell funcional l'impacte de les variants 'r' (p.V60L, p.V92M i p.R163Q) en el SNC, els resultats presentats en el treball II i les evidències prèviament descrites, indiquen que l'alteració funcional del MC1R atribuïda a l'al·lel p.V92M pot tenir un impacte en la fisiologia de les cèl·lules del SNC, a través de la desregulació d'altres vies de senyalització diferents a la via canònica del MC1R.

En la malaltia d'Alzheimer es produeix una inflamació crònica en el cervell que desencadena en un dèficit cognitiu [249]. Les cèl·lules de la barrera hematoencefàlica (BHE) juguen un paper clau tant en el desencadenament com en el manteniment d'aquesta inflamació crònica [250]. L'augment de la producció de la proteïna $A\beta_{42}$ augmenta l'estrès oxidatiu en les cèl·lules del cervell [251], fet que comporta un dany microvascular que desencadena una resposta inflamatòria a traves de l'activació de NF-KB [91, 249]. En les cèl·lules endotelials microvasculars del cervell i en els astròcits s'ha identificat l'expressió del MC1R [80, 90]. L'activació del MC1R en aquestes cèl·lules desencadena una resposta antiinflamatòria a través de la infraregulació de NF-KB [91, 252]. Per tant, suggerim que l'alteració funcional del MC1R atribuïda a l'al·lel p.V92M pot disminuir la infraregulació de NF-KB, minvant aquesta resposta antiinflamatòria i contribuint a la inflamació crònica en el cervell.

Treballs previs han reportat una disminució dels nivells de α-MSH en les cèl·lules del cervell i en el LCR dels pacients d'Alzheimer, indicant que la producció d'aquest neuropèptid derivat de la POMC podria ser clau en la fisiopatologia i progressió de la malaltia d'Alzheimer [253, 254]. El fet que les cèl·lules portadores de l'al·lel p.V92M presentin un MC1R amb la meitat d'afinitat per la α-MSH, podria explicar perquè els portadors d'aquesta variant són més susceptibles a desenvolupar Alzheimer.

3. EL GEN *MC1R* COM A FACTOR MODIFICADOR DE L'EDAT D'APARICIÓ DE LES MALALTIES NEURODEGENERATIVES

Igual que succeeix en la malaltia de Parkinson i Alzheimer, nivells elevats de dany oxidatiu s'han identificat en les àrees de neurodegeneració en els pacients amb Huntington [255-257]. Aquest fet indica que l'estrès oxidatiu pot contribuir a la patogènesi de la malaltia. L'activació del MC1R s'ha associat a una resposta antioxidant [67] i en les cèl·lules cutànies amb un MC1R no funcional, s'ha identificat una desregulació constitutiva de gens implicats en les vies de senyalització de malalties neurodegeneratives (Annex I). Aquestes evidències suggereixen que malgrat la malaltia de Huntington és una malaltia monogènica, el MC1R pot estar implicat en la fisiopatologia de la malaltia i modificar l'edat de debut en els pacients.

En el Treball III, es va identificar que l'al·lel p.R151C de *MC1R* modifica significativament l'edat d'inici de la malaltia de Huntington (p-valor = 0,004) (Taula 2, Treball III). Els casos portadors de l'al·lel p.R151C presenten una disminució de 5,13 anys respecte a l'edat mitjana de debut identificada en els casos no portadors.(Taula 3, Treball III). Concretament, el percentatge de variació en l'edat de debut atribuït a l'al·lel p.R151C és del 1,42%, similar al prèviament atribuït a altres variants al·lèliques en els gens *UCHL1* (1,7%), *PCG-1α* (1,5%) i *ATG7* (1%) [181, 185, 186].

Aquest resultat reforça la hipòtesi de que el gen *MC1R* està implicat en la fisiopatologia de malalties neurodegeneratives, i igual que succeïx en el Parkinson [215], l'impacte del MC1R en la malaltia de Huntington s'associa a un al·lel LOF de *MC1R*. Els estudis funcionals realitzats en cèl·lules cutànies, mostren que l'al·lel p.R151C disminueix l'expressió del MC1R en la membrana cel·lular i disminueix notablement la capacitat de senyalització a través de la via canònica del MC1R [7, 8].

Els ratolins portadors d'un MC1R no funcional ($Mc1r^{e/e}$) mostren, a nivell basal, un augment del dany oxidatiu en la regió ventral del mesencèfal [237], fet que els fa més susceptibles a desenvolupar Parkinson. Un augment del dany oxidatiu s'ha identificat també en les àrees de neurodegeneració en els pacients amb Huntington i alguns dels gens identificats com a modificadors de l'edat d'inici de la malaltia (*PPARGC1A*, *MAP3K5* i *MAP2K6*), estan implicats en la regulació de l'estrès oxidatiu [180, 181, 258]. Concretament, el gen *PPARGC1A* (codi OMIM: #604517) que codifica per la proteïna PGC-1α implicada en la regulació de la biogènesi mitocondrial i la resposta antioxidant [259-261], s'ha proposat com una possible diana terapèutica, ja que variants al·lèliques comuns en *PPARGC1A*, retarden de 2 a 3 anys l'edat d'aparició i la progressió de la malaltia de Huntington [181, 258]. Models murins deficients en PGC-1α mostren trastorns del moviment i degeneració del nucli estriat [262], alteracions que s'identifiquen en els pacients amb Huntington. Estudis realitzats en cèl·lules cutànies, han descrit que l'activació del MC1R indueix *downstream* l'expressió de PGC-1α [263, 264]. En base a aquestes evidencies, suggerim que la pèrdua de funció del MC1R associada a la variant p.R151C, pot contribuir a la fisiopatologia de la malaltia de Huntington i avançar-ne la seva aparició, a través de la infraexpressió de PGC-1α i la infraregulació de la resposta antioxidant.

L'edat d'inici del Parkinson i de l'Alzheimer són caràcters poligènics amb un elevat grau d'heretabilitat [112, 155]. Variants al·lèliques en gens de risc mig i baix a desenvolupar aquestes malalties, s'han identificat com a factors modificadors de l'edat d'aparició de les formes esporàdiques d'aquestes malalties [117, 121, 160]. En base a aquestes evidències, varem hipotetitzar que el gen *MC1R* pot ser un factor modificador de l'edat d'aparició del Parkinson i de l'Alzheimer.

Les variants genètiques de *MC1R* no sinònimes i amb una freqüència al·lèlica > 0,01 en els casos, van ser analitzades com a variants modificadores de l'edat d'inici d'aquestes malalties. En el cas del Parkinson, cap d'aquestes variants al·lèliques, ni l'al·lel p.R151C ni el p.R160W, modifiquen l'edat d'aparició de la malaltia (Taula suplementària 1, Treball I). D'igual forma en l'Alzheimer, cap d'aquestes variants al·lèliques, tampoc l'al·lel p.V92M, modifiquen l'edat de debut en els pacients portadors (Taula suplementària 2, Treball II). En canvi, en els casos d'Alzheimer analitzats en el treball II, es confirma que l'APOEε4 avança l'edat d'inici de la malaltia (Taula 6, Treball II). Aquests resultats indiquen que el gen *MC1R* no és un factor modificador de l'edat d'aparició d'aquestes malalties neurodegeneratives complexes, almenys a la població espanyola.

Tot i que en el cas del melanoma, el gen *MC1R* s'ha identificat com un factor modificador de l'edat d'aparició de la malaltia, aquest efecte s'observa quan el gen *MC1R* interacciona amb un factor genètic d'alt risc. Concretament, s'ha descrit que la presencia de variants en *MC1R* augmenta la penetrància del melanoma en individus portadors de mutacions a *CDKN2A* [59, 60], i disminueix en 20 anys l'edat mitjana de debut en aquests individus [29, 59]. Diferents treballs han avaluat si les variants al·lèliques de *MC1R* modifiquen l'edat d'inici de la malaltia en els pacients amb melanomes esporàdics [265, 266], i els resultats han estat negatius. En base a aquestes evidències, calen futurs treballs en els que s'avaluï el gen *MC1R* com a modificador de l'edat d'aparició de les formes familiars del Parkinson i de l'Alzheimer.

4. IMPACTE DEL MC1R EN LA REGULACIÓ DE L'ESTRÈS OXIDATIU I L'AUTOFÀGIA

Els resultats obtinguts en el treballs I, II i III juntament amb les evidències discutides anteriorment (apartats 1, 2 i 3 de la Discussió), indiquen que el gen *MC1R* és un factor de risc a Parkinson i Alzheimer i que modifica l'edat d'aparició del Huntington. Tot i que l'impacte del MC1R en el SNC s'ha confirmat en models murins, calen estudis funcionals per elucidar a través de quines funcions biològiques, la pèrdua de funció del MC1R contribueix a la patogènesi d'aquestes malalties neurodegeneratives.

Amb l'objectiu d'elucidar quines poden ser les funcions biològiques alterades associades a la pèrdua de funció del MC1R, en el treball IV és va analitzar la signatura gènica identificada en els co-cultius de cèl·lules cutànies portadores de variants LOF de *MC1R* (Annex I), mitjançant un anàlisi de predicció d'interacció proteïna-proteïna (PPI). Es tracta d'un mètode computacional d'enriquiment funcional, basat en la cerca de mòduls funcionals en llistats de gens (trànscrits) segons la interacció existent entre les proteïnes per les quals codifiquen.

Tenint en compte que des d' una perspectiva biològica, els trànscrits sobreexpressats o infraexpressats en un fenotip determinat, tendeixen a estar connectats [267, 268]. L'anàlisi de PPI es va realitzar de forma independent amb el grup de gens sobreexpressats i el grup de gens infraexpressats. A partir d'aquest anàlisi, varem identificar dues xarxes estadísticament significatives (p-valor < 0,001) (Figura 1, Treball IV) (Figura 2, Treball IV). Assumint que existeix una forta relació entre la funció biològica i la interacció proteica, aquestes xarxes van ser analitzades en base a paràmetres topològics de la pròpia xarxa, per tal d'identificar els gens de major rellevància en aquestes (*hub genes*) i determinar quines funcions biològiques s'hi troben enriquides. En base aquests paràmetres es van identificar 23 *hub genes* (Figura 3, Treball IV i Figura 4, Treball IV).

Les cèl·lules cutànies i les cèl·lules del sistema nerviós presenten un origen embrionari comú. Aquest fet, juntament amb la idea que les malalties neurodegeneratives poden tenir un caràcter sistèmic en el que es veuen afectats altres teixits més enllà del sistema nerviós [269-271], ha propiciat l'ús de diversos models *in vitro*, generats a partir de biòpsies cutànies, per a estudiar la fisiopatologia de les malalties neurodegeneratives [272-276]. En particular, al ser cultius primaris generats a partir de cèl·lules dels pacients, mantenen l'efecte causat per l'envelliment, la interacció amb l'ambient i els factors genètics de risc del propi individu. Diferents treballs han identificat en fibroblast de pacients amb Parkinson, Alzheimer i Huntington, biomarcadors que poden ser útils tan per al diagnòstic, com per comprendre la fisiopatologia d'aquestes malalties [277-280].

Així doncs, els resultats obtinguts en el treball IV poden ser d'interès per comprendre quines són les funcions biològiques alterades, que fan als individus portadors de variants LOF de *MC1R* més susceptibles a desenvolupar malalties neurodegeneratives.

Entre els 23 *hub genes* identificats, els gens *(PRKAA1, CDK1, BUB1B, PCNA, RPA1* i *BRCA1*) estan implicats en el manteniment de l'homeòstasi cel·lular i la reparació del dany en l'ADN [281-285] i els gens (*GBAS* i *ICT1*) estan implicats en la regulació de la fosforilació oxidativa i la funció mitocondrial [286, 287]. Donat que la mitocòndria és una de les principal fonts de generació de ROS [288] i que un augment d'aquestes incrementa el dany en l'ADN i afecta directament a processos implicats en el manteniment de l'homeòstasi cel·lular [289, 290], l'alteració funcional identificada en aquestes cèl·lules correlaciona amb un augment de l'estrès oxidatiu. Aquest resultat coincideix amb l'augment del dany oxidatiu prèviament identificat en els ratolins portadors d'un MC1R no funcional ($Mc1r^{e/e}$) [237] i indica que la pèrdua de funció del MC1R pot contribuir a la neurodegeneració augmentant l'estrès oxidatiu en les cèl·lules del SNC.

De forma interessant, 12 dels gens que s'identifiquen com a *hub genes* en el model *in vitro* (*PIK3C3, ATG4C, ATG10, SNX2, TRAF2, SQSTM1, CLN3, WIPI2, GABARAPL1, GABARAPL2, MAP1LC3B* i *MAP1LC3A*) estan implicats en la regulació de l'autofàgia i 5 d'ells (*CLN3, ATG10, WIPI2, SNX2* i *GABARAPL2*) s'identifiquen diferencialment expressats també en les biòpsies de pell sana dels individus pèl-rojos (model *in vivo*). A més, en aquests biòpsies s'identifiquen diferencialment expressats altres gens implicats en l'autofàgia (*ATG12, ATG2A, ATG9A* i *ATG7*) (Treball IV, Taula suplementària 26). L'autofàgia és un procés catabòlic que permet a la cèl·lula degradar a través dels lisosomes els components innecessaris o no funcionals i perllongar la supervivència cel·lular en condicions d'estrès [291].

Una de les característiques comuns en la majoria de malalties neurodegeneratives, és l'acumulació anòmala de proteïnes (α-sinucleïna, Aβ, tau i Htt). La degradació d'aquests agregats a través del sistema ubiquitina-proteosoma és clau per a disminuir la toxicitat i l'estrès oxidatiu que té lloc en les cèl·lules on es dipositen aquests agregats de proteïnes neurotòxiques [292]. Nombrosos treballs han associat la presència d'aquests agregats a una disfunció del sistema ubiquitina-proteosoma en els pacients amb malalties neurodegeneratives [293-295]. Així doncs, una correcte autofàgia, procés secundari implicat en la degradació d'aquests agregats proteics, és clau per a disminuir l'estrès oxidatiu i impedir la neurodegeneració en els individus que presenten una alteració funcional del sistema ubiquitina-proteosoma [296]. No obstant, nombrosos treballs han reportat defectes en l'autofàgia en la majoria de casos de malalties neurodegeneratives [291, 297-300].

Recentment, un treball realitzat amb fibroblasts de pacients amb Parkinson ha identificat que aquests presenten un augment de les ROS, una disfunció mitocondrial i una desregulació de l'autofàgia respecte als dels individus control [301]. Per tant, aquest treball identifica alterats en aquests fibroblasts, processos biològics que es troben alterats en les neurones dels pacients amb Parkinson i confirma doncs el caràcter sistèmic d'aquesta malaltia. De forma interessant, els processos biològics alterats en els fibroblasts d'aquests pacients coincideixen amb els processos biològics identificats a partir de l'anàlisi PPI en les cèl·lules cutànies portadores d'un MC1R no funcional. Per tant, l'augment de l'estrès oxidatiu i l'alteració de l'autofàgia podrien explicar la susceptibilitat a desenvolupar malalties neurodegeneratives identificada en els individus portadors de variants al·lèliques *LOF* de MC1R.

.

CONCLUSIONS

1. Més enllà del seu rol en la pigmentació, el gen *MC1R* està implicat en la patogènesi de les malalties neurodegeneratives.

2. L'al·lel p.R160W de *MC1R* augmenta el risc a desenvolupar la malaltia de Parkinson a la població espanyola.

3. Les variants al·lèliques de pèrdua de funció de *MC1R* augmenten el risc a desenvolupar tant melanoma com Parkinson, fet que explica la relació epidemiològica bidireccional entre el risc a melanoma i el de Parkinson.

4. L'al·lel p.V92M de *MC1R* augmenta el risc a desenvolupar la malaltia d'Alzheimer a la població espanyola, especialment en aquells individus on el risc no pot atribuir-se a l'al·lel 4 de l'*APOE*.

5. L' al·lel p.R151C de *MC1R* disminueix l'edat d'aparició de la malaltia de Huntington a la població espanyola.

6. El gen *MC1R* no és un factor modificador de l'edat d'aparició de les formes esporàdiques de Parkinson i les formes esporàdiques d'Alzheimer.

7. La pèrdua de funció del MC1R pot contribuir a la patogènesi de les malalties neurodegeneratives a través d'un augment del dany oxidatiu, una disminució de la resposta antiinflamatòria i una desregulació de l'autofàgia.

BIBLIOGRAFIA

1. Chhajlani V, Wikberg JE. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett. 1992; 309: 417-20.

2. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science. 1992; 257: 1248-51.

3. Wolf Horrell EM, Boulanger MC, D'Orazio JA. Melanocortin 1 Receptor: Structure, Function, and Regulation. Front Genet. 2016; 7: 95.

4. Smith AG, Box NF, Marks LH, Chen W, Smit DJ, Wyeth JR, Huttley GA, Easteal S, Sturm RA. The human melanocortin-1 receptor locus: analysis of transcription unit, locus polymorphism and haplotype evolution. Gene. 2001; 281: 81-94.

5. Herraiz C, Garcia-Borron JC, Jimenez-Cervantes C, Olivares C. MC1R signaling. Intracellular partners and pathophysiological implications. Biochim Biophys Acta. 2017; 1863: 2448-61.

6. Herraiz C, Olivares C, Castejon-Grinan M, Abrisqueta M, Jimenez-Cervantes C, Garcia-Borron JC. Functional Characterization of MC1R-TUBB3 Intergenic Splice Variants of the Human Melanocortin 1 Receptor. PLoS One. 2015; 10: e0144757.

7. Garcia-Borron JC, Abdel-Malek Z, Jimenez-Cervantes C. MC1R, the cAMP pathway, and the response to solar UV: extending the horizon beyond pigmentation. Pigment Cell Melanoma Res. 2014; 27: 699-720.

8. Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ. Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol. 2011; 87: 978-87.

9. Gerstenblith MR, Goldstein AM, Fargnoli MC, Peris K, Landi MT. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat. 2007; 28: 495-505.

10. Naysmith L, Waterston K, Ha T, Flanagan N, Bisset Y, Ray A, Wakamatsu K, Ito S, Rees JL. Quantitative measures of the effect of the melanocortin 1 receptor on human pigmentary status. J Invest Dermatol. 2004; 122: 423-8.

11. Beaumont KA, Shekar SN, Newton RA, James MR, Stow JL, Duffy DL, Sturm RA. Receptor function, dominant negative activity and phenotype correlations for MC1R variant alleles. Hum Mol Genet. 2007; 16: 2249-60.

12. Newton RA, Roberts DW, Leonard JH, Sturm RA. Human melanocytes expressing MC1R variant alleles show impaired activation of multiple signaling pathways. Peptides. 2007; 28: 2387-96.

13. Scott MC, Wakamatsu K, Ito S, Kadekaro AL, Kobayashi N, Groden J, Kavanagh R, Takakuwa T, Virador V, Hearing VJ, Abdel-Malek ZA. Human melanocortin 1 receptor variants, receptor function and melanocyte response to UV radiation. J Cell Sci. 2002; 115: 2349-55.

14. Ito S, Wakamatsu K. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. 2003; 16: 523-31.

15. Barsh GS. What controls variation in human skin color? PLoS Biol. 2003; 1: E27.

16. Jimbow K, Quevedo WC, Jr., Fitzpatrick TB, Szabo G. Some aspects of melanin biology: 1950-1975. J Invest Dermatol. 1976; 67: 72-89.

17. Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. Postepy Dermatol Alergol. 2013; 30: 30-41.

18. http:// droualb.faculty.mjc.edu - Chapter 4 - Integumentary System.

19. Liu Y, Hong L, Wakamatsu K, Ito S, Adhyaru B, Cheng CY, Bowers CR, Simon JD. Comparison of structural and chemical properties of black and red human hair melanosomes. Photochem Photobiol. 2005; 81: 135-44.

20. Resistance to Targeted Anti-Cancer Therapeutics - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/11-Process-of-melanogenesis-and-resistance-mechanisms-of-melanoma-Melanosomes-mature_fig1_278679964.

21. Oren M, Bartek J. The sunny side of p53. Cell. 2007; 128: 826-8.

22. van Schanke A, Jongsma MJ, Bisschop R, van Venrooij GM, Rebel H, de Gruijl FR. Single UVB overexposure stimulates melanocyte proliferation in murine skin, in contrast to fractionated or UVA-1 exposure. J Invest Dermatol. 2005; 124: 241-7.

23. Walker GJ, Kimlin MG, Hacker E, Ravishankar S, Muller HK, Beermann F, Hayward NK. Murine neonatal melanocytes exhibit a heightened proliferative response to ultraviolet radiation and migrate to the epidermal basal layer. J Invest Dermatol. 2009; 129: 184-93.

24. Sturm RA, Duffy DL. Human pigmentation genes under environmental selection. Genome Biol. 2012; 13: 248.

25. Nasti TH, Timares L. MC1R, eumelanin and pheomelanin: their role in determining the susceptibility to skin cancer. Photochem Photobiol. 2015; 91: 188-200.

26. Rees JL, Harding RM. Understanding the evolution of human pigmentation: recent contributions from population genetics. J Invest Dermatol. 2012; 132: 846-53.

27. Rees JL. Genetics of hair and skin color. Annu Rev Genet. 2003; 37: 67-90.

28. Raimondi S, Sera F, Gandini S, Iodice S, Caini S, Maisonneuve P, Fargnoli MC. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer. 2008; 122: 2753-60.

29. Sturm RA. Skin colour and skin cancer - MC1R, the genetic link. Melanoma Res. 2002; 12: 405-16.

30. Garcia-Borron JC, Sanchez-Laorden BL, Jimenez-Cervantes C. Melanocortin-1 receptor structure and functional regulation. Pigment Cell Res. 2005; 18: 393-410.

31. Scherer D, Bermejo JL, Rudnai P, Gurzau E, Koppova K, Hemminki K, Kumar R. MC1R variants associated susceptibility to basal cell carcinoma of skin: interaction with host factors and XRCC3 polymorphism. Int J Cancer. 2008; 122: 1787-93.

32. Andresen PA, Nymoen DA, Kjaerheim K, Leivestad T, Helsing P. Susceptibility to cutaneous squamous cell carcinoma in renal transplant recipients associates with genes regulating melanogenesis independent of their role in pigmentation. Biomark Cancer. 2013; 5: 41-7.

33. http://www.mdpi.com/2079-9284/3/2/18/htm.

34. Bertolotto C. Melanoma: from melanocyte to genetic alterations and clinical options. Scientifica (Cairo). 2013; 2013: 635203.

35. Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, Herlyn M, Marchetti MA, McArthur G, Ribas A, Roesch A, Hauschild A. Melanoma. Nat Rev Dis Primers. 2015; 1: 15003.

36. Ossio R, Roldan-Marin R, Martinez-Said H, Adams DJ, Robles-Espinoza CD. Melanoma: a global perspective. Nat Rev Cancer. 2017; 17: 393-4.

37. Schadendorf D, Hauschild A. Melanoma in 2013: Melanoma--the run of success continues. Nat Rev Clin Oncol. 2014; 11: 75-6.

38. https://seer.cancer.gov/statfacts/html/melan.html.

39. Potrony M, Badenas C, Aguilera P, Puig-Butille JA, Carrera C, Malvehy J, Puig S. Update in genetic susceptibility in melanoma. Ann Transl Med. 2015; 3: 210.

40. Whiteman DC, Whiteman CA, Green AC. Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. Cancer Causes Control. 2001; 12: 69-82.

41. Gandini S, Sera F, Cattaruzza MS, Pasquini P, Picconi O, Boyle P, Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. Eur J Cancer. 2005; 41: 45-60.

42. Kanavy HE, Gerstenblith MR. Ultraviolet radiation and melanoma. Semin Cutan Med Surg. 2011; 30: 222-8.

43. Dennis LK, Vanbeek MJ, Beane Freeman LE, Smith BJ, Dawson DV, Coughlin JA. Sunburns and risk of cutaneous melanoma: does age matter? A comprehensive meta-analysis. Ann Epidemiol. 2008; 18: 614-27.

44. Premi S, Wallisch S, Mano CM, Weiner AB, Bacchiocchi A, Wakamatsu K, Bechara EJ, Halaban R, Douki T, Brash DE. Photochemistry. Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure. Science. 2015; 347: 842-7.

45. Amaro-Ortiz A, Yan B, D'Orazio JA. Ultraviolet radiation, aging and the skin: prevention of damage by topical cAMP manipulation. Molecules. 2014; 19: 6202-19.

46. Beani JC. [Ultraviolet A-induced DNA damage: role in skin cancer]. Bull Acad Natl Med. 2014; 198: 273-95.

47. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol. 1988; 124: 869-71.

48. Gandini S, Sera F, Cattaruzza MS, Pasquini P, Abeni D, Boyle P, Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. Eur J Cancer. 2005; 41: 28-44.

49. Gandini S, Sera F, Cattaruzza MS, Pasquini P, Zanetti R, Masini C, Boyle P, Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. Eur J Cancer. 2005; 41: 2040-59.

50. Mucci LA, Hjelmborg JB, Harris JR, Czene K, Havelick DJ, Scheike T, Graff RE, Holst K, Moller S, Unger RH, McIntosh C, Nuttall E, Brandt I, et al. Familial Risk and Heritability of Cancer Among Twins in Nordic Countries. JAMA. 2016; 315: 68-76.

51. Florell SR, Boucher KM, Garibotti G, Astle J, Kerber R, Mineau G, Wiggins C, Noyes RD, Tsodikov A, Cannon-Albright LA, Zone JJ, Samlowski WE, Leachman SA. Population-based analysis of prognostic factors and survival in familial melanoma. J Clin Oncol. 2005; 23: 7168-77.

52. Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, Azon A, Gonzalez U, Castel T, Campoy A, Herrero J, Marti R, Brunet-Vidal J, et al. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol. 2005; 23: 3043-51.

53. Goldstein AM, Chan M, Harland M, Gillanders EM, Hayward NK, Avril MF, Azizi E, Bianchi-Scarra G, Bishop DT, Bressac-de Paillerets B, Bruno W, Calista D, Cannon Albright LA, et al. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. Cancer Res. 2006; 66: 9818-28.

54. Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Albright LA, Chaudru V, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet. 2007; 44: 99-106.

55. Read J, Wadt KA, Hayward NK. Melanoma genetics. J Med Genet. 2016; 53: 1-14.

56. Tsao H, Chin L, Garraway LA, Fisher DE. Melanoma: from mutations to medicine. Genes Dev. 2012; 26: 1131-55.

57. Ward KA, Lazovich D, Hordinsky MK. Germline melanoma susceptibility and prognostic genes: a review of the literature. J Am Acad Dermatol. 2012; 67: 1055-67.

58. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, McCarthy MI, Ramos EM, Cardon LR, Chakravarti A, Cho JH, Guttmacher AE, Kong A, et al. Finding the missing heritability of complex diseases. Nature. 2009; 461: 747-53.

59. Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA, Hayward NK. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 2001; 69: 765-73.

60. Demenais F, Mohamdi H, Chaudru V, Goldstein AM, Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, et al. Association of MC1R variants and host phenotypes with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010; 102: 1568-83.

61. Williams PF, Olsen CM, Hayward NK, Whiteman DC. Melanocortin 1 receptor and risk of cutaneous melanoma: a meta-analysis and estimates of population burden. Int J Cancer. 2011; 129: 1730-40.

62. Tagliabue E, Fargnoli MC, Gandini S, Maisonneuve P, Liu F, Kayser M, Nijsten T, Han J, Kumar R, Gruis NA, Ferrucci L, Branicki W, Dwyer T, et al. MC1R gene variants and non-melanoma skin cancer: a pooled-analysis from the M-SKIP project. Br J Cancer. 2015; 113: 354-63.

63. Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, Bavinck JN, Leiden Skin Cancer Study T. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001; 68: 884-94.

64. Pasquali E, Garcia-Borron JC, Fargnoli MC, Gandini S, Maisonneuve P, Bagnardi V, Specchia C, Liu F, Kayser M, Nijsten T, Nagore E, Kumar R, Hansson J, et al. MC1R variants increased the risk of sporadic cutaneous melanoma in darker-pigmented Caucasians: a pooled-analysis from the M-SKIP project. Int J Cancer. 2015; 136: 618-31.

65. Cao J, Wan L, Hacker E, Dai X, Lenna S, Jimenez-Cervantes C, Wang Y, Leslie NR, Xu GX, Widlund HR, Ryu B, Alani RM, Dutton-Regester K, et al. MC1R is a potent regulator of PTEN after UV exposure in melanocytes. Mol Cell. 2013; 51: 409-22.

66. Kadekaro AL, Kavanagh R, Kanto H, Terzieva S, Hauser J, Kobayashi N, Schwemberger S, Cornelius J, Babcock G, Shertzer HG, Scott G, Abdel-Malek ZA. alpha-Melanocortin and endothelin-1 activate antiapoptotic pathways and reduce DNA damage in human melanocytes. Cancer Res. 2005; 65: 4292-9.

67. Abdel-Malek ZA, Knittel J, Kadekaro AL, Swope VB, Starner R. The melanocortin 1 receptor and the UV response of human melanocytes--a shift in paradigm. Photochem Photobiol. 2008; 84: 501-8.

68. Phin S, Moore MW, Cotter PD. Genomic rearrangements of PTEN in prostate cancer. Front Oncol. 2013; 3: 240.

69. http://terrymcgarty.blogspot.com.es/2012/12/mitf-and-melanoma-targeting-specific.html.

70. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491: 449-53.

71. Morgan AM, Lo J, Fisher DE. How does pheomelanin synthesis contribute to melanomagenesis?: Two distinct mechanisms could explain the carcinogenicity of pheomelanin synthesis. Bioessays. 2013; 35: 672-6.

72. Bustamante J, Bredeston L, Malanga G, Mordoh J. Role of melanin as a scavenger of active oxygen species. Pigment Cell Res. 1993; 6: 348-53.

73. Brash DE. UV-induced melanin chemiexcitation: a new mode of melanoma pathogenesis. Toxicol Pathol. 2016; 44: 552-4.

74. Abdel-Malek ZA, Cassidy P. Dark CPDs and photocarcinogenesis: the party continues after the lights go out. Pigment Cell Melanoma Res. 2015; 28: 373-4.

75. Robles-Espinoza CD, Roberts ND, Chen S, Leacy FP, Alexandrov LB, Pornputtapong N, Halaban R, Krauthammer M, Cui R, Timothy Bishop D, Adams DJ. Germline MC1R status influences somatic mutation burden in melanoma. Nat Commun. 2016; 7: 12064.

76. Puig-Butille JA, Escamez MJ, Garcia-Garcia F, Tell-Marti G, Fabra A, Martinez-Santamaria L, Badenas C, Aguilera P, Pevida M, Dopazo J, del Rio M, Puig S. Capturing the biological impact of CDKN2A and MC1R genes as an early predisposing event in melanoma and non melanoma skin cancer. Oncotarget. 2014; 5: 1439-51.

77. Raposo G, Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic. 2002; 3: 237-48.

78. Raposo G, Marks MS. Melanosomes--dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol. 2007; 8: 786-97.

79. Xia Y, Wikberg JE, Chhajlani V. Expression of melanocortin 1 receptor in periaqueductal gray matter. Neuroreport. 1995; 6: 2193-6.

80. Wong KY, Rajora N, Boccoli G, Catania A, Lipton JM. A potential mechanism of local anti-inflammatory action of alpha-melanocyte-stimulating hormone within the brain: modulation of tumor necrosis factor-alpha production by human astrocytic cells. Neuroimmunomodulation. 1997; 4: 37-41.

81. Teare KA, Pearson RG, Shakesheff KM, Haycock JW. Alpha-MSH inhibits inflammatory signalling in Schwann cells. Neuroreport. 2004; 15: 493-8.

82. Christiansen JH, Coles EG, Wilkinson DG. Molecular control of neural crest formation, migration and differentiation. Curr Opin Cell Biol. 2000; 12: 719-24.

83. Mogil JS, Bailey AL. Sex and gender differences in pain and analgesia. Prog Brain Res. 2010; 186: 141-57.

84. Chartoff EH, Mavrikaki M. Sex Differences in Kappa Opioid Receptor Function and Their Potential Impact on Addiction. Front Neurosci. 2015; 9: 466.

85. Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KV, Lariviere WR, Groce MK, Wallace MR, Kaplan L, Staud R, Ness TJ, Glover TL, Stankova M, et al. The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. Proc Natl Acad Sci USA. 2003; 100: 4867-72.

86. Chen J, Zhang R, Chen X, Wang C, Cai X, Liu H, Jiang Y, Liu C, Bai B. Heterodimerization of human orexin receptor 1 and kappa opioid receptor promotes protein kinase A/cAMP-response element binding protein signaling via a Galphas-mediated mechanism. Cell Signal. 2015; 27: 1426-38.

87. Caruso V, Lagerstrom MC, Olszewski PK, Fredriksson R, Schioth HB. Synaptic changes induced by melanocortin signalling. Nat Rev Neurosci. 2014; 15: 98-110.

88. Randall CL, McNeil DW, Shaffer JR, Crout RJ, Weyant RJ, Marazita ML. Fear of pain mediates the association between MC1R genotype and dental fear. J Dent Res. 2016; 95: 1132-7.

89. Liem EB, Lin CM, Suleman MI, Doufas AG, Gregg RG, Veauthier JM, Loyd G, Sessler DI. Anesthetic requirement is increased in redheads. Anesthesiology. 2004; 101: 279-83.

90. de Angelis E, Sahm UG, Ahmed AR, Olivier GW, Notarianni LJ, Branch SK, Moss SH, Pouton CW. Identification of a melanocortin receptor expressed by murine brain microvascular endothelial cells in culture. Microvasc Res. 1995; 50: 25-34.

91. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 2008; 31: 353-60.

92. Parkinson J. An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci. 2002; 14: 223-36; discussion 222.

93. Lewy FH. Paralysis agitans. I. Pathologische Anatomie. Lewandowsky’s Handbuch der Neurologie, 3 Band: Spez Neurologie II. Berlin: Springer; 1912. p 920–933.

94. Tretiakoff (1919) Contribution a l’étude de l’anatomie pathologique de locus niger de soemmerling. Université de Paris, Paris.

95. http://www.sci-news.com/medicine/stem-cell-transplants-recovery-parkinsons-disease-03932.html.

96. Tanner CM, Goldman SM. Epidemiology of Parkinson's disease. Neurol Clin. 1996; 14: 317-35.

97. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE. Parkinson disease. Nat Rev Dis Primers. 2017; 3: 17013.

98. Beitz JM. Parkinson's disease: a review. Front Biosci (Schol Ed). 2014; 6: 65-74.

99. Lee A, Gilbert RM. Epidemiology of Parkinson Disease. Neurol Clin. 2016; 34: 955-65.

100. Twelves D, Perkins KS, Counsell C. Systematic review of incidence studies of Parkinson's disease. Mov Disord. 2003; 18: 19-31.

101. Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, Nelson LM. Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. Am J Epidemiol. 2003; 157: 1015-22.

102. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. Lancet Neurol. 2006; 5: 525-35.

103. Nussbaum RL, Ellis CE. Alzheimer's disease and Parkinson's disease. N Engl J Med. 2003; 348: 1356-64.

104. Baldereschi M, Di Carlo A, Rocca WA, Vanni P, Maggi S, Perissinotto E, Grigoletto F, Amaducci L, Inzitari D. Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging. Neurology. 2000; 55: 1358-63.

105. Caudle WM, Guillot TS, Lazo CR, Miller GW. Industrial toxicants and Parkinson's disease. Neurotoxicology. 2012; 33: 178-88.

106. Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Richardson RJ. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology. 1998; 50: 1346-50.

107. Park M, Ross GW, Petrovitch H, White LR, Masaki KH, Nelson JS, Tanner CM, Curb JD, Blanchette PL, Abbott RD. Consumption of milk and calcium in midlife and the future risk of Parkinson disease. Neurology. 2005; 64: 1047-51.

108. Ross GW, Abbott RD, Petrovitch H, Morens DM, Grandinetti A, Tung KH, Tanner CM, Masaki KH, Blanchette PL, Curb JD, Popper JS, White LR. Association of coffee and caffeine intake with the risk of Parkinson disease. JAMA. 2000; 283: 2674-9.

109. Chapman MA. Does smoking reduce the risk of Parkinson's disease through stimulation of the ubiquitin-proteasome system? Med Hypotheses. 2009; 73: 887-91.

110. Dahodwala N, Siderowf A, Xie M, Noll E, Stern M, Mandell DS. Racial differences in the diagnosis of Parkinson's disease. Mov Disord. 2009; 24: 1200-5.

111. Rybicki BA, Johnson CC, Peterson EL, Kortsha GX, Gorell JM. A family history of Parkinson's disease and its effect on other PD risk factors. Neuroepidemiology. 1999; 18: 270-8.

112. Hamza TH, Payami H. The heritability of risk and age at onset of Parkinson's disease after accounting for known genetic risk factors. J Hum Genet. 2010; 55: 241-3.

113. Lesage S, Brice A. Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum Mol Genet. 2009; 18: R48-59.

114. Kalinderi K, Bostantjopoulou S, Fidani L. The genetic background of Parkinson's disease: current progress and future prospects. Acta Neurol Scand. 2016; 134: 314-26.

115. He X, Zhang Y, Chen J, Xie C, Gan R, Yang R, Wang L, Nie K, Wang L. The patterns of EEG changes in early-onset Parkinson's disease patients. Int J Neurosci. 2017; 127: 1028-35.

116. Lill CM. Genetics of Parkinson's disease. Mol Cell Probes. 2016; 30: 386-96.

117. Davis AA, Andruska KM, Benitez BA, Racette BA, Perlmutter JS, Cruchaga C. Variants in GBA, SNCA, and MAPT influence Parkinson disease risk, age at onset, and progression. Neurobiol Aging. 2016; 37: 209.e1-209.e7.

118. Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, DeStefano AL, Kara E, Bras J, Sharma M, Schulte C, Keller MF, Arepalli S, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat Genet. 2014; 46: 989-93.

119. Hill-Burns EM, Ross OA, Wissemann WT, Soto-Ortolaza AI, Zareparsi S, Siuda J, Lynch T, Wszolek ZK, Silburn PA, Mellick GD, Ritz B, Scherzer CR, Zabetian CP, et al. Identification of genetic modifiers of age-at-onset for familial Parkinson's disease. Hum Mol Genet. 2016; 25: 3849-62.

120. Diamond SG, Markham CH, Hoehn MM, McDowell FH, Muenter MD. Effect of age at onset on progression and mortality in Parkinson's disease. Neurology. 1989; 39: 1187-90.

121. Brockmann K, Schulte C, Hauser AK, Lichtner P, Huber H, Maetzler W, Berg D, Gasser T. SNCA: major genetic modifier of age at onset of Parkinson's disease. Mov Disord. 2013; 28: 1217-21.

122. Botta-Orfila T, Ezquerra M, Pastor P, Fernandez-Santiago R, Pont-Sunyer C, Compta Y, Lorenzo-Betancor O, Samaranch L, Marti MJ, Valldeoriola F, Calopa M, Fernandez M, Aguilar M, et al. Age at onset in LRRK2-associated PD is modified by SNCA variants. J Mol Neurosci. 2012; 48: 245-7.

123. Gan-Or Z, Bar-Shira A, Mirelman A, Gurevich T, Giladi N, Orr-Urtreger A. The age at motor symptoms onset in LRRK2-associated Parkinson's disease is affected by a variation in the MAPT locus: a possible interaction. J Mol Neurosci. 2012; 46: 541-4.

124. Trinh J, Gustavsson EK, Vilarino-Guell C, Bortnick S, Latourelle J, McKenzie MB, Tu CS, Nosova E, Khinda J, Milnerwood A, Lesage S, Brice A, Tazir M, et al. DNM3 and genetic modifiers of age of onset in LRRK2 Gly2019Ser parkinsonism: a genome-wide linkage and association study. Lancet Neurol. 2016; 15: 1248-56.

125. Nalls MA, Escott-Price V, Williams NM, Lubbe S, Keller MF, Morris HR, Singleton AB, International Parkinson's Disease Genomics C. Genetic risk and age in Parkinson's disease: Continuum not stratum. Mov Disord. 2015; 30: 850-4.

126. Alzheimer A. Über einen eigenartigen schweren Erkrankungsprozeβ der Hirnrincle. Neurol Central. 1906; 25:1134.

127. Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol. 2010; 23: 213-27.

128. Anoop A, Singh PK, Jacob RS, Maji SK. CSF Biomarkers for Alzheimer's Disease Diagnosis. Int J Alzheimers Dis. 2010; pii: 606802.

129. Corrada MM, Brookmeyer R, Paganini-Hill A, Berlau D, Kawas CH. Dementia incidence continues to increase with age in the oldest old: the 90+ study. Ann Neurol. 2010; 67: 114-21.

130. Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. Alzheimer's disease. Nat Rev Dis Primers. 2015; 1: 15056.

131. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984; 34: 939-44.

132. Blennow K, Dubois B, Fagan AM, Lewczuk P, de Leon MJ, Hampel H. Clinical utility of cerebrospinal fluid biomarkers in the diagnosis of early Alzheimer's disease. Alzheimers Dement (Amst). 2015; 11: 58-69.

133. Forlenza OV, Radanovic M, Talib LL, Aprahamian I, Diniz BS, Zetterberg H, Gattaz WF. Cerebrospinal fluid biomarkers in Alzheimer's disease: Diagnostic accuracy and prediction of dementia. Alzheimers Dement (Amst). 2015; 1: 455-63.

134. Welge V, Fiege O, Lewczuk P, Mollenhauer B, Esselmann H, Klafki HW, Wolf S, Trenkwalder C, Otto M, Kornhuber J, Wiltfang J, Bibl M. Combined CSF tau, p-tau181 and amyloid-beta 38/40/42 for diagnosing Alzheimer's disease. J Neural Transm (Vienna). 2009; 116: 203-12.

135. Beach TG, Monsell SE, Phillips LE, Kukull W. Accuracy of the clinical diagnosis of Alzheimer disease at National Institute on Aging Alzheimer Disease Centers, 2005-2010. J Neuropathol Exp Neurol. 2012; 71: 266-73.

136. Molinuevo JL, Gispert JD, Dubois B, Heneka MT, Lleo A, Engelborghs S, Pujol J, de Souza LC, Alcolea D, Jessen F, Sarazin M, Lamari F, Balasa M, et al. The AD-CSF-index discriminates Alzheimer's disease patients from healthy controls: a validation study. J Alzheimers Dis. 2013; 36: 67-77.

137. Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, Kimmel LH, Bergeson J, Manetti GJ, Zimmermann M, Tang B, Bartko JJ, Cohen RM. Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. JAMA. 2003; 289: 2094-103.

138. Riedel BC, Thompson PM, Brinton RD. Age, APOE and sex: Triad of risk of Alzheimer's disease. J Steroid Biochem Mol Biol. 2016; 160: 134-47.

139. Norton S, Matthews FE, Barnes DE, Yaffe K, Brayne C. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. Lancet Neurol. 2014; 13: 788-94.

140. Reitz C, Mayeux R. Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem Pharmacol. 2014; 88: 640-51.

141. Van Cauwenberghe C, Van Broeckhoven C, Sleegers K. The genetic landscape of Alzheimer disease: clinical implications and perspectives. Genet Med. 2016; 18: 421-30.

142. Gatz M, Reynolds CA, Fratiglioni L, Johansson B, Mortimer JA, Berg S, Fiske A, Pedersen NL. Role of genes and environments for explaining Alzheimer disease. Arch Gen Psychiatry. 2006; 63: 168-74.

143. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993; 261: 921-3.

144. Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA. 1997; 278: 1349-56.

145. Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV. apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain. J Clin Invest. 2008; 118: 4002-13.

146. Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, DeStafano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thorton-Wells TA, Jones N, et al. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat Genet. 2013; 45: 1452-8.

147. Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, et al.

Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet. 2009; 41: 1094-9.

148. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet. 2009; 41: 1088-93.

149. Seshadri S, Fitzpatrick AL, Ikram MA, DeStefano AL, Gudnason V, Boada M, Bis JC, Smith AV, Carassquillo MM, Lambert JC, Harold D, Schrijvers EM, Ramirez-Lorca R, et al. Genome-wide analysis of genetic loci associated with Alzheimer disease. JAMA. 2010; 303: 1832-40.

150. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, Gallins PJ, Buxbaum JD, Jarvik GP, Crane PK, Larson EB, Bird TD, Boeve BF, et al. Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet. 2011; 43: 436-41.

151. Hollingworth P, Harold D, Sims R, Gerrish A, Lambert JC, Carrasquillo MM, Abraham R, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Jones N, Stretton A, et al. Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. Nat Genet. 2011; 43: 429-35.

152. Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, Cruchaga C, Sassi C, Kauwe JS, Younkin S, Hazrati L, Collinge J, Pocock J, et al. TREM2 variants in Alzheimer's disease. N Engl J Med. 2013; 368: 117-27.

153. Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, et al. Variant of TREM2 associated with the risk of Alzheimer's disease. N Engl J Med. 2013; 368: 107-16.

154. Sims R, Williams J. Defining the genetic architecture of Alzheimer's disease: where next. Neurodegener Dis. 2016; 16: 6-11.

155. Naj AC, Jun G, Reitz C, Kunkle BW, Perry W, Park YS, Beecham GW, Rajbhandary RA, Hamilton-Nelson KL, Wang LS, Kauwe JS, Huentelman MJ,

Myers AJ, et al. Effects of multiple genetic loci on age at onset in late-onset Alzheimer disease: a genome-wide association study. JAMA Neurol. 2014; 71: 1394-404.

156. Velez JI, Lopera F, Patel HR, Johar AS, Cai Y, Rivera D, Tobon C, Villegas A, Sepulveda-Falla D, Lehmann SG, Easteal S, Mastronardi CA, Arcos-Burgos M. Mutations modifying sporadic Alzheimer's disease age of onset. Am J Med Genet B Neuropsychiatr Genet. 2016; 171: 1116-30.

157. Blue EE, Yu CE, Thornton TA, Chapman NH, Kernfeld E, Jiang N, Shively KM, Buckingham KJ, Marvin CT, Bamshad MJ, Bird TD, Wijsman EM. Variants regulating ZBTB4 are associated with age-at-onset of Alzheimer's disease. Genes Brain Behav. 2017.

158. Lambert JC, Sleegers K, Gonzalez-Perez A, Ingelsson M, Beecham GW, Hiltunen M, Combarros O, Bullido MJ, Brouwers N, Bettens K, Berr C, Pasquier F, Richard F, et al. The CALHM1 P86L polymorphism is a genetic modifier of age at onset in Alzheimer's disease: a meta-analysis study. J Alzheimers Dis. 2010; 22: 247-55.

159. Khachaturian AS, Corcoran CD, Mayer LS, Zandi PP, Breitner JC, Cache County Study I. Apolipoprotein E epsilon4 count affects age at onset of Alzheimer disease, but not lifetime susceptibility: The Cache County Study. Arch Gen Psychiatry. 2004; 61: 518-24.

160. Thambisetty M, An Y, Tanaka T. Alzheimer's disease risk genes and the age-at-onset phenotype. Neurobiol Aging. 2013; 34: 2696 e1-5.

161. Huntington, G. 1872. On chorea. Med. Surg. Rep. 26: 320–321.

162. Morrison PJ, Harding-Lester S, Bradley A. Uptake of Huntington disease predictive testing in a complete population. Clin Genet. 2011; 80: 281-6.

163. Ross CA, Tabrizi SJ. Huntington's disease: from molecular pathogenesis to clinical treatment. Lancet Neurol. 2011; 10: 83-98.

164. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell. 1993; 72: 971-83.

165. Bates GP, Dorsey R, Gusella JF, Hayden MR, Kay C, Leavitt BR, Nance M, Ross CA, Scahill RI, Wetzel R, Wild EJ, Tabrizi SJ. Huntington disease. Nat Rev Dis Primers. 2015; 1: 15005.

166. https://neuroscienceireland.com/the-huntingtons-disease-drug-trial-why-all-the-hope.

167. Roos RA. Huntington's disease: a clinical review. Orphanet J Rare Dis. 2010; 5: 40.

168. Semaka A, Creighton S, Warby S, Hayden MR. Predictive testing for Huntington disease: interpretation and significance of intermediate alleles. Clin Genet. 2006; 70: 283-94.

169. Keum JW, Shin A, Gillis T, Mysore JS, Abu Elneel K, Lucente D, Hadzi T, Holmans P, Jones L, Orth M, Kwak S, MacDonald ME, Gusella JF, et al. The HTT CAG-Expansion Mutation Determines Age at Death but Not Disease Duration in Huntington Disease. Am J Hum Genet. 2016; 98: 287-98.

170. Hendricks AE, Latourelle JC, Lunetta KL, Cupples LA, Wheeler V, MacDonald ME, Gusella JF, Myers RH. Estimating the probability of *de novo* HD cases from transmissions of expanded penetrant CAG alleles in the Huntington disease gene from male carriers of high normal alleles (27-35 CAG). Am J Med Genet A. 2009; 149A: 1375-81.

171. Govert F, Schneider SA. Huntington's disease and Huntington's disease-like syndromes: an overview. Curr Opin Neurol. 2013; 26: 420-7.

172. Tsai YC, Metzger S, Riess O, Soehn AS, Nguyen HP. Genetic analysis of polymorphisms in the kalirin gene for association with age-at-onset in European Huntington disease patients. BMC Med Genet. 2012; 13: 48.

173. Lee JM, Ramos EM, Lee JH, Gillis T, Mysore JS, Hayden MR, Warby SC, Morrison P, Nance M, Ross CA, Margolis RL, Squitieri F, Orobello S, et al. CAG repeat expansion in Huntington disease determines age at onset in a fully dominant fashion. Neurology. 2012; 78: 690-5.

174. Andresen JM, Gayan J, Djousse L, Roberts S, Brocklebank D, Cherny SS, Group US-VCR, Group HMCR, Cardon LR, Gusella JF, MacDonald ME, Myers

RH, Housman DE, et al. The relationship between CAG repeat length and age of onset differs for Huntington's disease patients with juvenile onset or adult onset. Ann Hum Genet. 2007; 71: 295-301.

175. Gusella JF, MacDonald ME, Lee JM. Genetic modifiers of Huntington's disease. Mov Disord. 2014; 29: 1359-65.

176. Wexler NS, Lorimer J, Porter J, Gomez F, Moskowitz C, Shackell E, Marder K, Penchaszadeh G, Roberts SA, Gayan J, Brocklebank D, Cherny SS, Cardon LR, et al. Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. Proc Natl Acad Sci USA. 2004; 101: 3498-503.

177. Li JL, Hayden MR, Almqvist EW, Brinkman RR, Durr A, Dode C, Morrison PJ, Suchowersky O, Ross CA, Margolis RL, Rosenblatt A, Gomez-Tortosa E, Cabrero DM, et al. A genome scan for modifiers of age at onset in Huntington disease: The HD MAPS study. Am J Hum Genet. 2003; 73: 682-7.

178. van Dellen A, Hannan AJ. Genetic and environmental factors in the pathogenesis of Huntington's disease. Neurogenetics. 2004; 5: 9-17.

179. Georgiou N, Bradshaw JL, Chiu E, Tudor A, O'Gorman L, Phillips JG. Differential clinical and motor control function in a pair of monozygotic twins with Huntington's disease. Mov Disord. 1999; 14: 320-5.

180. Arning L, Monte D, Hansen W, Wieczorek S, Jagiello P, Akkad DA, Andrich J, Kraus PH, Saft C, Epplen JT. ASK1 and MAP2K6 as modifiers of age at onset in Huntington's disease. J Mol Med (Berl). 2008; 86: 485-90.

181. Taherzadeh-Fard E, Saft C, Andrich J, Wieczorek S, Arning L. PGC-1alpha as modifier of onset age in Huntington disease. Mol Neurodegener. 2009; 4: 10.

182. Metzger S, Rong J, Nguyen HP, Cape A, Tomiuk J, Soehn AS, Propping P, Freudenberg-Hua Y, Freudenberg J, Tong L, Li SH, Li XJ, Riess O. Huntingtin-associated protein-1 is a modifier of the age-at-onset of Huntington's disease. Hum Mol Genet. 2008; 17: 1137-46.

183. Dhaenens CM, Burnouf S, Simonin C, Van Brussel E, Duhamel A, Defebvre L, Duru C, Vuillaume I, Cazeneuve C, Charles P, Maison P, Debruxelles S, Verny

C, et al. A genetic variation in the ADORA2A gene modifies age at onset in Huntington's disease. Neurobiol Dis. 2009; 35: 474-6.

184. Arning L, Saft C, Wieczorek S, Andrich J, Kraus PH, Epplen JT. NR2A and NR2B receptor gene variations modify age at onset in Huntington disease in a sex-specific manner. Hum Genet. 2007; 122: 175-82.

185. Metzger S, Saukko M, Van Che H, Tong L, Puder Y, Riess O, Nguyen HP. Age at onset in Huntington's disease is modified by the autophagy pathway: implication of the V471A polymorphism in Atg7. Hum Genet. 2010; 128: 453-9.

186. Metzger S, Bauer P, Tomiuk J, Laccone F, Didonato S, Gellera C, Soliveri P, Lange HW, Weirich-Schwaiger H, Wenning GK, Melegh B, Havasi V, Baliko L, et al. The S18Y polymorphism in the UCHL1 gene is a genetic modifier in Huntington's disease. Neurogenetics. 2006; 7: 27-30.

187. Pinto RM, Dragileva E, Kirby A, Lloret A, Lopez E, St Claire J, Panigrahi GB, Hou C, Holloway K, Gillis T, Guide JR, Cohen PE, Li GM, et al. Mismatch repair genes Mlh1 and Mlh3 modify CAG instability in Huntington's disease mice: genome-wide and candidate approaches. PLoS Genet. 2013; 9: e1003930.

188. Tabares-Seisdedos R, Rubenstein JL. Inverse cancer comorbidity: a serendipitous opportunity to gain insight into CNS disorders. Nat Rev Neurosci. 2013; 14: 293-304.

189. Driver JA. Inverse association between cancer and neurodegenerative disease: review of the epidemiologic and biological evidence. Biogerontology. 2014; 15: 547-57.

190. Driver JA, Beiser A, Au R, Kreger BE, Splansky GL, Kurth T, Kiel DP, Lu KP, Seshadri S, Wolf PA. Inverse association between cancer and Alzheimer's disease: results from the Framingham Heart Study. BMJ. 2012; 344: e1442.

191. Driver JA, Kurth T, Buring JE, Gaziano JM, Logroscino G. Prospective case-control study of nonfatal cancer preceding the diagnosis of Parkinson's disease. Cancer Causes Control. 2007; 18: 705-11.

192. Ferreira JJ, Neutel D, Mestre T, Coelho M, Rosa MM, Rascol O, Sampaio C. Skin cancer and Parkinson's disease. Mov Disord. 2010; 25: 139-48.

193. Liu R, Gao X, Lu Y, Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology. 2011; 76: 2002-9.

194. Bajaj A, Driver JA, Schernhammer ES. Parkinson's disease and cancer risk: a systematic review and meta-analysis. Cancer Causes Control. 2010; 21: 697-707.

195. Olsen JH, Friis S, Frederiksen K. Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology. 2006; 17: 582-7.

196. Dalvin LA, Damento GM, Yawn BP, Abbott BA, Hodge DO, Pulido JS. Parkinson Disease and Melanoma: Confirming and reexamining an association. Mayo Clin Proc. 2017; 92: 1070-9.

197. Huang P, Yang XD, Chen SD, Xiao Q. The association between Parkinson's disease and melanoma: a systematic review and meta-analysis. Transl Neurodegener. 2015; 4: 21.

198. Skibba JL, Pinckley J, Gilbert EF, Johnson RO. Multiple primary melanoma following administration of levodopa. Arch Pathol. 1972; 93: 556-61.

199. Przybilla B, Schwab U, Landthaler M, Braun-Falco O. Development of two malignant melanomas during administration of levodopa. Acta Derm Venereol. 1985; 65: 556-7.

200. Sandyk R. Accelerated growth of malignant melanoma by levodopa in Parkinson's disease and role of the pineal gland. Int J Neurosci. 1992; 63: 137-40.

201. Rosin MA, Braun M, 3rd. Malignant melanoma and levodopa. Cutis. 1984; 33: 572-4.

202. Slominski A, Zmijewski MA, Pawelek J. L-tyrosine and L-dihydroxyphenylalanine as hormone-like regulators of melanocyte functions. Pigment Cell Melanoma Res. 2012; 25: 14-27.

203. Pan T, Li X, Jankovic J. The association between Parkinson's disease and melanoma. Int J Cancer. 2011; 128: 2251-60.

204. Zecca L, Casella L, Albertini A, Bellei C, Zucca FA, Engelen M, Zadlo A, Szewczyk G, Zareba M, Sarna T. Neuromelanin can protect against iron-mediated oxidative damage in system modeling iron overload of brain aging and Parkinson's disease. J Neurochem. 2008; 106: 1866-75.

205. Brenner M, Hearing VJ. The protective role of melanin against UV damage in human skin. Photochem Photobiol. 2008; 84: 539-49.

206. Lo RY, Tanner CM, Van Den Eeden SK, Albers KB, Leimpeter AD, Nelson LM. Comorbid cancer in Parkinson's disease. Mov Disord. 2010; 25: 1809-17.

207. Olsen JH, Tangerud K, Wermuth L, Frederiksen K, Friis S. Treatment with levodopa and risk for malignant melanoma. Mov Disord. 2007; 22: 1252-7.

208. Vermeij JD, Winogrodzka A, Trip J, Weber WE. Parkinson's disease, levodopa-use and the risk of melanoma. Parkinsonism Relat Disord. 2009; 15: 551-3.

209. Elbaz A, Peterson BJ, Bower JH, Yang P, Maraganore DM, McDonnell SK, Ahlskog JE, Rocca WA. Risk of cancer after the diagnosis of Parkinson's disease: a historical cohort study. Mov Disord. 2005; 20: 719-25.

210. Siple JF, Schneider DC, Wanlass WA, Rosenblatt BK. Levodopa therapy and the risk of malignant melanoma. Ann Pharmacother. 2000; 34: 382-5.

211. Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A. Family history of melanoma and Parkinson disease risk. Neurology. 2009; 73: 1286-91.

212. Wright Willis A, Evanoff BA, Lian M, Criswell SR, Racette BA. Geographic and ethnic variation in Parkinson disease: a population-based study of US Medicare beneficiaries. Neuroepidemiology. 2010; 34: 143-51.

213. Wu XC, Eide MJ, King J, Saraiya M, Huang Y, Wiggins C, Barnholtz-Sloan JS, Martin N, Cokkinides V, Miller J, Patel P, Ekwueme DU, Kim J. Racial and ethnic variations in incidence and survival of cutaneous melanoma in the United States, 1999-2006. J Am Acad Dermatol. 2011; 65: S26-37.

214. Herrero Hernandez E. Pigmentation genes link Parkinson's disease to melanoma, opening a window on both etiologies. Med Hypotheses. 2009; 72: 280-4.

215. Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009; 65: 76-82.

216. Schmidt SA, Ording AG, Horvath-Puho E, Sorensen HT, Henderson VW. Non-melanoma skin cancer and risk of Alzheimer's disease and all-cause dementia. PLoS One. 2017; 12: e0171527.

217. Dong J, Gao J, Nalls M, Gao X, Huang X, Han J, Singleton AB, Chen H, International Parkinson's Disease Genomics Consortium. Susceptibility loci for pigmentation and melanoma in relation to Parkinson's disease. Neurobiol Aging. 2014; 35: 1512 e5- e10.

218. Scherer D, Nagore E, Bermejo JL, Figl A, Botella-Estrada R, Thirumaran RK, Angelini S, Hemminki K, Schadendorf D, Kumar R. Melanocortin receptor 1 variants and melanoma risk: a study of 2 European populations. Int J Cancer. 2009; 125: 1868-75.

219. Cordoba-Lanus E, Hernandez-Jimenez JG, Medina-Coello C, Espinoza-Jimenez A, Gonzalez A, Rodriguez-Perez MD, Carretero-Hernandez G, Almeida P, Suarez-Hernandez J, Perera-Molinero A, Fernandez-de-Misa R. MC1R gene variants and sporadic malignant melanoma susceptibility in the Canary Islands population. Arch Dermatol Res. 2014; 306: 51-8.

220. Foo JN, Zhao Y, Liu J, Tan EK. Nonsynonymous variants in MC1R are rare in Chinese Parkinson disease cases. Ann Neurol. 2015; 78: 152-3.

221. Jiang Q, Liu G. Lack of association between MC1R variants and Parkinson's disease in European descent. Ann Neurol. 2016.

222. Lubbe SJ, Escott-Price V, Brice A, Gasser T, Hardy J, Heutink P, Sharma M, Wood NW, Nalls M, Singleton AB, Williams NM, Morris HR, International Parkinson's Disease Genomics Consortium. Is the MC1R variant p.R160W associated with Parkinson's? Ann Neurol. 2016; 79: 159-61.

223. Lorenzo-Betancor O, Wszolek ZK, Ross OA. Rare variants in MC1R/TUBB3 exon 1 are not associated with Parkinson's disease. Ann Neurol. 2016; 79: 331.

224. Gan-Or Z, Mohsin N, Girard SL, Montplaisir JY, Ambalavanan A, Strong S, Mallett V, Laurent SB, Bourassa CV, Boivin M, Langlois M, Arnulf I, Hogl B, et al. The role of the melanoma gene MC1R in Parkinson disease and REM sleep behavior disorder. Neurobiol Aging. 2016; 43: 180 e7- e13.

225. Makova K, Norton H. Worldwide polymorphism at the MC1R locus and normal pigmentation variation in humans. Peptides. 2005; 26: 1901-8.

226. Savage SA, Gerstenblith MR, Goldstein AM, Mirabello L, Fargnoli MC, Peris K, Landi MT. Nucleotide diversity and population differentiation of the melanocortin 1 receptor gene, MC1R. BMC Genet. 2008; 9: 31.

227. Seldin MF, Shigeta R, Villoslada P, Selmi C, Tuomilehto J, Silva G, Belmont JW, Klareskog L, Gregersen PK. European population substructure: clustering of northern and southern populations. PLoS Genet. 2006; 2: e143.

228. Tell-Marti G, Puig-Butille JA, Potrony M, Badenas C, Mila M, Malvehy J, Marti MJ, Ezquerra M, Fernandez-Santiago R, Puig S. Reply. Ann Neurol. 2016; 79: 161-3.

229. Gayan J, Galan JJ, Gonzalez-Perez A, Saez ME, Martinez-Larrad MT, Zabena C, Rivero MC, Salinas A, Ramirez-Lorca R, Moron FJ, Royo JL, Moreno-Rey C, Velasco J, et al. Genetic structure of the Spanish population. BMC Genomics. 2010; 11: 326.

230. Bauchet M, McEvoy B, Pearson LN, Quillen EE, Sarkisian T, Hovhannesyan K, Deka R, Bradley DG, Shriver MD. Measuring European population stratification with microarray genotype data. Am J Hum Genet. 2007; 80: 948-56.

231. Yuasa I, Umetsu K, Harihara S, Kido A, Miyoshi A, Saitou N, Dashnyam B, Jin F, Lucotte G, Chattopadhyay PK, Henke L, Henke J. Distribution of two Asian-related coding SNPs in the MC1R and OCA2 genes. Biochem Genet. 2007; 45: 535-42.

232. Yamaguchi K, Watanabe C, Kawaguchi A, Sato T, Naka I, Shindo M, Moromizato K, Aoki K, Ishida H, Kimura R. Association of melanocortin 1 receptor gene (MC1R) polymorphisms with skin reflectance and freckles in Japanese. J Hum Genet. 2012; 57: 700-8.

233. Herraiz C, Journe F, Ghanem G, Jimenez-Cervantes C, Garcia-Borron JC. Functional status and relationships of melanocortin 1 receptor signaling to the cAMP and extracellular signal-regulated protein kinases 1 and 2 pathways in human melanoma cells. Int J Biochem Cell Biol. 2012; 44: 2244-52.

234. Puig-Butille JA, Carrera C, Kumar R, Garcia-Casado Z, Badenas C, Aguilera P, Malvehy J, Nagore E, Puig S. Distribution of MC1R variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. Br J Dermatol. 2013; 169: 804-11.

235. Healy DG, Falchi M, O'Sullivan SS, Bonifati V, Durr A, Bressman S, Brice A, Aasly J, Zabetian CP, Goldwurm S, Ferreira JJ, Tolosa E, Kay DM, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. Lancet Neurol. 2008; 7: 583-90.

236. Chen X, Feng D, Schwarzschild MA, Gao X. Red hair, MC1R variants, and risk for Parkinson's disease - a meta-analysis. Ann Clin Transl Neurol. 2017; 4: 212-6.

237. Chen X, Chen H, Cai W, Maguire M, Ya B, Zuo F, Logan R, Li H, Robinson K, Vanderburg CR, Yu Y, Wang Y, Fisher DE, et al. The melanoma-linked "redhead" MC1R influences dopaminergic neuron survival. Ann Neurol. 2017; 81: 395-406.

238. Schapira AH, Tolosa E. Molecular and clinical prodrome of Parkinson disease: implications for treatment. Nat Rev Neurol. 2010; 6: 309-17.

239. Herpin TF, Yu G, Carlson KE, Morton GC, Wu X, Kang L, Tuerdi H, Khanna A, Tokarski JS, Lawrence RM, Macor JE. Discovery of tyrosine-based potent and selective melanocortin-1 receptor small-molecule agonists with anti-inflammatory properties. J Med Chem. 2003; 46: 1123-6.

240. Balasa M, Sanchez-Valle R, Antonell A, Bosch B, Olives J, Rami L, Castellvi M, Molinuevo JL, Llado A. Usefulness of biomarkers in the diagnosis and prognosis of early-onset cognitive impairment. J Alzheimers Dis. 2014; 40: 919-27.

241. Campbell CD, Ogburn EL, Lunetta KL, Lyon HN, Freedman ML, Groop LC, Altshuler D, Ardlie KG, Hirschhorn JN. Demonstrating stratification in a European American population. Nat Genet. 2005; 37: 868-72.

242. Tian C, Gregersen PK, Seldin MF. Accounting for ancestry: population substructure and genome-wide association studies. Hum Mol Genet. 2008; 17: R143-50.

243. Casula M, Muggiano A, Cossu A, Budroni M, Caraco C, Ascierto PA, Pagani E, Stanganelli I, Canzanella S, Sini M, Palomba G, Italian Melanoma I, Palmieri G. Role of key-regulator genes in melanoma susceptibility and pathogenesis among patients from South Italy. BMC Cancer. 2009; 9: 352.

244. Kanetsky PA, Panossian S, Elder DE, Guerry D, Ming ME, Schuchter L, Rebbeck TR. Does MC1R genotype convey information about melanoma risk beyond risk phenotypes? Cancer. 2010; 116: 2416-28.

245. Fernandez L, Milne R, Bravo J, Lopez J, Aviles J, Longo M, Benitez J, Lazaro P, Ribas G. MC1R: three novel variants identified in a malignant melanoma association study in the Spanish population. Carcinogenesis. 2007; 28: 1659-64.

246. Ghiorzo P, Bonelli L, Pastorino L, Bruno W, Barile M, Andreotti V, Nasti S, Battistuzzi L, Grosso M, Bianchi-Scarra G, Queirolo P. MC1R variation and melanoma risk in relation to host/clinical and environmental factors in CDKN2A positive and negative melanoma patients. Exp Dermatol. 2012; 21: 718-20.

247. Wu GS, Luo HR, Dong C, Mastronardi C, Licinio J, Wong ML. Sequence polymorphisms of MC1R gene and their association with depression and antidepressant response. Psychiatr Genet. 2011; 21: 14-8.

248. Albert PR. Why is depression more prevalent in women? J Psychiatry Neurosci. 2015; 40: 219-21.

249. Zenaro E, Piacentino G, Constantin G. The blood-brain barrier in Alzheimer's disease. Neurobiol Dis. 2017; 107: 41-56.

250. Chakraborty A, de Wit NM, van der Flier WM, de Vries HE. The blood brain barrier in Alzheimer's disease. Vascul Pharmacol. 2017; 89: 12-8.

251. Liu F, Lau BH, Peng Q, Shah V. Pycnogenol protects vascular endothelial cells from beta-amyloid-induced injury. Biol Pharm Bull. 2000; 23: 735-7.

252. Li D, Taylor AW. Diminishment of alpha-MSH anti-inflammatory activity in MC1r siRNA-transfected RAW264.7 macrophages. J Leukoc Biol. 2008; 84: 191-8.

253. Rainero I, May C, Kaye JA, Friedland RP, Rapoport SI. CSF alpha-MSH in dementia of the Alzheimer type. Neurology. 1988; 38: 1281-4.

254. Ma K, McLaurin J. alpha-Melanocyte stimulating hormone as a potential therapy for Alzheimer`s disease. Curr Alzheimer Res. 2017; 14: 18-29.

255. Browne SE, Ferrante RJ, Beal MF. Oxidative stress in Huntington's disease. Brain Pathol. 1999; 9: 147-63.

256. Stack EC, Matson WR, Ferrante RJ. Evidence of oxidant damage in Huntington's disease: translational strategies using antioxidants. Ann N Y Acad Sci. 2008; 1147: 79-92.

257. Browne SE, Beal MF. Oxidative damage in Huntington's disease pathogenesis. Antioxid Redox Signal. 2006; 8: 2061-73.

258. Weydt P, Soyal SM, Gellera C, Didonato S, Weidinger C, Oberkofler H, Landwehrmeyer GB, Patsch W. The gene coding for PGC-1alpha modifies age at onset in Huntington's disease. Mol Neurodegener. 2009; 4: 3.

259. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 1999; 98: 115-24.

260. Scarpulla RC, Vega RB, Kelly DP. Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab. 2012; 23: 459-66.

261. Marmolino D, Manto M, Acquaviva F, Vergara P, Ravella A, Monticelli A, Pandolfo M. PGC-1alpha down-regulation affects the antioxidant response in Friedreich's ataxia. PLoS One. 2010; 5: e10025.

262. Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, Jager S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, et al. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. Cell. 2004; 119: 121-35.

263. Ronai Z. The masters talk: the PGC-1alpha-MITF axis as a melanoma energizer. Pigment Cell Melanoma Res. 2013.

264. Shoag J, Haq R, Zhang M, Liu L, Rowe GC, Jiang A, Koulisis N, Farrel C, Amos CI, Wei Q, Lee JE, Zhang J, Kupper TS, et al. PGC-1 coactivators regulate MITF and the tanning response. Mol Cell. 2013; 49: 145-57.

265. Stratigos AJ, Dimisianos G, Nikolaou V, Poulou M, Sypsa V, Stefanaki I, Papadopoulos O, Polydorou D, Plaka M, Christofidou E, Gogas H, Tsoutsos D, Kastana O, et al. Melanocortin receptor-1 gene polymorphisms and the risk of cutaneous melanoma in a low-risk southern European population. J Invest Dermatol. 2006; 126: 1842-9.

266. Matichard E, Verpillat P, Meziani R, Gerard B, Descamps V, Legroux E, Burnouf M, Bertrand G, Bouscarat F, Archimbaud A, Picard C, Ollivaud L, Basset-Seguin N, et al. Melanocortin 1 receptor (MC1R) gene variants may increase the risk of melanoma in France independently of clinical risk factors and UV exposure. J Med Genet. 2004; 41: e13.

267. Qiu T, Wang H, Wang Y, Zhang Y, Hui Q, Tao K. Identification of genes associated with melanoma metastasis. Kaohsiung J Med Sci. 2015; 31: 553-61.

268. Sun H. Identification of key genes associated with gastric cancer based on DNA microarray data. Oncol Lett. 2016; 11: 525-30.

269. Hoepken HH, Gispert S, Azizov M, Klinkenberg M, Ricciardi F, Kurz A, Morales-Gordo B, Bonin M, Riess O, Gasser T, Kogel D, Steinmetz H, Auburger G. Parkinson patient fibroblasts show increased alpha-synuclein expression. Exp Neurol. 2008; 212: 307-13.

270. Morris JK, Honea RA, Vidoni ED, Swerdlow RH, Burns JM. Is Alzheimer's disease a systemic disease? Biochim Biophys Acta. 2014; 1842: 1340-9.

271. Jellinger KA. The pathomechanisms underlying Parkinson's disease. Expert Rev Neurother. 2014; 14: 199-215.

272. Connolly GP. Fibroblast models of neurological disorders: fluorescence measurement studies. Trends Pharmacol Sci. 1998; 19: 171-7.

273. Israel MA, Yuan SH, Bardy C, Reyna SM, Mu Y, Herrera C, Hefferan MP, Van Gorp S, Nazor KL, Boscolo FS, Carson CT, Laurent LC, Marsala M, et al. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature. 2012; 482: 216-20.

274. Abernathy DG, Kim WK, McCoy MJ, Lake AM, Ouwenga R, Lee SW, Xing X, Li D, Lee HJ, Heuckeroth RO, Dougherty JD, Wang T, Yoo AS. MicroRNAs induce a permissive chromatin environment that enables neuronal subtype-specific reprogramming of adult human fibroblasts. Cell Stem Cell. 2017; 21: 332-48 e9.

275. Fernandez-Santiago R, Carballo-Carbajal I, Castellano G, Torrent R, Richaud Y, Sanchez-Danes A, Vilarrasa-Blasi R, Sanchez-Pla A, Mosquera JL, Soriano J, Lopez-Barneo J, Canals JM, Alberch J, et al. Aberrant epigenome in iPSC-derived dopaminergic neurons from Parkinson's disease patients. EMBO Mol Med. 2015; 7: 1529-46.

276. Auburger G, Klinkenberg M, Drost J, Marcus K, Morales-Gordo B, Kunz WS, Brandt U, Broccoli V, Reichmann H, Gispert S, Jendrach M. Primary skin fibroblasts as a model of Parkinson's disease. Mol Neurobiol. 2012; 46: 20-7.

277. Ambrosi G, Ghezzi C, Sepe S, Milanese C, Payan-Gomez C, Bombardieri CR, Armentero MT, Zangaglia R, Pacchetti C, Mastroberardino PG, Blandini F. Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease. Biochim Biophys Acta. 2014; 1842: 1385-94.

278. Smith GA, Jansson J, Rocha EM, Osborn T, Hallett PJ, Isacson O. Fibroblast biomarkers of sporadic Parkinson's disease and LRRK2 kinase inhibition. Mol Neurobiol. 2016; 53: 5161-77.

279. Mocali A, Della Malva N, Abete C, Mitidieri Costanza VA, Bavazzano A, Boddi V, Sanchez L, Dessi S, Pani A, Paoletti F. Altered proteolysis in

fibroblasts of Alzheimer patients with predictive implications for subjects at risk of disease. Int J Alzheimers Dis. 2014; 2014: 520152.

280. Marchina E, Misasi S, Bozzato A, Ferraboli S, Agosti C, Rozzini L, Borsani G, Barlati S, Padovani A. Gene expression profile in fibroblasts of Huntington's disease patients and controls. J Neurol Sci. 2014; 337: 42-6.

281. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell. 2007; 129: 665-79.

282. Zhao M, Geng R, Guo X, Yuan R, Zhou X, Zhong Y, Huo Y, Zhou M, Shen Q, Li Y, Zhu W, Wang J. PCAF/GCN5-mediated acetylation of RPA1 promotes nucleotide excision repair. Cell Rep. 2017; 20: 1997-2009.

283. Kanao R, Masutani C. Regulation of DNA damage tolerance in mammalian cells by post-translational modifications of PCNA. Mutat Res. 2017; 803-805: 82-8.

284. Tanaka S, Tak YS, Araki H. The role of CDK in the initiation step of DNA replication in eukaryotes. Cell Div. 2007; 2: 16.

285. Deng CX. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res. 2006; 34: 1416-26.

286. Smits P, Rodenburg RJ, Smeitink JA, van den Heuvel LP. Sequence variants in four candidate genes (NIPSNAP1, GBAS, CHCHD1 and METT11D1) in patients with combined oxidative phosphorylation system deficiencies. J Inherit Metab Dis. 2010; 33 Suppl 3: S13-9.

287. Richter R, Rorbach J, Pajak A, Smith PM, Wessels HJ, Huynen MA, Smeitink JA, Lightowlers RN, Chrzanowska-Lightowlers ZM. A functional peptidyl-tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome. EMBO J. 2010; 29: 1116-25.

288. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009; 417: 1-13.

289. Dan Dunn J, Alvarez LA, Zhang X, Soldati T. Reactive oxygen species and mitochondria: a nexus of cellular homeostasis. Redox Biol. 2015; 6: 472-85.

290. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012; 24: 981-90.

291. Nah J, Yuan J, Jung YK. Autophagy in neurodegenerative diseases: from mechanism to therapeutic approach. Mol Cells. 2015; 38: 381-9.

292. Zheng Q, Huang T, Zhang L, Zhou Y, Luo H, Xu H, Wang X. Dysregulation of ubiquitin-proteasomesystem in neurodegenerative diseases. Front Aging Neurosci. 2016; 8: 303.

293. Lim KL, Tan JM. Role of the ubiquitin proteasome system in Parkinson's disease. BMC Biochem. 2007; 8 Suppl 1: S13.

294. Upadhya SC, Hegde AN. Role of the ubiquitin proteasome system in Alzheimer's disease. BMC Biochem. 2007; 8 Suppl 1: S12.

295. Ortega Z, Lucas JJ. Ubiquitin-proteasome system involvement in Huntington's disease. Front Mol Neurosci. 2014; 7: 77.

296. Ciechanover A, Kwon YT. Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. Exp Mol Med. 2015; 47: e147.

297. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013; 19: 983-97.

298. Pan T, Kondo S, Le W, Jankovic J. The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease. Brain. 2008; 131: 1969-78.

299. Jaeger PA, Wyss-Coray T. Beclin 1 complex in autophagy and Alzheimer disease. Arch Neurol. 2010; 67: 1181-4.

300. Cortes CJ, La Spada AR. The many faces of autophagy dysfunction in Huntington's disease: from mechanism to therapy. Drug Discov Today. 2014; 19: 963-71.

301. Teves JMY, Bhargava V, Kirwan KR, Corenblum MJ, Justiniano R, Wondrak GT, Anandhan A, Flores AJ, Schipper DA, Khalpey Z, Sligh JE, Curiel-Lewandrowski C, Sherman SJ, et al. Parkinson's disease skin fibroblasts display

signature alterations in growth, redox homeostasis, mitochondrial function, and autophagy. Front Neurosci. 2017; 11: 737.

ANNEXOS

ANNEX I

www.impactjournals.com/oncotarget/ Oncotarget, Vol. 5, No. 6

Capturing the biological impact of *CDKN2A* and *MC1R* genes as an early predisposing event in melanoma and non melanoma skin cancer.

Joan Anton Puig-Butille[1,2], María José Escámez[3,4,5], Francisco Garcia-Garcia[6,7], Gemma Tell-Marti [1], Àngels Fabra[8], Lucía Martínez-Santamaría[3,4,5], Celia Badenas[1,2], Paula Aguilera[1,2], Marta Pevida[5,9], Joaquín Dopazo[6,7,10], Marcela del Río[3,4,5], Susana Puig[1,2]

[1] Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer), Barcelona, Spain

[2] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona, Spain.

[3] Regenerative Medicine Unit. Epithelial Biomedicine Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain.

[4] Department of Bioengineering. Universidad Carlos III (UC3M), Madrid, Spain.

[5] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain

[6] Functional Genomics Node, National Institute of Bioinformatics, CIPF Valencia, Spain

[7] Department of Bioinformatics, Centro de Investigación Príncipe Felipe, Valencia, Spain

[8] Biological Clues of the Invasive and Metastatic Phenotype Group. Molecular Oncology Lab, IDIBELL, Barcelona, Spain

[9] Tissue Engineering Unit. Centro Comunitario de Sangre y Tejidos del Principado de Asturias (CCST), Oviedo, Spain

[10] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Valencia, Spain.

Correspondence to: *Susana Puig,* ***email:*** *susipuig@gmail.com*

Keywords: Familial Melanoma, CDKN2A, MC1R, gene expression, p16, mutations

Received: *September 25, 2013* ***Accepted***: *December 16, 2013* ***Published***: *December 16, 2013*

ABSTRACT:

Germline mutations in *CDKN2A* and/or red hair color variants in *MC1R* genes are associated with an increased susceptibility to develop cutaneous melanoma or non melanoma skin cancer.

We studied the impact of the *CDKN2A* germinal mutation p.G101W and *MC1R* variants on gene expression and transcription profiles associated with skin cancer. To this end we set-up primary skin cell co-cultures from siblings of melanoma prone-families that were later analyzed using the expression array approach.

As a result, we found that 1535 transcripts were deregulated in *CDKN2A* mutated cells, with over-expression of immunity-related genes (*HLA-DPB1, CLEC2B, IFI44, IFI44L, IFI27, IFIT1, IFIT2, SP110* and *IFNK*) and down-regulation of genes playing a role in the Notch signaling pathway. 3570 transcripts were deregulated in *MC1R* variant carriers. In particular, genes related to oxidative stress and DNA damage pathways were up-regulated as well as genes associated with neurodegenerative diseases such as Parkinson's, Alzheimer and Huntington.

Finally, we observed that the expression signatures indentified in phenotypically normal cells carrying *CDKN2A* mutations or *MC1R* variants are maintained in skin cancer tumors (melanoma and squamous cell carcinoma). These results indicate that transcriptome deregulation represents an early event critical for skin cancer development.

INTRODUCTION

The worldwide incidence of skin cancer, including non melanoma skin cancer (NMSC) and cutaneous melanoma (CM), is rapidly increasing, being the most common human cancer[1]. While, both entities are influenced by the carcinogenic effect of UV exposure, their incidence and tumor aggressiveness differ considerably, CM being the least frequent and most aggressive form[2] .

Approximately, 10% of total melanoma cases originate in individuals belonging to high-risk melanoma pedigrees. To date, *CDKN2A* is the major gene associated with the high risk inherited in melanoma prone families [3] and in multiple primary melanoma patients [4]. *CDKN2A* acts as a tumor suppressor gene, negatively regulating cell cycle progression and promoting cellular senescence. Recently, a role for *CDKN2A* in cellular oxidative stress regulation has been suggested [5, 6]. Recurrent *CDKN2A* mutations have been identified in melanoma families [7-11], mutation p.G101W being the most frequent one detected in Mediterranean pedigrees [12]. Although heterozygous loss of *CDKN2A* is sufficient to confer a 67% lifetime risk of melanoma [13], the mechanisms responsible for tumor enhancement still have to be clarified [14]. In contrast, the role of high-penetrance *CDKN2A* mutations in NMSC susceptibility has not been clearly elucidated [15, 16].

Skin cancer epidemiology is complex due to the multigenic nature of the disease. In particular, the *MC1R* gene is a key regulator of skin pigmentation and melanocyte differentiation, playing a central role in determining the pigmentation phenotype, sun sensitivity and tanning ability[17]. Certain *MC1R* polymorphisms are responsible for the red hair color (RHC) phenotype (red hair, fair skin and poor tanning response)[18] which is associated with high UV-radiation sensitivity and skin cancer susceptibility (CM and NMSC)[19, 20]. Furthermore, *MC1R* variants are a modifying factor for melanoma risk in *CDKN2A* mutation carriers, [21-23] suggesting that multiple medium- and low-penetrance genes may influence the risk conferred by high penetrance melanoma genes.

To date, there is a lack of information about the mechanisms underlying the increased skin cancer risk in carriers of *CDKN2A* mutations in association with *MC1R* variant*s*. The effect of both genes has been separately investigated using murine-derived melanocytes and/ or focused on mono-cultured melanocytes [6, 24-26]. However, these mono-cultures exhibit phenotypic characteristics closely mimicking those observed in transformed melanocytes, such as elevated growth rate [27, 28] and acquired expression of melanoma-associated antigens[29, 30] which are abolished when they are co-cultured with keratinocytes[30-32]. This data indicates that melanocyte proliferation and behavior is controlled by surrounding basal keratinocytes which regulate epidermal homeostasis [33, 34]. Thus, the establishment and characterization of a melanocyte–keratinocyte co-culture system is essential for elucidating the early molecular events leading to skin cancer.

The aim of the present study was to investigate the global molecular effect of germinal *CDKN2A* mutations (p.G101W) and *MC1R* RHC variants in the transcriptome of primary skin cells from individuals belonging to skin cancer prone families (familial melanoma pedigrees).

RESULTS

Impact of *CDKN2A* germinal mutation p.G101W on Global Transcript Profiles of skin primary cultures:

Whole genome expression profiling was performed on melanoctye-keratinocyte co-cultures from *CDKN2A* mutation carriers (samples A1 and B1 described in Table 1) and wild-type *CDKN2A* individuals (samples A2 and B2). After stringent microarray data filtering, 1536 of the initial 19596 transcripts on the array were found to be differentially expressed in *CDKN2A* mutated cultured cells. Specifically, 60.7% of transcripts were found to be up-regulated (933/1536) and 39.3% of them down-regulated (603/1536).

Class comparison generated a signature of 108 unique genes that significantly discriminated between *CDKN2A* mutation and non-mutation (p-value <0.0001, listed in Table S1). Functional categorization of those transcripts belonging to known genes *(*Figure 1a, Figure 1b*)* showed that the mutant *CDKN2A* signature included deregulation of a vast number of genes involved in cell component and metabolism (37% up-regulated and 42% down-regulated,). Notably, immunomodulation and interferon response genes represented the second most prevalent subset of up-regulated transcripts (24%) and third down-regulated subgroup (13%) which may reflect a constitutively altered cytokine and chemokine profile in mutant cells. The set of deregulated genes involved in immunomodulation and interferon response includes: *CLEC2B, IFI44L, IFIT1, IFI44, IFI27, IFNK, DDX58, RNF182, IFIT2, IL15, SP110, BTN3A2, RFX2, IL17D* and *HLA-DPB1* (Figure 2). The molecular profile also included deregulation of genes which may confer a phenotype with the capability for malignant transformation since they are involved in cell adhesion, cell growth and proliferation. Over-expression of apoptosis related genes *CASP3*, *XAF1* and *SAMD9* was also detected. Transcription factors such as *CEBPZ, LEF1, MACC1, TBX1, ZNF404* and *ZNF8,* epidermal differentiation and melanogenesis genes such as *KRT2, TYPR1, LIMS3, EEA1* and *MFI2* were also components of the mutant *CDKN2A* signature.

Further examination by functional enrichment

Table 1: Genotype and phenotype features of four individuals from two melanoma pedigrees.

Pedigree A						
Individual	MM*	CDKN2A	MC1R	Skin phototype	Eye color	Hair color
A1	5	p.G101W	p.R160W, p.R151C	II	Green	Red
A2	0	Wild-type	p.R160W, p.R151C	II	Green	Red
Pedigree B						
Individual	MM*	CDKN2A	MC1R	Skin phototype	Eye color	Hair color
B1	0	p.G101W	Wild-type	II	Blue	Black
B2	0	Wild-type	Wild-type	II	Blue	Brown

Two siblings from each pedigree (A and B) were included. Each individual was considered as a "genomic condition" in the study. A total of two and three melanoma cases have been found in pedigree A and B, respectively. Abbreviations: *CDKN2A*: cyclin-dependent kinase inhibitor 2A; *MC1R*: melanocortin 1 receptor; MM: melanomas. * Number of melanomas in each individual.

analysis revealed an over-representation of down-regulated genes from the Notch signaling pathway (hsa_04330; p=0.038) in mutant *CDKN2A* skin cells including *NCOR2, PSEN, DLLC1, DVL3, NOTCH3,*

Fig 1a

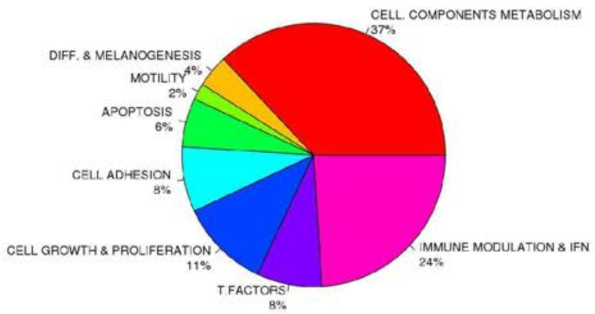


Fig 1b

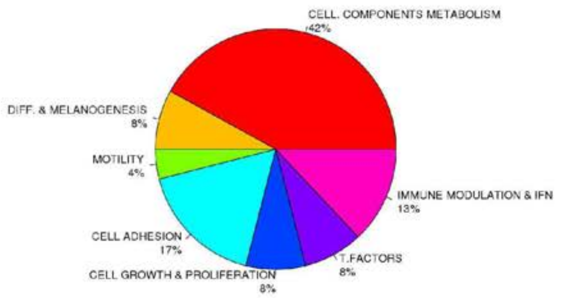


Figure 1: Impact of germinal CDKN2A mutation on global transcript profile in melanocyte-keratinocyte co-culture system. Functional classification of the set of 78 of 108 deregulated genes in *CDKN2A* mutants are indicated in Fig.1a Up-regulated genes and Fig.1b Down-regulated genes. T. Factors (transcription factors), Immune modulation & IFN (Immunomodulation and interferon related genes); Cell. Components and metabolism (Cellular components and metabolism), Diff & Melanogenesis (differentiation and melanogenesis)

RFNG and the co-activator *MAML1* which controls the growth-promoting effect of Notch1.

Impact of non-functional *MC1R* gene on Global Transcript Profiles of skin primary cultures:

MC1R analysis was carried out following the same workflow used in the *CDKN2A* analysis. Whole genome expression profiling displayed 3570 transcripts differentially expressed in RHC variant (A1 and A2) vs. wild-type MC1R skin cells (B1 and B2). Specifically, 54% of transcripts (1954/3570) were up-regulated while 46% (1616/3570) were down-regulated. By class comparison, 159 genes showed highly statistically significant differences ($p < 0.0000001$) (listed in Table S2). RHC skin cells showed a down-regulation of a subset of genes categorized as melanocyte differentiation and pigmentation genes. The transcription of most of these genes is regulated directly via the *MC1R* signaling pathway, or indirectly through keratinocyte differentiation genes *(TYRP1, MLANA, TYR, CRNN, PADI1, GDF15, MLPH, KIT, INHBB, DAB2, KLK6, FOXC1,* and *NDRG4)*. Furthermore, RHC variant skin cells exhibited up-regulation of DNA damage and/or DNA repair response genes (*BUB1, CHEK1, POLQ, RAD51AP1, RAD54B, RAD51C, BLM, UBE2T, GINS2, EXO1, FAM33A, PTTG2, PTTG1, RAD54L, DTL),* cell cycle and proliferation genes *(CDKN3, CDC25, CDK1, CCNA2, CDC20, CCNB2, CCNB1, CKS, CDCA3, PLK1, PRIM, CDCA8, GMNN, CDC6, VRK1, CKS1B, RFC4, CDC45, CENPN, HAUS8, PSRC, MCM6, MELK, AURKA, NCAPG)* and microtubule-based motors kinesin superfamily genes *(KIF11, KIF15, KIF2C, KIF4A,KIF23)*.

The functional enrichment analysis showed a considerably higher number of altered pathways in non-functional *MC1R* cells than in *CDKN2A* mutant cells. The RHC skin cells displayed over-expression of genes playing

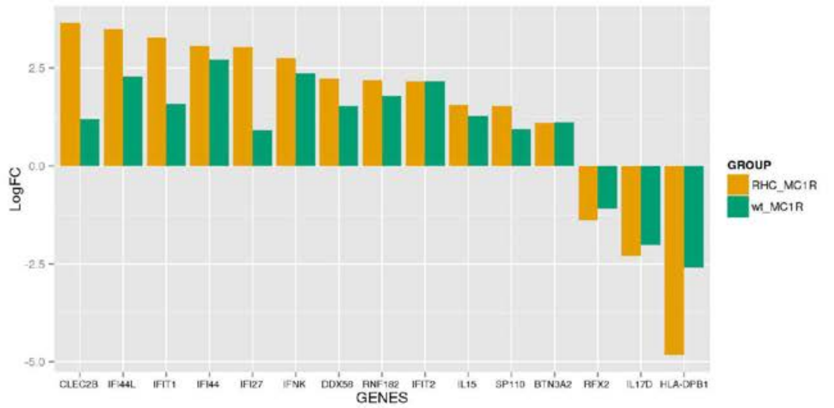


Figure 2: Set of immunomodulation and interferon response deregulated in mutant CDKN2A co-cultures. The logarithm of the Fold change (LogFC) for the set of genes is indicated for wild-type *MC1R* co-cultures (wt_MC1R; green) and for Red Hair Colour *MC1R* variants co-cultures (RHC_MC1R; yellow).

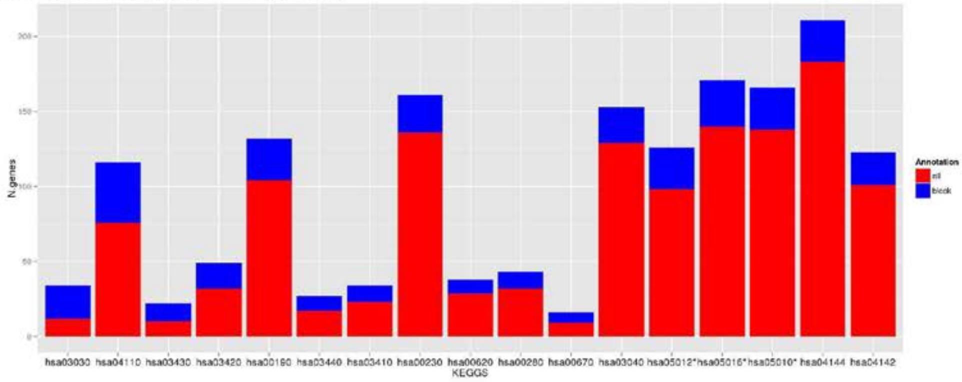


Figure 3: Deregulated pathways in RHC MC1R melanocyte-keratinocyte co-culture system. In blue are the number of total genes annotated within the pathway, in red are the number of deregulated genes in the RHC *MC1R* co-cultures. The KEGG term is indicated for each pathway (axis X). * Pathways related to neurodegenerative diseases.

Table 2: Deregulated pathways in RHC MC1R melanocyte-keratinocyte co-culture system.

KEGG Term	STATUS	Adj. P-Value
DNA replication (hsa03030)	UP	3.83E-14
Cell cycle (hsa04110)	UP	7.87E-14
Mismatch repair (hsa 03430)	UP	1.56E-06
Nucleotide excision repair (hsa03420)	UP	7.01E-06
Oxidative phosphorylation (hsa00190)	UP	6.91E-05
Homologous recombination (hsa03440)	UP	6.98E-04
Base excision repair (hsa03410)	UP	1.07E-03
Purine metabolism (hsa00230)	UP	1.80E-02
Pyruvate metabolism (hsa00620)	UP	3.47E-02
Valine. leucine and isoleucine degradation (hsa00280)	UP	8.13E-03
One carbon pool by folate (hsa00670)	UP	2.69E-03
Spliceosome (hsa03040)	UP	1.80E-02
Parkinson's disease (hsa05012)	UP	3.10E-05
Hugtington's disease (hsa05016)	UP	5.25E-04
Alzheimer's disease (hsa 05010)	UP	3.03E-03
Endocytosis (hsa04144)	DOWN	3.83E-02
Lysosome (hsa04142)	DOWN	4.70E-03

The KEGG term is indicated for each pathway (X-axis). Status: UP: pathway overrepresented in the group of up-regulated transcripts in RHC MC1R; DOWN: pathway over-represented in the group of down-regulated transcripts in RHC MC1R.

a role in major DNA repair and cell homeostasis pathways such as DNA replication, cell cycle and also oxidative phosphorylation which leads to production of reactive oxygen species (Figure 3 and Table 2). The lysosome and endocytosis pathways, which are intrinsically related to melanosome transfer from melanocytes to keratinocytes, were over-represented in the set of down-regulated transcripts as a direct consequence of low pigment production in RHC skin cells. Interestingly, RHC skin cells also exhibited an up-regulation of genes involved in neurodegenerative disorders such as Parkinson´s disease (hsa05012), Huntington´s disease (hsa5016) and Alzheimer´s disease (hsa05010). Although most of the genes were involved in several of these pathways, some of them were restricted to Huntington's disease (*AP2B1, CREB3L4, POLR2H* and *SOD1*), to Alzheimer´s disease (*NAE1, FAS*) or to Parkinson's disease (*PARK7).*

Besides the lack of functionality caused by p.R151C and p.R160W variants, we also analyzed a possible effect on *MC1R* levels, by the relative quantification of *MC1R* expression. We observed a statistically significant reduction in *MC1R* expression in RHC skin cells (ddCt=0.06±0.02) compared to wild-type *MC1R* skin cells (ddCt=0.50±0.07; P-value<0.0001). Furthermore, the expression of *FARP1, SLFN11, GFPT2, COL5A3, TYRP1, LEF1, KRT2, ST6GALNAC3, MLANA, MSMB, SILV, A2M,* and *ALOX5* was evaluated by RT-PCR confirming the results obtained in the array (data not shown).

We further examined the overlap between genes differentially expressed in both mutant *CDKN2A* (n=1536) and in non functional MC1R cells (n=3570). A total of 485 genes were altered in the same fashion in both mutant *CDKN2A* and non functional *MC1R* cells and the expression of 136 genes was found inversely correlated between both groups. Of these 136 genes (listed in Table S3), 30 were up-regulated in mutant *CDKN2A* and down-regulated in non functional MC1R cells and 106 genes were down-regulated in mutant *CDKN2A* and up-regulated in non functional *MC1R* cells (Figure 4). The functional analysis of these 136 genes did not reveal over-representation of any specific pathway.

In silico model evaluation

We hypothesized that if the altered gene signatures observed in non-lesional skin from *CDKN2A* mutation or RHC *MC1R* variant carriers are critical for malignant transformation, these patterns will be maintained across the carcinogenic process. To support our hypothesis, the expression pattern of the most deregulated genes in mutant *CDKN2A* or RHC *MC1R* variant skin cells was compared to their expression observed in two previously published skin cancer datasets. The GSE2503 dataset contained the whole genome expression of five squamous cell carcinomas (SCCs) and six healthy skin samples[35] and the GSE12391 dataset included 23 melanomas (CMs) at different stages (radial growth phase or vertical growth phase) and 18 common nevi[36]. Due to differences in the array platforms used among the studies, only the expression of genes present in all datasets was evaluated (Figure 5). Thus, *MC1R* expression pattern analyses included 76.1% (121/159) of the most deregulated genes. Primary co-cultures with the non-functional *MC1R* gene showed a statistically significant expression pattern (p-value <0.005) similar to those detected in SCCs (Pearson correlation=0.44) and CMs (Pearson correlation=0.25) (Figure 5a).

Because of platform disparities, the mutant *CDKN2A* expression pattern was evaluated analyzing 53.7% of the most deregulated transcripts (58/108). The expression pattern correlated to those observed in SCCs (Pearson correlation=0.35) and CMs (Pearson correlation=0.28) showing a statistical tendency among datasets (p-value <0.06), in which immunomodulation and interferon response genes were also up-regulated (*SP110*, *BTN3A2*, *IL15*, *IFIT2*, *IFI44*, *IFIT1*, *IFI44L* and

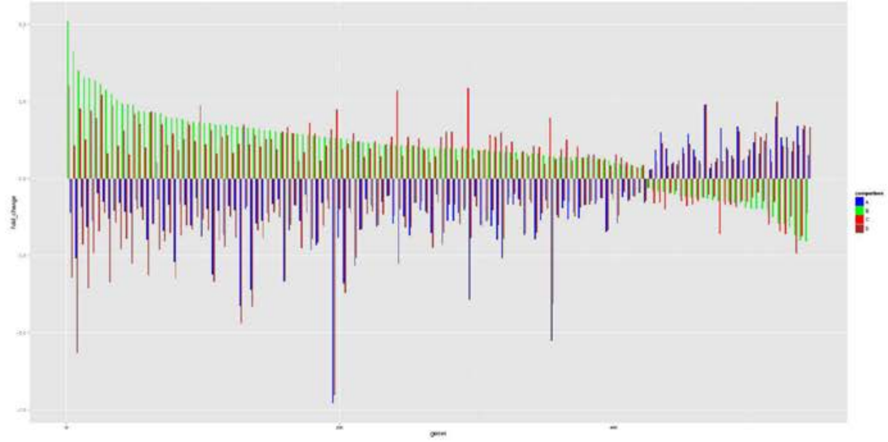


Figure 4: Genes inversely expressed in mutant CDKN2A and RHC MC1R. 136 genes were found inversely expressed between mutant *CDKN2A* and RHC *MC1R* genes (listed in Table S3). A: RHC *MC1R* vs wt *MC1R* (both mutant *CDKN2A*); B: mutant *CDKN2A* vs wt *CDKN2A* (both RHC *MC1R*); C: mutant *CDKN2A* vs wt *CDKN2A* (both wt *MC1R*); D: RHC MC1R vs wt MC1R (both wt *CDKN2A*).

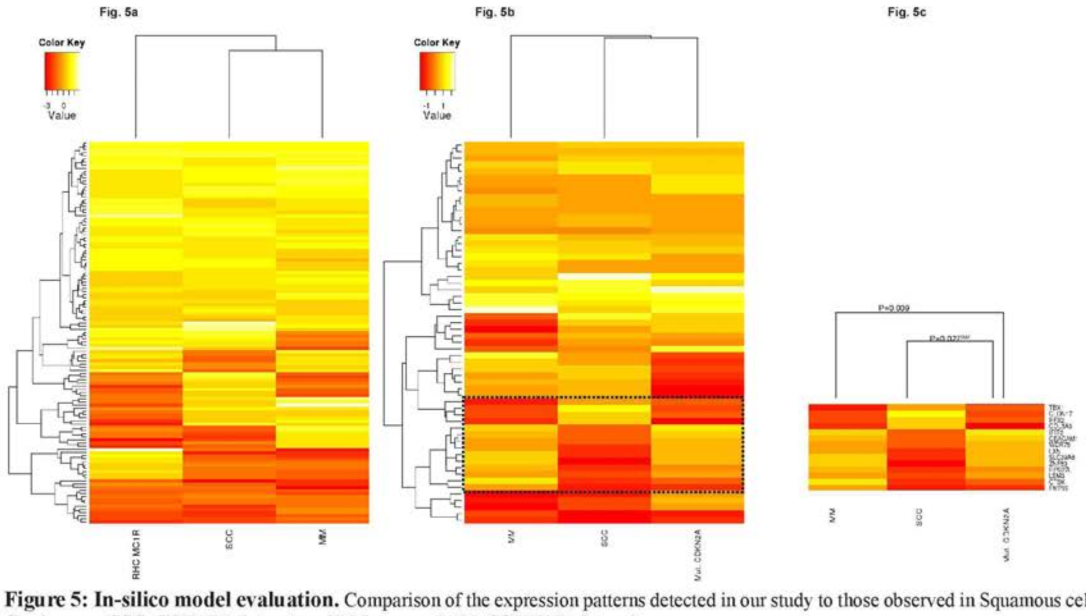


Figure 5: In-silico model evaluation. Comparison of the expression patterns detected in our study to those observed in Squamous cell Carcinomas (SSC; GSE2503 dataset) and Melanomas (MM; GSE12391 dataset).

Fig. 5A: Heatmap *MC1R* expression pattern showed a positive correlation among datasets (p-value <0.005).

Fig. 5B: Heatmap *CDKN2A* expression pattern showed a positive correlation among datasets (p-value <0.06).

Fig. 5C: Subset of genes with an inverse expression pattern between mutant *CDKN2A* skin cells and SSC and MM. [INV]: inversely correlated pattern.

CLEC2B; Figure 5b). A correlated expression pattern was found within a subset of genes between CMs and mutant *CDKN2A* skin cells (Pearson Correlation=0.66, P-value = 0.009; Figure 5c). Remarkably, this subset of genes presented an expression pattern that inversely correlated between SCCs and CMs (Pearson correlation= -0.88, p-value<0.001) and also between SCCs and mutant *CDKN2A* skin cells (Pearson correlation= -0.58, p-value=0.027). Specifically, the expression pattern of *TBX1, CLDN17, RFX2, COL5A3, IFIT2, CEACAM1, WDR78, LXN, SLC39A8, ZNF83, RPS27L, LSM3, CTSK,* and *PMP22* was exclusively correlated to MM and mutant *CDKN2A* MM prone skin cells, and inversely with NMSC.

DISCUSSION

The aim of the study was to determine the constitutional effect of germline *CDKN2A* mutations or *MC1R* variants in skin cancer, in order to identify early critical molecular targets implicated in the disease. To do so we analyzed co-cultured melanocyte-keratinocyte systems derived from two siblings belonging to two melanoma prone families with a founder *CDKN2A* mutation and/or carrying non-functional *MC1R* alleles to compare global gene expression profiles.

To date, *CDKN2A* is the major gene responsible for increased melanoma susceptibility in high-risk pedigrees. Germline high-penetrance mutations are found in 10% of melanoma prone families and somatic *CDKN2A* alterations are also recurrent events in primary melanomas[37] and melanoma cell lines[38]. Alterations in gene expression in as yet phenotypically normal cells but bearing single-hit mutations in tumor suppressor genes appear to be the earliest molecular change during cancer development[39].

Altered baseline expression signatures associated with *CDKN2*A mutations in cultured normal skin fibroblasts from familial melanoma patients have already been reported [40]. However, the transcriptome deregulation within keratinocytes and melanocytes where cutaneous carcinomas and melanomas arise have not been previously assessed. We found that transcriptome signatures were altered by single hit *CDKN2A* mutations in co-cultured melanocyte-keratinocyte systems. *CDKN2A* mutant cells exhibit mainly baseline differences in genes related to cell components, metabolism and immune response. Melanoma cells are addicted to oncogene-driven energy production which can be mediated by somatic *BRAF* mutations [41], our findings indicate that non transformed skin cells already have a deregulated metabolic profiling which may be necessary for the skin cancer initiation.

Previous studies suggest that genetic variation of some cytokines or their receptors influence melanoma susceptibility [42, 43] and even modify the risk in melanoma-prone families with *CDKN2A* mutations [44]. Consistent with this hypothesis, our data reflects the important role of innate immunity and immune response pathways which may be deregulated in early steps of melanoma development. Skin cells also exhibited deregulation of epidermal differentiation and melanogenesis genes. Thus, we found down-regulation of the *TYRP1* gene, a melanocyte differentiation marker and over expression of *MFI2* gene, a cell-surface glycoprotein playing a role in melanoma cell proliferation and tumorigenesis [45] and *EEA1* gene, a marker of early endosomes [46]. Interestingly, *EEA1* interacts with *MFI2* regulating endosome fusion and trafficking [47]. Early endosomes are direct precursors of melanosomes, which are the specialized organelles for the biosynthesis and storage of melanins[46]. These findings may indicate that constitutive disorganized melanosomal structures and changes in pigment production may favor malignant transformation which in turn, could be related to autoimmune response deregulation [48, 49].

Via functional analyses, we observed a down-regulation of the Notch signaling pathway in *CDKN2A* mutant cells. This pathway is essential in epidermal-melanocyte interactions [50, 51] and recent evidence suggests Notch pathway as a link between the control of epidermal differentiation and proliferation and skin homeostasis [52, 53]. Notch signaling has a dual action (as oncogene or as tumor suppressor) in skin cancer. While, up-regulation of the Notch pathway is observed in CMs or in SCCs at sun-protected sites, it is down-regulated in UV-related solar keratoses SCCs or in common melanocytic nevi (reviewed in Panelos and Massi, 2009 [54]). A high melanocytic nevi count is the strongest known risk factor for melanoma[55] and a potent predictor of mutant *CDKN2A* gene carrier status[56]. Thus, the baseline down-regulation of the Notch signaling pathway in *CDKN2A* mutation carriers may proffer a melanocyte proliferation advantage, which can trigger common nevi development in human skin.

The pigmentation related gene *MC1R* acts as a moderate melanoma risk gene and variants in this gene are modifying factors for melanoma risk in *CDKN2A* mutation carriers [21-23]. Furthermore, *MC1R* variants have also been clearly associated with elevated NMSC susceptibility [19, 20]. We observed that the number of genes differentially expressed in non-functional *MC1R* was considerably higher than in mutant *CDKN2A*. Both p.R151C and p.R160W alleles halted *MC1R* activity and also induced an altered cell surface molecule expression due to impaired export traffic [57] that could lead to a reduction in *MC1R* gene expression levels as observed in our study. We found that RHC *MC1R* skins cells differ from wild-type in the deregulation of genes involved in key physiological processes such as differentiation, cell adhesion and cell cycle progression which may be directly related to the etiology of skin cancer. We found that in non-functional *MC1R* cells, pathways associated with increased DNA damage without UV radiation exposure were up-regulated, indicating that RHC variants contribute to cancer risk in humans increasing the DNA damage mediated by intrinsic UV-independent mechanisms as recently observed in mice models [58]. Notably, RHC *MC1R* skin cells also showed up-regulated pathways related to neurodegenerative diseases. The *MC1R* gene is expressed in cells of the nervous system and its activation decreases oxidative stress and has anti-inflammatory and immunomodulatory effects [59]. Co-occurrence of Parkinson's disease (PD) and CMs [60, 61] has been reported in epidemiological studies. The diagnosis of CM is associated with an approximate 50% increased risk of subsequent PD[61] development whereas individuals with PD have a two-fold increase in risk of subsequent CM[62] development. Previous data suggests that this relation is associated with pigment-related genes[63] rather than Parkinson's-related genes[64, 65]. Our study further supports that *MC1R* is involved in the cross-link between both diseases. Thus, *MC1R* variants may increase the oxidative damage in brain cells and deregulate inflammatory processes which consequently, increase the susceptibility to neurodegenerative disorders.

Previous studies showed that many of the alterations associated with transcriptome and proteome signatures of as yet phenotypically normal cells bearing inherited alterations are also present in the corresponding form of cancer [39]. Thus, in our study, expression patterns detected in MM prone cells from non-lesional areas of the skin were compared with those observed in skin cancer tumors (SCCs and CMs) [35, 36]. Although differences in the experimental designs among studies (normal skin cells *vs* tumoral tissue or cultured cells *vs* fresh-frozen tissue) may interfere in the analysis, the expression patterns detected in our study correlate to those observed in skin tumors. Interestingly, the *MC1R* expression pattern was more similar among studies than the *CDKN2A* pattern (p-value<0.005 and p-value<0.06, respectively). These differences are caused in part by a subset of genes showing a converse pattern which allows us to distinguish between SSCs versus CMs and mutant *CDKN2A* skin cells (MM prone cells). Accordingly, previous studies have detected the inverse expression of two genes from this subset (*CEACAM1* and *CTSK)* between SSCs and MMs [66, 67]. Such differences may underlie the epidemiological differences observed between *MC1R* and *CDKN2A* and skin cancer susceptibility since *MC1R* polymorphisms are involved in both CMM and NMSC susceptibility while *CDKN2A* mutations are closely related to CM susceptibility rather than SCC susceptibility.

In conclusion, we have identified baseline expression signatures in skin cells carrying germline *CDKN2A* mutations and RHC *MC1R* variants which are maintained in skin tumors (melanoma and squamous cell carcinomas). The study identified a large catalogue of genes in *CDKN2A* mutant skin cells that are closely related to skin cancer, highlighting the role of genes involved in immune response, in melanosome biogenesis and the Notch signaling pathway Also, our data indicates that non functional *MC1R* variants promote DNA damage by intrinsic UV-independent mechanisms in human skin cells. Furthermore, our study revealed a role for *MC1R* in the susceptibility to neurodegenerative diseases which may be related to its role in oxidative stress and inflammatory processes.

METHODS

Study Design

To detect the effect of *CDKN2A* and *MC1R* genes, two melanoma families (A and B) were selected (Table I). The most frequent mutation, p.G101W, resulting from a common ancestor in Mediterranean pedigrees, was chosen in relation to the *CDKN2A* status. Regarding *MC1R* status, the presence of two red hair variants in each individual (RHC: p.R151C and p.R160W) was analyzed. To reduce the effect of intra-individual variability, two siblings from each family were selected, resulting in four different extreme genotypic conditions: a carrier of variants in both genes (sample A1), an individual without variants in any gene (sample B2) and two carriers of variants in one of these genes (samples A2 and B1), respectively.

The study was approved by the IRB and signed informed consents were obtained from all individuals.

Primary keratinocyte and melanocyte co-culture

Skin biopsies from non-lesional areas were taken and human keratinocytes and melanocytes were obtained by mechanical fragmentation and enzymatic digestion [68]. Briefly, skin biopsy fragments were digested with collagenase type I solution (2mg/ml) (Sigma–Aldrich, Gillingham, UK) for 90 minutes. The collagenase solution was then completely eliminated and the remaining skin biopsy was introduced into a mixture of 0.05% trypsin /0.02% EDTA (T/E) (Sigma). Every 30 min, T/E was changed for a fresh T/E mixture. Collected T/E was inactivated with serum containing culture medium and was centrifuged at 1400 rpm for 10 minutes. Primary keratinocytes and melanocytes obtained by this method were cultured on a feeder layer of lethally irradiated (X-ray; 50 Gy) 3T3-J2 cells (a gift from Dr J. Garlick, SUNY), as previously described [69, 70]. The seeding media was a 3:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, Barcelona, Spain) and HAM'S F12 (GIBCO-BRL) containing 10% FCS, 0.1 nM choleric toxin, 2 nM T3, 5 μg/mL insulin, and 0.4 μg/mL hydrocortisone. Cells were cultured at 37ºC in a humid atmosphere containing 5% CO2. The culture medium was changed every two days. This co-culture system has been extensively proven to preserve stemness of keratinocytes[69-71] as well as to maintain the physiological melanocyte:keratinocyte ratio (1:40)[72, 73]. Moreover, by using the skin cells from this type of co-culture, the donor's phenotypic pigmentation and UV-response features are preserved *in vivo* on a humanized skin mouse model[74].

RNA extraction

Total RNA isolation from primary cultures on passage 3-4 was performed using the Trizol extraction method (Invitrogen Life Technologies, Carlsbad, CA) followed by purification in commercial columns (Qiagen, Valencia, CA). Total isolated RNA was further purified using an RNeasy kit (Qiagen, Valencia, CA). RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific) and integrity of the RNA was verified by Bioanalyzer 2100 (Agilent, USA). The RNA integrity number was in all cases higher than 8.

Expression array

Analysis of global expression was performed using the Whole Human Genome (4x44k) Oligo Microarray kit (G4112F, Agilent, US). The microarray contains probes from over 41,000 unique human genes and transcripts, all with public OMIM annotations (RefSeq, Goldenpath, Ensembl, Unigene Human Genome and GenBank databases). Overall, 50 ng of RNA were labeled using Low input Quickamp Labeling kit (Agilent, US). In all samples 10 commercial controls probes were added in order to standardize the results (RNA Spike-in kit, one color, Agilent, US). The arrays were scanned using the DNA Microarray Scanner G2565CA (Agilent, US). Finally, Feature Extraction Software (FES, Agilent, USA) was used both to perform the quality control process and to extract the information. Three replicates from each primary culture were analyzed.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) of selected genes

To confirm the microarray results, the expression of 13 genes (*FARP1, SLFN11, GFPT2, COL5A3, TYRP1, LEF1, KRT2, ST6GALNAC3, MLANA, MSMB, SILV, A2M,* and *ALOX5*) was validated by RT-PCR. Also, *MC1R* expression was evaluated. The *ACTB* gene was used for normalization. RNA derived from donors' healthy skin was used as a calibrator in each reaction. A total of 200 ng of RNA was retrotranscribed to cDNA using Taqman PCR Core Reagent kit (Roche Applied Science, Penzbergf, Germany). Real-time PCR was performed using Taqman Universal PCR master Mix (Roche Applied Science, Penzbergf, Germany). Reaction was performed in an ABI Step One plus RT-PCR sequence detection instrument (Applied Biosystems, CA, US). Primer design and gene-specific probe selection were carried out by the Universal Probe Library software (UPL, Roche, Mannheim, Germany).

Data was evaluated using the relative quantification method of ddCt[75]. Expression values were evaluated by T-test for equality means using the SPSS 17.0. P-values less than or equal to 0.05 were considered statistically significant.

Microarray data and statistical analyses

The Agilent Processed Signal was standardized across arrays using quantile normalization[76]. Differential gene expression analysis was carried out using the limma package from Bioconductor. Multiple testing adjustments of p-values were performed according to Benjamini and Hochberg methodology[77].

Gene set analysis was carried out for the Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) using FatiScan[78] in Babelomics[79]. This tool detects significantly up- or down-regulated blocks of functionally related genes in lists of genes ordered by different criteria such as differential expression, KEGG pathways and others. The core of the method is based on an algorithm to test whether a set of genes, labeled with terms (biological information), contain significant enrichments of one or several of these terms with respect to another set of reference genes. FatiScan uses a Fisher's exact test for 2×2 contingency tables for comparing two groups of genes and extracting a list of KEGG terms whose distribution among the groups is significantly different. Given that many KEGG terms are simultaneously tested, the results of the test are corrected for multiple testing to obtain an adjusted p-value. FatiScan returns adjusted p-values based on the False Discovery Rate (FDR) method[77]. KEGG Pathways for the genes in the microarray where taken from the KEGG web.

In order to gain insight into the co-regulation patterns on transcript expression profiles, data from our microarray experiments were clustered together with expression data obtained from two previous experiments focused on NMSC (GSE2503 dataset[35]) and MM (GSE12391 dataset[36]). Raw data were obtained from the Gene Expression Omnibus. For each dataset we analyzed the differential expression and determined the fold-changes. We selected two subsets: genes differentially expressed in our study for the *MC1R* comparison (RHC alleles vs. wild-type *MC1R*) and a second group of genes differentially expressed in our study for the *CDKN2A* comparison (p.G101W vs. wild-type *CDKN2A*). In each scenario, fold-changes were normalized using the quantile method[76] to compare differential expression results in all experiments. We performed a hierarchical clustering analysis of differentially expressed transcripts and the graphical representation showed the relationship between experiments. The correlation analysis quantified the linear relationship between the different studies m

Supplemental Data Description

Supplemental Data includes two tables with the list of the most deregulated genes associated with *CDKN2A* mutations or *MC1R* variants; and one table containing the list of genes inversely deregulated between CDKN2A and MC1R.

ACKNOWLEDGMENTS

The present project was founded by a grant from CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain (INTRA/07/726.2). The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (P.I. 09/01393 and P.I. 12/00840, co-funded by "Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una manera de hacer Europa"); by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain; by the European Commission under the 6th Framework Programme, Contract nr: LSHC-CT-2006-018702 (GenoMEL) and by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115). This work is also partly supported by grants BIO2008-04212 and SAF2010-16976 from the Spanish Ministry of Science and Innovation (MICINN), PROMETEO/2010/001 from the GVA-FEDER and S2010/BMD-2420 (CELLCAM) from Comunidad de Madrid.

Web Resources:

Bioconductor (http://www.bioconductor.org)

Babelomics (http://babelomics.bioinfo.cipf.es/)

Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) (http://www.genome.jp/kegg/)

Ensembl 56 release (http://www.ensembl.org)

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/)

Accession Numbers The GEO accession number for the expression microarray data reported in this paper is GSE44805.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ptorruosgomwqds&acc=GSE44805

Conflict of Interest:

The authors state no conflict of interest.

REFERENCES

1. Siegel R, Naishadham D and Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62(1):10-29.
2. Thompson JF, Scolyer RA and Kefford RF. Cutaneous melanoma in the era of molecular profiling. Lancet. 2009; 374(9687):362-365.
3. Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Albright LA, Chaudru V, Chompret A, Cuellar F, Elder DE, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet. 2007; 44(2):99-106.
4. Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, Azon A, Gonzalez U, Castel T, Campoy A, Herrero J, Marti R, Brunet-Vidal J and Mila M. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol. 2005; 23(13):3043-3051.
5. Liontos M, Pateras IS, Evangelou K and Gorgoulis VG. The Tumor Suppressor Gene ARF as a Sensor of Oxidative Stress. Curr Mol Med. 2012.
6. Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG, Samadashwily G and Grossman D. The p16(INK4A) tumor suppressor regulates cellular oxidative stress. Oncogene. 2011; 30(3):265-274.
7. Lang J, Boxer M and MacKie RM. CDKN2A mutations in Scottish families with cutaneous melanoma: results from 32 newly identified families. Br J Dermatol. 2005; 153(6):1121-1125.
8. Larre Borges A, Cuellar F, Puig-Butille JA, Scarone M, Delgado L, Badenas C, Mila M, Malvehy J, Barquet V, Nunez J, Laporte M, Fernandez G, Levrero P, Martinez-Asuaga M and Puig S. CDKN2A mutations in melanoma families from Uruguay. Br J Dermatol. 2009; 161(3):536-541.
9. Yakobson E, Eisenberg S, Isacson R, Halle D, Levy-Lahad E, Catane R, Safro M, Sobolev V, Huot T, Peters G, Ruiz A, Malvehy J, Puig S, Chompret A, Avril MF, Shafir R, et al. A single Mediterranean, possibly Jewish, origin for the Val59Gly CDKN2A mutation in four melanoma-prone families. Eur J Hum Genet. 2003; 11(4):288-296.
10. Majore S, De Simone P, Crisi A, Eibenschutz L, Binni F, Antigoni I, De Bernardo C, Catricala C and Grammatico P. CDKN2A/CDK4 molecular study on 155 Italian subjects with familial and/or primary multiple melanoma. Pigment Cell Melanoma Res. 2008; 21(2):209-211.
11. Eliason MJ, Larson AA, Florell SR, Zone JJ, Cannon-Albright LA, Samlowski WE and Leachman SA. Population-based prevalence of CDKN2A mutations

in Utah melanoma families. J Invest Dermatol. 2006; 126(3):660-666.

12. Ciotti P, Struewing JP, Mantelli M, Chompret A, Avril MF, Santi PL, Tucker MA, Bianchi-Scarra G, Bressac-de Paillerets B and Goldstein AM. A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families. Am J Hum Genet. 2000; 67(2):311-319.
13. Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, Chompret A, Ghiorzo P, Gruis N, Hansson J, Harland M, Hayward N, Holland EA, Mann GJ, Mantelli M, Nancarrow D, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 2002; 94(12):894-903.
14. Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D and Wang Y. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res. 2005; 11(20):7234-7242.
15. Jefferies S, Edwards SM, Hamoudi RA, A'Hern R, Foulkes W, Goldgar D and Eeles R. No germline mutations in CDKN2A (p16) in patients with squamous cell cancer of the head and neck and second primary tumours. Br J Cancer. 2001; 85(9):1383-1386.
16. Cabanillas R, Astudillo A, Valle M, de la Rosa J, Alvarez R, Duran NS and Cadinanos J. Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. Head Neck. 2011.
17. Rees JL. The genetics of sun sensitivity in humans. Am J Hum Genet. 2004; 75(5):739-751.
18. Valverde P, Healy E, Jackson I, Rees JL and Thody AJ. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nat Genet. 1995; 11(3):328-330.
19. Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ and Bavinck JN. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001; 68(4):884-894.
20. Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, Parsons PG, Green AC and Sturm RA. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. J Invest Dermatol. 2001; 116(2):224-229.
21. Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA and Hayward NK. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 2001; 69(4):765-773.
22. Goldstein AM, Chaudru V, Ghiorzo P, Badenas C, Malvehy J, Pastorino L, Laud K, Hulley B, Avril MF, Puig-Butille JA, Miniere A, Marti R, Chompret A, Cuellar F, Kolm I, Mila M, et al. Cutaneous phenotype and MC1R variants as modifying factors for the development of melanoma in CDKN2A G101W mutation carriers from 4 countries. Int J Cancer. 2007; 121(4):825-831.
23. Demenais F, Mohamdi H, Chaudru V, Goldstein AM, Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, Bergman W, Bianchi-Scarra G, Bressac-de Paillerets B, et al. Association of MC1R variants and host phenotypes with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010; 102(20):1568-1583.
24. Herraiz C, Jimenez-Cervantes C, Zanna P and Garcia-Borron JC. Melanocortin 1 receptor mutations impact differentially on signalling to the cAMP and the ERK mitogen-activated protein kinase pathways. FEBS Lett. 2009; 583(19):3269-3274.
25. Le Pape E, Passeron T, Giubellino A, Valencia JC, Wolber R and Hearing VJ. Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r. Proc Natl Acad Sci U S A. 2009; 106(6):1802-1807.
26. Sviderskaya EV, Hill SP, Evans-Whipp TJ, Chin L, Orlow SJ, Easty DJ, Cheong SC, Beach D, DePinho RA and Bennett DC. p16(Ink4a) in melanocyte senescence and differentiation. J Natl Cancer Inst. 2002; 94(6):446-454.
27. Eisinger M and Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci U S A. 1982; 79(6):2018-2022.
28. Herlyn M, Rodeck U, Mancianti M, Cardillo FM, Lang A, Ross AH, Jambrosic J and Koprowski H. Expression of melanoma-associated antigens in rapidly dividing human melanocytes in culture. Cancer Res. 1987; 47(12):3057-3061.
29. Elder DE, Rodeck U, Thurin J, Cardillo F, Clark WH, Stewart R and Herlyn M. Antigenic profile of tumor progression stages in human melanocytic nevi and melanomas. Cancer Res. 1989; 49(18):5091-5096.
30. Valyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhasz I and Herlyn M. Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. Lab Invest. 1993; 69(2):152-159.
31. Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE and Herlyn M. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol. 2000; 156(5):1515-1525.
32. Phillips J, Gawkrodger DJ, Caddy CM, Hedley S, Dawson RA, Smith-Thomas L, Freedlander E and Mac Neil S. Keratinocytes suppress TRP-1 expression and reduce cell number of co-cultured melanocytes - implications for grafting of patients with vitiligo. Pigment Cell Res. 2001; 14(2):116-125.
33. Haass NK, Smalley KS, Li L and Herlyn M. Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res. 2005; 18(3):150-159.
34. Hirobe T. Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of

mammalian epidermal melanocytes. Pigment Cell Res. 2005; 18(1):2-12.

35. Nindl I, Dang C, Forschner T, Kuban RJ, Meyer T, Sterry W and Stockfleth E. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. Mol Cancer. 2006; 5:30.

36. Scatolini M, Grand MM, Grosso E, Venesio T, Pisacane A, Balsamo A, Sirovich R, Risio M and Chiorino G. Altered molecular pathways in melanocytic lesions. Int J Cancer. 2010; 126(8):1869-1881.

37. de Snoo FA and Hayward NK. Cutaneous melanoma susceptibility and progression genes. Cancer Lett. 2005; 230(2):153-186.

38. Ogbah Z, Puig-Butille JA, Simonetta F, Badenas C, Cervera R, Mila J, Benitez D, Malvehy J, Vilella R and Puig S. Molecular characterization of human cutaneous melanoma-derived cell lines. Anticancer Res. 2012; 32(4):1245-1251.

39. Herbert BS, Chanoux RA, Liu Y, Baenziger PH, Goswami CP, McClintick JN, Edenberg HJ, Pennington RE, Lipkin SM and Kopelovich L. A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. Oncotarget. 2012; 1(6):405-422.

40. Fan M, Pfeffer SR, Lynch HT, Cassidy P, Leachman S, Pfeffer LM and Kopelovich L. Altered transcriptome signature of phenotypically normal skin fibroblasts heterozygous for CDKN2A in familial melanoma: relevance to early intervention. Oncotarget. 2013; 4(1):128-141.

41. Hall A, Meyle KD, Lange MK, Klima M, Sanderhoff M, Dahl C, Abildgaard C, Thorup K, Moghimi SM, Jensen PB, Bartek J, Guldberg P and Christensen C. Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the (V600E)BRAF oncogene. Oncotarget. 2013; 4(4):584-599.

42. Gu F, Qureshi AA, Niu T, Kraft P, Guo Q, Hunter DJ and Han J. Interleukin and interleukin receptor gene polymorphisms and susceptibility to melanoma. Melanoma Res. 2008; 18(5):330-335.

43. Howell WM, Turner SJ, Bateman AC and Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001; 2(1):25-31.

44. Yang XR, Pfeiffer RM, Wheeler W, Yeager M, Chanock S, Tucker MA and Goldstein AM. Identification of modifier genes for cutaneous malignant melanoma in melanoma-prone families with and without CDKN2A mutations. Int J Cancer. 2009; 125(12):2912-2917.

45. Guo J, Wen DR, Huang RR, Paul E, Wunsch P, Itakura E and Cochran AJ. Detection of multiple melanoma-associated markers in melanoma cell lines by RT in situ PCR. Exp Mol Pathol. 2003; 74(2):140-147.

46. Raposo G and Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic. 2002; 3(4):237-248.

47. Ramanathan HN and Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. Cell Res. 2011; 22(2):346-359.

48. Sakai C, Kawakami Y, Law LW, Furumura M and Hearing VJ, Jr. Melanosomal proteins as melanoma-specific immune targets. Melanoma Res. 1997; 7(2):83-95.

49. Hearing VJ. Biochemical control of melanogenesis and melanosomal organization. J Investig Dermatol Symp Proc. 1999; 4(1):24-28.

50. Schouwey K, Delmas V, Larue L, Zimber-Strobl U, Strobl LJ, Radtke F and Beermann F. Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner. Dev Dyn. 2007; 236(1):282-289.

51. Okuyama R, Tagami H and Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci. 2008; 49(3):187-194.

52. Xin Y, Lu Q and Li Q. IKK1 control of epidermal differentiation is modulated by notch signaling. Am J Pathol. 2011; 178(4):1568-1577.

53. Lin HY, Kao CH, Lin KM, Kaartinen V and Yang LT. Notch signaling regulates late-stage epidermal differentiation and maintains postnatal hair cycle homeostasis. PLoS One. 2011; 6(1):e15842.

54. Panelos J and Massi D. Emerging role of Notch signaling in epidermal differentiation and skin cancer. Cancer Biol Ther. 2009; 8(21):1986-1993.

55. Chang YM, Newton-Bishop JA, Bishop DT, Armstrong BK, Bataille V, Bergman W, Berwick M, Bracci PM, Elwood JM, Ernstoff MS, Green AC, Gruis NA, Holly EA, Ingvar C, Kanetsky PA, Karagas MR, et al. A pooled analysis of melanocytic nevus phenotype and the risk of cutaneous melanoma at different latitudes. Int J Cancer. 2009; 124(2):420-428.

56. Bishop JA, Wachsmuth RC, Harland M, Bataille V, Pinney E, Mac KP, Baglietto L, Cuzick J and Bishop DT. Genotype/phenotype and penetrance studies in melanoma families with germline CDKN2A mutations. J Invest Dermatol. 2000; 114(1):28-33.

57. Sanchez-Laorden BL, Herraiz C, Valencia JC, Hearing VJ, Jimenez-Cervantes C and Garcia-Borron JC. Aberrant trafficking of human melanocortin 1 receptor variants associated with red hair and skin cancer: Steady-state retention of mutant forms in the proximal golgi. J Cell Physiol. 2009; 220(3):640-654.

58. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491(7424):449-453.

59. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 2008; 31(7):353-360.

60. Liu R, Gao X, Lu Y and Chen H. Meta-analysis of the

relationship between Parkinson disease and melanoma. Neurology. 2011; 76(23):2002-2009.

61. Olsen JH, Friis S and Frederiksen K. Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology. 2006; 17(5):582-587.
62. Olsen JH, Friis S, Frederiksen K, McLaughlin JK, Mellemkjaer L and Moller H. Atypical cancer pattern in patients with Parkinson's disease. Br J Cancer. 2005; 92(1):201-205.
63. Gao X, Simon KC, Han J, Schwarzschild MA and Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009; 65(1):76-82.
64. Meng S, Song F, Chen H, Gao X, Amos CI, Lee JE, Wei Q, Qureshi AA and Han J. No association between Parkinson disease alleles and the risk of melanoma. Cancer Epidemiol Biomarkers Prev. 2011; 21(1):243-245.
65. Hassin-Baer S, Laitman Y, Azizi E, Molchadski I, Galore-Haskel G, Barak F, Cohen OS and Friedman E. The leucine rich repeat kinase 2 (LRRK2) G2019S substitution mutation. Association with Parkinson disease, malignant melanoma and prevalence in ethnic groups in Israel. J Neurol. 2009; 256(3):483-487.
66. Shinozuka K, Uzawa K, Fushimi K, Yamano Y, Shiiba M, Bukawa H, Yokoe H and Tanzawa H. Downregulation of carcinoembryonic antigen-related cell adhesion molecule 1 in oral squamous cell carcinoma: correlation with tumor progression and poor prognosis. Oncology. 2009; 76(6):387-397.
67. Quintanilla-Dieck MJ, Codriansky K, Keady M, Bhawan J and Runger TM. Cathepsin K in melanoma invasion. J Invest Dermatol. 2008; 128(9):2281-2288.
68. Rheinwald JG and Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975; 6(3):331-343.
69. Llames SG, Del Rio M, Larcher F, Garcia E, Garcia M, Escamez MJ, Jorcano JL, Holguin P and Meana A. Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation. 2004; 77(3):350-355.
70. Del Rio M, Larcher F, Serrano F, Meana A, Munoz M, Garcia M, Munoz E, Martin C, Bernad A and Jorcano JL. A preclinical model for the analysis of genetically modified human skin in vivo. Hum Gene Ther. 2002; 13(8):959-968.
71. Larcher F, Dellambra E, Rico L, Bondanza S, Murillas R, Cattoglio C, Mavilio F, Jorcano JL, Zambruno G and Del Rio M. Long-term engraftment of single genetically modified human epidermal holoclones enables safety pre-assessment of cutaneous gene therapy. Mol Ther. 2007; 15(9):1670-1676.
72. De Luca M, Franzi AT, D'Anna F, Zicca A, Albanese E, Bondanza S and Cancedda R. Coculture of human keratinocytes and melanocytes: differentiated melanocytes are physiologically organized in the basal layer of the cultured epithelium. Eur J Cell Biol. 1988; 46(1):176-180.
73. Guerra L, Capurro S, Melchi F, Primavera G, Bondanza S, Cancedda R, Luci A, De Luca M and Pellegrini G. Treatment of "stable" vitiligo by Timedsurgery and transplantation of cultured epidermal autografts. Arch Dermatol. 2000; 136(11):1380-1389.
74. Garcia M, Llames S, Garcia E, Meana A, Cuadrado N, Recasens M, Puig S, Nagore E, Illera N, Jorcano JL, Del Rio M and Larcher F. In vivo assessment of acute UVB responses in normal and Xeroderma Pigmentosum (XP-C) skin-humanized mouse models. Am J Pathol. 2010; 177(2):865-872.
75. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402-408.
76. Bolstad BM, Irizarry RA, Astrand M and Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19(2):185-193.
77. Benjamini Y, Drai D, Elmer G, Kafkafi N and Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001; 125(1-2):279-284.
78. Al-Shahrour F, Arbiza L, Dopazo H, Huerta-Cepas J, Minguez P, Montaner D and Dopazo J. From genes to functional classes in the study of biological systems. BMC Bioinformatics. 2007; 8:114.
79. Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, Tarraga J, Pascual-Montano A, Nogales-Cadenas R, Santoyo J, Garcia F, Marba M, Montaner D and Dopazo J. Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. Nucleic Acids Res. 2010; 38(Web Server issue):W210-213.

Supplementary Material:

Table S1: List of the most significant deregulated transcripts regarding *CDKN2A* status (p.G101W vs wild-type), adjusted P-value< 0.0001. Only genes identified in both *MC1R* wild-type and *MC1R* RHC variant carriers are included (N=108)
Abbreviations: FC: Fold Change; P-value: adjusted P-Value of such comparison; wt: wild-type.
*Statistical values of mutant *CDKN2A* vs wild-type in *MC1R* RHC skin cells (individual A1 vs individual A2).
**Statistical values of mutant *CDKN2A* vs wild-type in wild-type *MC1R* skin cells (individual B1 vs individual B2)

Gene ID	Gene Name	FC in MC1R RHC *	P-value in MC1R RHC *	FC in wt MC1R**	P-value in wt MC1R**
NM_002121	*HLA-DPB1*	-4.83	4.05E-13	-2.60	5.79E-10
NM_005766	*FARP1*	-3.6	1.32E-11	-1.53	5.80E-07
NM_152270	*SLFN11*	-3.4	1.87E-06	-2.08	7.47E-07
NM_005110	*GFPT2*	-3.2	5.49E-06	-2.50	5.64E-05
NM_015719	*COL5A3*	-3.1	1.87E-06	-2.28	3.91E-05
NM_000550	*TYRP1*	-2.76	1.43E-06	-2.34	6.40E-06
NM_016269	*LEF1*	-2.69	1.73E-09	-1.64	6.46E-07
NM_000423	*KRT2*	-2.45	7.93E-05	-2.70	1.58E-05
NM_000304	*PMP22*	-2.41	9.22E-10	-1.80	3.50E-08
NM_152996	*ST6GALNAC3*	-2.38	1.38E-08	-1.44	4.65E-06
NM_138284	*IL17D*	-2.31	7.53E-06	-2.02	2.08E-05
AF086044	*unknown*	-2.23	1.74E-10	-3.42	5.23E-13
NM_198951	*TGM2*	-2.08	4.10E-06	-2.42	4.33E-07
NM_138375	*CABLES1*	-2.08	5.78E-09	-3.31	8.92E-12
NM_006426	*DPYSL4*	-2.03	3.01E-05	-3.92	6.81E-09
NM_032048	*EMILIN2*	-1.83	5.82E-06	-2.04	1.00E-06
AK074473	*unknown*	-1.83	6.09E-06	-2.43	1.29E-07
NM_001129	*AEBP1*	-1.81	2.43E-07	-1.40	3.91E-06
NM_014070	*unknown*	-1.78	2.71E-07	-1.31	8.02E-06
BC004343	*C21orf122*	-1.66	5.99E-06	-1.47	1.48E-05
NM_148897	*SDR9C7*	-1.63	3.91E-05	-1.83	6.51E-06
AF131834	*unknown*	-1.53	2.07E-06	-2.60	1.81E-09
NM_080647	*TBX1*	-1.53	4.46E-06	-1.21	3.80E-05
NM_012131	*CLDN17*	-1.47	4.17E-05	-1.79	2.57E-06
NM_000635	*RFX2*	-1.38	2.98E-06	-1.08	2.98E-05
ENST00000224809	*unknown*	-1.28	6.59E-06	-1.81	6.37E-08
NM_032534	*KRBA1*	-1.21	2.92E-06	-0.97	2.25E-05
NM_152359	*CPT1C*	-1.08	1.08E-05	-1.48	1.50E-07
NM_001077263	*TMPRSS13*	-0.87	4.43E-05	-2.81	9.51E-12
NM_000396	*CTSK*	-0.74	7.83E-05	-1.81	1.05E-09
NM_003798	*CTNNAL1*	0.71	6.15E-05	0.81	9.91E-06
NM_015920	*RPS27L*	0.77	4.43E-05	0.86	8.34E-06
NM_001015509	*unknown*	0.79	1.13E-05	0.68	3.46E-05
THC2657938	*unknown*	0.81	4.28E-05	2.08	2.37E-10
NM_000022	*ADA*	0.87	8.65E-06	1.08	3.92E-07
THC2698177	*unknown*	0.89	6.21E-05	1.75	1.38E-08
NM_005760	*CEBPZ*	0.90	3.14E-05	2.20	3.14E-10
NM_004346	*CASP3*	0.91	1.50E-05	1.31	1.23E-07
NM_004487	*GOLGB1*	0.95	5.49E-06	1.17	2.84E-07
BC027178	*PRPF40A*	0.99	8.74E-05	2.62	3.74E-10
NM_004280	*EEF1E1*	1.00	7.97E-05	1.06	2.48E-05
NM_004986	*KTN1*	1.04	2.90E-06	2.53	1.59E-11
ENST00000377156	*unknown*	1.07	1.97E-05	1.09	9.97E-06
NM_007047	*BTN3A2*	1.09	1.77E-05	1.11	9.31E-06

1

NM_020169	*LXN*	1.11	3.10E-05	1.97	1.97E-08
ENST00000306024	*LSM3*	1.12	2.45E-05	0.99	5.33E-05
NM_001033719	*ZNF404*	1.13	4.49E-05	2.13	1.58E-08
NM_016010	*FAM164A*	1.13	1.23E-05	0.99	3.29E-05
NM_001007234	*unknown*	1.15	1.63E-06	1.42	7.87E-08
NM_001012334	*MDK*	1.16	6.19E-05	1.20	2.48E-05
NM_181453	*GCC2*	1.19	6.48E-05	1.56	1.83E-06
NM_033514	*LIMS3*	1.20	8.85E-07	1.16	8.49E-07
NM_033316	*MFI2*	1.20	9.60E-05	1.87	3.91E-07
NM_002890	*RASA1*	1.22	1.18E-06	0.86	3.86E-05
NM_018300	*ZNF83*	1.23	5.73E-05	1.70	8.63E-07
NM_201612	*unknown*	1.23	1.19E-05	2.28	4.38E-09
NM_207014	*WDR78*	1.30	6.81E-05	1.50	8.56E-06
NM_002078	*GOLGA4*	1.31	4.03E-05	1.21	5.59E-05
AK075484	*SAMD4B*	1.31	1.52E-05	3.52	3.01E-11
NM_003566	*EEA1*	1.33	9.48E-05	2.36	7.47E-08
NM_144777	*SCEL*	1.33	5.55E-07	1.01	9.54E-06
CB050071	*unknown*	1.41	5.52E-05	1.53	1.31E-05
NM_022154	*SLC39A8*	1.43	8.09E-05	1.54	2.19E-05
CR603437	*unknown*	1.44	1.33E-06	1.35	1.75E-06
NM_018169	*C12orf35*	1.48	2.17E-05	2.02	3.71E-07
NM_207331	*unknown*	1.49	9.36E-06	1.28	3.07E-05
AB075826	*FAM171B*	1.49	5.31E-05	1.47	3.83E-05
NM_004510	*SP110*	1.53	1.66E-07	0.94	3.63E-05
NM_172174	*IL15*	1.55	5.30E-08	1.29	4.08E-07
NM_000888	*ITGB6*	1.55	3.99E-05	2.52	8.05E-08
NM_005059	*RLN2*	1.58	1.97E-05	1.38	5.37E-05
NM_025114	*CEP290*	1.67	1.90E-05	2.70	3.88E-08
AK096536	*unknown*	1.70	3.16E-06	1.36	2.52E-05
THC2683448	*unknown*	1.72	2.01E-06	1.91	3.87E-07
NM_001013728	*unknown*	1.74	1.34E-05	2.23	4.47E-07
BC029919	*unknown*	1.78	4.62E-06	1.40	4.30E-05
NM_001712	*CEACAM1*	1.80	1.35E-06	1.56	4.49E-06
NM_003020	*SCG5*	1.83	2.10E-08	2.37	5.14E-10
NM_004921	*unknown*	1.86	8.41E-05	1.74	9.87E-05
NM_018429	*BDP1*	1.90	1.48E-05	2.05	3.60E-06
THC2676635	*unknown*	1.93	2.93E-08	1.27	3.49E-06
NM_178445	*CCRL1*	1.99	1.68E-05	1.98	1.08E-05
THC2740317	*unknown*	2.04	7.85E-06	1.75	2.63E-05
NM_182762	*MACC1*	2.05	5.66E-09	0.89	8.55E-05
XR_015158	*unknown*	2.15	6.40E-10	1.91	2.32E-09
NM_001547	*IFIT2*	2.16	4.68E-08	2.17	3.56E-08
NM_152737	*RNF182*	2.19	1.05E-05	1.78	5.97E-05
NM_014314	*DDX58*	2.22	3.99E-08	1.53	2.66E-06
AK022351	*unknown*	2.26	4.01E-05	2.74	2.66E-06
THC2526509	*unknown*	2.26	6.31E-08	1.19	7.99E-05
NM_181501	*ITGA1*	2.38	1.07E-08	1.09	8.38E-05
NM_018284	*GBP3*	2.40	1.80E-07	2.10	6.58E-07
AI078143	*unknown*	2.60	2.11E-07	1.52	7.46E-05
THC2510656	*unknown*	2.62	8.54E-06	2.91	1.51E-06
THC2549494	*unknown*	2.72	4.74E-06	2.48	8.65E-06
NM_020124	*IFNK*	2.74	7.98E-07	2.36	3.02E-06
NM_152703	*SAMD9L*	2.98	8.72E-06	2.94	6.15E-06
NM_005532	*IFI27*	3.03	3.42E-11	0.92	5.22E-05
NM_017654	*SAMD9*	3.04	1.08E-07	1.98	1.26E-05
NM_006417	*IFI44*	3.07	3.28E-12	2.71	7.74E-12
THC2679528	*unknown*	3.18	4.40E-07	2.89	9.26E-07
AK023743	*unknown*	3.18	1.52E-10	1.95	7.56E-08
NM_001548	*IFIT1*	3.28	6.99E-11	1.59	5.54E-07
BF213738	*unknown*	3.46	3.06E-11	1.34	2.66E-06
NM_006820	*IFI44L*	3.49	6.99E-11	2.28	1.49E-08
D89479	*SULT1B1*	3.56	1.70E-08	3.24	4.36E-08

2

NM_005127	*CLEC2B*	3.66	1.61E-13	1.20	1.33E-07
NM_017523	*XAF1*	5.11	1.98E-08	3.00	9.40E-06

Table S2: List of the most significant deregulated transcripts regarding *MC1R* status (p. R106W and p.R151C vs wild-type) identified in both comparisons.
Adjusted P-value < 0.0000001. Only genes identified in both analyses are included (N=159)
Abbreviations: FC: Fold Change; P-value: adjusted P-Value of such comparison; wt: wild-type.
*Statistical values of *MC1R* RHC vs wild-type in mutant *CDKN2A* skin cell (individual A1 vs individual B1).
**Statistical values of *MC1R* RHC vs wild-type in wild-type *CDKN2A* skin cell (individual A2 vs individual B2).

Gene ID	Gene Name	FC in mutant *CDKN2A**	P-value in mutant *CDKN2A**	FC in wt *CDKN2A***	P-value in wt *CDKN2A***
NM_005672	*PSCA*	-7.30	2.39E-12	-7.02	1.20E-12
NM_000550	*TYRP1*	-7.24	2.85E-12	-6.82	2.14E-12
NM_000095	*COMP*	-6.79	3.34E-13	-5.08	2.95E-12
NM_005511	*MLANA*	-5.74	2.51E-09	-6.96	7.05E-11
NM_000372	*TYR.TYRL*	-5.59	1.29E-12	-7.19	1.62E-14
NM_002443	*MSMB*	-5.30	1.03E-14	-4.05	1.47E-13
NM_006928	*SILV*	-4.79	1.66E-08	-6.05	3.02E-10
NM_000166	*GJB1*	-4.72	2.43E-10	-2.76	9.95E-08
NM_000014	*A2M*	-4.52	3.06E-08	-7.21	2.40E-11
NM_000698	*ALOX5*	-4.17	3.63E-11	-2.19	6.05E-08
NM_016190	*CRNN*	-4.14	8.50E-10	-4.68	5.89E-11
THC2711870	*unknown*	-3.98	8.16E-09	-6.00	1.27E-11
THC2675163	*unknown*	-3.73	6.81E-12	-6.37	5.96E-15
NM_032588	*TRIM63*	-3.70	6.23E-10	-5.36	1.70E-12
NM_004750	*CRLF1*	-3.70	1.31E-10	-2.14	6.30E-08
NM_013358	*PADI1*	-3.61	1.08E-08	-4.16	6.97E-10
ENST00000366930	*TGFB2*	-3.56	3.31E-10	-2.27	4.78E-08
NM_004864	*GDF15*	-3.31	8.19E-11	-4.23	1.09E-12
NM_020182	*PMEPA1*	-3.18	2.39E-12	-1.98	4.44E-10
NM_001712	*CEACAM1*	-3.12	6.03E-10	-3.36	7.97E-11
NM_005512	*LRRC32*	-3.11	8.78E-08	-3.21	2.37E-08
NM_144497	*AKAP12*	-3.10	8.04E-08	-2.91	7.23E-08
NM_130808	*CPNE4*	-2.97	4.58E-09	-2.69	6.33E-09
NM_001901	*CTGF*	-2.94	9.98E-10	-4.13	4.12E-12
NM_001039212	*unknown*	-2.87	9.12E-11	-3.14	9.05E-12
NM_024101	*MLPH*	-2.86	1.13E-08	-4.17	3.06E-11
ENST00000264554	*SHC2*	-2.80	1.77E-08	-2.97	3.32E-09
NM_000201	*ICAM1*	-2.75	2.83E-09	-2.45	4.99E-09
NM_000222	*KIT*	-2.75	6.97E-09	-5.47	3.15E-13
NM_201631	*TGM5*	-2.69	6.67E-09	-2.57	4.59E-09
NM_002193	*INHBB*	-2.61	3.47E-10	-2.46	2.87E-10
NM_001001547	*CD36*	-2.60	1.37E-08	-2.58	6.28E-09
NM_001945	*HBEGF*	-2.59	4.84E-08	-2.56	2.20E-08
NM_002463	*MX2*	-2.59	2.72E-09	-5.66	4.69E-14
NM_173198	*NR4A3*	-2.45	2.44E-10	-4.42	5.15E-14
NM_001343	*DAB2*	-2.42	2.64E-08	-3.27	1.92E-10
NM_138375	*CABLES1*	-2.23	1.35E-09	-3.46	1.49E-12
NM_001012964	*KLK6*	-2.19	2.71E-09	-4.55	8.27E-14
NM_016463	*CXXC5*	-2.10	3.00E-08	-2.26	4.70E-09
NM_001453	*FOXC1*	-1.94	1.02E-09	-1.72	2.04E-09
NM_003793	*CTSF*	-1.92	4.54E-08	-3.15	2.38E-11
NM_022910	*NDRG4*	-1.84	9.53E-09	-1.55	3.44E-08
NM_006989	*RASA4*	-1.67	7.49E-08	-2.15	1.08E-09
NM_145804	*ABTB2*	-1.45	7.91E-08	-2.40	3.58E-11

NM_003565	*ULK1*	-1.39	7.35E-08	-1.75	1.36E-09
NM_030912	*TRIM8*	-1.25	5.80E-08	-1.13	8.84E-08
NM_206836	*PECI*	1.33	6.12E-08	1.23	6.13E-08
NM_005915	*MCM6*	1.33	9.39E-08	1.69	1.62E-09
AY163812	*C17orf80*	1.34	6.62E-08	1.47	7.72E-09
CR600585	*unknown*	1.45	8.28E-09	1.35	7.88E-09
NM_033514	*LIMS3*	1.46	3.69E-08	1.42	2.13E-08
NM_016126	*HSPB11*	1.47	6.42E-08	1.48	2.29E-08
NM_001079525	*PAICS*	1.50	1.12E-08	2.03	7.45E-11
NM_016448	*DTL*	1.53	5.00E-09	1.85	1.41E-10
NM_003579	*RAD54L*	1.54	6.67E-09	1.87	1.86E-10
NM_032636	*PSRC1*	1.59	5.94E-08	1.67	1.21E-08
NM_033417	*HAUS8*	1.59	6.77E-08	1.71	1.05E-08
NM_018455	*CENPN*	1.61	2.25E-08	2.40	4.13E-11
NM_030771	*CCDC34*	1.64	3.02E-09	3.26	1.47E-13
NM_017868	*TTC12*	1.67	7.74E-10	2.18	7.74E-12
NM_003258	*TK1*	1.72	8.63E-08	1.83	1.53E-08
NM_003504	*CDC45L*	1.75	6.55E-09	1.99	4.57E-10
NM_002916	*RFC4*	1.76	1.32E-09	2.19	2.50E-11
ENST00000367003	*C1orf97*	1.77	2.25E-08	2.65	4.11E-11
NM_145231	*C14orf143*	1.84	1.04E-08	1.86	3.46E-09
NM_003798	*CTNNAL1*	1.85	2.44E-10	1.94	4.44E-11
NM_014317	*PDSS1*	1.86	3.58E-08	2.19	1.71E-09
NM_004219	*PTTG1*	1.87	9.03E-09	1.92	2.48E-09
NM_001159	*AOX1*	1.87	8.16E-09	2.23	3.05E-10
NM_018186	*C1orf112*	1.94	5.99E-09	1.94	2.12E-09
NM_001826	*CKS1B*	1.96	5.59E-09	2.00	1.55E-09
BX640843	*unknown*	1.99	5.94E-09	2.51	9.73E-11
NM_182620	*FAM33A*	2.01	1.76E-09	1.72	5.18E-09
NM_003686	*EXO1*	2.01	2.93E-10	2.19	3.22E-11
NM_004282	*BAG2*	2.02	9.45E-10	2.61	1.14E-11
NM_014051	*TMEM14A*	2.09	1.45E-08	2.14	4.46E-09
NM_016095	*GINS2*	2.12	7.82E-10	2.14	2.35E-10
NM_145014	*HYLS1*	2.13	5.04E-08	2.66	1.08E-09
NM_024037	*C1orf135*	2.13	8.41E-10	2.22	1.78E-10
NM_003384	*VRK1*	2.15	6.03E-10	2.37	5.77E-11
BC040051	*unknown*	2.15	5.79E-08	2.00	5.83E-08
NM_001254	*CDC6*	2.20	8.16E-08	2.57	4.42E-09
NM_014176	*UBE2T*	2.23	1.12E-11	2.18	5.39E-12
NM_203467	*PPIL5*	2.23	9.58E-10	1.82	5.22E-09
NM_000057	*BLM*	2.26	7.59E-08	2.85	1.45E-09
NM_024808	*C13orf34*	2.27	8.38E-08	2.54	7.74E-09
NM_006607	*PTTG2*	2.27	2.24E-10	1.92	7.20E-10
NM_001928	*CFD*	2.30	3.69E-08	2.45	6.65E-09
NM_015895	*GMNN*	2.31	1.29E-10	1.77	1.51E-09
NM_018101	*CDCA8*	2.34	7.82E-10	2.36	2.52E-10
AK027541	*unknown*	2.35	2.05E-08	2.34	8.84E-09
NM_001766	*CD1D*	2.36	6.03E-09	2.53	9.33E-10
NM_002876	*RAD51C*	2.37	4.47E-10	2.20	4.50E-10
NM_000946	*PRIM1*	2.38	2.14E-09	2.22	1.94E-09
NM_005030	*PLK1*	2.40	8.38E-08	2.41	3.33E-08
NM_031299	*CDCA3*	2.41	2.46E-09	2.09	6.11E-09
NM_012415	*RAD54B*	2.43	6.39E-08	2.34	4.24E-08
NM_006479	*RAD51AP1*	2.46	3.02E-08	3.80	3.48E-11
NM_138555	*KIF23*	2.48	8.16E-09	2.87	4.69E-10
NM_018193	*FANCI*	2.50	4.23E-11	2.38	2.70E-11
NM_014791	*MELK*	2.50	1.91E-09	2.50	7.39E-10
NM_175617	*MT1E*	2.52	6.20E-10	2.12	2.36E-09
NM_012177	*FBXO5*	2.54	1.32E-09	2.45	8.26E-10
NM_001012271	*BIRC5*	2.55	9.58E-10	2.63	2.35E-10
NM_001012267	*CENPP*	2.56	2.85E-12	1.76	1.78E-10
NM_001827	*CKS2*	2.57	1.36E-10	2.22	3.37E-10

4

BC044246	*TMEM200A*	2.57	7.02E-09	2.04	5.61E-08
NM_012310	*KIF4A*	2.57	3.81E-09	2.48	2.30E-09
NM_199420	*POLQ*	2.58	6.02E-08	3.45	5.20E-10
NM_001274	*CHEK1*	2.58	1.29E-10	3.00	5.77E-12
NM_001005414	*unknown*	2.62	6.03E-10	1.95	1.10E-08
NM_182751	*MCM10*	2.62	2.54E-08	3.10	1.09E-09
NM_001010897	*SERP2*	2.65	3.23E-09	2.27	9.59E-09
NM_001034	*RRM2*	2.67	1.05E-08	2.47	1.16E-08
NM_145018	*C11orf82*	2.71	1.68E-10	2.95	1.85E-11
NM_031966	*CCNB1*	2.74	2.63E-11	2.84	5.39E-12
NM_005059	*RLN2*	2.78	9.81E-09	2.58	1.05E-08
NM_006845	*KIF2C*	2.80	1.74E-09	2.78	7.12E-10
NM_001211	*BUB1B*	2.81	4.88E-08	3.45	1.32E-09
NM_198433	*AURKA*	2.85	1.71E-08	2.81	8.31E-09
NM_004701	*CCNB2*	2.92	2.85E-12	3.11	5.71E-13
NM_020242	*KIF15*	2.93	5.10E-09	4.62	4.38E-12
NM_004523	*KIF11*	2.96	1.80E-08	3.18	2.81E-09
NM_001255	*CDC20*	3.05	4.70E-09	2.92	3.13E-09
BC013418	*C13orf3*	3.10	3.10E-09	3.42	3.24E-10
AL834537	*CCDC150*	3.11	3.65E-08	4.61	7.45E-11
NM_004336	*BUB1*	3.13	2.15E-10	3.42	2.35E-11
NM_004117	*FKBP5*	3.14	4.53E-08	3.43	5.67E-09
NM_052913	*TMEM200A*	3.14	8.40E-08	2.96	7.23E-08
NM_001067	*TOP2A*	3.14	9.65E-11	3.93	1.70E-12
NM_005733	*KIF20A*	3.15	1.78E-11	2.96	1.38E-11
NM_001813	*CENPE*	3.18	6.03E-10	4.69	1.24E-12
NM_152515	*CKAP2L*	3.18	8.97E-08	3.74	4.49E-09
NM_001237	*CCNA2*	3.19	2.93E-10	3.25	8.24E-11
NM_018136	*ASPM*	3.20	1.92E-09	3.55	1.78E-10
NM_007280	*OIP5*	3.21	1.51E-11	2.87	2.37E-11
NM_138419	*FAM54A*	3.23	3.04E-11	3.07	2.01E-11
NM_020675	*SPC25*	3.26	1.59E-12	3.45	1.87E-13
NM_018492	*PBK*	3.28	3.26E-09	3.95	9.92E-11
NM_022346	*NCAPG*	3.29	5.95E-13	3.50	7.81E-14
NM_024094	*DSCC1*	3.31	3.47E-10	3.99	1.02E-11
NM_032117	*MND1*	3.35	6.71E-12	3.77	5.71E-13
NM_001809	*CENPA*	3.38	7.61E-11	3.90	3.72E-12
NM_001786	*CDC2*	3.38	2.39E-12	3.27	1.24E-12
NM_145697	*NUF2*	3.40	8.28E-09	4.81	3.22E-11
NM_021186	*ZP4*	3.49	1.03E-14	4.91	3.21E-17
NM_016343	*CENPF*	3.52	1.25E-09	2.85	7.88E-09
NM_001790	*CDC25C*	3.60	3.47E-08	3.67	1.06E-08
NM_018131	*CEP55*	3.63	4.13E-10	3.89	5.89E-11
NM_016195	*KIF20B*	3.68	4.08E-10	2.62	1.36E-08
NM_014750	*DLGAP5*	3.71	2.39E-12	4.17	1.87E-13
NM_005192	*CDKN3*	4.04	7.94E-08	4.03	3.28E-08
NM_018365	*MNS1*	4.09	5.55E-10	3.31	3.38E-09
NM_012484	*HMMR*	4.51	2.85E-12	5.25	1.87E-13
NM_080672	*PHACTR3*	4.53	8.83E-10	3.81	3.34E-09
NM_001956	*EDN2*	4.78	2.38E-09	4.67	1.19E-09
NM_153046	*TDRD9*	4.83	1.87E-12	4.59	1.08E-12
NM_032935	*MT4*	5.04	2.85E-12	5.54	4.26E-13
BC004565	*unknown*	5.05	1.16E-11	2.65	2.23E-08

5

HGNC_symbol	Ensembl_link	mutant *CDKN2A* vs wt *CDKN2A* (both RHC *MC1R*) log F.C	adj.p.value	mutant *CDKN2A* vs wt *CDKN2A* (both wt *MC1R*) log F.C	adj.p.value	RHC *MC1R* vs wt *MC1R* (both mutant *CDKN2A*) log F.C	adj.p.value	RHC *MC1R* vs wt *MC1R* (both wt *CDKN2A*) log F.C	adj.p.value
XAF1	NM_017523	5.11	1.98E-08	3.00	9.40E-06	-1.11	0.041921124	-3.22	1.59E-06
MX2	NM_002463	4.14	1.32E-11	1.06	0.000133758	-2.59	2.72E-09	-5.66	4.69E-14
IFI44L	NM_006820	3.49	6.99E-11	2.28	1.49E-08	-0.93	0.000400236	-2.14	9.59E-09
IFIT3	NM_001549	3.26	8.18E-09	1.26	0.00036059	-1.57	3.37E-05	-3.57	5.21E-10
CAPN14	AK092257	3.25	3.91E-05	2.20	0.00113099	-1.37	0.032844763	-2.42	0.0002558
	AK023743	3.18	1.52E-10	1.95	7.56E-08	-0.49	0.039372674	-1.71	1.27E-07
IFI44	NM_006417	3.07	3.28E-12	2.71	7.74E-12	-0.76	5.31E-05	-1.12	2.60E-07
KRT19	NM_002276	2.86	3.59E-10	0.81	0.000744197	-1.31	4.49E-06	-3.36	8.78E-12
IFNK	NM_020124	2.74	7.98E-07	2.36	3.02E-06	-1.04	0.007725554	-1.43	0.000326988
IFI6	NM_022873	2.56	3.45E-08	1.05	0.000601236	-0.79	0.00606156	-2.30	2.96E-08
BTN3A3	NM_006994	2.42	1.07E-05	1.55	0.000669858	-1.08	0.011856791	-1.95	3.31E-05
LMO2	NM_005574	2.41	1.43E-10	0.79	1.00E-04	-1.12	1.67E-06	-2.75	5.51E-12
GBP3	NM_018284	2.40	1.80E-07	2.10	6.58E-07	-0.70	0.020231435	-1.00	0.000961856
RNF182	NM_152737	2.19	1.05E-05	1.78	5.97E-05	-0.93	0.015597153	-1.33	0.000585284
RHOBTB3	NM_014899	2.17	1.04E-05	1.01	0.008209935	-1.98	1.59E-05	-3.14	2.33E-08
IFIT2	NM_001547	2.16	4.68E-08	2.17	3.56E-08	-1.48	3.08E-06	-1.47	1.46E-06
USP18	NM_017414	2.14	1.55E-08	0.52	0.018990885	-0.69	0.002714314	-2.31	1.21E-09
EPSTI1	NM_033255	2.12	3.99E-05	1.77	0.000160602	-1.70	0.0002321	-2.05	1.60E-05
	A_32_P53976	2.01	3.17E-05	1.10	0.005270514	-0.86	0.026819981	-1.77	3.55E-05
RSAD2	NM_080657	1.96	0.001691807	1.44	0.012103521	-2.71	4.60E-05	-3.23	2.96E-06
	AK023660	1.96	4.65E-08	0.92	0.000195518	-0.81	0.000669435	-1.85	2.23E-08
	THC2676635	1.93	2.93E-08	1.27	3.49E-06	-0.86	0.000221405	-1.52	1.43E-07
CLCA3	NM_004921	1.86	8.41E-05	1.74	9.87E-05	-1.54	0.000333218	-1.65	8.58E-05
PARP9	NM_031458	1.83	2.21E-06	1.21	0.000138412	-0.63	0.024137843	-1.26	4.46E-05
SCG5	NM_003020	1.83	2.10E-08	2.37	5.14E-10	-1.87	8.28E-09	-1.32	2.93E-07
HS3ST1	NM_005114	1.80	2.00E-05	1.12	0.001411596	-1.00	0.003458263	-1.69	1.14E-05
CEACAM1	NM_001712	1.80	1.35E-06	1.56	4.49E-06	-3.12	6.03E-10	-3.36	7.97E-11
OAS1	NM_002534	1.76	0.000121473	0.79	0.039773363	-1.04	0.007622258	-2.01	7.90E-06
	L06175	1.75	0.000889078	1.36	0.004950943	-1.82	0.000403092	-2.22	2.73E-05
	AK124281	1.75	5.68E-05	1.40	0.00033138	-0.90	0.011971445	-1.25	0.000538014
C5orf56	ENST00000378953	1.73	0.000157045	0.84	0.030463445	-1.03	0.008749111	-1.93	1.47E-05
CRNN	NM_016190	1.67	0.000105303	1.13	0.002501087	-4.14	8.50E-10	-4.68	5.89E-11

	AK025669	1.67	0.000315954	1.77	0.000112469	-1.00	0.013197622	-0.90	0.017900911
PADI1	NM_013358	1.66	0.000201315	1.12	0.004289502	-3.61	1.08E-08	-4.16	6.97E-10
	THC2640099	1.63	0.003962305	1.41	0.008167973	-1.45	0.006843443	-1.67	0.001407815
TNFSF10	NM_003810	1.61	3.76E-05	1.04	0.001525958	-1.37	0.000112711	-1.93	1.06E-06
F3	NM_001993	1.56	0.007308907	1.28	0.019400875	-1.14	0.039539591	-1.42	0.007227404
IL15	NM_172174	1.55	5.30E-08	1.29	4.08E-07	-0.82	5.92E-05	-1.09	1.10E-06
SP110	NM_004510	1.53	1.66E-07	0.94	3.63E-05	-0.67	0.000916414	-1.26	4.53E-07
BST2	NM_004335	1.49	0.00349873	1.52	0.00211498	-3.36	4.60E-07	-3.33	2.09E-07
CLCA4	NM_012128	1.49	0.001612926	1.67	0.000362464	-1.67	0.000354497	-1.48	0.000637245
	BX090412	1.48	0.001281109	1.47	0.000930605	-0.87	0.037340541	-0.88	0.025448576
	BC045163	1.45	3.62E-05	0.58	0.040103551	-1.39	3.12E-05	-2.25	3.73E-08
HOXA10	NM_018951	1.43	4.44E-06	0.86	0.000579266	-0.53	0.022134445	-1.10	2.33E-05
TLR2	NM_003264	1.42	0.000310032	1.80	1.61E-05	-2.33	7.17E-07	-1.95	2.78E-06
	THC2657493	1.38	0.000134482	1.47	4.25E-05	-2.16	4.46E-07	-2.07	3.14E-07
OASL	NM_003733	1.34	1.66E-05	0.58	0.01676382	-0.78	0.001967307	-1.54	7.42E-07
	CR625594	1.34	0.000464881	1.05	0.002484941	-0.70	0.03890031	-0.98	0.002738176
PSCA	NM_005672	1.32	0.002415497	1.59	0.000301512	-7.30	2.39E-12	-7.02	1.20E-12
	A_24_P476718	1.31	0.000870782	2.24	1.89E-06	-1.93	9.86E-06	-1.00	0.00360047
SPINK1	NM_003122	1.29	0.005034924	0.96	0.024954425	-3.40	1.10E-07	-3.73	1.34E-08
	CR612518	1.28	0.006785661	1.14	0.011308172	-0.96	0.032090928	-1.11	0.009489293
	A_32_P169353	1.20	0.045002273	1.48	0.010727271	-2.84	2.98E-05	-2.56	4.67E-05
	BC062324	1.19	0.01579769	1.22	0.010153956	-1.67	0.00082046	-1.64	0.000563989
DHX58	NM_024119	1.18	5.99E-06	0.71	0.000741093	-0.68	0.001151375	-1.15	2.09E-06
	BM989848	1.16	0.011329228	0.98	0.023865075	-0.89	0.042562201	-1.07	0.010191815
MDK	NM_001012334	1.16	6.19E-05	1.20	2.48E-05	-1.55	1.14E-06	-1.51	7.03E-07
S100A4	NM_002961	1.12	0.000446466	0.72	0.010561535	-0.77	0.007225432	-1.17	0.000100805
BTN3A2	NM_007047	1.09	1.77E-05	1.11	9.31E-06	-0.57	0.00559599	-0.55	0.004476681
ALDH1L1	NM_012190	1.09	0.006504537	1.35	0.000801896	-1.46	0.000386161	-1.20	0.001386589
SEPP1	NM_005410	1.08	0.017795719	2.85	1.26E-06	-2.78	1.49E-06	-1.00	0.01532286
	THC2671679	1.07	3.76E-05	0.73	0.001017835	-1.24	3.55E-06	-1.58	7.50E-08
HCP5	NM_006674	1.06	0.007759651	1.33	0.000898615	-1.84	3.33E-05	-1.57	9.87E-05
	AK026517	1.05	0.002902491	1.06	0.001900336	-0.80	0.015527585	-0.79	0.011579443
	THC2754005	1.02	0.000456872	1.29	2.63E-05	-1.03	0.000244525	-0.77	0.002241279
ITGA2	NM_002203	1.01	0.022552265	0.94	0.026376749	-1.06	0.012631859	-1.13	0.005479826
WNT7A	NM_004625	0.99	0.000464881	0.53	0.033957447	-1.77	4.27E-07	-2.24	8.20E-09

NEBL	NM_006393	0.99	3.91E-05	0.73	0.000558386	-0.52	0.008729268	-0.78	0.000141682
PLAT	NM_000930	0.98	0.000875467	1.35	2.25E-05	-2.14	8.14E-08	-1.77	3.78E-07
EIF2AK2	NM_002759	0.98	0.020075442	1.52	0.000512242	-1.38	0.001188256	-0.84	0.027870165
	AF086052	0.98	0.009230344	1.53	0.000138634	-1.38	0.000353702	-0.83	0.013843783
ALDH1A3	NM_000693	0.96	0.000531565	0.60	0.014677759	-1.10	8.25E-05	-1.46	1.54E-06
	BC056662	0.96	0.014386956	1.04	0.006439966	-1.04	0.006569774	-0.96	0.007518063
FMO2	NM_001460	0.96	0.037434212	2.94	1.09E-06	-3.92	2.36E-08	-1.94	5.37E-05
	A_32_P35031	0.95	0.000214111	0.62	0.005152449	-0.59	0.00820823	-0.91	0.000104052
	K03200	0.93	0.005429992	0.89	0.005220606	-1.51	3.96E-05	-1.55	1.55E-05
	AK130514	0.91	0.017250913	0.93	0.011913737	-1.39	0.000462002	-1.38	0.00028677
ZSCAN4	NM_152677	0.90	0.014937773	1.41	0.000279708	-1.54	0.000105108	-1.03	0.002697114
APOL6	NM_030641	0.89	0.018487069	1.35	0.000505697	-1.98	8.89E-06	-1.51	9.06E-05
STS	NM_000351	0.87	0.014650689	1.50	0.000108672	-2.58	1.92E-07	-1.95	2.61E-06
	THC2543120	0.86	0.009788415	1.06	0.001361535	-0.83	0.009051891	-0.63	0.035366686
PPARG	NM_138711	0.86	0.001039611	1.19	2.54E-05	-0.84	0.000819453	-0.50	0.023893587
IFITM1	NM_003641	0.85	0.002663548	0.62	0.018223872	-0.67	0.012012196	-0.90	0.000693909
	CA314451	0.84	0.016043784	0.89	0.00853883	-1.83	9.45E-06	-1.79	6.00E-06
	THC2551769	0.84	0.004358863	0.73	0.008331505	-0.69	0.012821531	-0.79	0.003004577
ENC1	NM_003633	0.83	0.002044084	1.05	0.000153929	-1.96	1.05E-07	-1.74	2.01E-07
KCNN4	NM_002250	0.82	0.006830609	1.03	0.000785576	-1.34	5.13E-05	-1.14	0.000158723
SPINK5	NM_006846	0.76	0.001825778	0.47	0.033705902	-0.70	0.002501485	-0.99	5.08E-05
MSMB	NM_002443	0.73	0.000832073	1.96	6.81E-09	-5.28	1.03E-14	-4.05	1.47E-13
PARP12	NM_022750	0.73	0.001230432	0.63	0.002667999	-1.18	4.95E-06	-1.27	8.66E-07
	THC2545454	0.71	0.02260588	0.95	0.002276893	-0.95	0.002311169	-0.71	0.012721232
LYN	NM_002350	0.71	0.003114957	1.27	7.07E-06	-1.32	3.92E-06	-0.76	0.000708175
	AK022020	0.71	0.004745127	0.59	0.012998879	-1.12	4.23E-05	-1.25	6.53E-06
LAYN	NM_178834	0.71	0.01514181	1.04	0.000500006	-1.28	5.82E-05	-0.95	0.000706483
PPP1R12B	NM_032105	0.68	0.009014906	0.68	0.00660204	-0.86	0.00096841	-0.86	0.000563989
ATP1B1	NM_001677	0.67	0.040190654	0.78	0.013887884	-0.84	0.008541899	-0.73	0.014330567
CAMK2G	NM_172171	0.66	0.009630083	0.77	0.001946373	-0.83	0.001095948	-0.71	0.002542575
	AK023696	0.64	0.030845413	0.63	0.025358095	-0.64	0.025179173	-0.64	0.017239992
TMEM132A	NM_017870	0.60	0.015740285	0.64	0.007383258	-1.72	3.16E-07	-1.68	1.76E-07
TRIM2	NM_015271	0.59	0.004377494	0.42	0.029710532	-0.50	0.009901857	-0.67	0.000579911
LRAT	NM_004744	0.51	0.043998571	0.78	0.00198053	-1.46	2.88E-06	-1.19	1.50E-05
CCNDBP1	NM_037370	0.48	0.009949949	0.67	0.00049834	-0.61	0.001068743	-0.43	0.01171219

SMAD3	U68019	0.45	0.047091148	0.52	0.017762701	-0.74	0.001205253	-0.68	0.001635126
TMEM51	NM_018022	0.45	0.028015892	0.43	0.029372403	-0.56	0.005094908	-0.58	0.002388652
	AK025166	0.39	0.028250766	0.37	0.030166685	-0.45	0.008515843	-0.47	0.004029612
NPEPPS	NM_006310	0.38	0.044356931	0.45	0.014074734	-0.79	0.000114668	-0.72	0.000155666
ALG9	NM_001077691	-0.31	0.031798838	-0.29	0.034627663	0.28	0.044702714	0.30	0.023463075
	BC002724	-0.42	0.014772148	-0.81	3.29E-05	0.93	6.18E-06	0.54	0.000991685
MPP1	NM_002436	-0.42	0.047666353	-0.78	0.000483518	1.51	2.71E-07	1.16	3.00E-06
MYO19	NM_001033580	-0.43	0.026532957	-1.00	1.21E-05	0.98	1.38E-05	0.41	0.02114696
FANCC	NM_000136	-0.51	0.011609739	-0.47	0.014584719	0.48	0.012959276	0.52	0.004986322
OVOL2	NM_021220	-0.52	0.001084197	-0.39	0.007292061	0.47	0.001844654	0.60	0.000100716
SIN3B	BC025026	-0.57	0.011569948	-0.75	0.00103124	1.01	5.47E-05	0.83	0.000227367
CDT1	NM_030928	-0.58	0.019198248	-0.90	0.000479465	1.44	2.66E-06	1.13	2.30E-05
PON3	NM_000940	-0.62	0.005105333	-0.84	0.000276722	0.93	8.48E-05	0.72	0.000647845
ADRB2	NM_000024	-0.63	0.001524882	-0.73	0.000236914	0.59	0.001792781	0.48	0.005629305
PLK1	NM_005030	-0.63	0.042808398	-0.63	0.034901844	2.40	8.38E-08	2.41	3.33E-08
	CR611847	-0.66	0.000634076	-0.51	0.003687258	0.35	0.044627807	0.50	0.002990718
	A_23_P216071	-0.70	0.00958912	-0.58	0.022202131	0.54	0.033583338	0.66	0.00706467
CTSK	NM_000396	-0.74	7.83E-05	-1.81	1.05E-09	1.64	2.75E-09	0.58	0.000331696
ASF1B	NM_018154	-0.76	0.048590758	-0.83	0.022912812	1.00	0.007299284	0.92	0.008832468
NMNAT3	NM_178177	-0.76	0.000552736	-0.87	9.23E-05	0.76	0.000345671	0.65	0.000881113
REEP2	NM_016606	-0.78	0.005759911	-0.93	0.001039901	1.69	1.66E-06	1.54	2.21E-06
ABCF2	NM_007189	-0.80	0.000944624	-0.74	0.001305162	0.60	0.00667932	0.66	0.001912744
LMCD1	NM_014583	-0.95	0.002645754	-0.76	0.010312363	0.74	0.012418953	0.93	0.001307708
NRP2	NM_201264	-0.97	0.002390052	-0.63	0.031835409	1.17	0.000297324	1.51	9.74E-06
ST7OT1	NR_002330	-0.99	0.000144723	-0.44	0.048099669	0.80	0.00068922	1.36	1.07E-06
PKMYT1	NM_182687	-0.99	0.001983455	-0.74	0.012491031	1.23	0.000180492	1.48	1.20E-05
CPT1C	NM_152359	-1.08	1.08E-05	-1.48	1.50E-07	0.94	2.56E-05	0.54	0.003391166
BEX5	NM_001012978	-1.26	0.001348714	-0.76	0.032521061	2.00	7.09E-06	2.50	2.08E-07
DHRS13	NM_144683	-1.45	0.00088615	-1.72	0.000114616	1.32	0.001366222	1.05	0.005333513
CLDN17	NM_012131	-1.47	4.17E-05	-1.79	2.57E-06	1.34	6.23E-05	1.01	0.000593411
STAC	NM_003149	-1.57	0.001093743	-1.23	0.005257075	0.88	0.043609023	1.22	0.003788264
	AK074473	-1.83	6.09E-06	-2.43	1.29E-07	1.71	7.39E-06	1.11	0.000405428
	CR602075	-2.00	0.007381353	-1.87	0.008773947	1.59	0.024722861	1.73	0.010382231
CD248	NM_020404	-2.04	2.85E-05	-1.14	0.004130168	0.77	0.049370648	1.67	7.16E-05

ANNEX II

Carta de resposta a Lubbe *et al*. [222] Revista: *Annals of Neurology*

Programme, Biomedicum, University of Helsinki), T. Peuralinna (Department of Neurology, Helsinki University Central Hospital), L. Myllykangas (Folkhalsan Institute of Genetics and Department of Pathology, University of Helsinki), and R. Sulkava (Department of Public Health and General Practice Division of Geriatrics, University of Eastern Finland) for the Finnish controls (Vantaa85 + GWAS data). We used genome-wide association data generated by the Wellcome Trust Case-Control Consortium 2 (WTCCC2) from UK patients with Parkinson's disease and UK control individuals from the 1958 Birth Cohort and National Blood Service.

We thank Jeffrey Barrett for assistance with the design of the ImmunoChip.

Author Contributions

S.J.L. and H.R.M. were responsible for conception and design of the study. S.J.L., M.S., V.E.P., N.M.W., and H.R.M. were responsible for data analysis and editing. J.M.B., A.B., T.G., J.H., P.H., N.W.W., A.B.S., and H.R.M. were responsible for sample and/or data collection.

Potential Conflicts of Interest

Dr Morris reports grants from Medical Research Council UK, grants from Wellcome Trust, grants from Parkinson's UK, and grants from Ipsen Fund during the conducting of the study; grants from Motor Neuron Disease Association, grants from Welsh Assembly Government, personal fees from Teva, personal fees from AbbVie, personal fees from Teva, personal fees from UCB, personal fees from Boerhinger Ingelheim, and personal fees from GlaxoSmithKline outside the submitted work.

[1]*Department of Clinical Neuroscience, Institute of Neurology, University College London, London, United Kingdom,* [2]*Institute of Psychological Medicine and Clinical Neurosciences, Medical Research Council Center for Neuropsychiatric Genetics and Genomics, Department of Psychological Medicine and Neurology, Cardiff University School of Medicine, Cardiff, United Kingdom,* [3]*Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Université Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, F-75013, Paris, France,* [4]*Department for Neurodegenerative Diseases, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany and Genome Biology for Neurodegenerative Diseases, German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany,* [5]*Department of Molecular Neuroscience, Institute of Neurology, University College London, London, United Kingdom,* [6]*Reta Lila Weston Institute, University College London Institute of Neurology, London, United Kingdom,* [7]*Genome Biology for Neurodegenerative Diseases, German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany,* [8]*UCL Genetics Institute, University College London, London, United Kingdom,* [9]*Laboratory of Neurogenetics, National Institute on Aging, Bethesda, MD, and* [10]*see Supplementary Text*

References

1. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 2008; 122:2753–2760.
2. Tell-Marti G, Puig-Butille JA, Potrony M, et al. The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol 2015;77:889–894.
3. Dong J, Gao J, Nalls M, et al. Susceptibility loci for pigmentation and melanoma in relation to Parkinson's disease. Neurobiol. Aging 2014;35:1512.e5–10.
4. Gerstenblith MR, Goldstein AM, Fargnoli MC, et al. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 2007;28:495–505.
5. The 1000 Genomes Project Consortium. A global reference for human genetic variation. Nature 2015;526:68–74.
6. Gayán J, Galan JJ, González-Pérez A, et al. Genetic structure of the Spanish population. BMC Genomics 2010;11:326.

DOI: 10.1002/ana.24527

Reply

Gemma Tell-Martí, MSc,[1,2]
Joan Anton Puig-Butille, PhD,[2,3] Miriam Potrony, MSc,[1]
Celia Badenas, PhD,[2,3] Montserrat Milà, PhD,[2,3]
Josep Malvehy, MD, PhD,[1,2] María José Martí, MD,[4]
Mario Ezquerra, PhD,[4] Rubén Fernández-Santiago, PhD,[4]
Susana Puig, MD, PhD[1,2,5]

Based on the epidemiological co-occurrence of cutaneous melanoma (CM) and Parkinson disease (PD) reported in Caucasians,[1] we recently sequenced the *MC1R* gene, which is involved in CM susceptibility, in 870 patients with PD and 736 controls from Spain and found a significant association of the p.R160W variant with PD (Table).[2] More recently, this association has not been detected in an international cohort of 5,944 PD cases and 4,642 controls from the International PD Genetic Consortium (IPDGC). In this study, Lubbe et al stated that our selection of controls could act as a potential source of bias because (1) we specifically excluded melanoma patients as controls for our PD patients; and (2) our controls were from a wider Spanish population, whereas the PD cases were from Barcelona. First, we would like to point out that the noninclusion of melanoma patients as part of the control population should be a mandatory eligibility criterion in such comorbidity studies to avoid any confounding overrepresentation of *MC1R* risk variants.[3] Second, we have re-evaluated the minor allele frequency (MAF) of p.R160W in our controls[2] from the wider Spanish area (MAF = 0.011; n = 605) and from Barcelona (MAF = 0.015; n = 131) and found similar MAFs that were also largely similar to those reported in other Spanish studies (see Table).[4,5] In accordance with previous reports showing large homogeneity and minor stratification in the Spanish population,[6] these results indicate that our overall control sample lacks signs of genetic heterogeneity. In addition, it is well known from the melanoma field that the frequency of *MC1R* variants varies considerably across populations,[7] as also observed in the IPDGC study (see Table). Thus, in this pooled cohort

TABLE 1. Allele Frequency of p.R160W *MC1R* Variant Reported in Different Case–Control Studies

	p.R160W in PD Studies				p.R160W in Different Control Populations	
Population	MAF, Controls (N)	MAF, PD (N)	OR [95% CI]	*p*	Population	MAF, Controls (N)
Spain[2]	0.012 (736)	0.024 (870)	2.10 [1.18–3.73]	0.009[a]	Spain[4] Spain[5]	0.014 (544) 0.010 (491)
Letter IPDGC						
Greece	0.026 (882)	0.034 (965)	1.34 [0.92–1.95]	0.131	Greece[8] Greece[9]	0.010 (155) 0.012 (166)
France	0.040 (451)	0.036 (508)	1.00 [0.61–1.66]	0.989	France[10] France[11]	0.024 (210) 0.030 (869)
Germany	0.068 (880)	0.075 (1,296)	1.10 [0.87–1.41]	0.425	Germany[4]	0.060 (1038)
The Netherlands	0.075 (409)	0.066 (312)	0.86 [0.57–1.30]	0.469	The Netherlands[12]	0.105 (385)
United States	0.077 (1,437)	0.074 (1,756)	0.92 [0.76–1.12]	0.385	United States[13] United States[14]	0.060 (166) 0.062 (179)
United Kingdom	0.080 (583)	0.073 (1,107)	0.95 [0.72–1.24]	0.624	United Kingdom[15] United Kingdom[16]	0.091 (93) 0.066 (332)
Pooled population[b]	0.062 (4,642)	0.064 (5,944)	1.01 [0.90–1.13]	0.853	Pooled population[c]	0.053 (3,593)

[a]Statistically significant.
[b]Includes all populations assessed in the IPDGC study.
[c]Includes all control populations listed in this table, except those from Spain.
CI = confidence interval; IPDGC = International PD Genetic Consortium; MAF = minor allele frequency; N = number of individuals included in each study; OR = odds ratio; PD = Parkinson disease.

different subpopulation stratification could be limiting the replication of our findings. Moreover, it should be mentioned that the MAF of p.R160W in Greek control populations previously reported in skin cancer studies (MAF = 0.010–0.012) (1) differs to that observed by Lubbe et al (MAF = 0.026), (2) is considerably lower than the MAF reported in their Greek PD patients (MAF = 0.034), and (3) is similar to that observed in our Spanish controls. Therefore, results from the Greek population of the IPDGC study should be interpreted with caution. We conclude that future comorbidity studies aiming to validate our association findings of *MC1R* and PD should be performed in larger and homogeneous populations in which control samples should specifically not include subjects with CM or family history of CM so as to avoid an overrepresentation of risk variants in the controls.

Author Contributions

R.F.-S. and S.P. contributed equally as senior authors.

Potential Conflicts of Interest

Nothing to report.

[1]*Dermatology Department, Melanoma Unit, Hospital Clinic and August Pi i Sunyer Biomedical Research Institute,* [2]*Center for Networked Biomedical Research on Rare Diseases, Carlos III Health Institute,* [3]*Biochemical and Molecular Genetics Service, Hospital Clinic and August Pi i Sunyer Biomedical Research Institute,* [4]*Laboratory of Neurodegenerative Disorders, Department of Clinical and Experimental Neurology, August Pi i Sunyer Biomedical Research Institute, Hospital Clinic of Barcelona, Center for Networked Biomedical Research in Neurodegenerative Diseases, and* [5]*Medicine Department, University of Barcelona, Barcelona, Spain*

References

1. Liu R, Gao X, Lu Y, Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology 2011;76: 2002–2009.
2. Tell-Marti G, Puig-Butille JA, Potrony M, et al. The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol 2015;77:889–894.
3. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 2008; 122:2753–2760.
4. Scherer D, Nagore E, Bermejo JL, et al. Melanocortin receptor 1 variants and melanoma risk: a study of 2 European populations. Int J Cancer 2009;125:1868–1875.
5. Cordoba-Lanus E, Hernandez-Jimenez JG, Medina-Coello C, et al. MC1R gene variants and sporadic malignant melanoma susceptibility in the Canary Islands population. Arch Dermatol Res 2014; 306:51–58.

6. Gayan J, Galan JJ, Gonzalez-Perez A, et al. Genetic structure of the Spanish population. BMC Genomics 2010;11:326.
7. Gerstenblith MR, Goldstein AM, Fargnoli MC, et al. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 2007;28:495–505.
8. Stratigos AJ, Dimisianos G, Nikolaou V, et al. Melanocortin receptor-1 gene polymorphisms and the risk of cutaneous melanoma in a low-risk southern European population. J Invest Dermatol 2006;126:1842–1849.
9. Dessinioti C, Sypsa V, Kypreou K, et al. A case-control study of MC1R variants in Greek patients with basal cell carcinoma: increased risk independently of pigmentary characteristics. Exp Dermatol 2015;24:476–478.
10. Matichard E, Verpillat P, Meziani R, et al. Melanocortin 1 receptor (MC1R) gene variants may increase the risk of melanoma in France independently of clinical risk factors and UV exposure. J Med Genet 2004;41:e13.
11. Hu HH, Benfodda M, Dumaz N, et al. A large French case-control study emphasizes the role of rare Mc1R variants in melanoma risk. Biomed Res Int 2014;2014:925716.
12. Bastiaens MT, ter Huurne JA, Kielich C, et al. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet 2001;68:884–894.
13. Council ML, Gardner JM, Helms C, et al. Contribution of genetic factors for melanoma susceptibility in sporadic US melanoma patients. Exp Dermatol 2009;18:485–487.
14. Kanetsky PA, Ge F, Najarian D, et al. Assessment of polymorphic variants in the melanocortin-1 receptor gene with cutaneous pigmentation using an evolutionary approach. Cancer Epidemiol Biomarkers Prev 2004;13:808–819.
15. Flanagan N, Healy E, Ray A, et al. Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation. Hum Mol Genet 2000;9:2531–2537.
16. Winney B, Boumertit A, Day T, et al. People of the British Isles: preliminary analysis of genotypes and surnames in a UK-control population. Eur J Hum Genet 2012;20:203–210.

DOI: 10.1002/ana.24526

ANNEX III

Carta de resposta a Foo *et al*. [220] **Revista: *Annals of Neurology***

individuals, consistent with its absence or extremely low frequencies in 1000 Genomes East Asians.

To identify novel variants in *MC1R* that may be specific to the Chinese population, we sequenced *MC1R* in 198 Chinese samples from Singapore (99 early onset PD cases and 99 controls). We identified 5 nonsynonymous variants, including 2 common variants p.V92M (rs2228479, allele frequency 27.5%) and p.R163Q (rs885479, 56.9%) and 3 rare variants p.R67Q (rs34090186, 1.26%), p.I120T (rs33932559, 4.04%), and p.R307S (novel, 0.25%). With the exception of the R307S variant, which was found in only 1 control subject, we genotyped the other 4 recurrent variants, along with the pigmentation and skin cancer risk variant p.R151C (rs1805007),[5] in the full set of 778 cases and 517 controls. The p.R151C variant was also not found in any of our case or control individuals. None of the 4 polymorphic variants showed any evidence of association with PD in our Chinese samples (Table).

Our results show that both the p.R160W and p.R151C variants are extremely rare in the Chinese population. None of the other rare or common variants in *MC1R* identified in our Chinese samples is associated with PD. We conclude that nonsynonymous variants in *MC1R* are rare in the Chinese population and not a major risk factor for PD.

Acknowledgment

Supported by the National Medical Research Council (STaR award and Translational Clinical Research programme in Parkinson's disease), Duke-NUS Graduate Medical School, and Singapore Millennium Foundation.

Potential Conflicts of Interest

Nothing to report.

[1]*Human Genetics, Genome Institute of Singapore, A*STAR, Singapore*
[2]*Department of Neurology, Singapore General Hospital, National Neuroscience Institute, Singapore*
[3]*Duke-NUS Graduate Medical School, Singapore*

References

1. Tell-Martí G, Puig-Butille JA, Potrony M, et al. The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol 2015;77:889–894.
2. Bertoni JM, Arlette JP, Fernandez HH, et al. Increased melanoma risk in Parkinson disease: a prospective clinicopathological study. Arch Neurol 2010;67:347–352.
3. Holmes D. The cancer that rises with the sun. Nature 2014;515:S110–S111.
4. Sng J, Koh D, Siong WC, Choo TB. Skin cancer trends among Asians living in Singapore from 1968 to 2006. J Am Acad Dermatol 2009;61:426–432.
5. Zhang M, Song F, Liang L, et al. Genome-wide association studies identify several new loci associated with pigmentation traits and skin cancer risk in European Americans. Hum Mol Genet 2013;22:2948–2959.

DOI: 10.1002/ana.24419

Reply

Gemma Tell-Martí, MSc,[1,2]
Joan Anton Puig-Butille, PhD,[2,3] Miriam Potrony, MSc,[1]
Celia Badenas, PhD,[2,3] Montserrat Milà, PhD,[2,3]
Josep Malvehy, MD, PhD,[1,2] María José Martí, PhD,[4]
Mario Ezquerra, PhD,[4] Rubén Fernández-Santiago, PhD,[4†]
and Susana Puig, MD, PhD[1,2,5†]

Recessive genetic loss-of-function variants in the melanocortin 1 receptor (*MC1R*) gene (p.D84E, p.R142H, p.R151C, p.R160W, p.I155T, and p.D294H) are responsible for the red hair color (RHC) phenotype (red hair, fair skin, poor tanning ability, and heavy freckling) and increase the risk of developing cutaneous melanoma.[1] Based on the epidemiological comorbidity of melanoma and Parkinson's disease (PD) reported in Caucasians, we sequenced the entire *MC1R* gene in one Spanish population encompassing 870 PD patients and 736 healthy controls.[2] We found an association between PD and the *MC1R* loss-of-function p.R160W variant, which showed an allelic frequency of 2% in PD patients and 1% in controls (odds ratio = 2.10).

Subsequently, Foo et al. investigated whether nonsynonymous variants in *MC1R* influence the PD risk in one Chinese population ascertained from Singapore by studying 778 PD cases and 517 controls. The entire coding region of *MC1R* was sequenced in a subset of 99 PD cases and 99 controls, and then top frequent variants, together with the PD-associated loss-of-function variants, p.R160W[2] and p.R151C,[3] were genotyped in the entire sample. Foo et al. did not detect the variants, p.R160W and p.R151C, in any of the subjects of study and the most frequent variant, p.R163Q (allelic frequency of 61.12% in PD patients), was not associated with PD. These findings are not surprising given that (1) melanoma occurs at a much lower incidence in Asians and comorbidity with PD in these populations is not as well documented as in Caucasians; (2) previous reports showed that p.R160W and p.R151C are absent or extremely rare in Asians[4] and thus unlikely to modulate PD or melanoma in these ethnicities; and (3) the most frequent *MC1R* variant reported, p.R163Q, is not associated with the RHC phenotype and causes only a slight reduction of *MC1R* function.[1] Yet, p.R163Q has been shown to increase melanoma risk after chronic sun exposure owing to its role in photodamage and/or photoaging, but independently of the RHC risk phenotype.[5]

Akin to this *MC1R* study, the effect of genetic variants located in other PD-associated genes, including PD pathogenic mutations, have been shown to be uncommon or absent in Asians. For instance, missense mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are the most frequent cause of familial PD[6,7] and also sporadic PD. The *LRRK2* G2019S mutation alone explains up to 6% of familial and 3% of sporadic PD cases in Europeans[8,9] and up to 20% of total cases among Ashkenazi Jews[10] or 40% in North African Berbers.[11] However, the G2019S mutation has rarely been detected in Asians (<0.1%).[12]

In conclusion, results from studies published to date on PD and melanoma[2,3] reinforce the hypothesis that only loss-of-function *MC1R* variants modulate the risk of PD and indicate a population-specific effect of *MC1R* variants in modulating the risk for PD, at least in Caucasians, but perhaps not in Asians.

Potential Conflicts of Interest

Nothing to report.

[1]*Dermatology Department, Melanoma Unit, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain*
[2]*Center for Networked Biomedical Research on Rare Diseases (CIBERER), Carlos III Health Institute (ISCIII), Barcelona, Spain*
[3]*Biochemical and Molecular Genetics Service, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain*
[4]*Laboratory of Neurodegenerative Disorders, Department of Clinical and Experimental Neurology, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Hospital Clínic of Barcelona, Center for Networked Biomedical Research in Neurodegenerative Diseases (CIBERNED), Barcelona, Spain*
[5]*Medicine Department, University of Barcelona (UB), Barcelona, Spain*
[†]*Contributed equally as senior authors.*

References

1. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 2008; 122:2753–2760.
2. Tell-Marti G, Puig-Butille JA, Potrony M, et al. The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol 2015;77:889–894.
3. Gao X, Simon KC, Han J, et al. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol 2009;65:76–82.
4. Savage SA, Gerstenblith MR, Goldstein AM, et al. Nucleotide diversity and population differentiation of the melanocortin 1 receptor gene, MC1R. BMC Genet 2008;9:31.
5. Puig-Butille JA, Carrera C, Kumar R, et al. Distribution of MC1R variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. Br J Dermatol 2013;169:804–811.
6. Paisan-Ruiz C, Jain S, Evans EW, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 2004;44:595–600.
7. Zimprich A, Biskup S, Leitner P, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 2004;44:601–607.
8. Di Fonzo A, Rohe CF, Ferreira J, et al. A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease. Lancet 2005;365:412–415.
9. Gilks WP, Abou-Sleiman PM, Gandhi S, et al. A common LRRK2 mutation in idiopathic Parkinson's disease. Lancet 2005;365:415–416.
10. Ozelius LJ, Senthil G, Saunders-Pullman R, et al. LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. N Engl J Med 2006;354:424–425.
11. Lesage S, Durr A, Tazir M, et al. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N Engl J Med 2006; 354:422–423.
12. Healy DG, Falchi M, O'Sullivan SS, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. Lancet Neurol 2008;7:583–590.

DOI: 10.1002/ana.24418

ANNEX IV

Carta de resposta a Jiang & Liu [221] Revista: *Annals of Neurology*

ANNALS *of Neurology*

4. Lubbe SJ, Escott-Price V, Brice A, et al. Is the MC1R variant p.R160W associated with Parkinson's? Ann Neurol 2016;79:159–161.
5. Nalls MA, Pankratz N, Lill CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat Genet 2014;46:989–993.

DOI: 10.1002/ana.24627

Reply

Gemma Tell-Martí, MSc,[1,2]
Joan Anton Puig-Butille, PhD,[2,3] Miriam Potrony, MSc,[1]
Celia Badenas, PhD,[2,3] Montserrat Milà, PhD,[2,3]
Josep Malvehy, MD, PhD,[1,2] María José Martí, MD,[4]
Mario Ezquerra, PhD,[4] Rubén Fernández-Santiago, PhD,[4]
and Susana Puig, MD, PhD[1,2,5]

Recessive genetic loss-of-function variants in the melanocortin 1 receptor (*MC1R*) gene (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, and p.D294H) are responsible for the red hair color (RHC) phenotype (red hair, fair skin, high UV sensitivity, and poor tanning ability) and increase the risk of cutaneous melanoma (CM).[1] Based on the epidemiological co-occurrence of CM and Parkinson's disease (PD) reported in Caucasians,[2] and the over-representation of RHC individuals in PD patients,[3] we recently investigated whether *MC1R* genetic variants could also be associated with PD risk. We sequenced the entire *MC1R* gene in 870 patients with PD and 736 controls from Spain and found a marginal significant association of the p.R160W variant with PD.[4] In a recent study, Jiang and Liu analyzed *MC1R* variants publically available from PD genome-wide association studies (GWAS). They reported that none of the 26 *MC1R* variants investigated in these GWAS was associated with PD. However, none of these 26 markers included the well-known loss-of-function *MC1R* variants involved in CM, including p.D84E (rs1805006), p.R142H (rs11547464), p.R151C (rs1805007), p.I155T (rs1110400), p.D294H (rs1805009), or p.R160W (rs1805008), which was the only variant showing association with PD in our study. This is important given that association reports suggest that, yet with possible population-specific effects, only loss-of-function *MC1R* variants modulate the risk of PD.[3,4]

Author Contributions

R.F.-S. and S.P. contributed equally as senior authors.

Potential Conflict of Interest

Nothing to report.

[1]*Dermatology Department, Melanoma Unit, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain,* [2]*Center for Networked Biomedical Research on Rare Diseases (CIBERER), Carlos III Health Institute (ISCIII), Barcelona, Spain,* [3]*Biochemical and Molecular Genetics Service, Hospital Clínic and August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain,* [4]*Laboratory of Neurodegenerative Disorders, Department of Clinical and Experimental Neurology, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Hospital Clínic of Barcelona, Center for Networked Biomedical Research in Neurodegenerative Diseases (CIBERNED), Barcelona, Spain, and* [5]*Medicine Department, University of Barcelona (UB), Barcelona, Spain*

References

1. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 2008;122:2753–2760.
2. Liu R, Gao X, Lu Y, Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology 2011;76:2002–2009.
3. Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol 2009;65:76–82.
4. Tell-Martí G, Puig-Butille JA, Potrony M, et al. The MC1R melanoma risk variant p.R160W is associated with Parkinson disease. Ann Neurol 2015;77:889–894.

DOI: 10.1002/ana.24629

ANNEX V

Supplementary Table: Comparison of allelic frequencies of MC1R variants among two independent controls cohorts

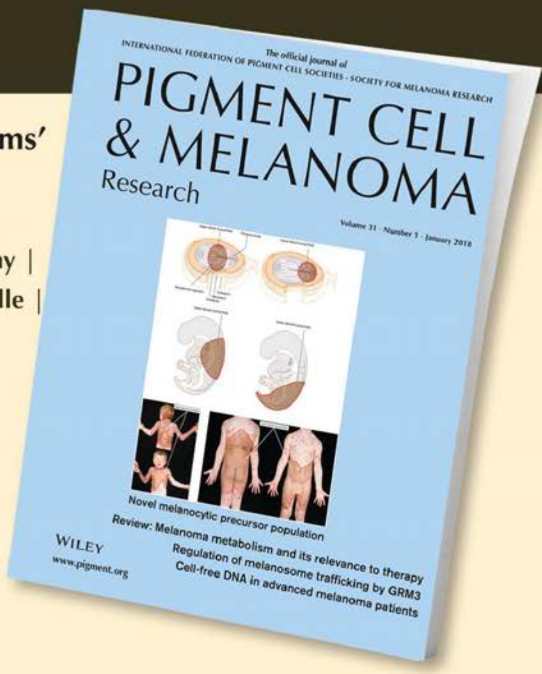
AA change	*Controls (N=160)				^Controls (N=736)				P-value
	[a]MAF	[a]Genotype frequency			MAF	Genotype frequency			
	2	1-1	1-2	2-2	2	1-1	1-2	2-2	
p.V60L	0.16	0.72	0.25	0.03	0.15	0.734	0.242	0.024	0.63
p.V92M	0.05	0.9	0.1	0	0.05	0.895	0.102	0.003	0.62
p.R163Q	0.01	0.98	0.02	0	0.02	0.96	0.04	0	0.21
p.R151C	0.03	0.94	0.06	0	0.02	0.958	0.039	0.003	0.54
p.R160W	0.01	0.975	0.025	0	0.01	0.98	0.02	0	0.89

*Controls reported in Tell-Martí G, et al. doi:10.3233/JAD-161113
^ Controls reported in Tell-Martí G, et al. doi: 10.1002/ana.24373
MAF: Minor allele frequency
[a]Allele described as 1 (wild type allele) or 2 (variant allele)

ANNEX VI

The official journal of
INTERNATIONAL FEDERATION OF PIGMENT CELL SOCIETIES · SOCIETY FOR MELANOMA RESEARCH

PIGMENT CELL & MELANOMA Research



Melanocortin 1 receptor (*MC1R*) polymorphisms' influence on size and dermoscopic features of nevi

María Gabriela Vallone | Gemma Tell-Marti | Miriam Potrony | Aida Rebollo-Morell | Celia Badenas | Joan Anton Puig-Butille | Pol Gimenez-Xavier | Cristina Carrera | Josep Malvehy | Susana Puig

DOI: 10.1111/pcmr.12646
Volume 31, Issue 1, Pages 39–50

If you wish to order reprints of this article, please see the guidelines **here**

Supporting Information for this article is freely available **here**

EMAIL ALERTS
Receive free email alerts and stay up-to-date on what is published in Pigment Cell & Melanoma Research – **click here**

Received: 15 May 2017 | Accepted: 1 September 2017
DOI: 10.1111/pcmr.12646

ORIGINAL ARTICLE

WILEY

Melanocortin 1 receptor (*MC1R*) polymorphisms' influence on size and dermoscopic features of nevi

María Gabriela Vallone[1,2,†] | Gemma Tell-Marti[1,3,†] | Miriam Potrony[1] | Aida Rebollo-Morell[1] | Celia Badenas[3,4] | Joan Anton Puig-Butille[3,4] | Pol Gimenez-Xavier[1,3] | Cristina Carrera[1,3] | Josep Malvehy[1,3,5] | Susana Puig[1,3,5]

[1]Dermatology Department, Melanoma Unit, Hospital Clínic, IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain

[2]Dermatology Department, Hospital Alemán, Buenos Aires, Argentina

[3]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain

[4]Biochemical and Molecular Genetics Service, Hospital Clínic, IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain

[5]Medicine Department, Universitat de Barcelona, Barcelona, Spain

Correspondence
Susana Puig
Emails: susipuig@gmail.com and spuig@clinic.ub.es

Funding information
The research at the Melanoma Unit in Barcelona is partially funded by Spanish Fondo de Investigaciones Sanitarias grants PI12/00840, PI15/00716, and PI15/00956; CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain, co-financed by European Development Regional Fund "A way to achieve Europe" EDRF; AGAUR 2014_SGR_603 of the Catalan Government, Spain; European Commission under the 6th Framework Programme, Contract No. LSHC-CT-2006-018702 (GenoMEL) and by the European Commission under the 7th Framework Programme, Diagnoptics; A grant from "Fundació La Marató de TV3, 201331-30," Catalonia, Spain; a grant from Telemaraton of Spain "Todos somos raros" and a grant from "Asociación Española Contra el Cáncer (AECC)." The work was carried out at the Esther Koplowitz Center, Barcelona. Miriam Potrony is the recipient of a PhD Fellowship FI14/00231 (PFIS) from Instituto de Salud Carlos III, Spain.

Summary

The melanocortin 1 receptor (*MC1R*) is a highly polymorphic gene. The loss-of-function *MC1R* variants ("R") have been strongly associated with red hair color phenotype and an increased melanoma risk. We sequenced the *MC1R* gene in 175 healthy individuals to assess the influence of *MC1R* on nevus phenotype. We identified that *MC1R* variant carriers had larger nevi both on the back [*p*-value = .016, adjusted for multiple parameters (adj. *p*-value)] and on the upper limbs (adj. *p*-value = .007). Specifically, we identified a positive association between the "R" *MC1R* variants and visible vessels in nevi [*p*-value = .033, corrected using the FDR method for multiple comparisons (corrected *p*-value)], dots and globules in nevi (corrected *p*-value = .033), nevi with eccentric hyperpigmentation (corrected *p*-value = .033), a high degree of freckling (adj. *p*-value = .019), and an associative trend with presence of blue nevi (corrected *p*-value = .120). In conclusion, the *MC1R* gene appears to influence the nevus phenotype.

KEYWORDS
blue nevi, dermoscopy, melanocortin 1 receptor (*MC1R*) gene, nevogenesis, nevus

†Contributed equally to this work.

1 | INTRODUCTION

Melanoma is a complex and heterogeneous disease which results from the interaction between UV radiation exposure (Gandini, Sera, Cattaruzza, Pasquini, Picconi et al., 2005) and intrinsic host factors. Phenotypic characteristics such as hair and eye color, skin phototype, presence of freckles, number of nevi or presence of atypical nevi have been associated with melanoma risk (Gandini, Sera, Cattaruzza, Pasquini, Abeni et al., 2005; Gandini, Sera, Cattaruzza, Pasquini, Zanetti et al., 2005; Potrony et al., 2015). The melanocortin 1 receptor (*MC1R*) gene is a key regulator of human pigmentation traits (Raimondi et al., 2008). The presence of loss-of-function *MC1R* variants correlates with red hair color (RHC) phenotype which is characterized by fair skin, red hair, presence of freckles, high sun sensitivity, and poor tanning response (Raimondi et al., 2008). To date, six loss-of-function *MC1R* variants, defined as "R" alleles, have been associated with the RHC phenotype (Raimondi et al., 2008) and a higher risk of developing melanoma (Raimondi et al., 2008). Additionally, those *MC1R* variants not associated with the RHC phenotype, defined as "r" variants, have been associated with a slightly increased risk of melanoma (Williams, Olsen, Hayward, & Whiteman, 2011) and may be linked to specific melanoma subtypes (Puig-Butille et al., 2013).

Beyond pigmentation-related features, the most significant melanoma risk factor is a high nevus count as well as presence of clinically atypical nevi (Bataille et al., 1996; Carrera et al., 2011; Gandini, Sera, Cattaruzza, Pasquini, Abeni et al., 2005). Large nevi (5 mm or more in diameter) are indicative of a highly proliferative melanocytic population and have been particularly associated with increased melanoma risk (Newton-bishop et al., 2010). Therefore, identifying molecular mechanisms involved in nevogenesis is crucial to improve knowledge of melanocyte biology and to identify individuals with high melanoma risk. The understanding of nevi development has increased with the use of dermoscopy, which has improved the diagnosis and the management of nevi (Malvehy, Puig, Argenziano, Marghoob, & Soyer, 2007). The dermoscopic classification of acquired melanocytic nevi is based on the assessment of three main criteria: (i) global pattern (globular, globular reticular, globular homogeneous, reticular, reticular homogeneous, and homogeneous); (ii) pigment distribution (uniform, central hyperpigmentation, central hypopigmentation, eccentric hyperpigmentation, eccentric hypopigmentation, and multifocal hypopigmentation or hyperpigmentation); and (iii) color (white, red, light brown, dark brown, blue, gray, and black) (Zalaudek et al., 2007). Specifically, dermoscopy shows that the majority of common acquired nevi have a regular overall architecture with reticular global pattern (Hofmann-Wellenhof & Soyer, 2007; Zalaudek, Docimo, & Argenziano, 2009) and uniform color (Hofmann-Wellenhof & Soyer, 2007), black and brown being the most common ones (Zalaudek et al., 2007). In contrast, uncommon dermoscopic patterns such as eccentric hyperpigmentation and multicomponent (reticular, globular, and homogeneous areas in a single lesion) patterns are significant predictors of melanoma (Arevalo, Altamura, Avramidis, Blum, & Menzies, 2008) and also, certain colors such as dark blue (Popadic, Sinz, & Kittler, 2017) can be present in nevi simulating melanoma (Arevalo et al., 2008; Hofmann-Wellenhof et al., 2002). Moreover, nevi with a complex dermoscopic pattern (multicomponent or reticular–globular pattern) have been reported to be more prevalent in melanoma patients (Lipoff et al., 2008).

Significance

This study contributes to the identification of the phenotypical characteristics of nevi associated with *MC1R* variants and reinforces the role of the *MC1R* gene in nevogenesis, increasing the risk of developing larger nevi and modifying their clinical and dermoscopic characteristics. Notably, this study showed a positive trend between carriers of "R" *MC1R* variants and the presence of blue nevi.

The dermoscopic pattern of nevi might be influenced by extrinsic factors such as UV radiation exposure (Hofmann-Wellenhof et al., 1998) and intrinsic host factors such as age (Zalaudek et al., 2006), skin phototype (Tuma, Yamada, Atallah, Mendes Araujo, & Hirata, 2015) and history of melanoma (Lipoff et al., 2008). Furthermore, nevus count and specific dermoscopic nevi pattern are associated with genetic variants (Orlow et al., 2015) suggesting that nevi development is modulated by host genetic variants and that dermoscopically defined subsets of acquired nevi are molecularly distinct. To date, an association between "R" *MC1R* variants and a hypopigmented melanoma appearance with fewer dermoscopic features has been shown, making early diagnosis more difficult (Carrera et al., 2011) and indicating the impact of *MC1R* on dermoscopic melanoma features. It was observed that in this "tricky-to-diagnose" melanoma, the number of colors and the number of structures decrease when the number of "R" variants increase (Cuéllar et al., 2009; Zalaudek, Meiklejohn, Argenziano, Thurber, & Sturm, 2009). Moreover, atypical nevi with structureless areas were more frequently observed in individuals with two RHC variants while pigment network and dark brown color were more frequently observed in individuals without "R" variants (Quint et al., 2012). To date, the study of the impact of *MC1R* variants on dermoscopic features has been evaluated in melanoma and atypical nevi (Carrera et al., 2011; Cuéllar et al., 2009; Quint et al., 2012; Zalaudek, Meiklejohn, Argenziano, Thurber et al., 2009). Thus, their impact on dermoscopic features of common acquired nevi is still limited. This study aims to evaluate the role of the *MC1R* gene in nevogenesis in terms of number, size, and dermoscopic features of common nevi.

2 | RESULTS AND DISCUSSION

Overall, 185 healthy individuals from the Hospital Clínic of Barcelona were included in the study (demographic and phenotypic characteristics are indicated in Table S1). Participants were, predominantly, white (85.4%), had phototype III-V (60.5%), brown eyes (76.2%), brown hair (79.5%) and a varying degree of sun exposure and presence of sunburns. The sun-related behavior, specific dermatological aspects and dermoscopic nevi features are summarized in Tables S2–S4.

2.1 | Analysis of clinical and dermoscopic nevi features according to host factors

Phenotype and number of nevi were assessed in the cohort of 185 individuals. We considered all nevi with a diameter of at least 1 mm and discarded all smaller ones. Overall, 65.9% of individuals had ≤50 nevi and 34.1% had >50 nevi on total body nevus count (TBNC). We observed that 21.10% of the individuals had at least one clinically atypical nevus or Clark nevus (Table S3). The predominant nevus pattern among 184 of 185 studied individuals, as one individual only had one nevus, was as follows: reticular (81%), globular (12.5%), globular reticular (3.8%), homogeneous (1.6%), reticular homogeneous (0.5%) and globular homogeneous (0.5%). When considering nevus on the back and upper limbs, the mean maximum nevi diameter was 5.28 ± 2.88 mm on the back (ranging from 1 to 20 mm) and 3.87 ± 1.76 mm on the upper limbs (ranging from 1 to 12 mm).

In our cohort, age was inversely correlated with high total body nevus count (TBNC) (*p*-value = .008) in agreement that nevi number increases from puberty to midlife and thereafter decreases (Bataille, Snieder, MacGregor, Sasieni, & Spector, 2000; Zalaudek, Catricalà, Moscarella, & Argenziano, 2011). As previously reported, higher TBNC was observed in males (OR: 1.45, 95% CI [1.25–1.68], adj. *p*-value <.001), in light-skinned individuals (OR: 2.76, 95% CI [1.48–5.18], *p*-value = .001) (Aalborg, Morelli, Byers, Mokrohisky, & Crane, 2010; Aguilera et al., 2009; Blake et al., 2014; Echeverría, Bulliard, Guillén, & Nagore, 2014) and correlates with height (OR: 1.06, 95% CI [1.02–1.10], *p*-value = .003) (Ribero et al., 2016).

We observed previously reported findings related to clinical and dermoscopic nevi features, such as more red/pink nevi and more nevi with central hypopigmentation and eccentric hyperpigmentation in light-skinned individuals (Table 1) (Zalaudek et al., 2007) and higher TBNC in individuals with a higher degree of sun exposure (>50 nevi)

TABLE 1 Evaluation of nevi characteristics and presence of freckles according to Fitzpatrick phototype

	Phototype I-II *N* (%)	Phototype III-V *N* (%)	OR (95% CI)	*p*-value
Color of nevi				
White nevi	8 (11)	6 (5.4)	2.17 (0.72–6.55)	.159
Red/pink nevi	26 (35.6)	23 (20.5)	**2.14 (1.10–4.15)**	**.023**
Light brown nevi	73 (100)	87 (77.7)	UND	**<.001**
Dark brown nevi	30 (41.1)	54 (48.2)	0.75 (0.41–1.36)	.342
Visible vessels	32 (43.8)	37 (33.0)	1.58 (0.86–2.90)	.138
Dermoscopic patterns				
Globular pattern	31 (42.5)	41 (36.6)	1.28 (0.70–2.33)	.424
Reticular pattern	70 (95.9)	102 (91.1)	2.28 (0.61–8.61)	.21
Homogeneous pattern	4 (5.5)	13 (11.6)	0.44 (0.14–1.41)	.16
Globular homogeneous pattern	7 (9.6)	6 (5.4)	1.87 (0.60–5.82)	.27
Globular reticular pattern	17 (23.3)	22 (19.6)	1.24 (0.61–2.54)	.55
Reticular homogeneous pattern	10 (13.7)	8 (7.1)	2.06 (0.77–5.50)	.14
Pigment distribution				
Central hyperpigmentation	17 (23.3)	27 (24.1)	0.96 (0.47–1.91)	.90
Central hypopigmentation	7 (9.6)	2 (1.8)	**5.83 (1.17–28.92)**	**.016**
Eccentric hyperpigmentation	19 (26)	11 (9.8)	**3.23 (1.43–7.28)**	**.003**
Eccentric hypopigmentation	3 (4.1)	0 (0)	UND	.031
Multifocal hypo/hyperpigmentation	6 (8.2)	7 (6.3)	1.34 (0.43–4.17)	.61
Uniform	69 (94.5)	106 (94.6)	0.98 (0.26–3.59)	.97
Dermoscopic structures				
Pigmented network	73 (100)	109 (97.3)	UND	.16
Dots and globules	39 (53.4)	51 (45.5)	1.37 (0.76–2.48)	.29
Freckles	26 (35.6)	14 (12.5)	**3.87 (1.85–8.09)**	**.001**
Clark nevi	21 (28.8)	18 (16.1)	**2.11 (1.03–4.32)**	**.039**
Blue nevus	2 (2.7)	7 (6.3)	0.423 (0.085–2.09)	.278

UND: Undetermined due to lack of an individual in one of the categories of the 2 × 2 contingency table.

Significant values are in bold.

TABLE 2 Evaluation of number and size of nevi according to gender and phototype

	Gender					Phototype				
	Female		Male			I-II		III-V		
Nevi location	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*p*-value	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*p*-value
Back nevi										
Total nevus count	126	13.87 (33.09)	38	26.32 (20.10)	**<.001**	59	18.78 (15.85)	105	15.62 (15.80)	.221
Total nevus count ≥2 mm	139	9.30 (9.32)	46	19.37 (15.44)	**<.001**	73	14.71 (12.50)	112	9.91 (11.22)	**.007**
Maximum nevi diameter (mm)	134	4.97 (2.7)	44	6.23 (3.29)	**.010**	71	5.80 (2.93)	107	4.93 (2.82)	**.049**
Upper limb nevi										
Total nevus count	131	27.03 (21.49)	38	39.63 (32.10)	.107	63	36.24 (28.95)	106	26.08 (21.11)	**.009**
Total nevus count ≥2 mm	139	11.62 (12.83)	46	22.63 (21.56)	**<.001**	73	18.79 (19.42)	112	11.46 (12.83)	**.002**
Maximum nevi diameter (mm)	137	3.70 (1.61)	45	4.38 (2.10)	**.031**	73	4.27 (1.89)	109	3.60 (1.63)	**.011**

Significant values are in bold.

(OR: 2.03, 95% CI [1.08–3.82], *p*-value = .003)(Baron et al., 2014) or individuals who presented sunburns during childhood (OR: 2.26, 95% CI [1.15–4.44], *p*-value = .003)(Carli, Naldi, Lovati, and Vecchia (2002). These results indicate that despite the reduced sample size, the cohort is representative of the general population.

Notably, we detected that gender and phototype also influence nevi size. We observed that males and light-skinned individuals present larger nevi on the back and upper limbs (Table 2), suggesting that gender-related factors and pigment synthesis may impact on the proliferative capacity of melanocytes. Gender differences have been reported in the etiology and prognosis of melanoma (Autier et al., 2004; Scoggins et al., 2006) as males have more melanomas on the trunk and worst melanoma prognosis. Pigment-related factors such as loss-of-function *MC1R* variants have been also associated with melanoma development (Bertolotto, 2013; Nan, Kraft, Hunter, & Han, 2009).

Age, phototype, sun exposure degree and presence of sunburns during childhood influence the presence of Clark nevi (Naeyaert & Brochez, 2003; Titus-Ernstoff et al., 1991). These associations were also confirmed in our cohort where Clark nevi were more prevalent in those individuals with light skin (Table 1), younger age (*p*-value = .041), highest sun exposure degree (OR: 3.83, 95% CI [1.69–8.67], *p*-value = .001) and presence of sunburns during childhood (OR: 2.27, 95% CI [1.15–4.44], *p*-value = .003).

Finally, we evaluated the presence of freckles and we found that light-skinned individuals were prone to them (Table 1). Although degree of freckling is largely genetically determined (Bastiaens et al., 2001; Flanagan et al., 2000; Rees, 2004), it is also induced by sunlight (Praetorius, Sturm, & Steingrimsson, 2014). In accordance with this evidence, we observed that degree of freckling is higher in individuals with a high degree of sun exposure (OR: 12.44, 95% CI [1.58–97.90], *p*-value = .003) and in those who presented sunburns during childhood (OR: 4.92, 95% CI [1.52–15.87], *p*-value = .004).

2.2 | Analysis of size and dermoscopic features of nevi according to *MC1R* gene

The *MC1R* gene was sequenced and assessed in 94.6% (175/185) of individuals. Overall, 56.6% of individuals carried at least one non-synonymous *MC1R* polymorphism. Based on the *MC1R* polymorphism classification, we found two (1.1%) *R/R*, eight (4.6%) *R/r*, 25 (14.3%) *R/-*, 12 (6.9%) *r/r*, 52 (29.7%) *r/-*, and 76 (43.4%) wild-type (WT) individuals. When we compared *MC1R* genotype frequencies between white and other ethnicity subsets, no statistically significant differences were detected (*p*-value = .474), indicating that all included individuals were representative of a homogeneous control population.

We assessed whether the presence of *MC1R* polymorphisms influences dermoscopic nevi features, adjusting for all the variables which have been associated with these dermoscopic features in our cohort (Tables 3 and 4). The *MC1R* variants, especially "R" variants, modulate color (hypopigmentation) and dermoscopic patterns (less "structures") of melanomas and nevi, resulting in more discernible vessels (Bassoli et al., 2013; Cuéllar et al., 2009; Quint et al., 2012). We detected an overrepresentation of visible vessels in nevi and a strong association with eccentric hyperpigmentation pattern associated with "R" *MC1R* variants carriers (Table 4).

Notably, we detected that *MC1R* variant carriers (Table 3) especially those "R" *MC1R* variants carriers, were prone to show nevi with dots and globules. These dermoscopic structures correlate with aggregations of pigmented melanocytes, melanophages or clumps of melanin within the cornified layer, the epidermis, the dermoepidermal junction or the papillary dermis (Neila & Soyer, 2011). Moreover, nevi with globular/cobblestone pattern derive from immature dermal melanoblasts which proliferate during early childhood in the dermis (Zalaudek et al., 2011). Based on this evidence and considering that *MC1R* is crucial for melanocyte proliferation, regulation and differentiation (D'Mello, Finlay, Baguley, & Askarian-amiri, 2016), we suggest

TABLE 3 Evaluation of nevi dermoscopic features according to *MC1R* genotype

	WT	Any *MC1R* variant[a]	WT versus any *MC1R* variant		
	N (%)	*N* (%)	OR (95% CI)	Adj. *p*-value	Corrected *p*-value*
Color of nevi					
White nevi	5 (6.6)	8 (8.1)	1.15 (0.36–3.72)[b]	.815[b]	.922[b]
Red/pink nevi	15 (19.7)	32 (32.3)	1.81 (0.89–3.07)[b]	.102[b]	.408[b]
Light brown nevi	63 (82.9)	88 (88.9)	1.36 (0.55–3.39)[b]	.504[b]	.704[b]
Dark brown nevi	35 (46.1)	44 (44.4)	0.96 (0.52–1.76)[b]	.898[b]	.945[b]
Visible vessels	21 (27.6)	44 (44.4)	**2.00 (1.05–3.82)**[b]	**.036**[b]	.240[b]
Dermoscopic patterns					
Globular pattern	25 (32.9)	43(43.4)	1.57 (0.84–2.92)	.157	.523
Reticular pattern	69 (90.8)	93 (93.9)	1.57 (0.51–4.89)	.434	.668
Homogeneous pattern	5 (6.6)	12 (12.1)	1.96 (0.66–5.82)	.226	.565
Globular homogeneous pattern	4 (5.3)	9 (9.1)	1.80 (0.53–6.08)	.344	.668
Globular reticular pattern	13 (17.1)	22 (22.2)	1.38 (0.65–2.97)	.403	.668
Reticular homogeneous pattern	5 (6.6)	12 (12.1)	1.96 (0.66–5.82)	.226	.565
Pigment distribution					
Central hyperpigmentation	16 (21.1)	27 (27.3)	1.40 (0.69–2.86)[b]	.350[b]	.668[b]
Central hypopigmentation	2 (2.6)	6 (6.1)	2.01 (0.38–10.48)[b]	.410[b]	.668[b]
Eccentric hyperpigmentation	7 (9.2)	22 (22.2)	**2.55 (1.01–6.44)**[b]	**.048**[b]	.240[b]
Eccentric hypopigmentation	0 (0)	3 (3.0)	–	–	
Multifocal hypo/hyperpigmentation	7 (7.1)	5 (6.6)	1.014 (0.30–3.37)[b]	.980[b]	.980[b]
Uniform	72 (94.7)	93 (93.9)	0.87 (0.23–3.22)[b]	.830[b]	.922[b]
Dermoscopic structures					
Pigmented network	75 (98.7)	97 (98)	0.44 (0.034–5.70)[c]	.528[c]	.704[c]
Dots and globules	28 (36.8)	58 (58.6)	**2.85 (1.44–5.66)**[c]	**.003**[c]	.060[c]
Clark nevi	13 (17.1)	23 (23.2)	1.12 (0.46–2.77)[d]	.799[d]	.922[d]
Blue nevi	1 (1.3)	8 (8.1)	**6.59 (0.81–53.91)**	**.045**	.240

*Corrected by FDR method for multiple comparisons.
[a]Includes all genotypes (*R/R, R/r, R/-, r/r, r/-*) except wild-type individuals (WT).
[b]Adjusted for phototype.
[c]Adjusted for age, gender, and phototype.
[d]Adjusted for age, phototype, total nevus count, sun exposure degree, and presence of sunburns during childhood (from 0 to 10 years old).
Significant values are in bold.

that a decrease in *MC1R* function could induce a lack of maturation of melanocytes and hence dots and globules in nevi. In addition, we assessed whether the *MC1R* gene may influence the number or size of nevi. All the analyses included in Table 5 were adjusted for those variables associated with TBNC in our cohort. We identified that *MC1R* variant carriers had larger nevi than wild-type individuals independent of the body site assessed (back or upper limbs) (Table 5). Although this association was detected independently to the *MC1R* variant subtype, this association reached the highest statistical significance in "R" *MC1R* variant individuals (Table 5). A previous study suggests that sunburns are associated with nevi larger than 2 mm in *MC1R* variant carriers (Baron et al., 2014). However, our analyses were adjusted for sun exposure degree and presence of sunburns, indicating that *MC1R* variants influence nevi size as well as UV radiation effects. An association between *MC1R* variants and greater size of melanomas (Carrera et al., 2011) and congenital melanocytic nevi (Kinsler et al., 2012) has been described; our results indicate that such an effect is also observed in common acquired nevi. Altogether, these data suggest that *MC1R* has a growth-promoting effect on melanocytes.

In contrast, when considering total nevus count on upper limbs and back, association between *MC1R* variants and a higher number of nevi was restricted to those individuals harboring "R" *MC1R* variants and to those lesions located on the upper limbs (Table 5). In addition, we observed an association between "R" *MC1R* variant carriers and a higher TBNC (OR: 2.34, 95% CI [1.001–5.65], adj. *p*-value = .046) adjusting for gender, age, phototype, height, degree of sun exposure and presence of sunburns as previously observed (Kanetsky et al., 2010).

TABLE 4 Evaluation of nevi dermoscopic features according to different subtypes of *MC1R* variants

	WT	r/-, r/r				≥1 R variant[a]			
	N (%)	*N* (%)	OR (95% CI)	Adj. *p*-value	Corrected *p*-value*	*N* (%)	OR (95% CI)	Adj. *p*-value	Corrected *p*-value*
Color of nevi									
White nevi	5 (6.6)	4 (6.2)	0.86 (0.22–3.40)[b]	.829[b]	.829[b]	4 (11.4)	1.64 (0.40–6.66)[b]	.490[b]	.610[b]
Red/pink nevi	15 (19.7)	18 (28.1)	1.54 (0.70–3.41)[b]	.280[b]	.598[b]	14 (40)	2.45 (0.99–6.07)[b]	.052[b]	.208[b]
Light brown nevi	63 (82.9)	59 (92.2)	2.13 (0.69–6.62)[b]	.190[b]	.598[b]	29 (82.9)	0.72 (0.23–2.29)[b]	.580[b]	.610[b]
Dark brown nevi	35 (46.1)	26 (40.6)	0.82 (0.42–1.62)[b]	.573[b]	.745[b]	18 (51.4)	1.28 (0.57–2.87)[b]	.554[b]	.610[b]
Visible vessels	21 (27.6)	24 (37.5)	1.54 (0.75–3.16)[b]	.236[b]	.598[c]	20 (57.1)	**3.33 (1.43–7.75)**[b]	**.005**[b]	**.033**[b]
Dermoscopic patterns									
Globular pattern	25 (32.9)	28 (43.8)	1.59 (0.80–3.16)	.188	.598	15 (42.9)	1.53 (0.67–3.48)	.311	.478
Reticular pattern	69 (90.8)	59 (92.2)	1.20 (0.36–3.97)	.769	.829	34 (97.1)	3.45 (0.41–29.17)	.256	.465
Homogeneous pattern	5 (6.6)	8 (12.5)	2.03 (0.63–6.54)	.236	.598	4 (11.4)	1.83 (0.46–7.29)	.390	.557
Globular homogeneous pattern	4 (5.3)	4 (6.2)	1.20 (0.29–5.00)	.802	.829	5 (14.3)	3.00 (0.75–11.95)	.119	.297
Globular reticular pattern	13 (17.1)	14 (21.9)	1.36 (0.58–3.15)	.477	.5981	8 (22.9)	1.44 (0.53–3.86)	.474	.610
Reticular homogeneous pattern	5 (6.6)	6 (9.4)	1.47 (0.43–5.06)	.542	.745	6 (17.1)	2.94 (0.83–10.39)	.094	.297
Pigment distribution									
Central hyperpigmentation	16 (21.1)	21 (32.8)	1.80 (0.84–3.86)[b]	.132[b]	.598[b]	6 (17.1)	0.83 (0.29–2.36)[b]	.722[b]	.722[b]
Central hypopigmentation	2 (2.6)	3 (4.7)	1.64 (0.26–10.32)[b]	.596[b]	.745[b]	3 (8.6)	2.78 (0.42–18.17)[b]	.293[b]	.478[b]
Eccentric hyperpigmentation	7 (9.2)	10 (15.6)	1.70 (0.60–4.81)[b]	.319[b]	.598[b]	12 (34.3)	**4.70 (1.60–13.79)**[b]	**.005**[b]	**.033**[b]
Eccentric hypopigmentation	0 (0)	0 (0)	–	–		3 (8.6)	–	–	
Multifocal hypo/ hyperpigmentation	7 (7.1)	1 (1.6)	0.22 (0.025–2.00)[b]	.181[b]	.598[b]	6 (17.1)	2.82 (0.79–10–10)[b]	.111[b]	.297[b]
Uniform	72 (94.7)	59 (92.2)	0.64 (0.16–2.52)[b]	.526[b]	.745[b]	34 (97.1)	1.96 (0.21–18.54)[b]	.557[b]	.610[b]
Dermoscopic structures									
Pigmented network	75 (98.7)	63 (98.4)	0.59 (0.03–11.54)[c]	.727[c]	.745[c]	34 (97.1)	0.08 (0.001–5.79)[c]	.253[c]	.465[c]
Dots and globules	28 (36.8)	35 (54.7)	**2.21 (1.05–4.66)**[c]	**.037**[c]	.598[c]	23 (65.7)	**4.63 (1.73–12.40)**[c]	**.002**[c]	**.033**[c]
Clark nevi	13 (17.1)	10 (15.6)	1.73 (0.57–5.20)[d]	.329[d]	.598[d]	13 (37.1)	2.27 (0.78–6.61)[d]	.134[d]	.299[d]
Blue nevi	1 (1.3)	3 (4.7)	3.69 (0.37–36.36)	.264	.598	5 (14.3)	**12.50 (1.40–111.51)**	**.024**	.120

*Corrected by FDR method for multiple comparisons.
[a]Includes all individuals with at least 1 "R" *MC1R* variant (*R/R, R/r, R/-*).
[b]Adjusted for phototype.
[c]Adjusted for age, gender, and phototype.
[d]Adjusted for age, phototype, total nevus count, sun exposure degree, and presence of sunburns during childhood (from 0 to 10 years old).
Significant values are in bold.

TABLE 5 Evaluation of number and size of nevi according to MC1R genotype

Nevi location	WT N	WT Mean (SD)	Any MC1R variant[a] N	Any MC1R variant[a] Mean (SD)	Any MC1R variant[a] Adj. p-value[c]	r/-, r/r N	r/-, r/r Mean (SD)	r/-, r/r Adj. p-value[c]	≥1 R variant[b] N	≥1 R variant[b] Mean (SD)	≥1 R variant[b] Adj. p-value[c]
Back nevi											
Total nevus count	72	14.58 (14.17)	82	18.49 (17.35)	.240	54	18.56 (17.77)	.356	28	18.36 (16.84)	.330
Total nevus count ≥2 mm	76	9.00 (8.93)	99	13.95 (13.71)	**.016**	64	12.30 (11.18)	**.046**	35	16.97 (17.20)	.068
Maximum nevi diameter	72	4.56 (2.36)	98	5.82 (3.21)	**.004**	64	5.61 (3.05)	**.015**	34	6.21 (3.49)	**.021**
Upper limbs nevi											
Total nevus count	73	25.05 (20.34)	86	32.66 (24.86)	.061	56	29.84 (24.76)	.366	30	37.93 (24.58)	**.007**
Total nevus count ≥2 mm	76	10.61 (13.76)	99	16.12 (15.72)	**.007**	64	12.95 (12.03)	.083	35	21.91 (19.79)	**.002**
Maximum nevi diameter	74	3.58 (1.64)	98	3.99 (1.73)	.192	63	3.94 (1.88)	.449	35	4.09 (1.42)	.127

[a]Includes all genotypes (*R/R, R/r, R/-, r/r, r/-*) except wild-type individuals (WT).
[b]Includes all individuals with at least 1 "R" MC1R variants (*R/R, R/r, R/-*).
[c]Adjusted for gender, age, phototype, height, sun exposure degree, and presence of sunburns during childhood (from 0 to 10 years old). All statistical analyses included in this table were performed relative to wild-type individuals (WT).
Significant values are in bold.

Interestingly, we identified a positive trend between "R" *MC1R* variant and presence of blue nevi (Table 4). Even though the most common somatic mutations in acquired nevi did not match the somatic mutations identified in blue nevi (Roh, Eliades, Gupta, & Tsao, 2015), both types of lesions could share similar genetic susceptibility and *MC1R* could be a genetic factor involved in the development of both of them.

The *MC1R* gene has been shown to be a major contributor to freckle formation (Bastiaens et al., 2001; Flanagan et al., 2000; Rees, 2004). We identified a positive association between "R" *MC1R* variant carriers and both presence of freckles and higher degree of freckling, even after adjusting for phototype, age, gender, sun exposure and presence of sunburns during childhood (Table 6). When we focused the analysis on number of *MC1R* variants, we observed that compound heterozygotes (*R/r*) and homozygotes (*R/R* and *r/r*) had an eightfold increased risk of developing freckles, also being strongly associated with a higher degree of freckling (Table 7). These findings correlate with a previously described "dosage effect," in which degree of freckling was associated with number of *MC1R* variants (Bastiaens et al., 2001).

The major limitation of our study is the sample size of our cohort. However, our work provides positive results from our hypothesis and highlights a putative role for *MC1R* in the genetic susceptibility of developing larger melanocytic nevi, some specific dermoscopic patterns, blue nevi, and freckles. Another minor limitation is that "sun-related behavior" was based exclusively on adult controls, self-reporting habits at age 0–10, which is susceptible to recall and reporting bias, and that sun-related behaviors after 10 years of age were not considered.

In conclusion, our results reinforce the role of the *MC1R* gene as a genetic modifier of nevi size. Specifically, this is the first time that this influence is described in common nevi. Our study provides further evidence supporting that *MC1R* influences clinical and dermoscopic nevi features and suggests an associative trend between *MC1R* "R" variants and the presence of blue nevi. Further studies should be conducted to validate our findings in other populations with a higher frequency of *MC1R* "R" variants.

3 | METHODS

We used a cross-sectional study design to analyze the impact of the *MC1R* gene on nevus phenotype in 185 healthy individuals collected at the Hospital Clínic of Barcelona (HCB), Spain. All gave their written informed consent according to the declaration of Helsinki, and the study was approved by the local ethics committee of the Hospital Clínic of Barcelona.

The inclusion criteria were as follows: adults (≥18 years old) considered healthy (able to perform normal activities and in case of presenting a chronic condition, this was treated and under control) who were spouses of melanoma patients at the Melanoma Unit (Service of Dermatology of the Hospital Clínic, Barcelona) or volunteers.

The exclusion criteria were as follows: (i) personal history of melanoma, non-cutaneous malignancies, immunosuppression, or

TABLE 6 Evaluation of presence and degree of freckling according to *MC1R* genotype

	WT	Any *MC1R* variant[a]			r/-, r/r			≥1 R variant[b]		
	N (%)	N (%)	OR (95% CI)[c]	Adj. *p*-value[c]	N (%)	OR (95% CI)[c]	Adj. *p*-value[c]	N (%)	OR (95% CI)[c]	Adj. *p*-value[c]
Freckles	8 (10.5)	28 (28.3)	**3.02 (1.21–7.55)**	**.018**	14 (21.9)	1.94 (0.71–5.28)	.194	14 (40)	**6.38 (1.88–21.66)**	**.003**
Degree of freckling (moderate–severe)[d]	2 (2.6)	10 (10.1)	3.01 (0.56–16.15)	.197	3 (4.7)	2.07 (0.17–24.80)	.566	7 (20)	**15.53 (1.58–152.91)**	**.019**

[a]Includes all genotypes (R/R, R/r, R/-, r/r, r/-) except wild-type individuals (WT).
[b]Includes all individuals with at least 1 "R" MC1R variants (R/R, R/r, R/-).
[c]Adjusted for phototype, age, gender, sun exposure degree, and presence of sunburns during childhood (from 0 to 10 years old).
[d]The degree of freckling was classified as none, mild, moderate, or severe. In this analysis, it was categorized into two different categories (1 = none–mild versus 2 = moderate–severe) and we presented the results relative to moderate–severe.
Significant values are in bold.

genodermatosis predisposing to skin cancer (i.e., Xeroderma pigmentosum, albinism, and Gorlin syndrome), (ii) familial history of melanoma in first-degree relatives, (iii) pregnant women, and (iv) relatives of another control individual in the same study.

3.1 | Clinical data

Two dermatologists trained in dermoscopy performed data collection (questionnaire and skin examination). Participants completed a detailed questionnaire regarding demographic characteristics (age, gender, and ethnicity), phenotype (phototype and eye and hair color), and sun-related behavior (sun exposure degree and presence of sunburns) during childhood (from 0 to 10 years old). The phototype was assigned according to the classification by Fitzpatrick from I to V (Fitzpatrick, 1988). For statistical analyses, phototype was divided into two categories: light-skinned individuals (phototype I-II) and dark-skinned individuals (phototype III-V). The sun exposure degree between 10 a.m. and 4 p.m. from 0 to 10 years old was graded as low (<50 hr/year), moderate (51–120 hr/year), or intense (>120 hr/year). For the statistical analyses, exposure was categorized into two categories: low exposure (none–mild) and high exposure (moderate–intense). History of sunburns during childhood was considered as the presence of long-lasting erythema (≥12 hr)(Holman, Berkowitz, Guy, Hartman, & Perna, 2014) and/or blisters after sun exposure from 0 to 10 years old. For the statistical analyses, this was categorized into two categories: absence (no type of sunburn) and presence (any type of sunburn).

Afterward, each participant underwent a whole-skin examination and dermoscopic examination. We recorded the following data: Freckles were considered as absent (none) or present, and the degree of freckling was classified as none, mild, moderate, or severe considering coverage and density of freckles. For the statistical analyses, this was categorized into two categories: low degree (none–mild) and high degree (moderate–severe). As freckles appear early in childhood and partly vanish with age, direct interrogation about freckles during childhood and sun exposure complemented the physical examination. Total body nevus count (TBNC): This included whole-body nevi and was categorized into four different ranges: 0–50, 51–100, 101–200, 201 or more. For the statistical analyses, this was categorized into two categories: low TBNC (≤50 nevi) and high TBNC (>50 nevi). In addition, we specifically assessed the total nevus count on two different anatomical localisations (back and upper limbs) and we categorized them into two groups according to nevus diameter: the first group included all melanocytic lesions with diameters ≥ 1 mm and the second group included all nevi with diameters ≥ 2 mm. The maximum and the minimum diameter were recorded from all the nevi evaluated on the back and the upper limbs.

Based on dermoscopy examination, the following data were considered: (i) presence of Clark nevi (atypical melanocytic nevi) was defined as previously reported (Garbe et al., 1994) and (ii) presence of blue nevi (well-circumscribed, homogeneous, relatively confluent blue-gray to blue-black pigmented lesions) (Malvehy, Puig, Braun, Marghoob, & Kopt, 2006). Dermoscopic characteristics of nevus: (i) global patterns of nevi and predominant nevus pattern: All the global

TABLE 7 Evaluation of presence and degree of freckling according to number of *MC1R* variants

	WT	1 variant (r/-, R/-)			2 variants (r/r, R/R, R/r)		
	N (%)	*N* (%)	OR (95% CI)[a]	Adj. *p*-value[a]	*N* (%)	OR (95% CI)[a]	Adj. *p*-value[a]
Freckles	8 (10.5)	16 (20.8)	2.07 (0.77–5.58)	.148	12 (54.5)	**8.31 (2.31–29.93)**	**.001**
Degree of freckling (moderate–severe)[b]	2 (2.6)	3 (3.9)	1.04 (0.13–8.09)	.969	7 (31.8)	**36.76 (2.08–649.58)**	**.014**

[a]Adjusted for age, gender, phototype, sun exposure degree, and presence of sunburns during childhood (from 0 to 10 years old).
[b]The degree of freckling was classified as none, mild, moderate, or severe. In this analysis, it was divided into two different categories (1 = none–mild versus 2 = moderate–severe) and we presented the results relative to moderate–severe.
Significant values are in bold.

patterns were registered as present or absent and the predominant pattern was specified. The predominant nevus pattern was defined as the pattern being present in more than 40% of all the individual's nevi as outlined previously for Clark nevi (Hofmann-Wellenhof et al., 2001). Global patterns were classified as globular, reticular, homogeneous, globular homogeneous, globular reticular, reticular homogeneous, and unclassified (Hofmann-Wellenhof et al., 2001). (ii) Type of dermoscopic structures: pigment network, dots and globules, projections, blotches, peppering, regression structures, blue-whitish veil, shining white sticks, and rosettes were recorded (Malvehy et al., 2006). (iii) Colors were classified as present or absent in the following categories: white, red/pink, light brown, dark brown, blue, and black. When a nevus exhibits more than one color, the one observed in >50% of the lesion was recorded as "prevalent color" (Zalaudek et al., 2007). (iv) Pigment distribution was classified as uniform, central hyperpigmentation, central hypopigmentation, eccentric hyperpigmentation, eccentric hypopigmentation, or multifocal (Hofmann-Wellenhof et al., 2001; Zalaudek et al., 2007). Eccentric hyperpigmentation included true and pseudo-eccentric hyperpigmentation (Arevalo et al., 2008). (v) Presence of visible vessels (Giacomel & Zalaudek, 2013).

Whole-body melanocytic lesions were examined with a DermLite II Pro HR dermoscope. Images of back and upper limbs and nevus were taken solely for illustration proposes.

3.2 | *MC1R* molecular screening

Genomic DNA from peripheral blood lymphocytes or cells in saliva of controls was isolated by standard methods. Saliva was collected using Oragene DNA collection Kits. Polymerase chain reaction (PCR) amplification of the *MC1R*-coding sequence was conducted using the previously reported primers (Chaudru et al., 2005), performing an initial denaturizing step at 95°C for 5 min, followed by 35 PCR cycles (94°C for 1 min, 55°C for 1 min, 72°C for 3 min) and a final extension at 72°C for 10 min. PCR products were purified using Multiscreen Filter plates (Millipore, Billerica, MA). We sequenced the entire coding region of *MC1R* using the following internal primers (TM-F: 5′AACCTGCACTCACCCATGTA3′ and TM-R: 5′TTTAAGGCCAAAGCCCTGGT3′) and the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequences were run on an ABI3100 automatic sequencer (Applied Biosystems) and analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems, Ettenheim, Germany).

3.3 | Statistical analysis

MC1R non-synonymous variants were classified as red hair color "R" (complete loss-of-function) or non-red hair color "r" (partial loss-of-function) according to previously reported criteria (Davies et al., 2012; Duffy et al., 2004; Raimondi et al., 2008; Wendt et al., 2016). Therefore, *MC1R* variants classified as "R" were p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, and p.D294H. All other non-synonymous *MC1R* variants were classified as "r." Synonymous variants were considered as wild-type *MC1R* alleles "-." According to this classification, we determined the following genotypes: *R/R*, *R/r*, *R/-*, *r/r*, *r/-*, *-/-* (WT). We defined "R" variant carriers as those individuals who presented *R/R*, *R/r*, or *R/-* genotypes, and "r" variant carriers those who presented *r/r* or *r/-* genotypes. Descriptive analysis was performed, including mean ± standard deviation (*SD*) and/or percentages for categorical variables. Pearson's chi-square test and *t* test were used to compare the categorical and continuous variables, respectively. For variables not normally distributed, for example, maximum nevi size and number of nevi of each size (total nevus count both on the back and on the upper limbs, total nevus count ≥ 2 mm both on the back and on the upper limbs), the values were log-transformed for parametric testing.

We used the logistic regression model to assess all the relationships between the categorical dependent variables and one or more independent variables. Thus, we used the logistic regression model to analyze the association between the two phototype categories (Fitzpatrick phototypes I-II versus III-V) and many independent variables such as dermoscopic patterns, dermoscopic structures, nevi color, pigment distribution, and visible vessels (Table 1).

We also used this statistical method to evaluate the relationship between the *MC1R* genotype and total nevus count, categorizing this variable into two groups (≤50 nevi versus >50 nevi), and adjusting this logistic regression model for age, gender, height, and Fitzpatrick skin phototype of the volunteers at the time the nevi were counted, as all of these variables are statistically associated with nevi number.

In Table 2, we evaluated the effect of gender and Fitzpatrick skin phototype on nevi size using a linear regression model, defining the maximum nevi size or the nevi number of each size category as dependent variables.

In Tables 3 and 4, we evaluated possible associations between *MC1R* genotype and 20 different nevi dermoscopic features (also included in Table 1) adjusting for all the variables statistically associated with each one of these nevi characteristics.

In Table 5, we evaluated the effect of the *MC1R* genotype on nevi size by a multiple linear regression model, using maximum nevi size or the nevi number of each size category as dependent variables. The effect of the *MC1R* genotype on nevi size was adjusted for all the variables statistically associated with nevi number and size.

In Table 6, we evaluated the effect of the *MC1R* genotype on the presence and degree of freckling using a logistic regression model which was adjusted for all the variables statistically associated with freckling. In Table 7, we assessed the same dependent variables according to the number of *MC1R* variants adjusting again for all the variables statistically associated with freckling.

All statistical analyses were performed using the SPSS software version 22.0 (IBM Corp. Released 2013. Armonk, NY). Although some multiple comparisons were performed in Tables 5–7, none of the reported *p*-values were corrected for multiple testing as the different comparisons were complementary between them and were focused on the same basic question (Ridker et al., 2008; Rothman, 1990; Saville, 1990). However, 20 different non-complementary questions were assessed in Tables 3 and 4; therefore, all the reported *p*-values shown in Tables 3 and 4 were corrected using the FDR multiple comparisons method (Benjamini & Hochberg, 1995; Glickman, Rao, & Schultz, 2014).

All definitive *p*-values (*p*-value, Adj. *p*-value or corrected *p*-value) $<.05$ were considered statistically significant.

ACKNOWLEDGEMENTS

We are grateful to the participants for taking part in the study and to Lorena Taméz Peña for helping with data collection.

ORCID

María Gabriela Vallone http://orcid.org/0000-0002-7346-8638
Josep Malvehy http://orcid.org/0000-0002-6998-914X
Susana Puig http://orcid.org/0000-0003-1337-9745

REFERENCES

Aalborg, J., Morelli, J., Byers, T., Mokrohisky, S., & Crane, L. (2010). Effect of hair color and sun sensitivity on nevus counts in white children in colorado. *Journal of the American Academy of Dermatology*, *63*, 430–439.

Aguilera, P., Puig, S., Guilabert, A., Julià, M., Romero, D., Vicente, A., ... Malvehy, J. (2009). Prevalence study of nevi in children from Barcelona. Dermoscopy, constitutional and environmental factors. *Dermatology*, *218*, 203–314.

Arevalo, A., Altamura, D., Avramidis, M., Blum, A., & Menzies, S. (2008). The significance of eccentric and central hyperpigmentation, multifocal hyper/hypopigmentation, and the multicomponent pattern in melanocytic lesions lacking specific dermoscopic features of melanoma. *Archives of Dermatology*, *144*, 1440–1444.

Autier, P., Boniol, M., Severi, G., Pedeux, R., Grivegnée, A., Doré, J., & European Organisation for Research and Treatment of Cancer Melanoma Group (2004). Sex differences in numbers of nevi on body sites of young European children: Implications for the Etiology of Cutaneous melanoma. *Cancer Epidemiology, Biomarkers & Prevention*, *13*, 2003–2005.

Baron, A. E., Asdigian, N. L., Gonzalez, V., Aalborg, J., Terzian, T., Stiegmann, R. A., ... Box, N. F. (2014). Interactions between ultraviolet light and MC1R and OCA2 variants are determinants of childhood nevus and freckle phenotypes. *Cancer Epidemiology, Biomarkers & Prevention*, *23*, 2829–2839.

Bassoli, S., Maurichi, A., Rodolfo, M., Casari, A., Frigerio, S., Pupelli, G., ... Pellacani, G. (2013). CDKN2A and MC1R variants influence dermoscopic and confocal features of benign melanocytic lesions in multiple melanoma patients. *Experimental Dermatology*, *22*, 411–416.

Bastiaens, M., ter Huurne, J., Gruis, N., Bergman, W., Westendorp, R., Vermeer, B. J., & Bouwes Bavinck, J. N. (2001). The melanocortin-1-receptor gene is the major freckle gene. *Human Molecular Genetics*, *10*, 1701–1708.

Bataille, V., Newton Bishop, J., Sasieni, P., Swerdlow, A., Pinney, E., Griffiths, K., & Cuzick, J. (1996). Risk of cutaneous melanoma in relation to the numbers, types and sites of naevi: A case -control study. *British Journal of Cancer*, *73*, 1605–1611.

Bataille, V., Snieder, H., MacGregor, A., Sasieni, P., & Spector, T. (2000). Genetics of risk factors for melanoma: An adult twin study of nevi and freckles. *Journal of the National Cancer Institute*, *92*, 457–463.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B*, *57*, 289–300.

Bertolotto, C. (2013). Melanoma: from Melanocyte to genetic alterations and clinical options. *Scientifica*, *2013*, 22. Articule ID 635203.

Blake, T., Mc Clenahan, P., Duffy, D., Schaider, H., McEniery, E., & Soyer, H. P. (2014). Distribution analyses of acquired melanocytic naevi on the trunk. *Dermatology*, *228*, 269–275.

Carli, P., Naldi, L., Lovati, S., Vecchia, C. L., & Oncology Cooperative Group of the Italian Group for Epidemiologic Research in Dermatology (GISED) (2002). The density of melanocytic nevi correlates with constitutional variables and history of sunburns: A prevalence study among Italian schoolchildren. *International Journal of Cancer*, *101*, 375–379.

Carrera, C., Palou, J., Malvehy, J., Segura, S., Aguilera, P., Salerni, G., ... Puig, S. (2011). Early stages of melanoma on the limbs of high-risk patients: Clinical, dermoscopic, reflectance confocal microscopy and histopathological characterization for improved recognition. *Acta Dermato Venereologica*, *91*, 137–146.

Chaudru, V., Laud, K., Avril, M. F., Minière, A., Chompret, A., Bressac-De Paillerets, B., ... The French Familial Melanoma Study Group (2005). Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. *Cancer Epidemiology, Biomarkers & Prevention*, *14*, 2384–2390.

Cuéllar, F., Puig, S., Kolm, I., Puig-Butille, J., Zaballos, P., Martí-Laborda, R., ... Malvehy, J. (2009). Dermoscopic features of melanomas associated with MC1R variants in Spanish CDKN2A mutation carriers. *British Journal of Dermatology*, *160*, 48–53.

Davies, J. R., Randerson-moor, J., Kukalizch, K., Harland, M., Kumar, R., Madhusudan, S., ... Newton-bishop, J. (2012). Inherited variants in the MC1R gene and survival from cutaneous melanoma: A BioGenoMEL study. *Pigment Cell & Melanoma Research*, *25*, 384–394.

D'Mello, S. A., Finlay, G. J., Baguley, B. C., & Askarian-amiri, M. E. (2016). Signaling pathways in melanogenesis. *International Journal of Molecular Sciences*, *17*, 1144.

Duffy, D. L., Box, N. F., Chen, W., Palmer, J. S., Montgomery, G. W., James, M. R., ... Sturm, R. A. (2004). Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Human Molecular Genetics*, *13*, 447–461.

Echeverría, B., Bulliard, J.-L., Guillén, C., & Nagore, E. (2014). Indicators for the total number of melanocytic naevi: An adjunct for screening campaigns. Observational study on 292 patients. *British Journal of Dermatology*, *170*, 144–149.

Fitzpatrick, T. B. (1988). The validity and practicality of sun-reactive skin types I through VI. *Archives of Dermatology*, *124*, 869–871.

Flanagan, N., Healy, E., Ray, A., Philips, S., Todd, C., Jackson, I. J., ... Rees, J. L. (2000). Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation. *Human Molecular Genetics*, *9*, 2531–2537.

Gandini, S., Sera, F., Cattaruzza, M. S., Pasquini, P., Abeni, D., Boyle, P., & Melchi, C. F. (2005). Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. *European Journal of Cancer*, *41*, 28–44.

Gandini, S., Sera, F., Cattaruzza, M. S., Pasquini, P., Picconi, O., Boyle, P., & Melchi, C. F. (2005). Meta-analysis of risk factors for cutaneous melanoma: II Sun exposure. *European Journal of Cancer*, *41*, 45–60.

Gandini, S., Sera, F., Cattaruzza, M. S., Pasquini, P., Zanetti, R., Masini, C., ... Melchi, C. F. (2005). Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. *European Journal of Cancer*, *41*, 2040–2059.

Garbe, C., Büttner, P., Weiß, J., Soyer, H. P., Stocker, U., Krüger, S., ... Orfanos Constantin, E. (1994). Associated factors in the prevalence of more than 50 common melanocytic nevi, atypical Melanocytic nevi, and actinic Lentigines: Multicenter case-control study of the central malignant melanoma registry of the German dermatological society. *Journal of Investigative Dermatology*, *102*, 700–705.

Giacomel, J., & Zalaudek, I. (2013). Pink lesions. *Dermatologic Clinics*, *31*, 649–678.

Glickman, M. E., Rao, S. R., & Schultz, M. R. (2014). False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. *Journal of Clinical Epidemiology*, *67*, 850–857.

Hofmann-Wellenhof, R., Blum, A., Wolf, I. H., Piccolo, D., Kerl, H., Garbe, C., & Soyer, H. P. (2001). Dermoscopic classification of atypical melanocytic nevi (clark nevi). *Archives of Dermatology*, *137*, 1575–1580.

Hofmann-Wellenhof, R., Blum, A., Wolf, I. H., Zalaudek, I., Piccolo, D., Kerl, H., ... Soyer, H. P. (2002). Dermoscopic classification of Clark's nevi (atypical melanocytic nevi). *Clinics in Dermatology*, *20*, 255–258.

Hofmann-Wellenhof, R., & Soyer, H. (2007). Common nevus. In H. Marion Philipp (Ed.), *Color atlas of melanocytic lesions of the skin* (7th ed., pp. 102–104). Berlin, Heidelberg: Springer.

Hofmann-Wellenhof, R., Soyer, H., Wolf, I., Smolle, J., Reischle, S., Rieger, E., ... Kerl, H. (1998). Ultraviolet radiation of melanocytic nevi. A dermoscopic study. *Archives of Dermatology*, *134*, 845–850.

Holman, D. M., Berkowitz, Z., Guy, G. P., Hartman, A. M., & Perna, F. M. (2014). The association between demographic and behavioral characteristics and sunburn among U.S. adults — National Health Interview Survey, 2010. *Preventive Medicine*, *63*, 6–12.

Kanetsky, P. A., Panossian, S., Elder, D. E., Guerry, D., Ming, M. E., Schuchter, L., & Rebbeck, T. R. (2010). Does MC1R genotype convey information about melanoma risk beyond risk phenotypes? *Cancer*, *116*, 2416–2428.

Kinsler, V. A., Abu-Amero, S., Budd, P., Jackson, I. J., Ring, S. M., Northstone, K., ... Healy, E. (2012). Germline melanocortin-1-receptor genotype is associated with severity of cutaneous phenotype in congenital melanocytic nevi: A role for MC1R in human fetal development. *The Journal of Investigative Dermatology*, *132*, 2026–2032.

Lipoff, J. B., Scope, A., Dusza, S., Marghoob, A. A., Oliveria, S. A., & Halpern, A. C. (2008). Complex dermoscopic pattern: A potential risk marker for melanoma. *British Journal of Dermatology*, *158*, 821–824.

Malvehy, J., Puig, S., Argenziano, G., Marghoob, A. A., Soyer, H. P., & International Dermoscopy Society Board members (2007). Dermoscopy report : Proposal for standardization. Results of a consensus meeting of the International Dermoscopy Society. *Journal of the American Academy of Dermatology*, *57*, 84–95.

Malvehy, J., Puig, S., Braun, R. P., Marghoob, A. A., & Kopt, A. W. (2006). *Handbook of dermoscopy*. New York: Taylor & Francis.

Naeyaert, J., & Brochez, L. (2003). Dysplastic nevi. *New England Journal of Medicine*, *349*, 2233–2240.

Nan, H., Kraft, P., Hunter, D. J., & Han, J. (2009). Genetic variants in pigmentation genes, pigmentary phenotypes, and risk of skin cancer in Caucasians. *International Journal of Cancer*, *125*, 909–917.

Neila, J., & Soyer, H. P. (2011). Key points in dermoscopy for diagnosis of melanomas, including difficult to diagnose melanomas, on the trunk and extremities. *Journal of Dermatology*, *38*, 3–9.

Newton-bishop, J. A., Newton-Bishop, J. A., Chang, Y.-M., Iles, M. M., Taylor, J. C., Bakker, B., ... Bishop, D. T. (2010). Melanocytic nevi, nevus genes and melanoma risk in a large case-control study in the United Kingdom. *Cancer Epidemiology, Biomarkers & Prevention*, *19*, 2043–2054.

Orlow, I., Satagopan, J. M., Berwick, M., Enriquez, H. L., White, K. A., Cheung, K., ... Halpern, A. C. (2015). Genetic factors associated with naevus count and dermoscopic patterns: Preliminary results from the Study of Nevi in Children (SONIC). *British Journal of Dermatology*, *172*, 1081–1089.

Popadic, M., Sinz, C., & Kittler, H. (2017). The significance of blue color in dermatoscopy. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft*, *15*, 302–307.

Potrony, M., Badenas, C., Aguilera, P., Puig-butille, J. A., Carrera, C., Malvehy, J., & Puig, S. (2015). Update in genetic susceptibility in melanoma. *Annals of Translational Medicine*, *3*, 210.

Praetorius, C., Sturm, R. A., & Steingrimsson, E. (2014). Sun-induced freckling: Ephelides and solar lentigines. *Pigment Cell & Melanoma Research*, *27*, 339–350.

Puig-Butille, J. A., Carrera, C., Kumar, R., Garcia-Casado, Z., Badenas, C., Aguilera, P., ... Puig, S. (2013). Distribution of MC1R variants among melanoma subtypes: P.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. *British Journal of Dermatology*, *169*, 804–811.

Quint, K. D., Van Der Rhee, J. I., Gruis, N. A., Ter Huurne, J. A., Wolterbeek, R., Van Der Stoep, N., ... Kukutsch, N. A. (2012). Melanocortin 1 receptor (MC1R) variants in high melanoma risk patients are associated with specific dermoscopic ABCD features. *Acta Dermato Venereologica*, *92*, 587–592.

Raimondi, S., Sera, F., Gandini, S., Iodice, S., Caini, S., Maisonneuve, P., & Fargnoli, M. C. (2008). MC1R variants, melanoma and red hair color phenotype: A meta-analysis. *International Journal of Cancer*, *122*, 2753–2760.

Rees, J. L. (2004). The genetics of sun sensitivity in humans. *American Journal of Human Genetics*, *75*(5), 739–751.

Ribero, S., Zugna, D., Osella-Abate, S., Glass, D., Nathan, P., Spector, T., & Bataille, V. (2016). Prediction of high naevus count in a healthy UK population to estimate melanoma risk. *British Journal of Dermatology*, *174*, 312–318.

Ridker, P. M., Danielson, E., Fonseca, F. A., Genest, J., Gotto, A. M., Kastelein, J. J., ... Glynn, R. J. (2008). Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *New England Journal of Medicine*, *359*, 2195–2207.

Roh, M. R., Eliades, P., Gupta, S., & Tsao, H. (2015). Genetics of melanocytic nevi. *Pigment Cell & Melanoma Research*, *28*, 661–672.

Rothman, K. J. (1990). No adjustments are needed for multiple comparisons. *Epidemiology*, *1*, 43–46.

Saville, D. J. (1990). Multiple comparison procedures: The practical solution. *The American Statistician*, *44*, 174–180.

Scoggins, C. R., Ross, M. I., Reintgen, D. S., Noyes, R. D., Goydos, J. S., Beitsh, P. D., ... Trial, Sunbelt Melanoma (2006). Gender-related differences in outcome for melanoma patients. *Annals of Surgery*, *243*, 693–700.

Titus-Ernstoff, L., Ernstoff, M. S., Duray, P. H., Barnhill, R. L., Holubkov, R., & Kirkwood, J. M. (1991). A relation between childhood sun exposure and dysplastic nevus syndrome among patients with nonfamilial melanoma. *Epidemiology*, *2*, 210–214.

Tuma, B., Yamada, S., Atallah, A. N., Mendes Araujo, F. M., & Hirata, S. H. (2015). Dermoscopy of black skin: A cross-sectional study of clinical and dermoscopic features of melanocytic lesions in individuals with type V/VI skin compared to those with type I/II skin. *Journal of the American Academy of Dermatology*, *73*, 114–119.

Wendt, J., Rauscher, S., Burgstaller-Muehlbacher, S., Fae, I., Fischer, G., Pehamberger, H., & Okamoto, I. (2016). Human determinants and the role of melanocortin-1 receptor variants in melanoma risk independent of UV radiation exposure. *JAMA Dermatology*, *152*, 776–782.

Williams, P. F., Olsen, C. M., Hayward, N. K., & Whiteman, D. C. (2011). Meta-analysis and estimates of population burden. *International Journal of Cancer*, *129*, 1730–1740.

Zalaudek, I., Argenziano, G., Mordente, I., Moscarella, E., Corona, R., Sera, F., … Kerl, H. (2007). Nevus Type in Dermoscopy Is Related to Skin Type in White Persons. *Archives of Dermatology*, *143*, 351–356.

Zalaudek, I., Catricalà, C., Moscarella, E., & Argenziano, G. (2011). What dermoscopy tells us about nevogenesis. *Journal of Dermatology*, *38*, 16–24.

Zalaudek, I., Docimo, G., & Argenziano, G. (2009). Using dermoscopic criteria and patient-related factors for the management of pigmented melanocytic nevi. *Archives of Dermatology*, *145*, 816–826.

Zalaudek, I., Grinschgl, S., Argenziano, G., Marghoob, A. A., Blum, A., Richtig, E., … Hofmann-Wellenhof, R. (2006). Age-related prevalence of dermoscopy patterns in acquired melanocytic naevi I. *British Journal of Dermatology*, *154*, 299–304.

Zalaudek, I., Meiklejohn, W., Argenziano, G., Thurber, A. E., & Sturm, R. A. (2009). "White" Nevi and "Red" melanomas: Association with the RHC phenotype of the MC1R gene. *The Journal of Investigative Dermatology*, *129*, 1305–1307.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Vallone MG, Tell-Marti G, Potrony M, et al. Melanocortin 1 receptor (*MC1R*) polymorphisms' influence on size and dermoscopic features of nevi. *Pigment Cell Melanoma Res*.. 2018;31:39–50. https://doi.org/10.1111/pcmr.12646

Supplementary Table 1: Demographic and phenotypic characteristics of 185 studied subjects.

Age (years), mean ± SD	42.64 ± 16.21
Sex	**N (%)**
Female	139 (75.1)
Male	46 (24.9)
Ethnicity	**N (%)**
White	158 (85.4)
Other*	27 (14.6)
Phototype†	**N (%)**
I-II	73 (39.5)
III-V	112 (60.5)
Eye colour	**N (%)**
Blue	16 (8.6)
Green	20 (10.8)
Grey	1 (0.5)
Brown	141 (76.2)
Black	7 (3.8)
Hair colour	**N (%)**
Red	2 (1.1)
Blonde	20 (10.8)
Brown	147 (79.5)
Black	16 (8.6)
N	**185 (100)**

* "Other" included Latin, mixed and Arab ethnicities. †Phototype was determined according to the Fitzpatrick phototyping scale (ranging from I-VI). We grouped phototype variables into two groups (I-II *vs* III-V).

Supplementary Table 2: Sun-related behaviour during childhood (from 0 to 10 years old).

Sun exposure degree	**N (%)**
None	4 (2.2)
Low	81 (43.8)
Moderate	47 (25.4)
Intense	46 (24.9)
Unknown	7 (3.8)
Presence of sunburns	**N (%)**
Absence	131 (70.8)
Presence	49 (26.5)
Unknown	5 (2.7)
N	**185 (100)**

Supplementary Table 3: Specific dermatological aspects studied (N = 185).

Freckles	**N (%)**
None	145 (78.4)
Low	25 (13.5)
Moderate	12 (6.5)
Severe	3 (1.6)
Nevus total count	**N (%)**
<50	122 (65.9)
51-100	47 (25.4)
101-200	15 (8.1)
>200	1 (0.5)
Clark nevus	**N (%)**
Presence	39 (21.1)
Absence	146 (78.9)
Blue nevus	**N (%)**
Presence	9 (4.9)
Absence	176 (95.1)
N	**185 (100)**

Supplementary Table 4: Dermoscopic nevi features of 185 subjects studied (N= 185).

Dermoscopic pattern	**N (%)**
Reticular	172 (93)
Globular	72 (38.9)
Globular - reticular	39 (21.1)
Homogeneous	17 (9.2)
Reticular - homogeneous	18 (9.7)
Globular - homogeneous	13 (7)
Unclassified	1 (0.5)
Dermoscopic structures	**N (%)**
Pigment Network	182 (98.4)
Dots and globules	90 (48.6)
Projections	1 (0.5)
Blotches	3 (1.6)
Peppering	2 (1.1)
Regression structures	1 (0.5)
Blue-whitish veil	1 (0.5)
Shining white sticks	0 (0)
Rosettes	0 (0)
Colour	**N (%)**
Light brown	160 (86.5)
Dark brown	84 (45.4)
Red / pink	49 (26.5)
White	14 (7.6)
Blue	3 (1.6)
Black	3 (1.6)
Pigment distribution	**N (%)**
Uniform	175 (94.6)
Central hyperpigmentation	44 (23.8)
Central hypopigmentation	9 (4.9)
Eccentric hyperpigmentation	30 (16.2)
Eccentric hypopigmentation	3 (1.6)
Multifocal	13 (7)
Visible vessels	**N (%)**
Presence	69 (37.3)
Absence	116 (62.7)
N	**185 (100)**