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Graduate Program in Molecular Medicine

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PHENOTYPING AND GENOTYPING OF FAMILIAL MELANOMA AND MULTIPLE PRIMARY MELANOMA CASES IN MINAS GERAIS

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ATA DE DEFESA DE TESE

Às 10.00 horas do dia dois de agosto de cois mil vinte e um, por meio da plataforma Zoom, realizou-se a sessão pública para a defesa da Tese de **FRANCIELE ANTONIETA BIANCHI LEIDENZ**, número de registro 2016735869, graduada no curso de MEDICINA, como requisito parcial para a obtenção do grau de Doutor em MEDICINA MOLECULAR. A presidência da sessão coube ao professor Luiz Armando Cunha de Marco, Orientador. Inicialmente, o presidente fez a apresentação da Comissão Examinadora assim constituída: Luiz Armando Cunha de Marco - Orientador (UFMG), Flávia Vasques Bittencourt - Coorientadora (UFMG), Patrícia Gonçalves Pereira Couto (Instituto Hermes Pardini), Juliana Garcia Carneiro (Clínica Personal), Eitan Friedman (Universidade de Tel Aviv) e Allen Everett Bale (Universidade Yale). Em seguida, a candidata fez a apresentação do trabalho que constitui sua Tese de Doutorado, intitulada: **PHENOTYPING AND GENOTYPING OF FAMILIAL MELANOMA AND MULTIPLE PRIMARY MELANOMA CASES IN MINAS GERAIS**. Seguiu-se a arguição pelos examinadores e logo após, a Comissão reuniu-se, sem a presença da candidata e do público e decidiu considerar aprovada a Tese de Doutorado. O resultado final foi comunicado publicamente à candidata pelo presidente da Comissão. Nada mais havendo a tratar, o presidente encerrou a sessão e lavrou a presente ata que, depois de lida, se aprovada, será assinada pela Comissão Examinadora.

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To all my family and friends who were so understanding and supportive throughout these moments of my life. To all patients who suffer from melanoma and to all people engaged in medical research in Brazil.

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ABSTRACT

Background: Melanoma is the most aggressive skin cancer. About 15% of all melanoma patients have a positive family history, at times in a pattern that follows an autosomal dominant mode of transmission - hereditary melanoma. Some of these hereditary cases are associated with germline Pathogenic Sequence Variants (PSVs) in highly penetrance genes, especially *CDKN2A* (up to 40% of high-density melanoma families in some populations) and lower rates in a few additional genes (e.g., *CDK4*, *POT1*, *TERT*, *MITF*, *BAP1*, *MC1R*, *ACD*, *TERF2IP*). Notably the contribution of these latter genes to the overall burden of familial clusters of melanomas is less than 1%. Thus, most melanoma-prone families remain with no clear identified single highly penetrant gene that underlies melanoma predisposition. It is plausible that some of these inherited melanomas are oligogenic – co-carriership of multiple low penetrance risk variants (like variants in *MC1R)* rather than a monogenic or perhaps could be accounted for by environmental factors shared by family members. However, other high-penetrance genes likely exist and studies aiming to further establish the genetic basis of inherited melanoma focusing on phenotopically highly penetrant families may facilitate identifying these genes.

Objectives: Define the genetic basis of inherited melanoma in Minas Gerais.

Methods and patients: Applying whole-exome sequencing (WES) to genotype a large *CDKN2A* wild-type family with seven affected family members, as well as further study the contribution of *CDKN2A* and *MC1R* to melanoma predisposition by sequencing these genes in an additional subset of familial melanoma cases (four families with 11 affected individuals), single cases of multiple primary melanomas (MPM) (eight individuals) and phenotypically sporadic melanomas (25 individuals). Following the sequencing steps, correlating genotyping results with the specific phenotypes.

Results: WES revealed 187 sequence variants that were considered pathogenic or likely pathogenic. After filtering steps using Ingenuity and Mendel, MD bioinformatic tools, validation of selected seemingly pathogenic variants in *CDC27, ABCA4, GAMT, ELOVL5* and *ANKRD27* revealed that variants in these genes were unlikely to account for these familial cases of melanoma as co-segregation among affected family members was not found. Additionally, a missense variant p.Arg307Trp (c.919C>T) of *XPC* (NM_004628.5) was demonstrated in all affected individuals (*n*=5) of the three generations in family (*n*=15). Affected individuals are homozygous, non-affected individuals are either heterozygous or wild-type, confirming the diagnosis of atypical Xeroderma Pigmentosum (XP) Group C (XP-C). Unlike the majority of XP cases, these individuals showed late onset of tumors, modest skin changes, extraordinary number of melanomas in comparison to carcinomas (53 versus 9) and high prevalence of affected members (53,8%), mimicking a familial melanoma syndrome. The missense variant NM_002386.3:c.464T>C; NP_002377.4:p.Ile155Thr; (=I155T) in *MC1R* was detected in 60% (three of five) of affected members and is associated, in the genotyped family, with earlier age of melanoma diagnosis (27.6 yrs versus 40), higher number of tumors per person (14 versus five) and thicker melanomas (3 invasive versus none invasive melanomas) in XP carriers compared to non-carriers. Among all melanoma cases studied herein (n=44), this variant was detected at a much higher rate (18.2%) than in other melanoma cases from other populations (frequency of 0.00319 in 1000 Genome Project; 0.00527 in Exome Aggregation Consortium (ExAC); 0.00564 in exomes of The Genome Aggregation Database (gnomAD); 0.00659 in Trans-Omics for Precison Medicine (TOPMed) and 0.00793 in NHLBI Exome Sequencing Project (ESP) Exome Variant Server), suggesting a founder effect of this variant in the region of Minas Gerais. Sanger sequencing to genotype *CDKN2A* and *MC1R* in 44 phenotypically diverse melanoma cases and 102 non cancer controls revealed only one missense variant in the *CDKN2A* gene, the NM_058195.3:c.368G>C;

NP_478102.2:p.Cys123Ser AKA p.A109P, in a case of MPM with multiple atypical nevi. The rate of this variant in noncancer cases is ExAC 0.00001; gnomAD exomes 0.00002; The Genome Aggregation Database (gnomAD) 0.00006; TOPMed 0.00010; NHLBI Exome Sequencing Project (ESP) Exome Variant Server 0.00015. The functional consequences are yet unknown. Additionally a known polymorphism (NM_001195132.1:c.442G>A; NP_001182061.1:p.Ala148Thr; p.A148T, previously associated with a higher risk of melanoma in Southern Brazil, was detected in (5/44) 11.3% of melanoma patients in contrast to (2/102) 1.9% in controls (p = 0,02), suggesting it may also be a melanoma risk factor in this region. This polymorphism has a world populations frequency of 0.01885 in gnomAD; 0.02278 in ExAC; 0.02253 in NHLBI ESP; 0.00699 in 1000 Genomes Project; 0.02021 in TOPMed and 0.02089 in gnomAD exomes. Genotyping of *MC1R* revealed 11 nonsynonymous variants, two rare variants (Q23X and D184H) and one novel variant (M203T), the latter related to red hair color (RHC) phenotype (perhaps a novel RHC variant). The variant Q23X is clearly pathogenic as it generates a truncated protein and its frequency in gnomAD is 0.0003039. The variant D184H has a less obvious pathogenic impact and its frequency in gnomAD is 0.00003. Nonsynonymous variants (n=11) in the *MC1R* gene were significantly more frequent ($p < 0.001$; CI [0.32-0.63]) among melanoma patients compared with controls (61.4% vs 13.7%), with even higher rates in melanoma patients with a positive family history of melanoma or multiple primary melanomas (68.4%). Moreover, when all 44 individuals are considered, pathogenic variants were present in 61.1% (11/18) of patients who have had melanoma before the age of 40 years, in contrast to 42.1% (8/19) of patients who have had melanoma after the age of 50 years, suggesting *MC1R* has a role in early age of onset of melanoma independent of skin phenotype, although not statistically significant (p = 0.239).

Conclusion: WES was able to identify a family with several atypical features as Xeroderma Pigmentosum Group C with a rare mutation p.Arg307Trp (c.919C>T) not yet associated with

a clinical phenotype. Results also reinforce the role of *MC1R* in melanomagenesis irrespectively of the red hair phenotype and in a phenotypically heterogenous population like Brazilians. The pathogenic variant p.I155T has a much higher prevalence than in other populations, likely due to a founder effect in this region and is associated with a worse prognosis in XP patients. The clinical role of novel variant M203T, a possible novel RHC variant, and rare variants Q23X and D184H must be evaluated by further studies. Pathogenic sequence variants in *CDKN2A* are uncommon in this population, but the rare p.A109P might be implicated in a phenotype of multiple melanomas with early age of onset and atypical nevi.

Keywords: Melanoma, *CDKN2A*, *MC1R*, familial melanoma, multiple primary melanomas.

RESUMO

Introdução: O melanoma é o câncer de pele mais agressivo. Cerca de 15% de todos os pacientes com melanoma têm uma história familiar positiva, às vezes num padrão que segue um modo de transmissão autossômico dominante – melanoma hereditário. Alguns desses casos hereditários estão associados a variantes de sequência patogênica germinativa (PSVs) em genes de alta penetrância, especialmente CDKN2A (até 40% das famílias de melanoma de alta densidade em algumas populações) e taxas mais baixas em alguns genes adicionais (por exemplo, CDK4, POT1, TERT, MITF, BAP1, MC1R, ACD, TERF2IP). Notavelmente, a contribuição destes últimos genes para a carga global de grupos familiares de melanomas é inferior a 1%. Assim, a maioria das famílias propensas ao melanoma permanece sem um único gene altamente penetrante claramente identificado que esteja subjacente à predisposição ao melanoma. É plausível que alguns destes melanomas herdados sejam oligogénicos – co-transportadores de múltiplas variantes de risco de baixa penetrância (como variantes em MC1R) em vez de monogénicos ou talvez possam ser explicados por factores ambientais partilhados por membros da família. No entanto, provavelmente existem outros genes de alta penetrância e estudos que visam estabelecer ainda mais a base genética do melanoma hereditário com foco em famílias fenotopicamente altamente penetrantes podem facilitar a identificação desses genes.

Objetivos: Definir a base genética do melanoma hereditário em Minas Gerais.

Métodos: Realizou-se sequenciamento de exoma completo (WES) para genotipar uma grande família CDKN2A de tipo selvagem com sete membros da família afetados, bem como estudada ainda mais a contribuição de CDKN2A e MC1R para a predisposição ao melanoma, sequenciando esses genes em um subconjunto adicional de casos de melanoma familiar (quatro famílias com 11 indivíduos afetados), casos únicos de melanomas primários múltiplos (MPM) (oito indivíduos) e melanomas fenotipicamente esporádicos (25 indivíduos). Seguindo as

etapas de sequenciamento, correlacionando os resultados da genotipagem com os fenótipos específicos.

Resultados: O WES revelou 187 variantes de sequência que foram consideradas patogênicas ou provavelmente patogênicas. Após etapas de filtragem usando ferramentas de bioinformática Ingenuity e Mendel, MD, a validação de variantes aparentemente patogênicas selecionadas em CDC27, ABCA4, GAMT, ELOVL5 e ANKRD27 revelou que era improvável que variantes nesses genes explicassem esses casos familiares de melanoma como co-segregação entre os afetados. familiares não foram encontrados. Além disso, uma variante missense p.Arg307Trp (c.919C>T) de XPC (NM_004628.5) foi demonstrada em todos os indivíduos afetados (n=5) das três gerações da família (n=15). Os indivíduos afetados são homozigotos, os indivíduos não afetados são heterozigotos ou do tipo selvagem, confirmando o diagnóstico de Xeroderma Pigmentoso (XP) Grupo C (XP-C) atípico. Ao contrário da maioria dos casos de XP, estes indivíduos apresentaram tumores de início tardio, alterações cutâneas modestas, número extraordinário de melanomas em comparação aos carcinomas (53 versus 9) e alta prevalência de membros afetados (53,8%), mimetizando uma síndrome de melanoma familiar. . A variante missense NM_002386.3:c.464T>C; NP_002377.4:p.Ile155Thr; (=I155T) em MC1R foi detectado em 60% (três de cinco) dos membros afetados e está associado, na família genotipada, com idade mais precoce de diagnóstico de melanoma (27,6 anos versus 40), maior número de tumores por pessoa (14 versus cinco) e melanomas mais espessos (3 melanomas invasivos versus nenhum melanoma invasivo) em portadores de XP em comparação com não portadores. Entre todos os casos de melanoma aqui estudados (n = 44), esta variante foi detectada em uma taxa muito maior (18,2%) do que em outros casos de melanoma de outras populações (frequência de 0,00319 no 1000 Genome Project; 0,00527 no Exome Aggregation Consortium (ExAC) 0,00564 em exomas do Banco de Dados de Agregação de Genomas (gnomAD); Trans-Omics for Precison Medicine (TOPMed) e 0,00793 no NHLBI Exome

Sequencing Project (ESP) Exome Variant Server), sugerindo um efeito fundador desta variante na região de Minas Gerais. O sequenciamento Sanger para genótipo CDKN2A e MC1R em 44 casos de melanoma fenotipicamente diversos e 102 controles não cancerígenos revelou apenas uma variante missense no gene CDKN2A, o NM_058195.3:c.368G>C; NP_478102.2:p.Cys123Ser AKA p.A109P, em um caso de MPM com múltiplos nevos atípicos. A taxa desta variante em casos não oncológicos é ExAC 0,00001; exomas gnomAD 0,00002; Banco de dados de agregação de genoma (gnomAD) 0,00006; TOPMed 0,00010; NHLBI Exome Sequencing Project (ESP) Exome Variant Server 0.00015. As consequências funcionais ainda são desconhecidas. Além disso, um polimorfismo conhecido (NM_001195132.1:c.442G>A; NP_001182061.1:p.Ala148Thr; p.A148T, previamente associado a maior risco de melanoma no Sul do Brasil, foi detectado em (5/44) 11,3% dos pacientes com melanoma em contraste com (2/102) 1,9% nos controles (p = 0,02), sugerindo que também pode ser um fator de risco para melanoma nesta região. Este polimorfismo tem frequência na população mundial de 0,01885 no gnomAD; 0,02278 em ExAC; 0,02253 no NHLBI ESP; 0,00699 no Projeto 1000 Genomas; 0,02021 em TOPMed e 0,02089 em exomas gnomAD. A genotipagem do MC1R revelou 11 variantes não-sinônimas, duas variantes raras (Q23X e D184H) e uma nova variante (M203T), esta última relacionada ao fenótipo da cor do cabelo ruivo (RHC) (talvez uma nova variante do RHC). A variante Q23X é claramente patogênica pois gera uma proteína truncada e sua frequência no gnomAD é 0,0003039. A variante D184H tem um impacto patogénico menos óbvio e a sua frequência no gnomAD é de 0,00003. Variantes não sinônimas (n=11) no gene MC1R foram significativamente mais frequentes (p < 0,001; IC [0,32-0,63]) entre pacientes com melanoma em comparação com controles (61,4% vs 13,7%), com taxas ainda mais altas em pacientes com melanoma com história familiar positiva de melanoma ou múltiplos melanomas primários (68,4%). Além disso, quando todos os 44 indivíduos são considerados, variantes patogênicas estavam

presentes em 61,1% (11/18) dos pacientes que tiveram melanoma antes dos 40 anos, em contraste com 42,1% (8/19) dos pacientes que tiveram melanoma após os 50 anos de idade, sugerindo que o MC1R tem um papel na idade precoce de início do melanoma, independente do fenótipo da pele, embora não seja estatisticamente significativo (p = 0,239).

Conclusão: O WES conseguiu identificar uma família com diversas características atípicas como Xeroderma Pigmentoso Grupo C com uma mutação rara p.Arg307Trp (c.919C>T) ainda não associada a um fenótipo clínico. Os resultados também reforçam o papel do MC1R na melanomagênese, independentemente do fenótipo do cabelo ruivo e em uma população fenotipicamente heterogênea como a dos brasileiros. A variante patogênica p.I155T tem uma prevalência muito maior do que em outras populações, provavelmente devido a um efeito fundador nesta região e está associada a um pior prognóstico em pacientes XP. O papel clínico da nova variante M203T, uma possível nova variante do RHC e das variantes raras Q23X e D184H devem ser avaliados por estudos adicionais. Variantes de sequência patogênica em CDKN2A são incomuns nesta população, mas o raro p.A109P pode estar implicado em um fenótipo de melanomas múltiplos com início precoce e nevos atípicos.

Palavras-chave: Melanoma, *CDKN2A*, *MC1R*, melanoma maligno cutaneo, antígenos específicos de melanoma.

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	domain. Two figures below are magnification of the receptor's inner core.	

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LIST OF ABREVIATIONS

AJCC	American Joint Committee on Cancer
BRAF	Proto-oncogene *BRAF*
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CM	Cutaneous Melanoma
FAMMM	Familial Atypical Multiple Mole Melanoma
Kb	Kilobase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 Receptor
MEK or MAPK2	Mitogen-activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
MSH	Melanocyte Stimulating Hormone
MPM	Multiple Primary Melanoma
OMIM	**O**nline **M**endelian **I**nheritance in **M**an
PSV	Pathogenic Sequence Variant
RB	Retinoblastoma protein
RHC	Red hair color
SNP	Single-Nucleotide Polymorphism
SNV	Single-Nucleotide Variant
TNM	Tumor Nodes Metastases staging system by AJCC
UV	Ultraviolet
WES	Whole-Exome Sequencing

Summary

1. INTRODUCTION

1.1 Pathogenesis of cutaneous melanoma and major risk factors

Cutaneous melanoma (CM) is the deadliest of the common skin cancers (Potrony *et al*., 2015; Olsen, 2020). It is an aggressive malignant tumor originating from melanocytic cells of the basal epidermis or hair follicles (Figure 1). The main function of these cells is to produce and release melanin to the epidermis, a pigment that will serve as an ultraviolet (UV) radiation shield and prevent DNA damage (Sarkar and Gaddameedhi, 2020). This production is triggered initially when there is UV-induced DNA damage on keratinocytes, who will consequently produce melanocyte stimulating hormone (MSH) that binds to melanocortin receptor 1 (MC1R) on the melanocytes.

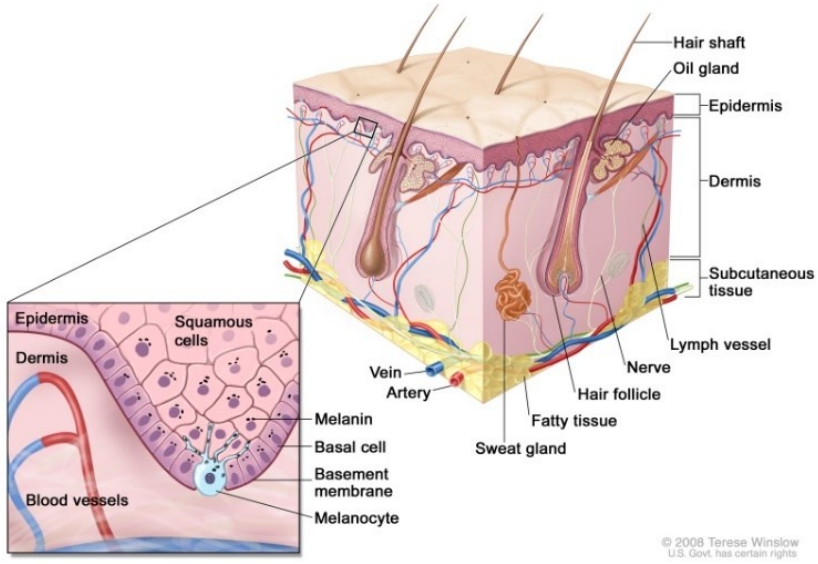


Figure 1. Schematic representation of normal skin. Melanocytes are cells present at the dermal-epidermal junction, at the basement membrane zone, located just below the basal cell keratinocytes (Source: PDQ® Melanoma Treatment: Heath Professional Version, 2020).

Melanoma is a multi-factorial disease that results from the interaction between genetic susceptibility and environmental exposure. Malignant melanomas share several unifying features, such as self-sufficiency of growth factors, insensitivity to growth inhibitors, evasion of cellular apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. These pathogenic characteristics are acquired through somatic activation of oncogenes or inactivation of tumor-suppression genes (Lugović-Mihić *et al*., 2019; Zeng *et al*.,

2019). These genetic alterations mainly occur by point mutations, deletions and translocations as well as other non genetic mechanisms like microRNA expression and promoter methylation (Latchana *et al*., 2016; Lugović-Mihić *et al.*, 2019; Gupta *et al*., 2020; Lee *et al.*, 2021).

The main environmental risk factor for the malignant transformation of melanocytes is UV radiation resulting from sun exposure, particularly the UV-B spectrum, because of its genotoxic effect (Sarkar and Gaddameedhi, 2020). This risk seems higher with intermittent sun exposure, compared with chronic sun exposure; therefore history of sunburns is extremely relevant in determing the risk for melanoma (Rastrelli *et al*., 2014). The stratospheric ozone layer plays a vital role in completely blocking the UV-C (the most dangerous type of UV radiation in carcinogenesis) and around 90-95% of the UV-B radiation. As ozone levels are depleted, the atmosphere loses more of its protective filter function and more solar UV radiation reaches the Earth's surface, increasing the chances of various UVR-mediated diseases, including skin cancer (Sarkar and Gaddameedhi, 2020). Limiting UVR exposure (for instance, avoiding external activities around noon and increasing protective measures) has the potential to reduce melanoma rates by at least 65% (Kanetsky & Hay, 2018).

UV-A exposure from artificial sources, such as sunbeds or phototherapy, has also been linked to a higher risk for developing melanoma (Rastrelli *et al*., 2014). Sunbeds, therefore, have been prohibited in Brazil since 2009. UV-A irradiation induces melanocytes malignant transformation through two different mechanisms: direct transformation of melanocytes into neoplastic cells by several mutations affecting both proto-oncogenes (*BRAF*, *CDK4*, etc.) and tumor suppressor genes (*TP53*, *NF1*, *PTEN*, etc.) or the transformation of melanocytes into benign nevi (of which 80% will harbor a *BRAF* V600E mutation). In this last scenario, after decades, depending on lack of immunological surveillance and additional acquired genetic mutations, such as *TERT* and *CDKN2A*, the nevi could undergo malignant transformation (Figure 2).

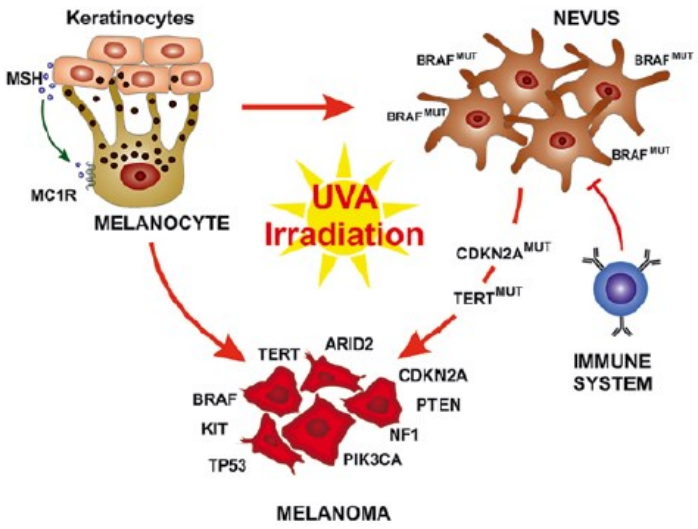


Figure 2. Two mechanisms of malignant transformation of melanocytes by UV-A radiation. Direct transformation of normal cells into neoplastic cells after several mutations affecting proto-oncogene and tumor suppressor genes (*TP53*, *NF1*, *PTEN*, etc.). Transformation of melanocytes into benign nevi, of which 80% harbor a *BRAF* V600E mutation. Host immune system can keep these nevi indolent for decades; however, depending on UV rays stimulus, additional genetic mutations, such as *TERT* and *CDKN2A*, may lead to malignant transformation of the nevi (Leonardi *et al*., 2018).

It is important to note that there are major differences in melanomas arising from chronically or from intermittently sun-exposed skin due to the degree of cumulative UV exposure (Leonardi *et al*., 2018). These include different sites of origin, ages at diagnosis, level of aggressiveness, types of oncogenic drivers and mutational loads. Melanomas in chronically sun-exposed areas usually occur in > 55-year-old individuals, on areas such as head, neck, dorsal region of upper limbs and the main genetic drivers are *BRAF* (50%), *NF1* and *NRAS* (20%). They typically have high mutational load related to UV exposure. In contrast, melanomas in intermittent sun-exposed areas related to sunburns (trunk and proximal extremities), frequently occur in younger individuals, are rarely associated with *BRAF* V600E and have a lower mutational load (Leonardi *et al*., 2018).

Mucosal and acral lentiginous melanomas usually have lower *BRAF* mutation rates (5-20%) and higher *C-KIT* activating mutation rates (5-10%) (Lugović-Mihić *et al*., 2019).

Interestingly, there is no significantly reported association between melanoma and tobacco smoking (Kessides *et al*., 2011; Dusingize *et al*., 2018).

Host risk factors also play an important role in the pathogenesis of cutaneous melanoma. The most important host risk factors are the number of melanocytic nevi, family history of melanoma and/or related cancer, and genetic susceptibility (Gandini *et al*., 2005; Duffy *et al*., 2019).

About 25% of all melanomas arise within a preexisting nevus. Therefore, there is a correlation between melanoma risk and number, size, and clinical characteristics of the nevi (Gandini *et al*., 2005; Duffy *et al.*, 2019). A high number of acquired melanocytic naevi is the strongest known phenotypic risk factor for melanoma, individuals with the highest numbers of naevi have a five- to 10-fold increase in melanoma risk, and this is associated with a younger age at diagnosis (Duffy *et al.*, 2019). The meta-analysis by Gandini *et al*. (2005), showed that patients with more than 100 nevi have a 7-fold increased risk for developing melanoma. Concerning the type of the nevi, the atypical ones (usually larger, at least 5 mm, with a flat component and atypical features such as multiple pigmentation, asymmetry, irregular borders) are associated with 29 to 49% of non-familial melanoma cases. The same group of reaerchers also reported that the presence of five atypical nevi leads to a 6-fold increase in melanoma occurrence (Gandini *et al*., 2005). Total body naevus count (TNC) is highly heritable and strongly affected by genetic polymorphisms within or near the *IRF4*, *MTAP*, *PLA2G6* and *MITF* genes. Also, high sun exposure is associated with development of more naevi (Duffy *et al.*, 2019; McMenimam *et al.*, 2020). In the families with cutaneous melanoma studied by Greene *et al*. (1983), subsequent studies (Bale *et al*. (1985, 1986)) showed that dysplastic nevus (DN), a lesion known to be a precursor of melanoma, also segregates in an autosomal dominant manner. Pascoe (1987) challenged the concept of a single dominant gene as proposed by Bale *et al*. (1986). Bale and Chakravarti (1987) defended their conclusion.

A positive family history of melanoma and/or cancer is also a strong melanoma risk factor. Approximately 7-15% of all melanoma patients have a positive family history; thus familial clustering of the disease could be accounted by the presence of an inherited genetic variant (Stahl *et al*, 2004; Leonardi *et al.*, 2018). The most common genes harboring germline pathogenic sequence variants in these families are the *CDKN2A* (or P16) and *CDK4,* but other genes and some recently discovered loci have been identified to be associated with melanoma predisposition: *POT1*, *TERT*, *BAP1*, *MITF*, *XPC*, *XPD*, *XPA*, *PTEN*, *TP53*) (Barrett *et al.*, 2011; Barrett *et al.*, 2015; Law *et al.*, 2015; Landi *et al*., 2020; Zanna *et al.*, 2021). Some of these genes are linked not only to melanomas, but to other types of cancer, consisting in complex cancer susceptibilty syndromes, such as Xeroderma Pigmentosum, Li-Fraumeni, and others (Rossi *et al.*, 2019).

Other host factor associated with a 50% increased risk for developing melanoma include: red hair, fair skin and light eyes, multiple freckles, sun sensitivity with inability to tan. This phenotype may be related to sequence alterations in the *MC1R* gene, which regulates skin pigmentation, but is also associated with DNA damage repair process and activation of survival pathways (Robles-Espinoza *et al.*, 2016; McMeniman *et al*., 2020; Toussi *et al.*, 2020). Some polymorphisms in this gene (known as *R* alleles) may generate a higher proportion of a different type of melanin other than the regular eumelanin, the so-called pheomelanin, which is less protective of the UV damage and therefore increase melanoma risk. There is crescent evidences that the loss in *MC1R* function, even in the absence of UV light, may be oncogenic (Robles-Espinoza *et al.*, 2016).

1.2 Epidemiology of melanoma

Melanoma is the most lethal of the common skin cancers, and its incidence is increasing (Potrony *et al.*, 2015; Siegel *et al*., 2020; Yang *et al*., 2020). Cutaneous melanoma is the 19th

most common type of cancer in men and women worldwide. According to the World Cancer Research Fund (https://www.wcrf.org/dietandcancer/cancer-trends/skin-cancer-statistics, accessed on 03/08/2021), there were nearly 300,000 new cases in 2018 and the top countries with the highest rates in 2018 are, in order, Australia (33,6 cases per 100,000 population), New Zealand (33,3 per 100,000), Norway (29,6 per 100,000), Denmark (27,6 per 100,000) and Netherlands (25,7 per 100,000). Brazil is not among the top 20 most affected countries.

In 2021, it is estimated that there will be **106,110** new cases of melanoma in the United States and **7,180** deaths from the disease (Figure 3). The worldwide incidence of CM has increased more rapidly than that reported for any other malignancy in Caucasian populations in a span of 30 years (De Giorgi *et al*., 2015).

Men and women are differently affected by melanoma (Olsen *et al*., 2020). There is a higher incidence in women until the age of 40 years; however, at the age of 75 years, the incidence in men is three times higher than in women. The overall lifetime risk of melanoma is believed to be 1.5 higher in men than women in most studies when age at diagnosis is not taken into consideration (Rastrelli *et al.*, 2014). In addition, mortality in men is almost twice higher than the mortality in women (4,600 versus 2,580) (https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html). The observed excess of male gender melanoma incidence in midlife in developed countries (US, Canada, Australia, New Zealand, UK, Sweden, Norway, and Denmark), is largely attributed to higher rates of melanoma of the head and neck (Olsen *et al*., 2020). Yet, overall, the anatomical location of melanoma in men is usually the trunk and, in women, on lower limbs (Olsen *et al*., 2020).

Ethnicity and skin color also play a role in CM incidence. CM is 20 times more common in Caucasians than in black people. Since 1960, the incidence of melanoma has been rising among Caucasians worldwide and is now posing a relevant socio-economic problem.

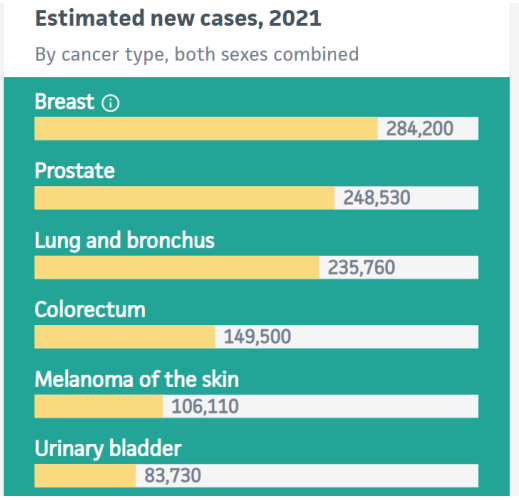


Figure 3. Number of estimated new cases of the six major types of cancer in the United States of America for the year 2021, excluding non-melanoma skin cancer. American Cancer Society, 2021. (Accessible in https://cancerstatisticscenter.cancer.org, accessed on 03/08/2021)

In Brazil, skin cancer of all histopathological types combined is the most common type of cancer, corresponding to 30% of all malignant tumors registered, but melanoma represents only 3% of the total skin cancer cases in the country. According to data last updated in 04/14/2021 from the Instituto Nacional do Cancer (INCA) website (https://www.inca.gov.br/tipos-de-cancer/cancer-de-pele-melanoma, accessed on 03/08/2021) a total of 8,450 new cases is estimated in the country for the year 2020 (4,200 men and 4,250 women). Although CM represents only approximately 3% of all skin cancers, it underlies ~75% of all skin cancer deaths (Raimondi, Suppa & Gandini, 2020). The total of CM related deaths in Brazil are estimated around 1,978 and the majority being male (1,159 deaths). Nonetheless, it is important to note that these numbers might be underestimated due to incomplete reporting of CM diagnosis, as stated in the Brazilian Minister of Health's official page DATASUS (http://tabnet.datasus.gov.br/cgi/idb2000/fqd05_10.htm).

Melanoma mortality seems to be stable in Brazil over the past two decades; however there was a significant increase in melanoma mortality rates among males and in the population

over sixty years of age, emphasizing the need to implement prevention strategies and expand access to effective therapy in the country (Nader Marta *et al*., 2020).

The age range with higher number of melanoma diagnosis is between 40 and 60 years. The median age at diagnosis and death are 57 and 67 years, respectively (Raimondi, Suppa & Gandini, 2020). It is the most common cancer found in young adult population, particularly women. In the year 2020, about 2,400 cases of melanoma will be diagnosed in people age 15 to 29, according to the American Society of Clinical Oncology (ASCO). This brings another important parameter of this disease, the so-called disability-adjusted life years (DALYs), which combines morbidity and mortality metrics. One DALY is equivalent to one year of healthy life lost and, together with incidence, mortality, and prevalence, is part of the Global Burden of Disease Study (GBD). In 2015, melanoma was responsible for 1,596,262 global DALYs (95% CI 1,293,447– 1,982,679) with an age-standardized rate of 23 DALYs per 100,000 persons (95% CI 18–28) (Karimkhani *et al*., 2017).

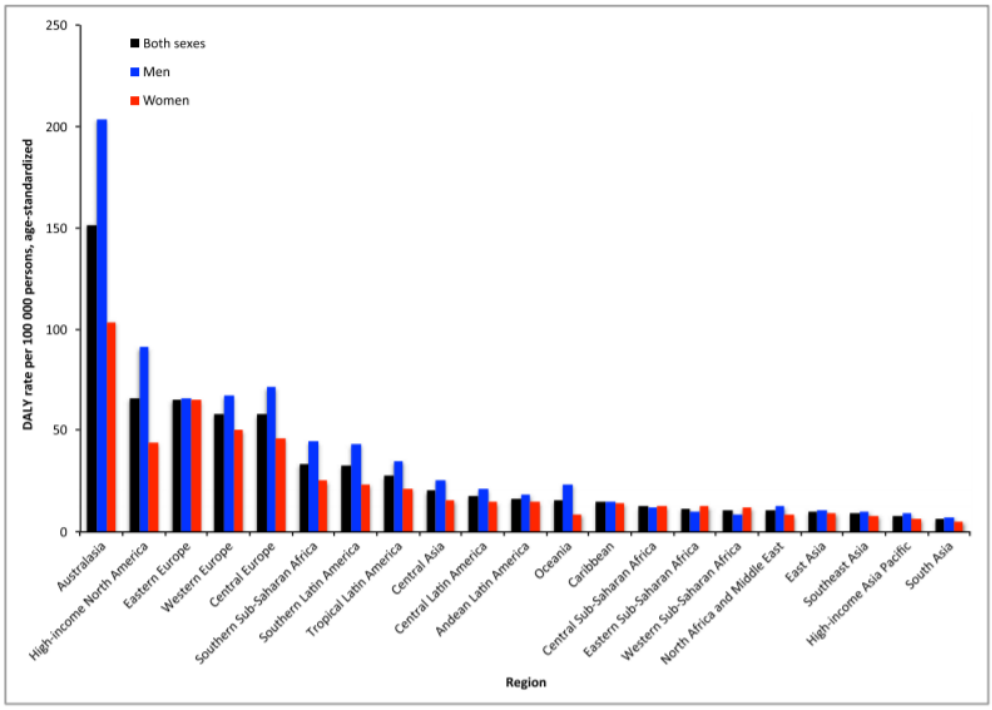


Figure 4. Age-standardized melanoma disability-adjusted life year (DALY) rates in 21 world regions, both by sex and total in the year 2015. (Karimkhani *et al*., 2017).

Data from ASCO (https://www.asco.org/search/site/MELANOMA) also shows that, after several consecutive years of rising mortality, from 2013 to 2017, deaths from melanoma have decreased by almost 6% in adults older than 50 and by 7% in those younger than 50. A recent report by Yang *et al*. (2020), using the WHO mortality database, showed an overall increase in mortality in men in all countries except the Czech Republic over the past 30 years, in contrast with stable or declining rates in women.

Among all CM diagnosed cases the 5-year survival is 93% (https://www.cancer.org/cancer/melanoma-skin-cancer/detection-diagnosis-staging/survival-rates-for-melanoma-skin-cancer-by-stage.html). 5-year and overall survival depends on the thickness of the primary melanoma, whether lymph nodes are involved, and whether there are distant metastases.

1.3 Genetics of melanoma

CM develops as a result of complex interaction between environmental factors (primarily sun exposure) and genetic factors (Bonadies & Bale, 2011; Olsen *et al*., 2020).

Comprehensive somatic sequencing studies (e.g., TCGA - https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga, COSMIC - https://cancer.sanger.ac.uk/cosmic) revealed that melanoma genomes are some of the most mutated genomes of all cancers, therefore, having a high neoantigen load, making melanoma significantly more immunogenic compared to other cancers (Gupta *et al*., 2020). Hence, melanoma is one of the cancer types most responsive to immune checkpoint blockage-based immunotherapies (Robert, 2020). Like all cancer types, a single somatic mutation is not sufficient to complete the malignant transformation process, and approximately 5–10 pathogenic alterations, which are spread across several signaling pathways, are needed to

accumulate in the melanocyte clone to trigger transformation to melanoma (Zeng *et al*., 2020). Based on massive somatic sequencing of multiple CM cases, CM can be classified into four main subtypes: BRAF-mutant, NRAS-mutant, NF1-deficient and triple wild-type (Gupta *et al*., 2020).

Most melanomas are sporadic, with no discearnable family history or other features suggesting genetic predisposition (e.g., early age at diagnosis, multiple CMs, diagnosis of multiple cancers in the same individual). Yet, ~15% of melanomas occur in patients with a family history and a subset of these patients have a germline pathogenic sequence variant (PSV) in a melanoma predisposing gene (Toussi *et al*., 2019). These CM predispostion genes are more commonly cell cycling regulators (Bonadies & Bale, 2011). The definition of "familial melanoma" is not well established, but commonly used criteria refer to a family with at least two first-degree relatives with melanoma or a family with three or more patients on the same side of the family (irrespective of degree of relationship) diagnosed with melanoma (Dêbniak, 2004). Uveal melanoma may also be overrepreseneted in CM families (Debniak *et al*., 2004, Soura *et al*., 2016), but it is still debatable if it could fit into the familial melanoma syndrome. It is important to note that most cases of familial melanoma are due to shared sun exposure experiences among family members with susceptible skin types and that hereditary melanoma features, such as unilateral lineage, multi-generational, multiple primary lesions and early onset of disease are in fact quite rare (Soura *et al*., 2016). The pattern of heritability in most CM families is usually autosomal dominant with incomplete penetrance (Rossi *et al.*, 2019). The major CM susceptibility gene is the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), believed to underlie 10 to 40% of familial cases (Maubec *et al*., 2012; Soura *et al*., 2016).

Multiple primary melanoma (MPM) is quite rare, with an estimated incidence ranging from 0.2% to 8.6% of all CM cases (De Giorgi *et al*., 2015). MPM has been defined when more

than one melanoma (not metastases) is presented in the same individual, synchronically or metachronically, the former occurring in ~ 40% of cases (De Giorgi *et al*., 2015), and the latter most commonly within 2 years of initial diagnosis. It is more commonly encountered in patients with a family history of CM (Bruno *et al*., 2016; Casula *et al.*, 2019; Peek *et al*., 2020). It is well known that patients diagnosed with a single primary melanoma are at elevated risk of developing a second primary melanoma during their lifetime. Prognosis has been shown to be worse for patients with MPM in comparison to patients with a single melanoma, with a mortality hazard ratio of 2.01 (95% CI=1.57-2.59; p<0.001) (Youlden *et al*., 2016).

The term "familial melanoma syndrome" (FMS) is used by some authors to describe the presence of two or more cases of melanoma in first- or second-degree relatives; or even the presence of two or more melanomas in the same individual (MPM). However, in areas with moderate to high incidence of melanomas, FMS would be diagnosed in individuals with three or more primary melanomas and in families with three or more cases in first- or second-degree relatives (Sá *et al*., 2018).

In this study, we chose to use the classification of hereditary/familial melanoma syndromes as proposed by Abdo *et al*. (2020), which uses as criteria the number of relatives diagnosed with cutaneous melanoma, the number of relatives with MPM or a combination of these two criteria. Early age of melanoma onset and presence of multiple typical or atypical nevi are also taken into consideration. Therefore, there are two distinct types of familial melanoma syndromes (Abdo *et al*., 2020):

a. Melanoma dominant: Melanoma is the first or the predominant clinical manifestation. These families are the object of this study.

b. Melanoma subordinate: Melanoma is not the most common, or the predominant presentation. These group include Cowden syndrome (*PTEN*), Li-Fraumeni (*TP53*) and BAP1 cancer syndrome (*BAP1*), among others.

1.3.1 Genetics of sporadic tumors

Approximately half of all sporadic melanoma have mutations in the proto-oncogene *B-Raf* or *BRAF* gene (OMIM, * 164757), which encodes a protein in the MAPK (mitogen-activated protein kinase cascade) signal pathway (Alqathalama, 2020; Schummer *et al*., 2020). This pathway regulates the cellular proliferation, growth, and migration and is excessively activated in almost all melanomas. The activation of MAPK begins when growth factor binds to the tyrosine-kinase receptor (TKR). This triggers an intracellular cascade of RAS family proteins, including BRAF, culminating in the activation of ERK (also known as MAPK). ERK is an enzyme that activates transcription factors on genes involved in cellular growth, proliferation, and migration. This pathway, as shown in figure 5, is important in melanomagenesis as in approximately 80% of all skin melanoma or melanocytic nevi there is either an activating mutation in *NRAS* (15-20%) or in *BRAF* (40-50%). Clinically, these molecular insights have led to a significant improvement in treatment outcomes of patients with metastatic and resected *BRAF* mutated melanomas, using combined therapy with BRAF and MEK inhibitors (vemurafenib + cobimetinib, dabrafenib + trametinib) *(*Lugović-Mihić *et al.*, 2019; Alqathama, 2020; Atkins *et al*., 2021; Moreira *et al*;. 2021; Mueller *et al*., 2021; Tarhini *et al*., 2021).

The major *BRAF* mutation, present in almost 70% of all mutations, is the transversion of thymidine into adenine (T/A), which results in Valine being replaced by Glutamate (V600E), causing activation of the kinase domain (Davies *et al*., 2002; Casula *et al*., 2019; Vicente *et al*., 2019). This mutation is more frequent in women and inversely proportional to age. Other substitutions can occur, such as V600K mutations (20% of *BRAF* mutations), which are more common in patients chronically exposed to sun. *BRAF* V600 mutations are an early event in melanoma development and are also seen in most benign and dysplastic nevi (80%).

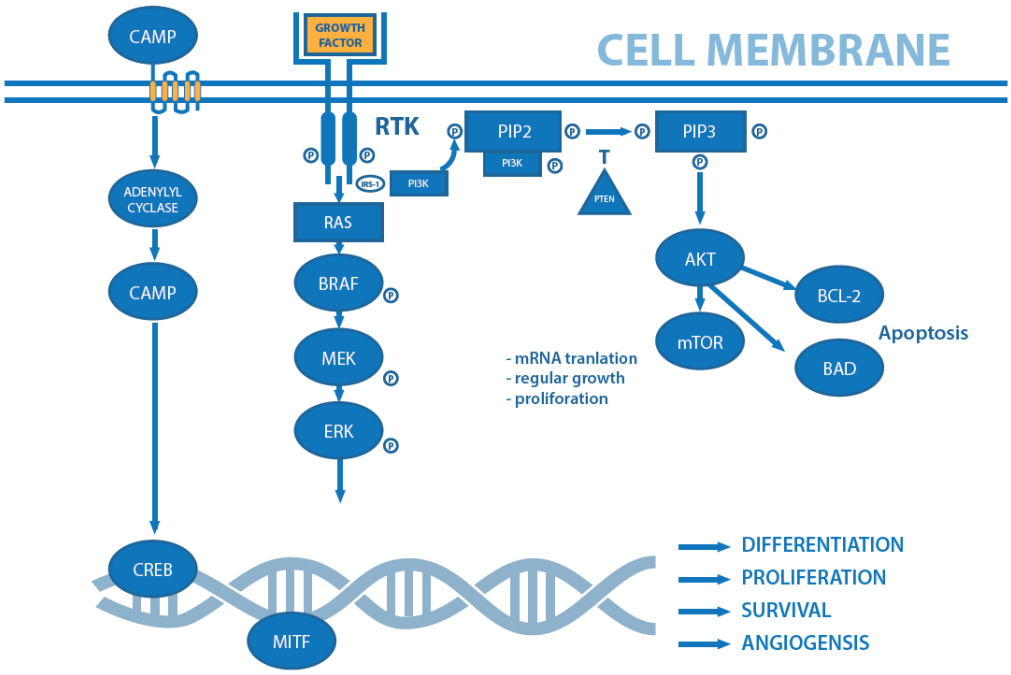


Figure 5. MAPK signal pathway. *RAS* and *BRAF* genes are mutated in approximately 80% of all melanomas, demonstrating how important this pathway is in melanomagenesis. The activating mutations will ultimately lead to enhanced cellular growth, proliferation, and migration. (Lugović-Mihić *et al*., 2019).

RAS activating mutations also promote cell proliferation but are a much rarer in melanomas (10-20%), with mostly *NRAS* mutations present in amelanotic nodular subtypes (Lugovic-Mihic *et al*., 2019). This mutation simultaneously activates the MAPK and the PI3K pathways (Lugovic-Mihic *et al*., 2019).

Noteworthy, *BRAF* and *RAS* mutations are almost mutually exclusive, suggesting that only one mutation in any of these genes is sufficient to activate the MAPK pathway and/or that sustained hyper-activation of the MAPK pathway leads to self-induced apoptosis (Raaijmakers *et al*., 2016).

PI3K-AKT (phosphatidylinositol-4,5-bisphosphate 3-kinase–protein kinase B) is another important signaling pathway involved in the regulation of cellular survival, growth, and apoptosis. A large number of melanomas (up to 80%) display increased activation of the PI3K signaling, usually by inactivation (by mutation, deletion, methylation of promoters) of the genes encoding PTEN inhibitor (Raaijmakers *et al*., 2016).

The tyrosine-kinase receptor and its ligand (stem cell factor) c-KIT usually causes insufficient pigmentation when mutated (Lugovic-Mihic *et al*., 2019). Loss of c-KIT expression is associated with the transition from a benign lesion to a melanoma. Activating mutations of *c-KIT*, that particularly activate the PI3K-AKT pathway, are seen in acral melanomas (palmoplantar, nail bed) and in chronically sun-exposed skin melanomas (Lugovic-Mihic *et al*., 2019).

The microphthalmia-associated transcription factor (MITF) is a transcription factor required for the differentiation of melanocytes which can promote malignant behavior in some melanomas (Tracey and Vij, 2019). MITF amplification occurs in 15-20% of all melanomas, but more often in metastatic melanomas. This event is probably later in the melanoma progression and tends to reduce the 5-year survival rate (Tracey and Vij, 2019). MITF increases the expression of genes involved in cell cycle progression, cellular proliferation, and survival. Melanocortin (ACTH, α-MSH) binds to the melanocortin 1-receptor (MC1R) in the melanocyte surface, which activates the expression of MITF. In consequence, an intracellular cascade mediated by adenylate cyclase and c-AMP, activates the protein kinase A (PKA), which will then activate CREB (cAMP response-element binding protein), a transcription factor that intensifies MITF expression (Garcia-Borron *et al*., 2014).

Other pathogenic somatic mutations in genes encoding for the many proteins involved in these pathways (shown in figure 5) can also lead to tumor development, but their frequency is significantly lower.

While clearly somatic PSV and other molecular events are a common thread in sporadic tumors, germline variants in the *MC1R* gene (OMIM # 155555) play a major role in predisposition to sporadic cutaneous melanoma and its association to CM has been replicated and confirmed by meta-analysis and genome-wide association studies (Raimondi *et al*., 2008;

Kanetsky *et al*., 2010; Pasquali *et al*., 2015; Tagliabue *et al*., 2018).This gene is further detailed in the next section, as it is also important in familial melanoma.

Germline genetic variations localizing to chromosome 11, identified through genome-wide association study (GWAS), have also reportedly been associated with increased primary melanoma tumor thickness at diagnosis, suggesting other germline variants have a role even in sporadic tumors (Mangantig *et al*., 2021).

1.3.2 Genetics of familial melanoma

Most CM families present with an autosomal-dominant pattern of inheritance, with various members affected in more than one generation. The most common gene mutated in these families is the tumor suppressor gene cyclin-dependent kinase inhibitor 2A - *CDKN2A* (OMIM # 600160; or *P16*) and much less commonly the gene *CDK4* (OMIM # 609048). Genetic predisposition is clinically suggested by the following features, in addition to family history: earlier age at diagnosis (< 40 years old), multiple primary melanomas, precursor lesion such as dysplastic nevi, and additional related tumors (e.g., pancreatic cancer). There are also other familial cancer syndromes that increase the risk of melanoma, such as Li-Fraumeni, Cowden syndrome and Xeroderma Pigmentosum (Abdo *et al.*, 2020).

The tumor-suppressor gene *CDKN2A*, located in chromosome 9p21, encodes two different proteins, p16INK4A (commonly referred to as p16) and p14ARF (commonly referred to as p14), both functioning in cell cycle regulation. The first regulates the transition from G1 to S phase of the cell cycle, functioning as a cyclin-dependent kinase (CDK) inhibitor and blocking the phosphorylation and inactivation of the Rb (retinoblastoma) protein at the same time, which is the key control point in the cell cycle. This pathway leads to senescence and regulation of damaged cells (Palmieri *et al*., 2009). On the other hand, p14ARF also acts anti proliferatively and inhibits the disintegration of the p53 tumor suppressor. The normal

expression of p53 puts a stop to the cell cycle in G2/M phase or induces apoptosis, for instance, as response to DNA damage caused by UV radiation (Figure 6).

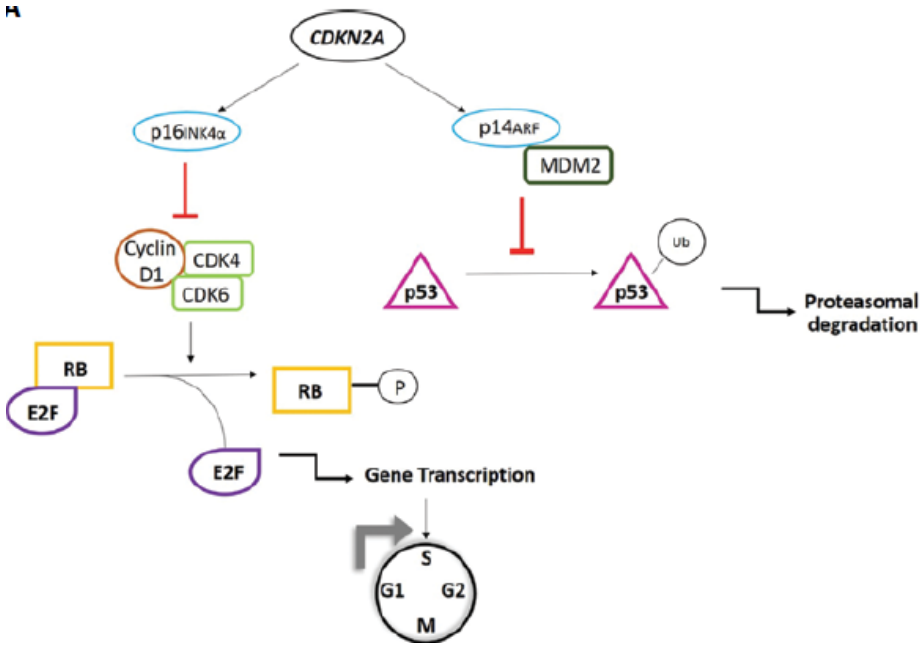


Figure 6. The major high-risk gene involved in melanoma susceptibility, *CDKN2A*, encodes 2 proteins (p16INK4α and p14ARF). The p16 INK4α inhibits the cyclin D1/CDK4/6 complex to release E2F through RB phosphorylation. The p14ARF, in the other hand, interacts with MDM2 blocking p53 ubiquitination, therefore, promoting apoptosis. Mutations in *CDKN2A* lead to 2 abnormal proteins that will allow cells to escape from cell cycle arrest and avoiding p53 degradation (Rossi *et al*., 2019).

The p16 and p14 mRNAs are transcribed from alternative first exons (1α and 1β) but use the same second and third exons (Figure 7). The two products share no amino acid sequence since they are translated in different reading frames (ARF = *alternative reading frame*). *CDKN2A* germline mutations in exon 1α affect only the p16 transcript, whereas some of those arising in exon 2 can affect both p16 and p14 (Aoude *et al*., 2015).

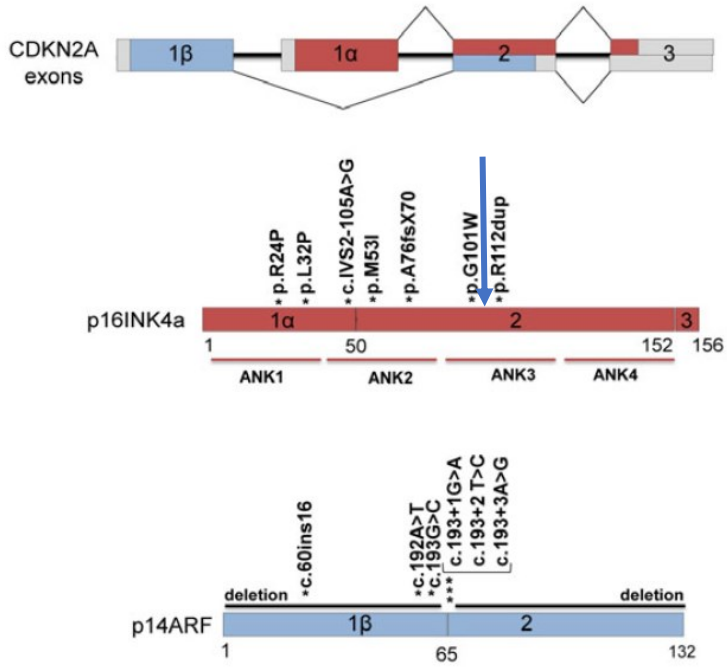


Figure 7. Schematic representation of *CDKN2A* gene and alternative splicing of p16INK4A and p14ARF. Exon 1α is spliced to INK4A exons 2 and 3, forming the p16 mature transcript whereas exon 1β is alternatively spliced to the same exons 2 and 3 generating the mature p14ARF transcript. This latter produces a different protein from p16 because translation occurs from an alternative reading frame. The arrow represents the approximately location of the genetic alteration found in the multiple primary melanoma patient, located in exon 2, therefore, affecting both transcripts (adapted from Aoude *et al*., 2015).

The p16INK4a structure consists of four ankyrin-type motifs. They form two antiparallel helices and a loop, with a cleft that binds and blocks the function of CDK4/6. Ankyrin repeats stack against one another forming a single domain. Each stack follows a multistate folding pathway that gives high structural plasticity to the p16INK4a protein and consequent high affinity for protein-protein interactions (Scaini *et al*., 2014) .This fold can also be seen as a continuum of semi-folded states where a single amino acid substitution may interrupt the p16INK4a specific ankyrin motif periodicity, severely perturbing the entire protein structure. Even though more than 60 different *CDKN2A* germline mutations have been identified to date, most of them missense mutations affecting p16INK4a, only 25% have been functionally assessed (McKenzie *et al*., 2010; Ming *et al*., 2020).

Genetic studies have shown that almost one-quarter of seemingly sporadic melanoma cases and half of familial melanoma cases harbot germline PSVs in *CDKN2A* (Tracey and Vij, 2019). More than 60 germline PSVs have been identified in the *CDKN2A* gene, the majority being missense in the p16 transcript, in 190 families worldwide (Rossi *et al*., 2019). Penetrance is usually remarkably high but varies according to the population incidence rate of melanoma; for instance, by the age of 80 years, penetrance is about 58% in Europe, 76% in the United States and 91% in Australia (Rossi *et al.*, 2019). Some clinical characteristics increase the likelihood of *CDKN2A*-associated familial melanoma: the number of affected members (10% chance in a family with two individuals affected, 30-40% of chance in a family of three or more affected individuals), the presence of at least one individual with multiple primary melanoma, a case of pancreatic cancer, and early age of onset of the tumors.

MPM patients, even without family history, may harbor *CDKN2A* PSVs with variable frequencies in different populations, such as 8.3% in the United States, 15% in North America and 57% in Greece (Rossi *et al*., 2019). In general, it is believed that high-risk high penetrace germline PSVs can be found in around 10% of sporadic patients with multiple primary melanoma (Longo *et al*., 2021).

CDKN2A-mutated melanoma families may also be associated with an increased incidence and risk for other malignancies, such as pancreatic cancer, but also breast, lung, and other tobacco-related tumors, therefore, being considered a hereditary cancer syndrome (Abdo *et al*., 2020).

There is a specific phenotype associated with *CDKN2A* mutations in some families that is known by the acronym FAMMM- **F**amilial **A**typical **M**ultiple **M**ole **M**elanoma syndrome. These families have a high total body melanocytic nevi count (usually more than 50) and multiple atypical nevi in proband, in addition to cutaneous melanoma. Patients with this

syndrome also are at an increased risk for pancreatic and central nervous system cancers (Abdo *et al*., 2020).

Selection criteria for eligibility for *CDKN2A* genetic testing varies by melanoma incidence, that can generally be classified into two main groups, geographic area/population with low melanoma incidence (Southern Europe) versus geographic area/population with high melanoma incidence (Northern Europe, United States, Australia). For the low incidence group, that includes Brazil, the criteria would be two (synchronous or metachronous) primary melanomas in a single individual and/or families with the following clinical features in first- or second-degree relatives on the same side of the family: two cases of melanoma (one invasive) or one case of melanoma and one case of pancreatic cancer (Rossi *et al*., 2019).

One study compared survival in germline *CDKN2A* mutated (n=96) and nonmutated familial melanoma cases (n=377), suggesting mutated cases have statistically significant worse survival from nonmelanoma cancers and also from melanoma, compared with melanoma cases without *CDKN2A* mutations (Helgadottir *et al*., 2016). Mutated cases had worse survival than melanoma (the hazard ratio [HR] was 2.5, 95% confidence interval [CI] 1.49 to 4.21) and than nonmelanoma cancers (HR 7.77, 95% CI 3.65-16.51).

Recently, patients with germline *CDKN2A* mutations were reported to have improved immunotherapy responses due to the increased tumor mutational load, which results in more neoantigens and stronger antitumorous immune responses (Helgadottir *et al*., 2020).

CDK4 (OMIM # 123829) is another gene mutated in rather small subset of melanoma families. It encodes one of the binding partners of p16 and, when mutated, p16 cannot inhibit the CDK4 kinase activity, which will increase the phosphorylation of RB bound to E2F transcription factors with higher E2F release. Through the activation of pro-S phase cell cycle genes, E2F promotes G1/S phase transition. Families harboring mutations in *CDK4* have a very

similar phenotype to *CDKN2A*-mutated families, with melanoma in early ages, MPMs and atypical nevi (Rossi *et al*., 2019).

The introduction of next generation sequencing techniques has allowed the discovery of novel genes associated with familial melanoma.

BAP1 (OMIM # 603089) gene germline PSVs are associated with CM and additionally a specific cancer phenotype. BAP1 protein regulates melanocyte differentiation and participates in the DNA damage response. BAP1 cancer susceptibility syndrome presents with a variable combination of uveal and/or cutaneous melanoma, multiple skin-colored melanocytic cutaneous tumors ("BAPomas") and also mesothelioma, renal cell carcinoma and basal cell carcinoma (Toussi *et al*., 2020).

TERT (OMIM # 187270) gene encodes the catalytic subunit of telomerase, responsible for the maintenance of telomere length. Therefore, PSVs in this gene can induce increased expression of telomerase, promoting telomere stabilization, which determines cell aging, turnover and senescence (Soura *et al*., 2016).

POT1 (OMIM # 606478) is also important for telomere maintenance, encoding a protein of the sheltering complex (Rossi *et al.*, 2019). Other genes associated with this complex, such as *ACD* (OMIM # 609377) and *TERF2IP* (OMIM # 605061), have also been reported to be mutated in some melanoma families less frequently.

Germline PSVs in *CDK4*, *BAP1*, *TERT*, *POT1*, *ACD*, *TERF21P* and *MITF* are rare, overall contributing to explain a further 10% of familial clustering of melanoma (Rossi *et al.*, 2019). The genes listed above are high penetrance genes and substantially increase CM lifetime risk compared with the general population. Intermediate penetrance genes are *MC1R* and *MITF* (Rossi *et al*., 2019). These increase CM risk by up to 4-fold.

MC1R (OMIM # 155555), located on chromosome 16q24.3, has only one exon with a total of 951 bp, encodes a seven-transmembrane domain 317 amino acid G-protein coupled

receptor for the α-melanocyte-stimulating hormone (α-MSH). This receptor is located on the cell surface of melanocytes and is related to cutaneous pigmentation. The normal result of the interaction between α-MSH and MC1R, through cAMP-induced tyrosinase activity, is eumelanin synthesis.

It is a highly polymorphic gene in the Caucasian population, having more than 100 variants identified (139 variants according to Leiden Open Variation Database - LOVD - https://databases.lovd.nl/shared/genes/MC1R); even in healthy controls the prevalence of variants can be about 60% (Tagliabue *et al*., 2018). In contrast, the variability of *MC1R* in African populations is small, with only three synonymous changes described: T314T, F300F, C272C (Fernandez *et al*., 2007). It is believed that *MC1R* variants conferred evolutionary advantage to our ancestors' migrations "out of Africa" as it facilitated vitamin D synthesis, as well as some variants are associated to higher birthweight, sexual selection, and camouflage in animals (Haddadeen *et al*., 2015).

Some specific variants (R142H, R151C, R160W and D294H) are known to reduce the receptor function, resulting in the production of higher proportion of pheomelanin instead of eumelanin. These variants, therefore, are classified as red hair color (RHC) or are called "R" variants and are associated with fair skin, freckling, UV radiation sensitivity and inability to tan (Rossi *et al.*, 2019). The "R" alleles have been associated with an increase in melanoma risk, independently of the skin phenotype, in fact, dark-skinned individuals harboring the R alleles were shown to have even higher risk (Tagliabue *et al.*, 2018).

It is well established that *MC1R* plays an important role in melanomagenesis independently of skin pigmentation or UV sensitivity, but also through other biological pathways, such as induction of antioxidant defenses, DNA repair mechanisms and melanocyte proliferation, regulation and differentiation (Rossi *et al*., 2019). Individuals of families

showing concomitant *MC1R* variants with *CDKN2A* PSVs have higher penetrance of melanomas.

Melanomas from R variants carriers tend to be more associated with hypopigmentation, structureless areas, atypia and vessels (Rossi *et al*., 2019).

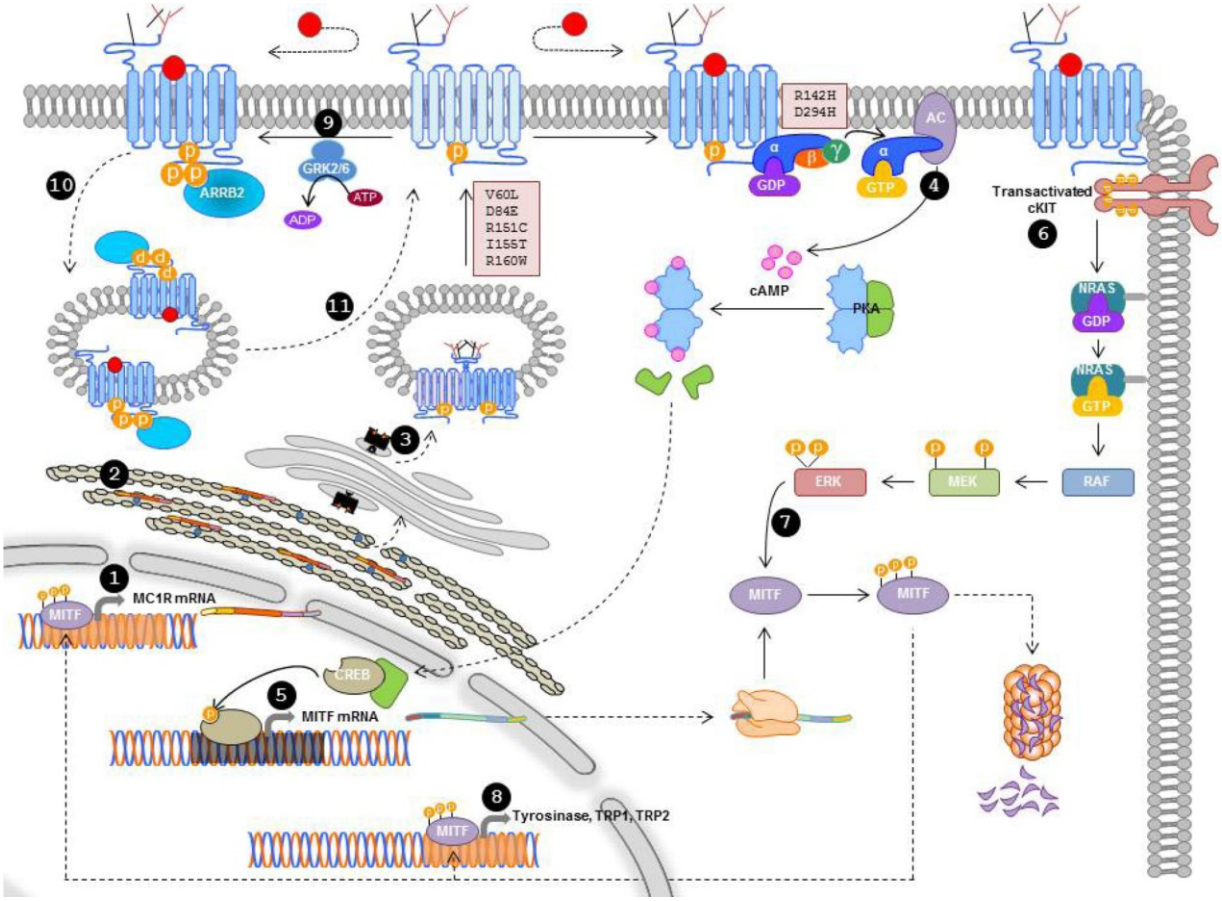


Figure 8. MC1R biosynthesis and functional coupling. Transcription of *MC1R* gene usually leads to the MC1R-001 transcript (1). In the cytoplasm, pos-translational modifications are performed (2). The oligomers proceed to the plasma membrane (3). Once the receptor binds to its agonist, MC1R activates AC via the Gs protein, through cAMP synthesis and PKA activation (4). Active PKA subunits go to the nucleus to activate CREB transcription factors, increasing MITF gene transcription rate (5). Activated MC1R also activates cKIT or RTK, triggering the NRAS-BRAF-MEK-ERK cascade (6). Active ERKs increases MITF transcription and its proteasome-dependent degradation (7). MITF then promotes transcription of melanogenic enzymes (8) and of MC1R. The receptor MC1R may be desensitized by GRK2 or GRK6-dependent phosphorylation and ARRB2 recruitment (9), then sequestrated in endocytic vesicles (10), which will be recycled to the cell surface most likely (11). From: (García-Borrón, Abdel-Malek and Jiménez-Cervantes, 2014).

MITF gene regulates melanocyte homeostasis, encoding a lineage-specific transcription factor involved in cell survival, differentiation, and proliferation. The rare p.E318K mutation has been implicated in families with melanoma and melanoma/renal cell carcinomas. The usual

phenotype of this mutation is high nevus count, MPM, melanoma before the age of 40 and non-blue eye color (Rossi *et al*., 2019).

Known genes or loci with identified mutations leading to increased risk of development of cutaneous malignant melanoma (CMM)

Gene	Inheritance	Syndrome	Type of Melanoma	Phenotype	Other Cancers	Clinical Significance
CDKN2A	AD	FAMMM	Cutaneous (CMM)	>50 atypical nevi, family history of CMM	Pancreatic and nervous system	6-month annual dermatologic evaluation; consider screening for pancreatic cancer
CDK4	AD				Pancreatic and nervous system	Follow CDKN2A recommendations
POT1	AD				Glioma, CLL	No specific recommendation, may follow CDKN2A recommendations
TERT	AD				Ovarian, renal, bladder, breast, lung	No specific recommendation, may follow CDKN2A recommendations
BRCA1&2	AD	Hereditary breast and ovarian cancer	Cutaneous and uveal		Breast, ovarian	Follow NCCN recommendations[63]
BAP1	AD	BAP1 Cancer Syndrome	Cutaneous and uveal	Atypical spitz nevi	Renal and mesothelioma	Excision of atypical spitz nevi, frequent screening for high-risk ocular melanomas
MITF	AD		Cutaneous	Increased nevus count	Renal	No specific recommendation
XPC, XPD, XPA	AR	Xeroderma pigmentosum	Cutaneous	Numerous lentigines at young age, freckling, keratitis	Nonmelanoma skin cancer	Isotretinoin tumor prophylaxis
PTEN	AD	PTEN hamartoma tumor, Cowden syndrome	Cutaneous	Multiple hamartomas and trichilemmomas	Breast, colorectal, thyroid, kidney, endometrial	Specific recommendations for p53 mutations, follow NCCN guidelines[48]
TP53	AD	Li-Fraumeni	Cutaneous and uveal	Early tumors	Breast, bone, soft tissue, Colorectal, leukemia	Yearly skin examination if positive history of skin malignancy

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; BAP1, BRCA1 associated protein; CDKN2A, cyclin dependent kinase inhibitor 2A; CLL, Chronic Lymphocytic Leukemia; FAMMM, familial atypical multiple mole melanoma syndrome; NCCN, National Comprehensive Cancer Network; PTEN, phosphatase and tensin homolog.

Figure 9. Main known genes related to familial melanomas, their form of inheritance, the typical phenotypes, the association with other types of cancers and the clinical significances of these findings. (Abdo *et al*., 2020).

XPC (XPC complex subunit, DNA damage recognition and repair factor, previously termed as XP, complementation group C) (Ali *et al*., 2020) gene (OMIM # 613208) is located on chromosome 3p25.1 and has 16 coding exons. The largest transcript encodes the XPC protein comprising 940 amino acids, the full-length protein contains eight domains, which is required for damage recognition in DNA photoproducts and to initiate the NER pathway (Ali *et al*., 2020). Mutations in *XPC* cause Xeroderma Pigmentosum group C (XP-C, OMIM # 278720), which is the most common form of XP in white population (www.omim.org).

Melanoma diagnosed in the context of hereditary cancer syndrome are overwhelmingly histopathologically indistinguishable from their sporadic counterparts, yet a small subset shows characteristic features such as those caused by *BAP1* germline mutations (in which case,

individuals often show a common specific type of melanocytic tumor called BAP1-inactivated nevi or BINs, typically presented as multiple, skin colored or reddish-brown, dome-shaped lesion with epitheliod melanocytes in histology) (Toussi *et al*., 2019).

Many of the genes, for instance *CDKN2A* and *TERT*, associated with hereditary melanoma with identifiable germline PSVs are also somatically mutated in melanoma (Toussi *et al*., 2019).

In up to 50% of highly dense melanoma families worldwide, the underlying genetic basis is still elusive with no germline PSV in any of the currently known genes. In these cases, the possibilities for the underlying genetic factors are rare PSVs in a few additional high-penetrance genes, a polygenic scenario of co-inheritance of multiple intermediate- and/or low-risk alleles, or a complex interaction between susceptibility genes and genetic modifiers, such as other genes, phenotypic characteristics, and/or environmental risk factors (Rossi *et al.*, 2019). These realities make study such as the current one of immense clinical importance.

Familial melanoma patients harbor distinct phenotypic traits that render them a special subset of individuals with a peculiar morphology of nevi and melanoma (Longo *et al.*, 2021). In these patients, melanomas are quite inconspicuous and often display dermoscopic features that overlap with nevi, like the presence of delicate network and structureless areas. Some genetic alterations, such as RHC polymorphisms in *MC1R*, increase the difficulties in detecting melanoma (Longo *et al.*, 2021). These patients, therefore, should be followed-up by a dermatologist with high level of expertise and multiple diagnostic tools (confocal microscopy, digital photography) must be used in order to obtain early melanoma diagnosis.

It is acknowledged that the clinical behavior and the morphological appearance of a tumor are the phenotypic expression of its genotype, and a high degree of genetic alterations is a hallmark of aggressiveness. Therefore, genomic alterations, together with patient and tumor characterizations could help distinct melanoma subtypes (Gandolfi *et al*., 2016).

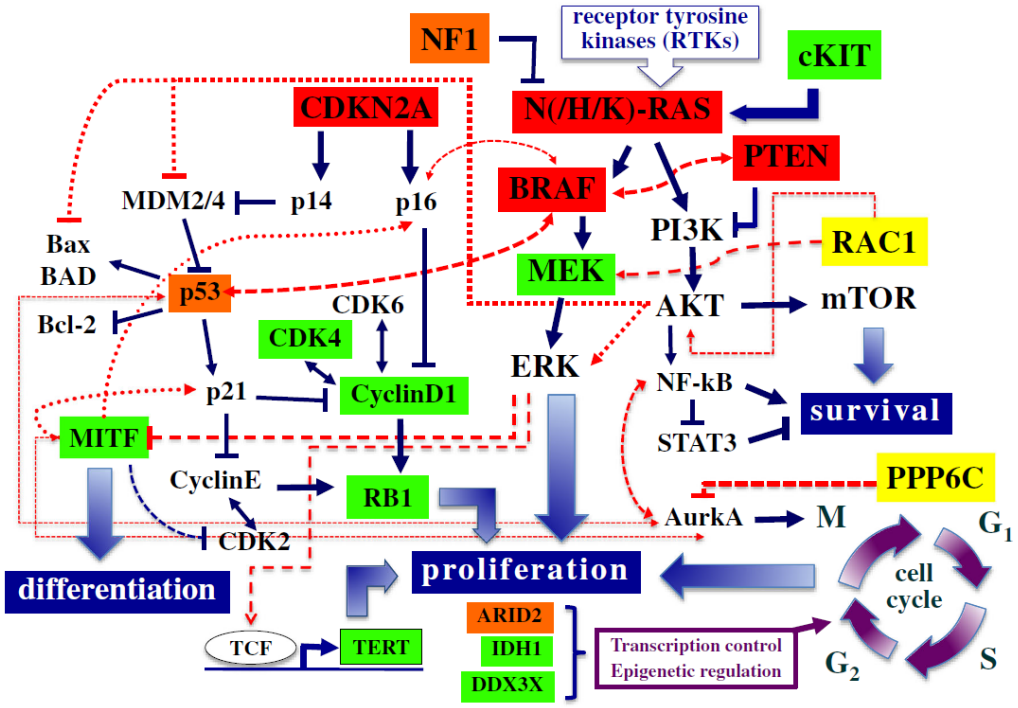


Figure 10. Multiple signaling pathways involved in melanomagenesis. Genes are evidenced according to the prevalence of alterations, including SNVs and CNVs, found in the study of 686 cutaneous melanoma samples: red ≥ 20% of cases; orange ≥ 10 to <20%; yellow ≥5 to <10; green ≥2 to <5%. From: (Palmieri *et al*., 2018).

1.3.2.1 Xeroderma pigmentosum (XP)

Xeroderma Pigmentosum (XP) is a rare autosomal recessive skin disorder characterized by hyperpigmentation, premature skin aging, ocular, and cutaneous photosensitivity, as well as increased risk of skin tumors (Ijaz *et al*., 2018). Skin cancers can also occur in unusual sites that get less UV radiation exposure, including the tongue (Ribeiro *et al*., 2018).

XP is a clinical heterogeneous disease, phenotype depending on patient's phototype as well as the environmental exposure to UV radiation since early childhood and conditions of life, but also, depends on the genetic alteration (Ribeiro *et al*., 2018; Espi *et al*., 2018). Consanguinity of parents is common in patients with rare autosomal recessive diseases and has been reported in about 30% of the XP cases (Lam *et al.*, 2005). The incidence of XP has been estimated in 1/250,000 in the United States, 1/20,000 in Japan and 2.3/1,000,000 in Western countries (Espi *et al*., 2018).

Common skin lesions in XP consist in hyperpigmented or hypopigmented macular lesions, xerosis, telangiectasia, poikiloderma, actinic keratoses, cutaneous tumors (BCC, SCC, and melanoma), ocular lesions (photophobia, conjunctivitis, keratitis, ectropion, pterigion and ocular neoplasms), neurological symptoms (sensorial deficiency, intellectual deficiency, ataxia, hyporeflexia and other symptoms) and visceral cancers (Espi *et al*., 2018). Neurological disorders such as microcephaly, diminished or absent deep tendon stretch reflexes, progressive sensorineural hearing loss and progressive cognitive impairment have been documented in 25% of XP patients (Ribeiro *et al.*, 2018). Neurological degeneration is observed in 20%–30% of homozygous patients with XPA or XPD mutations (Ribeiro *et al*., 2018).

There is an estimated 10,000-fold increased risk of non-melanoma skin cancer and 2,000-fold increased risk of melanoma under the age of twenty (Espi *et al*., 2018). The first skin lesions may appear at 8 years of age (Ribeiro *et al*., 2018). The median age of melanoma occurrence in patients with XP has been previously reported at 22 years (vs. 9 years for cutaneous carcinoma) (Espi *et al*., 2018). XP patient's life expectancy is about 30 years (Ribeiro *et al*., 2018). XP is a severe life-threatening condition, but its prognosis is widely influenced by patient's education, family awareness, effective photoprotection measures and access to care facilities (Espi *et al*., 2018). Ideally, genetic analyses should be proposed to patients, allowing presymptomatic diagnosis, antenatal diagnosis and/or counselling for future pregnancy planning (Espi *et al.*, 2018).

Only approximately 60% of XP patients show increased sunlight sensitivity in terms of severe sunburn following minor UV exposure, which usually represents the most conspicuous XP symptom during the first weeks of life. In contrast, the remaining 40 % of patients do not display any considerable sensitivity to sunlight (especially not complementation groups, XP-C, XP-E, and XP-V). Hyperpigmentation in sun-exposed areas at early age is present in all individuals. In XP patients, UV-induced skin cancers tend to arise at age 8, while in Caucasians

the mean age of these tumors is 60 years old (Lehmann *et al*., 2014). As a result of UV exposure, ophthalmologic changes usually arise only in the anterior part of the eye, affecting conjunctiva, cornea, lens as well as eyelids. While conjunctivitis, cataract, and pterygium represent frequent complications, tumors are less common.

XP has been categorized in seven complementation groups (XPA, XPB, XPC, XPD, XPE, XPF and XPG) with seven corresponding genes (*XPA*, *ERCC3*, *XPC*, *ERCC2*, *DDB2*, *ERCC4*, *ERCC5*) and one variant (XPV) with mutated *POLN* gene (Ijaz *et al*., 2018). The XP genes (*XPA*, *ERCC3*, *XPC*, *ERCC2*, *DDB2*, *ERCC4*, *ERCC5*) encode proteins that operate in nucleotide excision repair (NER) pathway and participate in recognition, demarcation, and removal of DNA damage. The XPV occurs when there is a defective polymerase η, normally implicated in the replication of DNA containing unrepaired damage (Espi *et al*., 2018). Many of the XPA, XPB, XPD, and XPG individuals exhibit a severe NER deficiency, whereas individuals with XPV variant show the least aggressive disease, have better prognosis, and enjoy higher life expectancy (Ribeiro *et al*., 2018). It appears that individuals who only have a partial NER defect (like XPF and XPC patients), tend not to develop neurologic symptoms at all or develop them later in life (Ribeiro *et al*., 2018).

The products of the XPA-G genes are involved in different steps of NER, namely damage recognition (XPE/DDB2 and XPC), DNA strand unwinding (XPB and XPD as components of transcription factor TFIIH), damage verification (XPA and XPD), and cleavage of the damaged site on the 5' (XPF) and 3' (XPG) sides of the damaged bases. The mechanism for recognition of damage depends on its location. If located on the transcribed strand of active genes, the recognition signal is the stalling of RNA polymerase by the damaged bases and its subsequent displacement by proteins that include the products of the defective genes in Cockayne Syndrome. This transcription-coupled NER (TC-NER) sub-pathway does not require the XPE or XPC proteins, whereas they are required for repair in the rest of the genome,

global genome-NER (GG-NER). Following damage recognition by either of these sub-pathways, the subsequent steps (involving the XPA, B, D, F and G proteins) are the same for both TC-NER and GG-NER (Lehmann *et al*., 2020). Those in which TC-NER remains functional (XP-C, E and V) are affected in the skin and eyes but do not have significant neurological problems as well as they do not usually present exaggerated sunburn reactions (Lehmann *et al*., 2020).

The literature on XP suggests that there are gradations in severity of the clinical features, dependent on the complementation group. XP-A is classically described as the XP group with the most severe phenotype. They usually have severe progressive neurological abnormalities and death before the age of 30 years. Even within this subgroup of patients, some mutations have been shown to cause a milder phenotype (Lehmann *et al*., 2020). XP-D patients tend to present extreme sunburn-sensitivity and most develop severe progressive neurological abnormalities. XP-F is a relatively rare XP group and individuals appear to have a lower susceptibility to ultraviolet-induced carcinogenesis than other groups. XP-C patients are particularly sensitive to sunlight-induced cancers (Lehmann *et al*., 2020). In summary, patients in groups XP-A, -D, and -G are generally considered to be the most severe, with early-onset neurological degeneration and abnormally severe sunburn reactions, whereas XP-C is thought to be intermediate, and XP-E and variant are the least severely affected (Fassihi *et al*., 2016). Even so, large cohorts of XP point out that there is significant heterogeneity of clinical features, not only between, but also within, complementation groups (Fassihi *et al*., 2016).

Whole genome scan has been used to reduce the number of possible disease-causing XP loci in some XP patients (Lam *et al*., 2005). Even when clinical diagnosis is likely, Whole-Exome sequencing (WES) has been used to confirm, precisely identify the genetic alteration, and categorize patients in specific subgroups of XP (Ali *et al*., 2020; Sugaya *et al*., 2021).

The treatment of Xeroderma Pigmentosum depends on early diagnosis, starting with immediate and strict prevention of sun exposure and other UV sources. This involves minimizing or avoiding staying outdoors without proper protection, even on cloudy days and specially in a tropical country where UV-radiation is constantly high. Even with a clinical suspicion of XP, sun protection measures must be initiated until confirmation or negative diagnosis. In addition, because of the extensive UV protection, patients should be supplemented with vitamin D, in addition to being given adequate nutritional guidance. A recent metanalysis regarding treatment of XP found most studies with low level of scientific evidence. Consensus exists regarding skin-sparing resections and the use of skin grafts from donor areas that were not exposed to the sun and/or were poorly pigmented. No study reported continuous, long-term follow-up to determine the incidence of recurrence or new lesions in the grafts or flap used (de Andrade *et al*., 2021). Pre-malignant lesions can be treated with cryotherapy, electrocautery and skin resurfacing, curettage, or surgical excision. Preference is given for non-invasive treatments. On cancerization field, topical preparations of 5-fluorouracil or imiquimod may be used. Isotretinoin or acitretin may be used for the prevention of skin neoplasms in patients who are actively developing many new skin tumors (de Andrade *et al*., 2021). When the neoplasia is inoperable, the therapeutic option is the use of radiotherapy, such as X radiation and electron therapy since most patients with XP are not sensitive to this radiation.

A randomized clinical trial with 20 patients tested T4 endonuclease V (a bacterial DNA repair enzyme) in a liposomal delivery vehicle applied topically (T4N5 liposome lotion) and demonstrated ability to lower the rate of new skin cancers in XP patients, without significant adverse effects, although currently this treatment is not available (Yarosh *et al*., 2001).

It has been estimated the existence of approximately 200 XP patients in Brazil, which would represent one case per million inhabitants (Castro *et al*., 2020). There is a cluster of 21

affected individuals in Araras (GO), central part of Brazil, where two independent mutations of the *POLH* gene have been identified, diagnosing these patients as Xeroderma Pigmentosum Variant syndrome, or XP-V (Castro *et al*., 2020). It is believed that two different families grouped together in the region decades ago, running from the social prejudice caused by the disease and fear that it would be contagious, leading to an elevated number of affected individuals due to inbreeding.

1.4 Clinical and histological features

Cutaneous melanoma is a clinical and molecularly heterogeneous skin cancer. It is categorized in four main subtypes: superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous melanoma (ALM) and lentigo malignant melanoma (LMM); (Rager *et al*., 2005). The main characteristics of each subtype are shown in table 1.

Histologically, SSM presents an epidermal lateral component with pagetoid spread of malignant melanocytes throughout the epidermis. Nodular areas representing vertical growth of the tumor can develop further. NM, in contrast, initially presents itself as a nodular, exophytic brown and/or black, often eroded or bleeding, tumor. Because of these characteristics, the warning signs of ABCDE (in English = **A**, asymmetry; **B**, border irregularities; **C**, color variegation; **D**, diameter > 6mm; **E**, evolution – how this lesion has evolved over the last years) further discussed in the section “Diagnosis”, are usually not applicable and early detection is more difficult. It has only a vertical growth phase; therefore, it is associated with greater Breslow thickness – a measurement with ocular micrometer from the top of the granular layer of the epidermis (or, if the surface is ulcerated, from the base of the ulcer) to the deepest invasive cell across the broad base of the tumor (Breslow, 1970). LMM is the progression of the slow-growing lentigo maligna (melanoma *in situ*) and is histologically characterized by a lentiginous proliferation of atypical melanocytes at the dermo epidermal

junction, confluence, dermal nests, and perifollicular concentration. ALM has an initial intraepidermal phase but later invasive growth can occur, leading to a nodular region (Garbe *et al*., 2020).

Other forms of cutaneous melanoma do exist, such as desmoplastic melanoma, melanoma arising from blue nevus, melanoma arising in a giant congenital nevus, nevoid melanoma, melanoma of childhood, balloon cell melanoma, myxoid melanoma, osteogenic melanoma and rhabdoid melanoma, but these were not investigated in the present work.

The micro stage of malignant melanoma is determined on histologic examination by the vertical thickness of the lesion in millimeters (Breslow classification, table 2) (Breslow, 1970) and/or the anatomic level of local invasion (Clark classification, table 3) (Clark *et al*., 1969). Accurate micro staging of the primary tumor requires careful histologic evaluation of the entire specimen by an experienced pathologist.

The Breslow thickness requires an optical micrometer fitted to the ocular position of a standard microscope. It is more reproducible and can accurately predict subsequent behavior of malignant melanoma in lesions thicker than 1.5 mm and must always be reported. It is the main information for the characterization of the tumor in the T (tumor) section of the TNM staging.

Table 1. The four major clinical subtypes of cutaneous melanoma.

CT subtype	Frequency (%)	Localization	Usual presentation	Characteristics	Genetics
Superficial Spreading Melanoma (SSM)	70	Back (in men) Posterior leg (in women)	Heterogenous pigmented flat lesion with or without elevated areas and sharp edges	Intermittent sun exposure; ABCDE rule applicable; *de novo (75%)* or associated with nevus (25%)	*BRAF* (40-50%); *RAS* (10-20%); *MITF* (15-20% of all melanomas, but more often in metastatic melanomas)
Nodular Melanoma (NM)	5	Trunk or limb	Nodule, elevated plaque or ulcerated polyp. Most common in 5th or 6th decade in men	Intermittent sun exposure; can be dark or even amelanotic; always *de novo*; only vertical growth phase with earlier metastasis	Mostly *NRAS* mutations present in amelanotic nodular subtypes
Acral Lentiginous Melanoma (ALM)	5 In Caucasians, also most common type among Asian, Hispanic and African patients	Glabrous skin (palmoplantar) or subungual	Flat, plaque or nodule of irregular pigmentation and poorly defined edges	Predominant in elderly women. In the nail apparatus, usually as a longitudinal dark line (melanonychia striata) in the hallux or toe	*BRAF* (15%); *NRAS* (15%); c-KIT mutations (10-23%)
Lentigo Maligna Melanoma (LMM).	4-15	Head and neck (sun-exposed areas)	Flat or slightly elevated irregular area of pigmentation usually light brown. More common in older individuals	Chronic sun exposition; usually surrounded by sun-damaged skin; slow growth and less invasive behavior	c-KIT mutations is common in chronic sun exposed lesions.

Adapted from Garbe *et al*., 2020.

Table 2. Breslow Classification (Tumor thickness in mm)

Breslow Depth	Tumor size
Not applicable	Tis (*in situ*)
< 1 mm	T1
1- 2 mm	T2
2.1- 4 mm	T3
> 4 mm	T4

Adapted from Breslow, 1970.

Table 3. Clark Classification (level of invasion in histology)

Level of Invasion	Description
Level I	Lesions involving only the epidermis (*in situ* melanoma); not an invasive lesion.
Level II	Invasion of the papillary dermis; does not reach the papillary-reticular dermal interface.
Level III	Invasion fills and expands the papillary dermis but does not penetrate the reticular dermis.
Level IV	Invasion into the reticular dermis but not into the subcutaneous tissue.
Level V	Invasion through the reticular dermis into the subcutaneous tissue.

Adapted from Melanoma Treatment (PDQ) Health Professional Version, 2020.

1.5 Diagnosis, staging and treatment of cutaneous melanoma

The gold-standard for the diagnosis of melanoma is pathological examination of a suspicious lesion through a specimen biopsy.

Dermoscope is a high-resolution optical handheld device that allows the magnification of the morphological structures of a skin lesion that are not visible to the naked eye. Dermoscopy has increased the accuracy of melanoma detection, since early microscopic changes can be visualized prior to the clinical alterations could be visualized with a naked eye. It allows the identification of many different lesion structures and colors and correlates them to histopathological findings (Cuéllar *et al*., 2009). There are some specific dermoscopic criteria for the diagnosis of melanoma: atypical pigment network, irregular dots/globules, irregular streaks, irregular pigmentation, regression structure, blue-whitish veil and vascular pattern. These criteria are shown in table 4.

An important and sophisticated tool to enhance the likelihood of detecting very early-stage melanomas is short-term surveillance with total-body or single-lesion photographic images. These devices (usually a high-quality camera and dermoscope attached to a computer harboring a dermatological software that enables comparison of photos, both macro and micro) is extremely useful in individuals with multiple nevi, atypical moles or those who do not want to be submitted to biopsy right away. Through the comparison between the photos, with a 3 to 6-month interval, minimal changes can be detected and therefore, encourage early biopsy procedure. Likewise, the absence of changes helps prevent unnecessary biopsies.

In vivo Reflectance Confocal Microscopy (RCM) is a valuable imaging tool that allows non-invasive *in vivo* examination of the suspected lesion at a nearly histological resolution. The images can show cellular alterations and help not only to encourage surgical excision but also stimulate follow-up of treatments and margin definitions. Although useful, it is not largely available, especially in Brazil.

Another relevant diagnostic technique is self-examination which is usually oriented with the ABCDE rule. The patient (or the non-dermatologist health-care provider) should pay attention to **A** (asymmetry), **B** (border irregularities), **C** (color variegation), **D** (diameter bigger than 6 mm) and **E** (evolution – how this lesion has evolved over the last years). This simple method can show between 57% and 90% of sensitivity (Rastrelli *et al*., 2014).

The "Ugly Duckling" sign is also useful to detect suspicious lesions. It means that a lesion that brings the examiner's attention to it, usually because it is somehow different from the neighbor lesions, should be better evaluated by a specialist.

Table 4. Dermoscopic criteria for melanoma diagnosis

Pigment network	Black, brown, or gray network. Irregular holes and thick lines irregularly distributed and ending abruptly at the periphery
Dots/globules	Irregular dots and globules for the shape and size
Streaks	Irregular and unevenly distributed
Irregular pigmentation	Black, brown, and gray pigmented areas with irregular shape and/or distribution
Regression structure	White scar-like areas, blue areas, or a combination of both
Blue-whitish veil	Confluent, irregular and structureless area of whitish-blue diffuse pigmentation associated with pigmented network, dots, globules, and streaks
Vascular pattern	Irregular hairpin vessels, dotted vessels, linear irregular vessels, or vessels within regression structures

Adapted from Rastrelli *et al*., 2014

To plan a proper treatment and assess prognosis in melanoma, it is necessary to determine melanoma staging right at the beginning. Clinical staging includes histologic data of the primary tumor plus information from the clinical and radiologic evaluation for metastatic disease (regional or distant).

The American Joint Committee on Cancer (AJCC) TNM (tumor, lymph node, metastasis) staging system is used worldwide and was been updated in 2017 (Gershenwald and Scolyer, 2018). Tumor (T) classification is based on the Breslow thickness; ulceration is classified as a high-risk feature for all tumors, and mitoses per square millimeter are a high-risk feature for tumors with a thickness less than 1 mm. The N (nodal) category addresses to metastatic disease both in regional lymph nodes and in non-nodal locoregional sites (i.e., microsatellites, satellites, and in-transit metastases). They are probably metastases of intralymphatic or possibly angiotrophic tumor spread (Gershenwald *et al.*, 2017). Satellite metastases are defined as clinically evident cutaneous and/or subcutaneous metastases occurring within 2 cm of the primary melanoma; microsatellites are defined as microscopic cutaneous and/or subcutaneous metastases found adjacent or deep to a primary melanoma on pathological examination and in-transit metastases have classically been defined as clinically evident cutaneous and/or subcutaneous metastases identified at a distance more than 2 cm from the primary melanoma in the region between the primary and the first echelon of regional lymph nodes (Keung and Gershenwald, 2018). The M (metastasis) category takes into consideration not only the anatomic site of the metastasis but also the level of serum lactate dehydrogenate (LDH).

After assessing all three categories (T, N and M) of the patient, both clinical and pathological classification are used in melanoma staging. The clinical staging (cTNM) includes micro staging of the primary melanoma after its biopsy and clinical/radiological assessment for regional or distant metastasis (and the necessary biopsies). There is no substage for clinical

stage III melanoma. The pathological staging (pTNM) is more detailed as it includes not only the clinical staging information as well as any additional staging information obtained from the wide surgical excision of the primary tumor and pathological information about the regional lymph nodes from the sentinel lymph node biopsy or completion lymph node dissection.

Localized disease (stages I and II) is the form of presentation in approximately 85% of patients with melanoma, 15% have regional nodal disease and only about 2% have distant metastasis at the time of diagnosis (Tracey and Vij, 2019).

Across all stages of melanoma, the average five-year survival rate in the U.S. is 92 percent. The estimated five-year survival rate for patients whose melanoma is detected early is about 99 percent. The survival rate falls to 65 percent when the disease reaches the lymph nodes and 25 percent when the disease metastasizes to distant organs (American Cancer Society https://www.cancer.org). The prognosis of the localized disease is directly connected to the depth of the tumor (Breslow thickness).

The number of positive lymph nodes is the most important prognostic factor for stage III melanomas.

When there are distant metastases, median survival is about 6 to 9 months. In stage IV, patients with non-visceral disease (such as skin, subcutaneous tissue, lymph nodes) have a better median survival rate (12 to 15 months) than patients with visceral metastasis and are more likely to respond to chemotherapy (Tracey and Vij, 2019).

Most melanoma patients present with early-stage disease, in which complete surgical excision of the tumor is curative.

About 10% of cases are diagnosed with advanced disease, being unresectable or already metastatic. For these patients, treatment will be based on *BRAF* mutation status and their clinical conditions. Since 2011, revolutionary therapeutic agents have been approved, such as RAF and MEK kinase inhibitors, as well as immune checkpoint inhibitors [anti-cytotoxic T-

lymphocyte-associated antigen 4 antibodies (anti-CTLA4) and anti-programmed cell death protein 1 antibody (anti-PD1)] (Seth *et al*., 2020). In the advanced-stage scenario, anti-PD1 and anti-CTLA4 antibodies (such as nivolumab, pembrolizumab and ipilimumab) or selective BRAF inhibitors (vemurafenib and dobrafenib) combined with MEK inhibitors (cobimetinib and trametinib) are showing promising results in clinical trials. In clinical practice, only *BRAF* V600E mutation is determined due to its relevance in guiding appropriate treatment strategy with the use of BRAF inhibitors with MEK inhibitors. This combination has led to up to 70% response rates and a rapid response induction and symptom control, with a 12-month progression-free survival interval. In case of BRAF inhibitor resistance on *BRAF* mutant melanoma, nivolumab and pembrolizumab seems effective (Seth *et al*., 2020).

Immunotherapy and kinase inhibitors are nowadays the gold-standard of systemic therapy, while chemotherapy is considered second-line. Anti-PD1 antibodies and, with lower magnitude anti-CTLA4 therapeutic agents, offer lower response rates, but potentially long durable responses. Melanoma therapeutic agents are rapidly evolving; therefore, it is encouraged the participation of patients in randomized clinical trials (Leonardi *et al.*, 2018; Seth *et al*., 2020; Tarhini *et al*., 2021).

1.6 Whole-exome sequencing – WES

The first report of applying WES to investigate the somatic landscape of a single cutaneous melanoma was published in 2010 (Pleasance *et al*., 2010). A subsequent study examined 14 matched normal and metastatic tumor DNAs from untreated individuals with melanoma (Wei *et al*., 2011) and from then on, several groups have applied WES and GWAS to unravel the underlying molecular basis of CMs (Barret *et al*., 2011; Barrett *et al*., 2015; Law *et al*., 2015; Landi *et al*., 2020; Yepes *et al*., 2020).

About 85% of disease-driving mutations occur in exons, specific protein coding regions of the genes that correspond to approximately 2% of the entire human genome (Choi *et al*., 2009). Mutations in exons of proto-oncogenes, tumor-suppressor genes, anti-apoptotic genes or genes associated with DNA repair are alterations associated to cancer development.

WES is a sequencing technique that implements a capture-based enrichment of the protein coding regions of genomic DNA (exons), using a set of oligonucleotide hybridization probes that target known exon sequences. The most common application of WES is mutational analysis: the detection of single-nucleotide variants (SNVs) or small insertions and deletions (Indels). There are some limitations of this method, beyond technicalities, as scientific knowledge of the function and clinical correlates of all truly protein-coding exons in the genome are still incomplete (Bamshad *et al*., 2011).

WES generates hundreds of millions of short sequences reads with an average length of 50 bp to 125 bp. Fragment sequencing has higher sequencing error rates than Sanger sequencing; thus, further validation using Sanger sequencing is essential. On average, exome sequencing identifies nearly 24,000 single nucleotide variants in African American samples and nearly 20,000 in European American samples, but more than 95% of these variants are known polymorphisms in human population (Bamshad *et al*., 2011).

The technique consists in a series of steps: genomic DNA fragmentation, hybridization of genomic areas of interest by specific probes, capture of hybridized probes, DNA fragments amplification, *sequencing by synthesis* of each fragment by a platform (figure 10).

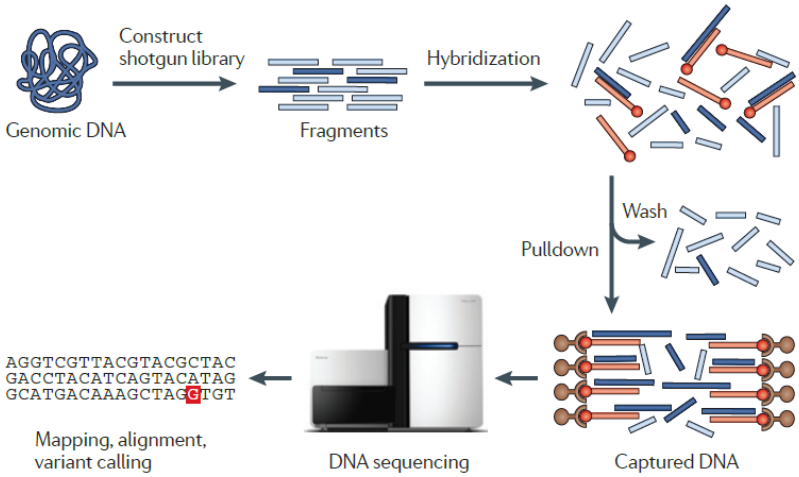


Figure 11. Basic steps for exome sequencing (From Bamshad *et al*., 2011).

The genetic landscape of melanoma (somatic and germline) were achieved using WES to define candidate genes associated with inherited predisposition to uveal melanoma (Abdel-Rahman *et al*., 2020), the potential oncogenic drivers of mucosal melanoma (Nassar and Tan, 2020), novel mutations in acral melanoma (Lim *et al*., 2020), germline *POT1* mutations in familial cases (Robles-Espinoza *et al.*, 2014; Wong *et al*., 2019; De Simone *et al*., 2020; Malińska *et al*., 2020) and to correlate between molecular data and clinical outcomes to PD-1 blockade treatment in metastatic patients (Zaretsky *et al.*, 2016; Liu *et al.*, 2019; Cui *et al*., 2021).

Since only around 50% of familial melanoma cases can be attributed to PSVs in known predisposition genes, primarilly *CDKN2A*, a substantial percentage of genetic risk for melanoma remains elusive (Robles-Espinoza *et al.*, 2014). It is reasonable to expect that additional genes may be discovered by applying WES to melanoma enriched families (Dębniak *et al.*, 2019; Campos *et al*., 2020). Therefore, in this study, we chose a large family with prominent clinically features suggestive of a melanoma dominant syndrome, as proposed by Abdo *et al*. (2020) and by Leachman *et al*. (2017), to perform WES.

2. OBJECTIVES

The overall objective of this study was to define the genetic basis of familial CM in Brazilian individuals from the state of Minas Gerais who present features suggestive of an inherited predisposition to CM. To further understand the genetic basis of these cases, sporadic melanoma cases were also studied.

To that end, the following specific aims were pursued:

2.1 Determine the presence of *CDKN2A* germline mutations in all study participants.

2.2 Determine the presence, frequency and types of variants or mutations in *MC1R* in study participants and compare that with non-CM Brazilian controls.

2.3 Perform WES on DNA samples from large kindred seemingly presenting inherited CM and select candidate PSVs and validate these by Sanger sequening.

2.4 Compare the frequency of rare/new PSVs in either seemingly familial or MPM and sporadic CM cases and population controls.

2.5 Correlate significant validated genetic alterations with clinical characteristics.

3. PATIENTS AND METHODS

3.1 Clinical cases study

Participant chracteristics

The study population encompassed four patients with a suggestive family history of melanoma with their available family members (a total of 11 individuals), eight individuals who have had more than one melanoma (Multiple Primary Melanoma) and 25 individuals who had one melanoma without family history were studied (sporadic melanoma cases). All cases attended the Dermatology Unit of Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG) or private offices in Belo Horizonte, Brazil. The exclusion criteria were poor-documented tumors or not confirmatory biopsy of melanoma and patients who fulfilled criteria for other genetic/inherited cancer syndromes, such as Li-Fraumeni or Xeroderma Pigmentosum (XP), in which melanoma is not the most common, first, or predominant presentation as proposed by Abdo *et al*. (2020).

This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais by the registration number CAAE-0472.0.203.240-11 (available in appendix 2).

All patients and their family members who agreed to participate in this study were interviewed (complete questionnaire available in appendix 1), clinically examined, had peripheral blood collected and signed written informed consent (Ethics registration number CAAE-0472.0.203.240-11). The main characteristics of these individuals are shown in table 5 (familial melanoma patients), table 6 (MPM patients) and table 7 (sporadic melanoma patients).

Family A is shown in figure 11. The deceased father had no known history of melanoma (I.1), and the 84-year-old healthy mother (I.2) had 13 offsprings (2nd generation), of whom seven have had one or more melanomas at the time of recruitment to the study. The proband (II.6) died of metastatic melanoma (primary tumor on the lower lip) when he was 38 years old (initially diagnosed at age 27), and his brother sought medical advice (II.11). Five of the remaining six affected siblings participated in the study (individuals II.6, II.7, II.8, II.11 and

II.13), along with the mother, five unaffected siblings (II.1, II.2, II.3, II.5 and II.12) and four offspring (3rd generation) of affected (III.12, III.15 and III.22) and unaffected individuals (III.24). The third generation of this family (ages range from 40 to 6) had no melanoma cases up to date. All 15 individuals participating in the study are Caucasian, with brown eyes and hair, Fitzpatrick phototypes II and III. In the melanoma patients, the majority has multiple atypical nevi (three have many (more than 20), one has some and one has none), all have a relevant history of non-protected sun exposure throughout life. Most of them have freckles or solar melanosis, confirming the sun-damaged skin. A total of 53 melanomas were diagnosed among the affected members. Four of the five melanoma individuals have had non-melanoma skin cancers (NMSC), all basal cell carcinomas (individual II.1 has also had one basosquamous carcinoma), a total of nine carcinomas.

Family B is composed by a presumably affected deceased father (no medical record confirming melanoma, but the family disclosed that he had melanoma), a healthy mother and their six offspring, three of whiom were also affected; however, only individuals II.1 and II.3 agreed to participate in this study. Each of the three affected individuals had only one melanoma and the age of diagnosis was 55, 53 and 59, respectively. Third generation has not been affected up to date and the family pedigree is shown in figure 12. Both affected individuals are fair skinned with multiple ephelides and solar lentigines, without atypical nevi.

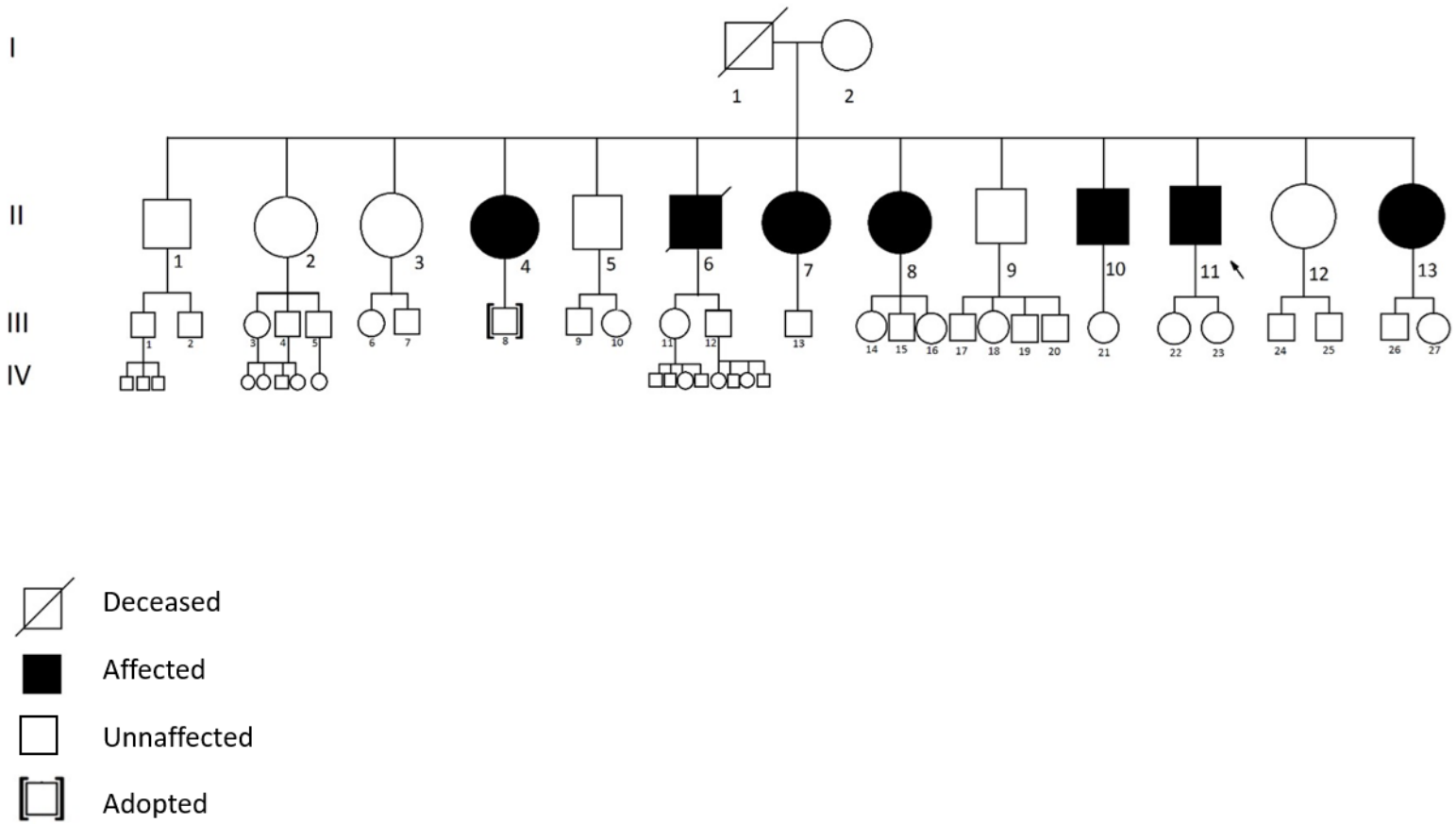


Figure 12. Familial melanoma family A. Black filled shapes represent affected siblings. White shapes are unaffected subjects. Third generation are young individuals and children, none of which are affected up to the present. The proband is represented by an arrow.

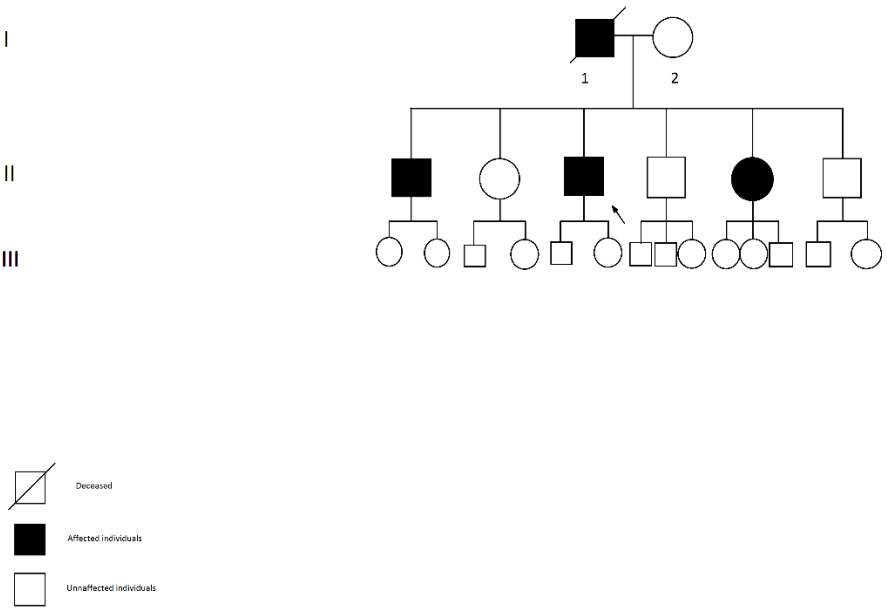


Figure 13. Familial melanoma family B. Black filled shapes represent affected subjects. White shapes are unaffected subjects.

Family C is composed by the proband (II.1), his affected father of 87 years of age, his healthy mother and his three children, none of which is affected up to the moment (figure 13). Third generation is young (range from 12 to 30) and up to the time of study end, not affected.

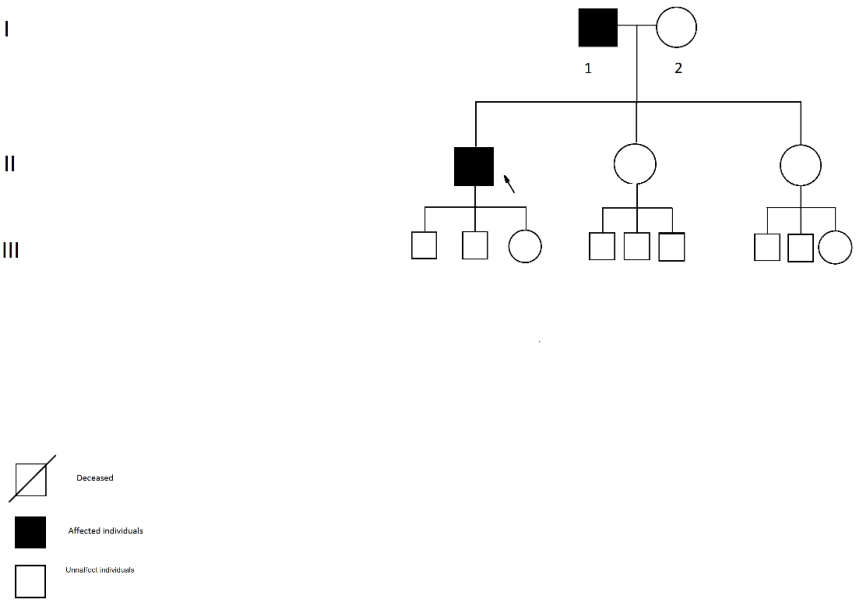


Figure 14. Familial melanoma family C. Black filled shapes represent affected father and son. White shapes are unaffected subjects.

Family D is composed by a deceased affected father (confirmed), a healthy mother and their six offsprings, two of them also affected. In the third generation, no individuals have been affected up to the end of the study. They are shown in figure 14.

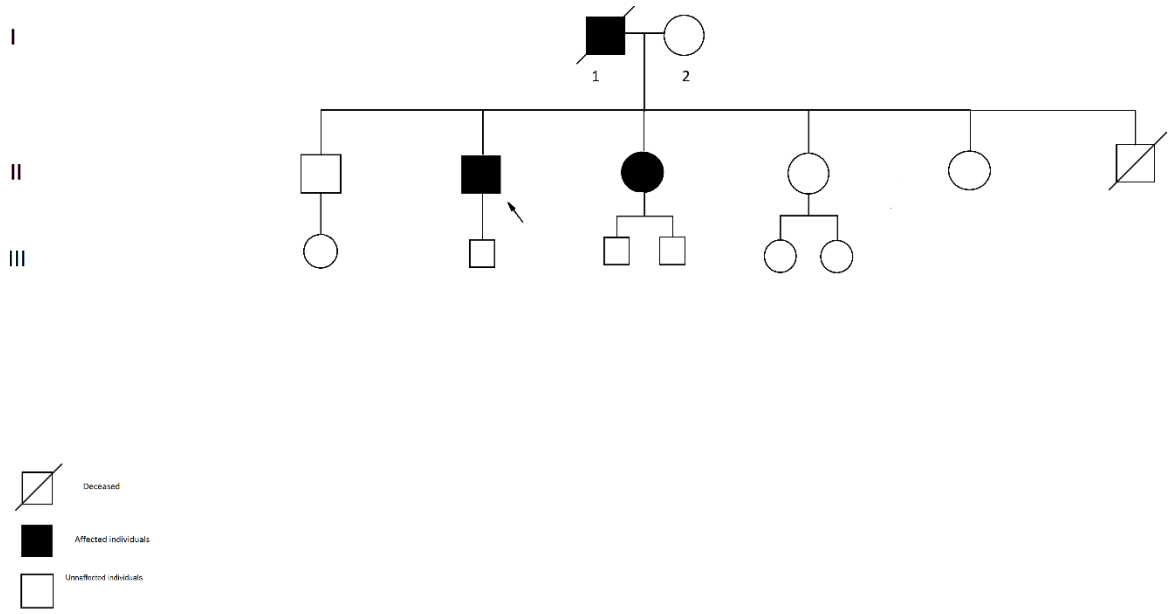


Figure 15. Familial melanoma family D. Black filled shapes represent affected subjects. White shapes are unaffected subjects.

Table 5. **Familial melanoma cases and their characteristics.**

Family	Affected individuals	Age of first melanoma	Number of melanomas	Tumor(s) localization(s)	Breslow thickness	Sun exposure	Total nevus body count	Other skin cancers
A	II.4	45 (now 62)	3	Head (3)	All *In situ*	Excessive unprotected	<20	None
	II.7	33 (now 53)	11	Head (3), neck (1), limbs (2) and upper trunk (5)	All *in situ*	Excessive unprotected	20-100	Two BCC
	II.8	35 (now 50)	7	Head (3), neck (2), and chest (2)	0.24mm and 6 *in situ*	Excessive unprotected	20-100	Three BCC
	II.10	48 (now 48)	1	Head (scalp)	*In situ*	Excessive unprotected	20-100	None
	II.11	28 (now 46)	24	Head (19), neck (2), and upper trunk (3)	0.45mm; 0.47mm; 0.9mm; 1.4mm; 2.0mm and 19 *in situ*	Excessive unprotected	>100	Two BCC, one basosquamous carcinoma
	II.13	22 (now 41)	7	Head (3), neck (3) and upper trunk (1)	0.23mm; 0.36 mm; 2.3 mm and 4 *in situ*	Excessive unprotected	>100	One BCC

B	II.1	55 (now 62)	1	Limb (Right arm)	0.65mm	Sunburn with blistering	20-100 Freckles	None
	II.3	53 (now 59)	1	Trunk (back)	0.8mm	Excessive unprotected	20-100 Freckles	None
	II.5	53 (now 58)	1	Trunk (back)	N/A	Excessive unprotected	20-100	N/A
C	I.1	50 (now 87)	1	Trunk (shoulder)	1.0 mm	Excessive unprotected	20-100 Phototype I	Tongue carcinoma
	II.1	40 (now 51)	1	Trunk (back)	0.7mm	None	20-100 Phototype II	No
D	I.1	> 50	1	N/A	N/A	N/A	N/A	N/A
	II.2	48 (now 57)	1	Posterior neck	0.3 mm	Sunburn with blistering	20-100	BCC on the back
	II.3	35 (now 56)	1	Upper trunk	*In situ*	Sunburn with blistering	20-100	None

N/A: Not available; BCC: basal cell carcinoma

A positive history of excessive unprotected sun exposure is present in 69.2% of familial melanoma cases. History of blistering is present in 23% and no excessive sun exposure is referred in 7.7%.

The eight patients with multiple primary melanomas are identified as individuals E, F, G, H, I, J, K e L. Their mean age of diagnosis of the first tumor is 39.4 years (range 16 to 63). All but one patient had superficial spreading melanoma (SSM), one had lentigo maligna at histopathological examination. Dermoscopy pattern of these patients showed unspecific pattern in the vast majority of lesions. None of them has a significant family history of melanoma or any other related tumor described in melanoma syndromes. Their relevant personal risk factors are pointed out in table 6.

Patient E is a 36-year-old Caucasian woman with a history of three melanomas, all *in situ*. The first melanoma showed up when she was 18 years old (at the right forearm), the second one by the age of 23 (at the fourth right chirodactyl) and third one by the age of 33 (at the pre-sternal area). She has fair skin and light hair, with Dysplastic Nevus Syndrome (multiple atypical nevi). She also has had two basal cell carcinomas on the forehead. She has healthy parents and one offspring of age 2.

Individual F is a 30-year-old man with a history of seven melanomas, the first one at age 18, all thin (<1mm), four *in situ*. He has multiple atypic nevi, no visible photodamage and no relevant sun exposure.

Individual G is a 62-year-old woman who has had three melanomas and has no other relevant personal or family history.

Individual H is a 71-year-old man who has had two melanomas, one superficial spreading melanoma (SSM) at the back with Breslow 0.6 mm at 59 years of age and the second one a nodular melanoma with Breslow 0.9 mm also on the back at age 60. He has had lymphatic metastasis and has done lymph node dissection. No family history of melanoma or other associated tumor was reported but a relevant history of sun damage was informed.

Individual I is 61-year-old woman with six previous melanomas, the first one at age 45. She also had 3 basal cell carcinomas, history of blistering due to sun exposure with visible skin photoaging and no other personal or family history of malignancies.

Individual J is a deceased woman who died of pancreatic cancer (not sure by medical records if primary or secondary) at age 83. She had multiple melanoma (at least 6 with precise histological confirmation) and multiple carcinomas (basal cell and squamous cell carcinomas - at least 10) since age 35, according to her recollection, but not all data could be confirmed. Of the tumors we could access histologically, she had at least two lentigo maligna (one suspicious of invasiveness) on the face and on the arm, one desmoplastic melanoma on the forearm with Breslow 3.0 mm, one nodular amelanotic melanoma on the other forearm with Breslow 8.0 mm and one nevoid melanoma on the trunk with Breslow 1.2 mm. She had systemic melanoma metastasis confirmed through PET-CT. She has light skin and red hair (photype II) and history of blistering since childhood. She refers that her deceased sister also had multiple carcinomas (and maybe even a melanoma – no confirmatory data). No other relevant personal or family history.

Individual K is a 67-year-old man who has had three melanomas, all of which *in situ* (one on the nose, one in the cervical region and one in the back), at the age 63, 64 and 65. He also had four basal cell carcinomas in the back and anterior trunk. His father has had two basal cell carcinomas but no melanomas or other relevant personal history. He reported an important history of sunburns of first (majority) and second (only a few) degrees in his youth. He was once a sporadic light smoker.

Individual L is a 58-year-old man who has had three melanomas on the back, the first at the age of 47 years old, a 0.3 mm deep superficial spreading melanoma and the other two *in situ* at age 51 and 58. Never smoked or had any sunburn. The only comorbidity is a mild

dyslipidemia and overweight. His sister has had three basal cell carcinomas and no melanomas. No other important family history.

Elderly individuals without personal history of cancer from our DNA databank were used as controls.

Table 6. Multiple Primary Melanoma patients' characteristics

Individual	Ages of melanomas	Actual age	Number of melanomas	Tumor(s) localization(s)	Breslow thickness	Sun exposure	Total nevus body count	Other skin cancers
E	18, 23, 33	36	3	All on the torso	All *in situ*	Sunburns	>100 (Dysplastic Nevus Syndrome)	Two BCC on the forehead
F	18 (3), 22, 28 (3)	30	7	Limbs (1) and trunk (6)	0.6mm; 0.28 mm; 0.55 mm and 4 *in situ*	Excessive unprotected	20-100	None
G	53, 56, 57	62	3	All on the trunk	2.15 mm; *in situ;* 0.38mm	Yes, blistering	<20	None
H	59, 60	71	2	All on the trunk	0.6mm and 0.9mm	Yes, blistering	<20	Two BCC
I	45,48, 47, 54, 57, 57	61	6	Face (1), limbs (1), trunk (4)	All *in situ*	Yes, blistering	<20	Three BCC
J	35 (?),79, 80, 82 (3)	84	6	Head (1), limbs (4) and torso (1)	1.2mm; 3.0mm; 8.0mm and *in situ*	Yes, blistering	20-100	Many BCC and SCC
K	63, 64, 65	68	3	Head, neck and back	All *in situ*	Yes, blistering	<20	Four BCC on the trunk (anterior and posterior)
L	47, 51, 58	58	3	All on the trunk	0.3 mm and two *in situ*	Sunburns	20-100	No

BCC: Basal cell carcinoma; SCC: Squamous cell carcinoma

3.2. DNA collection and extraction

Peripheral blood (5 ml) of all participants in this study was collected in vacuum tubes with EDTA. Genomic DNA was isolated using the saline concentration method of Lahiri and Nurnberger (Lahiri and Nurnberger, 1991). All participants gave informed consent as approved by the Ethics Committee of the Universidade Federal de Minas Gerais.

3.3 Sanger Sequencing

Genotyping for germline PSVs in the *CDKN2A* gene was carried out in all five affected family members of family A, two affected members of family B, two affected members of family C and all eight MPM individuals. Subsequently, *CDKN2A* was also genotyped in 25 phenotypiucally sporadic melanoma individuals. The gene *CDKN2A* has three exons but only two are coding exons. Exon-specific flanking primers (Table 7) were designed using PrimerBlast software, available at *http://www.ncbi.nlm.nih.gov/tools/primer-blast/*. The DNA extracted from blood samples was quantified and polymerase chain reactions (PCR) were performed using 50ng/ml DNA, 2.5ml of 10x Buffer IIB (40mM NaCl, 10mM TrisHCl, pH8.4, TritonX -100 0.1 %; 1.5MM MgCl2), dNTPs 2.5ml (0.2 mm), 0.5ml of each primer pair (10 pmol/ml) and 0.25ml of Taq polymerase (0.625U). The products were amplified in a thermocycler Eppendorf Mastercycler Gradient, using the following steps: denaturation at 94° C for 3 minutes, and 35 cycles of denaturation at 94ºC for 30 seconds. Annealing at 55-60ºC for 30 seconds and extension at 72° C for 30 seconds. At the end of the cycles, the reactions were subjected to a final extension at 72° C for 5 minutes. PCR reaction products were gel-verified and purified using the PCRLink^{TM} Quick PCR Purification Kit (Life technologies, Carlsbad, California), and then submitted to sequencing reaction with the ABI BigDye

Terminator Cycle Sequencing Kit v3.1 on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Table 7. *CDKN2A* primers list and PCR fragments.

Gene	Exon	Sequence (5'- 3')	Size (pb)
CDKN2A	1	**F,** TTCGCTAAGTGCTCGGAGTT **R,** GAGAATCGAAGCGCTACCT	486
	2	**F,** GGAAATTGGAAACTGGAAGC **R,** GCTGAACTTTCTGTGCTGGAAAATG	512

3.4. Whole-exome sequencing (WES)

There are different deep-sequencing platforms to choose from when performing whole exome sequencing. Hybridization is the most optimal and commonly used method for targeted exome. For this purpose, peripheral blood from a clinically affected individual from a family is collected and DNA of good quality extracted. Subsequently, DNA is subjected to whole exome capturing and sequencing using the Roche NimbleGen V2 chip (Madison, Wisconsin) or Nextera (San Diego, California) with the Illumina HiSeq2000 sequencing platform (San Diego, California).

The Illumina sequencing workflow begins with Nextera sample preparation, followed by cluster generation on a system flow cell, sequencing with Illumina's proprietary sequencing by synthesis technology and culminating with data analysis.

Samples consisting of longer fragments are first sheared into a random library of 100-300 base-pair long fragments. After fragmentation, the ends of the obtained DNA-fragments are repaired, and an A-overhang is added at the 3'-end of each strand. Afterwards, adaptors which are necessary for amplification and sequencing are ligated to both ends of the DNA-fragments. These fragments are then size selected and purified. The Cluster Generation is

performed on the Illumina cBot. Single DNA-fragments are attached to the flow cell by hybridizing to oligos on its surface that are complementary to the ligated adaptors. The DNA-molecules are then amplified by a so-called *bridge amplification* which results in a hundred of millions of unique clusters. Finally, the reverse strands are cleaved and washed away, and the sequencing primer is hybridized to the DNA-templates. During sequencing the huge number of generated clusters are sequenced simultaneously. The DNA-templates are copied base by base using the four nucleotides (ACGT) which are fluorescently labeled and reversibly terminated. After each synthesis step, the clusters are excited by a laser which causes fluorescence of the last incorporated base. After that, the fluorescence label and the blocking group are removed allowing the addition of the next base. The fluorescence signal after each incorporation step is captured by a built-in camera, producing images of the flow cell.

3.4.1 Variant Calling and annotation

Variant calling is the part of the process that is performed as the initial mapping of the reads, improvement of alignments and quality scores, variant identification, and recalibration of the variant's quality scores. In general, a coverage of 20X to 50X at each nucleotide is considered acceptable when identifying variations.

Raw sequence files were prepared using the Genome Analysis Tool Kit (GATK) for each of the sequenced samples. Each fastq file was aligned against the human hg19/GRCh37 reference genome. PCR duplicates were removed using Picard (*http://picard.sourceforge.net/)*, reads around known and detected indels were realigned, and base quality was recalibrated using GATK. In order to call variants from the processed BAM files, a variant calling pipeline from GATK was applied.

The VCF file was analyzed using two different tools. The first software used was *Mendel, MD,* developed by the Clinical Genomic Laboratory of Universidade Federal de Minas Gerais and available at *http://mendel.medicina.ufmg.br* (GCCL Cardenas *et al.*, 2017). VCF file was also analyzed by *Ingenuity® Variant Analysis™* software, available at *www.ingenuity.com/variants*. In these programs, the captured sequences were aligned with the human reference genome GRCh37 and a sequence of filters was applied.

For these analyses only variants with call quality of at least 40.0 and read depth of at least 20.0 were considered. Additionally, variants with allele frequency greater than or equal to 1.0% of the genomes reported in the 1000 genomes project (*www.1000genomes.org)*, the public Complete Genomics (*http://www.completegenomics.com/public-data/*) or NHLBI ESP exomes (*http://evs.gs.washington.edu/EVS/)* were also excluded from further analyses. In addition, through these bioinformatics tools, we used a filter to select only variants that were present in the patient's DNA, but absent in the two control exomes from our laboratory data bank (one adult individual with familial prolactinoma but no melanoma and one elderly individual with lung cancer but no melanoma). The selected genes carried homozygous or heterozygous sequence variants that occurred in the genotyped case and did not occurred in the controls.

3.4.2 Ingenuity® Variant AnalysisTM

In addition to the above listed confidence and frequency criteria, variants were selected if they fulfilled the following criteria: association with gain or loss of function, classified as nonsense, missense, *frameshift* or indels, homozygous or heterozygous; and were also either pathogenic or possibly pathogenic by Polyphen-2 (Choi *et al.*, 2009) and pathogenic or non-innocuous by SIFT, PROVEAN and CADD (Dong *et al.*, 2015).

Considering the biological context, the following key words were selected from *Ingenuity® Variant Analysis™* (Ingenuity biological analysis): melanoma, cutaneous melanoma, hereditary melanoma, early-onset familial melanoma, familial melanoma cancer, primary familial melanoma (autosomal dominant melanoma). The majority of familial cases up to date lack identifiable germ-line mutations in known susceptibility genes, so it is believed that a lot of potentially causative genes are yet to be discovered (Hawkes, Truong and Meyer, 2016). Therefore, the analysis was later extended to genes not classically associated with known diseases or genes whose biological role is still not clear.

3.4.3 Mendel, MD

The pathogenicity prediction of the variants using this algorithm was performed by selecting variants that were present in the patient, but not in the controls, and were assigned as pathogenic (or not benign) by all of the below listed prediction models PROVEAN (Choi *et al*., 2012), SIFT, PolyPhen and CADD (Dong *et al*., 2015).

3.4.4 Mutation Analysis

The results available from the *Ingenuity®* platform that considered a list of previously published proteins in melanoma was added to the list of genes selected through the *Ingenuity®* analysis that had no associated diseases and resulted in one mutated gene single list. This list was than combined to the analyses performed the same way using *Mendel, MD* and only those genes that were present in both analyses were further analyzed (an illustrative flowchart of this method is shown in figure 15, section 4.2).

The possible association between melanoma development and selected candidate variants was analyzed using the flowing parameters:

- Gene function and related pathways, using *Genecards* (http://www.genecards.org/), *OMIM* (https://www.omim.org/) and *UniProt* (http://www.uniprot.org/) databanks.
- Presence in COSMIC (http://cancer.sanger.ac.uk/cosmic) databank: for the mutations already included in COSMIC, it was stablished with types of cancers they were associated with.
- Presence in *Ensembl* databank: for the mutations already included in this databank, their frequency in populations and associated phenotype were stablished.
- Mutations effects observed in animal models in *Mouse Genome Informatics - MGI* (http://www.informatics.jax.org) and HomoloGene (http://www.ncbi.nlm.nih.gov/homologene) databanks.
- Carcinogenic effects described in Pubmed publications (https://www.ncbi.nlm.nih.gov/pubmed/).
- Deleterious effects, using bioinformatic tools through *SIFT*, *Polyphen, Provean* e *CAAD*. Their scores are shown in table 8. In this step, only variants predicted to be deleterious by most of these algoritms (3 of 4) were selected.

After these selection steps, a list of final candidate genes was generated (table 9, section 3.3.6).

Table 8. Tools for predicting the functional impact of a nonsynonymous genetic variation and their scores.

Substitution type	Scores			
	SIFT	***Polyphen***	***Provean***	***CAAD***
Intolerant or deleterious predicted substitutions[1]	< 0,05	> 0,43	< 2,5	>15
Benign or non-deleterious predicted substitutions	> 0,05	< 0,43	> 2,5	<15

[1]In Polyphen, the mutations with harmful potential are classified as possibly deleterious or probably deleterious.

3.3.5 WES sequence data validation

After performing WES and selecting variants for validation as outlined above, the alterations found were further validated using Sanger sequencing on an independent DNA sample from the same individual. These gene specific variants and their exon-specific flanking primers are described in table 9.

Furthermore, a comparison between the number and type of variants in *MC1R* in hereditary melanoma, multiple primary melanoma patients, sporadic melanoma patients diagnosed under 50 years of age and sporadic melanoma patients diagnosed over the age of 50 were also validated via Sanger sequencing, using the set of primers described in table 9.

DNA extracted from peripheral blood from all consenting family members as well as 102 ethnicity-matched controls without any discernible personal or family history of malignant tumors (recruited under an Ethics Committee approved protocol from amongst individuals who currently attend the Geriatric Clinic of the Universidade Federal de Minas Gerais) were tested for selected sequence variants that were seemingly pathogenic and causative. After DNA isolation all relevant genes were amplified by PCR with primers specific for each region (Table 9). PCR products were purified using PCRLink^{TM} Quick PCR Purification Kit (Life technologies, Carlsbad, California) following the manufacturer's protocol and visualized on a

silver-stained 6.5% polyacrylamide gel. Sequences were obtained on ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Bidirectional sequence data were analyzed using Sequencer 4.9 software.

Table 9. Primer list for whole-exome sequencing gene validation after WES.

Gene	Exon	Sequence (5'- 3')	Size (pb)
CDC27	14	**F,** AGTCTGCAACGGGAACATGA **R,** CCAGCACCATCAATACGACT	229
ABCA4	20	**F,** AGTTTGACTGACAGCCCCAG **R,** TCTCAGTTCCTGTGTCGCTT	278
ELOVL5	4	**F,** TCTACTCTTCTCCTGCTGTGC **R,** GGAGTGCCTCCTTCTGAGGAA	244
GAMT	5	**F,** CACTCCCCTTCCACAGAACCAC **R,** GGTCACTTCCTGGAGACCCAT	170
ANKRD27	21	**F,** TCAAGCCAATAGATTTTCAGAATG **R,** AGCCTGGGTGACAGAACAAG	210
OR4C3	1	**F,** GTGTTACAGAAAATAATCCTGACCA **R,** GTTTCTGTGCCTCTGCGTTC	184
MC1R	A (first part of the only exon)	**F,** CTGCCAGCACCATGAACTAA **R,** TCCAGCAGGAGGATGACG	481
	B (middle part of the only exon)	**F,** GAACCTGCACTCACCCATGT **R,** TTTAAGGCCAAAGCCCTGGT	514
	C (third part of the only exon)	**F,** GCTGTACGTCCACATGCTG **R,** ACAGGAACCAGACCACACAA	273
XPC	8	**F,** TTGAACAAGCACCATAACAAACAAC **R,** TGCCCAAGTCTTCCCTAACACAG	335

F, forward sequence; **R**, reverse sequence

3.5 Genotype-Phenotype correlations

Clinical data about the patients were studied through medical records, personal interview and physical examination when possible. Information regarding age of tumor onset,

number of tumors, localization, Breslow of tumors, personal history of cancer, family history of cancers, other possible personal risk factors (e.g., immunosuppression) and history of sun exposure were assessed.

3.6 Statistical analyses

The comparisons of proportions were performed using the chi-square test, Fisher's test and Monte Carlo adjustment when necessary. The level of significance used was 5% and the software used for all analyzes was Minitab version 18.

4. RESULTS

4.1 CDKN2A mutation analysis

Eleven familial melanoma patients, eight multiple primary melanoma patients and 25 sporadic melanoma patients, a total of 44 cases (clinical detailes are shown above, in table 5 on pages 71 and 72; in table 6 on page 76), were genotyped for defining the PSVs in the *CDKN2A* gene. All affected members of families A, B, C and D, and six multiple primary melanoma patients (F, G, H, I, J, K, L) harbored no sequence variants in both exons of *CDKN2A*. One MPM patient showed a mutation in exon 2 (detailed below in item 4.1.2). Five patients (one MPM and four sporadic melanoma patients) showed a polymorphism in exon 2 (detailed below in item 4.1.3).

4.1.2 *CDKN2A* Mutation A109P in a MPM patient

One individual (E) has a missense single nucleotide variant (SNV) in exon 2, p.Ala109Pro (c.325G>C), located in 9p21.3, transcript ENST00000304494.5, which was not detected in all 102 controls analyzed in this study (data not shown). This SNV (rs372481694) (Ensembl databank), has been classified as "Variant of Uncertain Significance" by a refinement of the ACMG–AMP variant classification criteria https://www.ncbi.nlm.nih.gov/clinvar/RCV000475093/ (Nykamp *et al*., 2017). The frequency of this variant in GnomAD is 0.00002 and this variant is not described in the Brazilian Genomic Variants databank (http://abraom.ib.usp.br/search.php) .

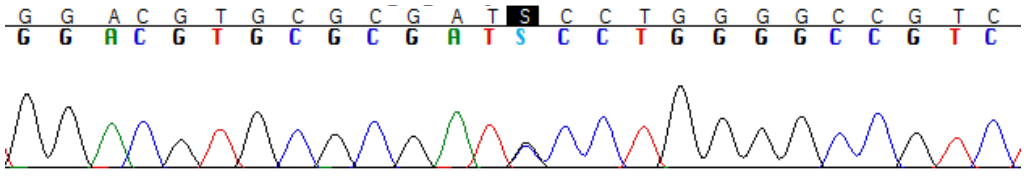


Figure 16. Representative electropherogram of the missense mutation A109P in heterozygosis in *CDKN2A* gene.

The patient bearing this variant is a 36-year-old woman, with Dysplastic Nevus Syndrome (more than 100 nevi, many atypical), fair skin and light hair (Fitzpatrick type II), who has had three thin cutaneous melanomas (< 1 mm Breslow) at ages 18, 23 and 33. She also has had two BCC on the face. She has one offspring who is 2 years old and her parents did not agree to participate in this study. There is no evidence of melanoma in both parents who are in their late sixties.

There is a personal history of some episodes of sunburn in childhood, including blisters. She is otherwise healthy and takes no medications. There is no history of cancer in her first-degree relatives.

Bioinformatic analysis of genomic sequence variants by some of the most utilized tools are described in table 10. Other tools that predict the effect of this amino acid change have also been applied, resulting in: "cancer" (score -5.58) in FATHMM (http://fathmm.biocompute.org.uk/); "disease" (score 0.552) in phD-SNP (https://snps.biofold.org/phd-snp/phd-snp.html ; score +24 in SNAP (score varies from -100 to + 100, both being the extremes of benign or deleterious, zero being neutral effect - https://rostlab.org/services/snap2web/). The Panther Classification System predicted "probably benign" (http://www.pantherdb.org); SNPs&Go predicted "neutral" (https://snps.biofold.org/cgi-bin/SNPs-and-GO.cgi) and Mutation Tester predicted protein might be affected but classified it as a polymorphism (http://www.mutationtaster.org/).

Table 10. Analyses of the single nucleotide variant (SNV) effect on protein structure and function by Polyphen, SIFT, PROVEAN and CADD.

Gene	Chr	Altered base	Altered amino acid	*PROVEAN*	*SIFT*	*Polyphen2*	*CADD*
CDKN2A	9	Gcc/Ccc	p.Ala109Pro	Deleterious - 2.57	Tolerated 0.29	Probably damaging (0.999)	Deleterious 22.2

Cutoff: PROVEAN (-2.5), SIFT (0.05), PPH2 (0.95) CADD (15).

Protein structure homology-modelling servers demonstrate that this SNV is located in a loop region within the CDKN2A protein (accessible in https://swissmodel.expasy.org/), as shown in figure 16.

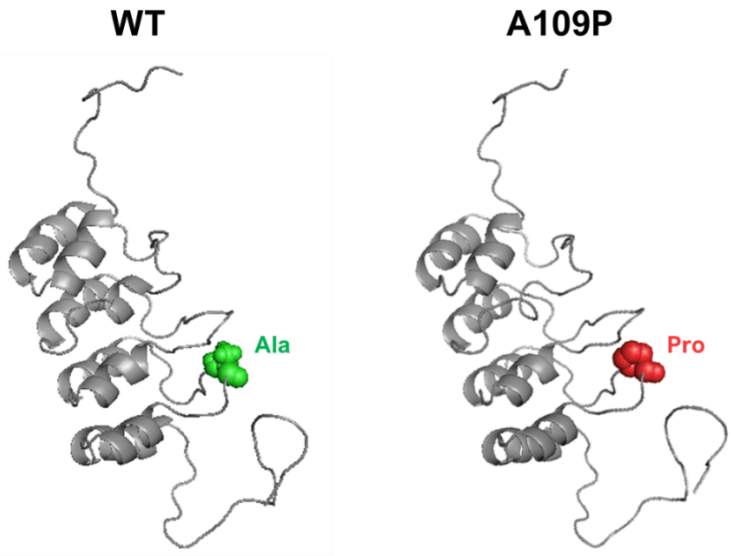


Figure 17. Swiss model protein structure homology-model represents the ALA109PRO located in a loop in the CDKN2A molecule (https://swissmodel.expasy.org/).

The clinical impact of this variant will be further understood with the follow-up of this patient and her descendents, but it is reasonable to assume that, as many *CDKN2A* mutations known up to the moment, this genetic alteration contributes to the phenotype of multiple atypical moles and early melanoma onset in this carrier.

4.1.3 *CDKN2A* polymorphism A148T

Among all 44 melanoma patients studied (19 familial/MPM and 25 sporadic melanoma patients), five (11.3%) presented the missense SNV in the *CDKN2A* gene: p.A148T; rs3731249 (Ensembl databank). Of these carriers, one is a MPM patient, two have had sporadic MM above the age of 50 and two have had MM prior to the age of 40. In control samples, the prevalence of this genomic alteration was 1.9% (two in 102). The percentage of p.A148T among cases is statistically significantly higher than the control group (p = 0,026; 95% CI [-0.0035-0.1916]).

This molecular alteration has been reported to be a polymorphism (present in roughly 2% of GnomAD) and previous functional assays have demonstrated normal protein activity in comparison to wild-type (McKenzie *et al*., 2010). In this case, prediction algorithms have classified it as either tolerant (SIFT 0.16) or benign (PolyPhen). It has been associated to elevated risk of ovarian cancer in a meta-analysis (Dong *et al.*, 2017). Additionally, it is important to note that this SNV has been associated with increased melanoma risk in some populations, including Brazilians. A population-based Polish study with 471 patients with MM has found a significantly increased frequency of this variant in comparison to 1,210 controls (7% vs 2.9%) and suggested the A148T seems to be associated with an increased risk of development of MM (Debniak *et al*., 2005). Bakos *et al*. (2011) showed that it is significantly more frequent in melanoma patients than in controls (12.6% vs 3.9%) among southern Brazilians (127 patients and 128 controls were enrolled), results similar to our findings. In the Brazilian Genomic Variants databank (http://abraom.ib.usp.br/search.php) it is described in 0.024765 of alleles.

4.2 Whole exome sequencing analysis

Variant calling from WES of the genotyped patient's DNA (II.13 of family A) resulted in 46,452 variants in 13,271 genes, the mean base call quality was 1,507 and average read depth was X96.

After the above specified filtering steps and analyzing only variations present in both softwares (Ingenuity® and Mendel, MD), a total of 187 sequence variants in 76 genes were targeted for further analysis. Excluding variants that were present in three control exomes from nonmelanoma patients (two familial prolactinomas and one lung cancer) – a total of 40 variants in 30 genes was defined. Then, manually, genes that were associated to known diseases (but not melanoma) in the literature were further excluded and the remaining variants were then analyzed according to the ACMG Standards and guidelines for the interpretation of sequence variants by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards *et al.*, 2015). This method is a sequence of steps with questions that should be answered by each type of variant after extensive literature and databank reviews, culminating in a classification of variants being one of the following five categories: "pathogenic", "likely pathogenic", "uncertain significance", "likely benign" or "benign" with greater than 90% certainty of a variant either being disease-causing or benign. The list of eight variants in genes that could not be excluded by any of the above steps and were classified as "pathogenic", "likely pathogenic" or "uncertain significance" by ACMG were then selected for subsequent validation by Sanger sequencing. Additionally, considering the possibility of an autosomal recessive trait in this family, a filter selecting only homozygous variants was used, resulting in only three variants in genes *CXarf22*, *OR6B1* and *XPC*, of which *XPC* was included.

Table 11. Sequence of filters used for both programs *Ingenuity Variant Analysis*™ and Mendel, MD.

Program	*Ingenuity Variant Analysis*™	Mendel, MD
1st filter	Variants with *read depth* ≥ 20 and *call quality* ≥ 40	Variants with *read depth* ≥ 20 and *call quality* ≥ 40
2nd filter	Variants with allelic frequency lower than 1% in *1000 Genome Project*, *ExAC*, *NHLBI ESP exomes* and *Allele Frequency Community*	Variants with allelic frequency lower than 1% in *1000 Genome Project* and dbSNP; and score between 0 to 0,05 in *SIFT* and 0,5 a 1,0 in *Polyphen*
3rd filter	Variants with pathogenic, possibly pathogenic, or uncertain phenotypes OR variants associated to gain of function in literature by BSIFT and *Ingenuity*, variants from genetic rearrangements or present in microRNA-ligand sites; OR variants that show insertion and deletion of DNA sequences.	Variants classified as *frameshift*, indel, missense or nonsense.
4th filter	Select homozygous or heterozygous variants that are present only in patient II.13 from family A but absent in two control exomes of our databank	Select homozygous or heterozygous variants that are present only in patient II.13 from family A but absent in two control exomes of our databank
5th filter	Variants already associated to melanoma in anyway and exclude variants associated to other biological condition not related to skin cancer	Variants with a high or moderate impact on protein structure

Two methods of whole-exome variant analyses were gathered together to produce a final list of genes, then a comparison with control exomes was done and the final list was then manually narrowed down both by literature research and by the ACMG guidelines on variant classification (Richards *et al.*, 2015), resulting in a final list of eight selected genes for subsequent study by Sanger Sequencing.

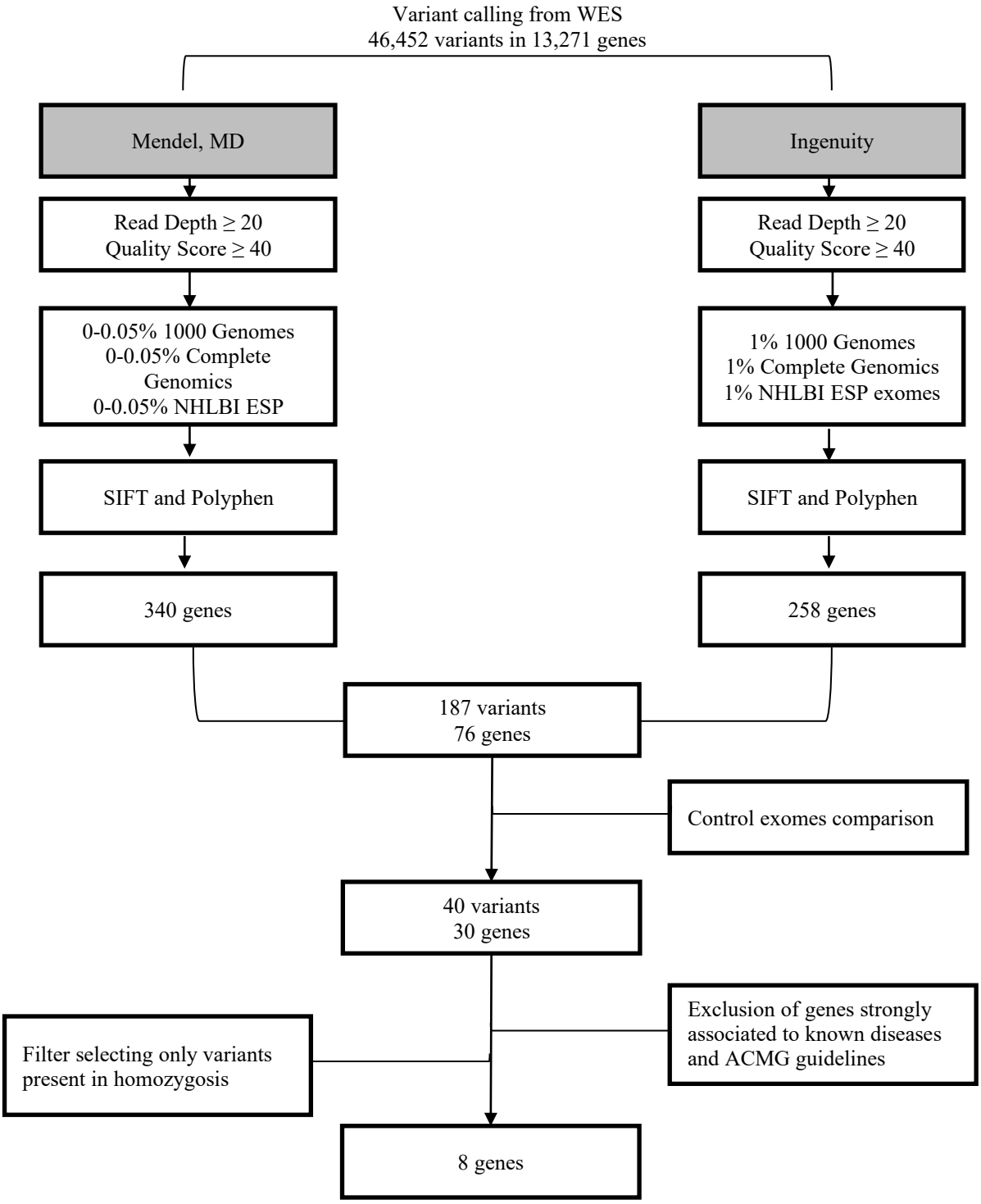


Figure 6. Flowchart of variant analyses schemes.

Table 12. Factors evaluated for gene/mutation selection in Family A after exome sequencing with an example of a candidate gene.

Genes (variants, analyzed criteria)	*MC1R (p.I155T)*
Are there studies of altered expression of this gene in tumors?	Yes
The protein synthetized by this gene is enrolled in DNA repair, genetic rearrangement, angiogenesis, melanogenesis or cellular proliferation?	Yes
The mutation has deleterious potential?[1]	Yes
The mutation has been described in any type of cancer?	Yes
The mutation has MAF <1%?	Yes
Are there animal models that evaluate this mutation effect?	No
Variant classification according to 2015 ACMG guidelines.	Likely pathogenic Strong evidence of pathogenicity (PS3) AND two supporting evidences of pathogenicity (PP3 and PP5)

Subtitle: [1]= At least in two of the following tools: *Provean*, *Polyphen2*, *SIFT* and *CADD*.

Table 13. Selected variants for validation with gene, dbSNP ID, type of genomic alteration, in silico prediction by SIFT/PolyPhen, available phenotypic information, frequency in gnomAD and frequency in AbraOM.

Gene/Variant	dbSNP ID	Type	SIFT; PolyPhen	Phenotypic information	GnomAD	AbraOM
ABCA4 p.T977M	rs148015012	M	D; PD	None reported	0.000147	0.000427
ANKRD27 p.D681G	N/A	M	D; PD	Albinism	N/A	N/A
CDC27 p.R625*	rs77685276	N	N/A; PD	None reported	N/A	N/A
ELOVL5 p.Q102*	rs150583340	N	D; PD	Spinocerebellar ataxia type38 (2019)	0.006355 (GMAF 0.00339)	0.00854
GAMT p.L171V	rs770110177	M	D; PD	None reported	0.000048	N/A
MC1R p.I155T	rs1110400	M	D; PD	MM susceptibility	0.005640	0.005551
OR4C3 p.P192L	rs73464001	M	D; N/A	None reported	N/A	N/A
XPC p.R307W	rs367961191	M	D; PD	Xeroderma Pigmentosum group C	0.00002422	N/A

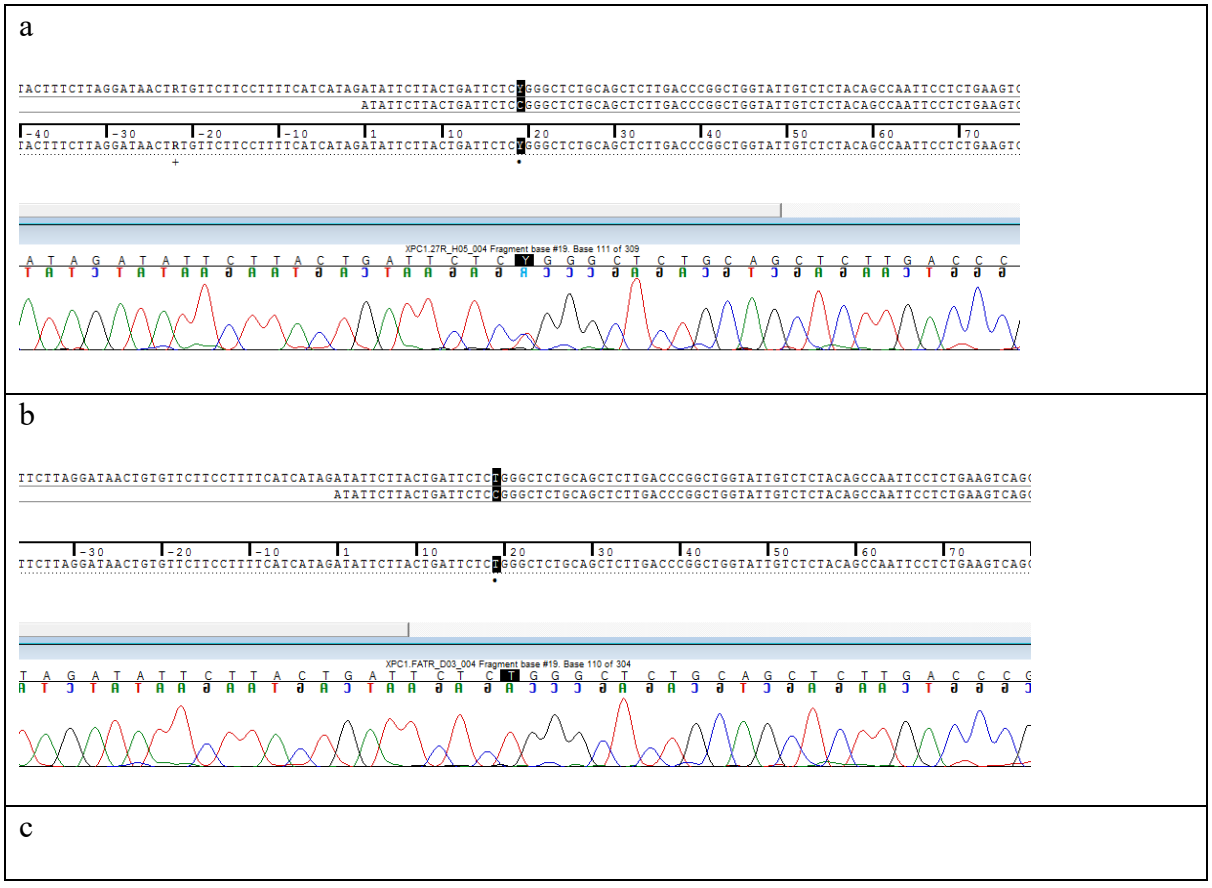
D: Damaging; PD: Probably Damaging; M: Missense; MM: Malignant Melanoma; N: Nonsense; N/A: Not available.

4.3 Exome detected pathogenic variants validation via Sanger sequencing

The nonsense variant found in *CDC27* was not validated by Sanger sequencing. Analyses of the allele distribution of *ABCA4, ANKRD27, ELOVL5, GAMT,* and *OR4C3* among carriers and their relatives in available family cases of families A, B, C and D demonstrated that variants in these genes were unlikely to account for these familial cases of melanoma. The

MC1R missense variant described was validated and the whole gene sequence was studied as described in section 4.4.

The variant p.Arg307Trp (c.919C>T) in exon 8 of *XPC* (NM_004628.5) was validated through Sanger sequencing in all available individuals of the three generations to confirm disease segregation (*n*=15). Sequencing demonstrated that the mother, who is not clinically affected, is heterozygous (C/T) for the referred mutation (fig.19a). All five affected individuals studied (individual II.10 did not want to participate in this study) are homozygous (T/T) for the mutation (fig.19b). Non-affected individuals II.1 and II.2 are heterozygous (C/T) and non-affected individual II.12 is homozygous with both wild-type alleles (C/C) (fig.19c). Offspring of affected individuals II.6, II.8 and II.11 are heterozygous (C/T) and offspring of II.12 is homozygous wild type (C/C).



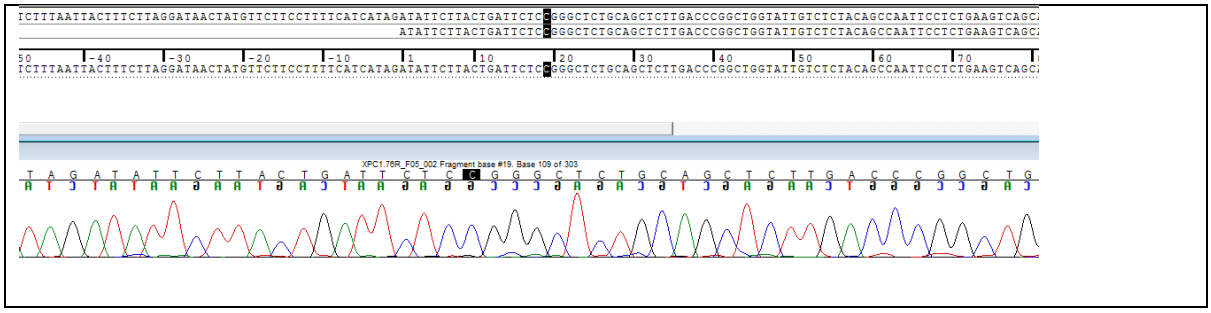


Fig.19. The genetic molecular studies identified a pathogenic missense mutation in *XPC* (c.919C>T; p.Arg307Trp). **A.** Heterozygosis (C/T) in the healthy mother. **B**. Homozygosis (T/T) in all affected individuals. **C.** Homozygosis wild type (C/C) in a normal sibling.

This variant has not been reported in the Brazilian Genomic variant database (ABraOM - https://abraom.ib.usp.br/search.php) and its frequency in international databanks is very low (0.000026 in TOPMED; 0.000024 in GnomAD_exome; 0.000036 in GnomAD; 0.00008 in ALFA). It had no clinical significance reported up to the present study.

The variant was analyzed by ten different mutation prediction tools and seven of them suggested it has significant negative effects on the protein, as shown in table 14.

Table 14. Mutation prediction algorithms consulted for the mutation A307T in *XPC* and the predicted consequence in the protein.

Software	Result
FATHMM	PASSENGER/OTHER (1.26)
Mutation Assessor	MEDIUM (2.6)
PANTHER	PROBABLY DAMAGING (preservation time: 1628 / Pdel 0.89)
PhdSNP	DISEASE (0.900)
SNPs&Go	NEUTRAL (0.421)
Mutation Taster	DISEASE CAUSING (amino acid sequence changed protein features (might be) affected splice site changes)
PROVEAN	DELETERIOUS (-6.872)
SNAP2	Effect Score: 70 (EFFECT)
SIFT	AFFECT PROTEIN FUNCTION (0.00)
PolyPhen	PROBABLY DAMAGING (1.00) sensitivity: 0.09; specificity: 0.99

4.4 *MC1R* gene analysis

Given the known association of *MC1R* gene and melanomagenesis as well as the uncertainty about the pathogenic role of the p.Ile155Thr variant, detected initially through WES, sequence analysis was extended to the entire 954-bp long exon of the gene and to the following study participants: 8 MPM, 11 familial melanoma affected individuals, 9 healthy familial melanoma members of family A, and 25 sporadic melanoma patients - 44 affected individuals - and 102 non affected controls.

MC1R nonsynonymous variants were detected in 27/44 (61.4%) of CM patients studied, compared with 14/102 (13.7%) in controls, being statiscally significant ($p < 0.001$; CI [0.32-0.63]). A total of thirteen different sequence variants were noted in the cases, 11 of which are nonsynonymous variants. Of these, one was a novel variant (M203T) and two are very rare variants (Q23X and D187H - frequency in genome databanks below 0.05%). One of the RHC variants (R151C, R160W, D294H) was found in 20,4% of cases, but none carried two RHC variants. A simplified list of all of them, with their incidences in the Genome Aggregation Database (gnomAD), in the Brazilian Genomic Variants Database (ABraOM), their incidence among the patients studied and in controls is shown in table 15.

Table 15. List of all *MC1R* variants found among melanoma patients, their incidence in gnomAD, ABraOM and controls.

Variant	GnomAD incidence	ABraOM* incidence	Familial melanoma	MPM	Sporadic MM < 50	Sporadic MM > 50	Frequency in cases	Frequency in controls
Q23X	0.0003039	0.000854	0	1	0	0	2.3%	Zero
V60L	0.06974	0.098207	0	2	2	1	9.0%	3.3%
V92M	0.07634	0.037575	0	0	1	0	2.3%	0.7%
L106L	0.002736	0.005551	0	0	0	0	2.3%	Zero
R142H	0.005323	0.003416	0	1	0	0	4.5%	Zero
R151C	0.044158	0.014091	0	0	0	2	4.5%	1.5%
I155T	0.005640	0.005551	5	1	1	1	18.2%	Zero
R160W	0.047166	0.022203	0	0	1	0	2.3%	Zero
R163Q	0.151540	0.093937	2	1	0	0	11.3%	6.1%
D184H	0.00003	Not reported	0	0	1	0	2.3%	Zero
M203T	0.000004	Not reported	0	0	1	0	2.3%	Zero
D294H	0.00972	0.014091	1	1	2	2	13.6%	Zero
T314T	0.142913	0.145175	4	0	2	1	22.7%	21.5%

*ABraOM: Brazilian Genomic Variants Database

Results of variants in the *MC1R* among affected individuals and their main clinical characteristics are shown in table 14 for familial melanoma members, table 15 for MPM patients and table 16 for sporadic melanoma patients, according to age of diagnosis (before or after 50 years of age).

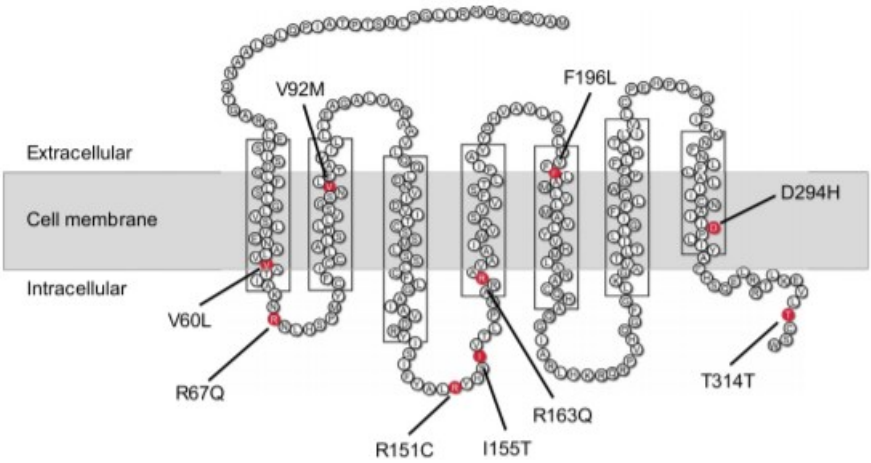


Figure 7. MC1R transmembrane protein, its amino acid composition, and the localization of some of the SNP found in this study. From (Carter *et al.*, 2018).

Table 14. *MC1R* results of all subjects of family A.

Subject	Phenotype	Skin Photo type	Age of onset	Breslow thickness of tumors	Tumor location	*MC1R* variants
Family A I.1	Non-affected mother of six affected individuals	III	N/A	None	N/A	None
Family A II.1	Non-affected sibling	III	N/A	None	N/A	None
Family A II.2	Non-affected sibling	III	N/A	None	N/A	None
Family A II.3	Non-affected sibling	III	N/A	None	N/A	p.Ile155Thr (155T)
Family A II.4	Affected sibling, 3 MM	III	45	All *in situ*	Head (3)	None
Family A II.5	Non-affected sibling	III	N/A	None	N/A	None
Family A II.7	Affected sibling, 11 MM	III	33	All *in situ*	Head (3), neck (1), limbs (2), trunck (5)	p.Ile155Thr (155T)
Family A II.8	Affected sibling, 7 MM	II	35	Six *in situ*, one 0.24mm	Head (3), neck (2) trunk (2)	None
Family A II.11	Affected sibling 24 MM (3 thin, 2 thick and 19 *in situ*)	II	28	1.4 mm; 0.9 mm; 0.45 mm; 0.47 mm; 2.0 mm and the rest *in situ* (Median Breslow thickness 0.217 mm – rage from *in situ* to 2 mm)	Head (19), neck (2), trunk (3)	p.Ile155Thr (I155T)

Subject	Phenotype	Skin Photo type	Age of onset	Breslow thickness of tumors	Tumor location	*MC1R* variants
Family A II.12	Non-affected sibling	III	N/A	None	N/A	None
Family A II.13	Affected sibling 7 MM	III	22	2.3 mm; 0.36 mm; 0.23 mm and 4 *in situ* (Median Breslow thickness 0.4128 mm – rage from *in situ* to 2.3 mm)	Head (3), neck (3), trunk (1)	p.Ile155Thr (155T) p.Thr314Thr (T314T)
Family A III. 12	Non-affected son of affected deceased metastatic melanoma patient II.6	III	N/A	None	N/A	None
Family A III.15	Non-affected son of affected sibling II.8	III	N/A	None	N/A	None
Family A III. 22	Non-affected daughter of affected sibling II.11	III	N/A	None	N/A	p.Ile155Thr (155T) p.Leu106Leu (L106L)
Family A III.24	Non-affected son of non-affected sibling II.12	III	N/A	None	N/A	None

MM: Malignant Melanoma; N/A: Non-Applicable.

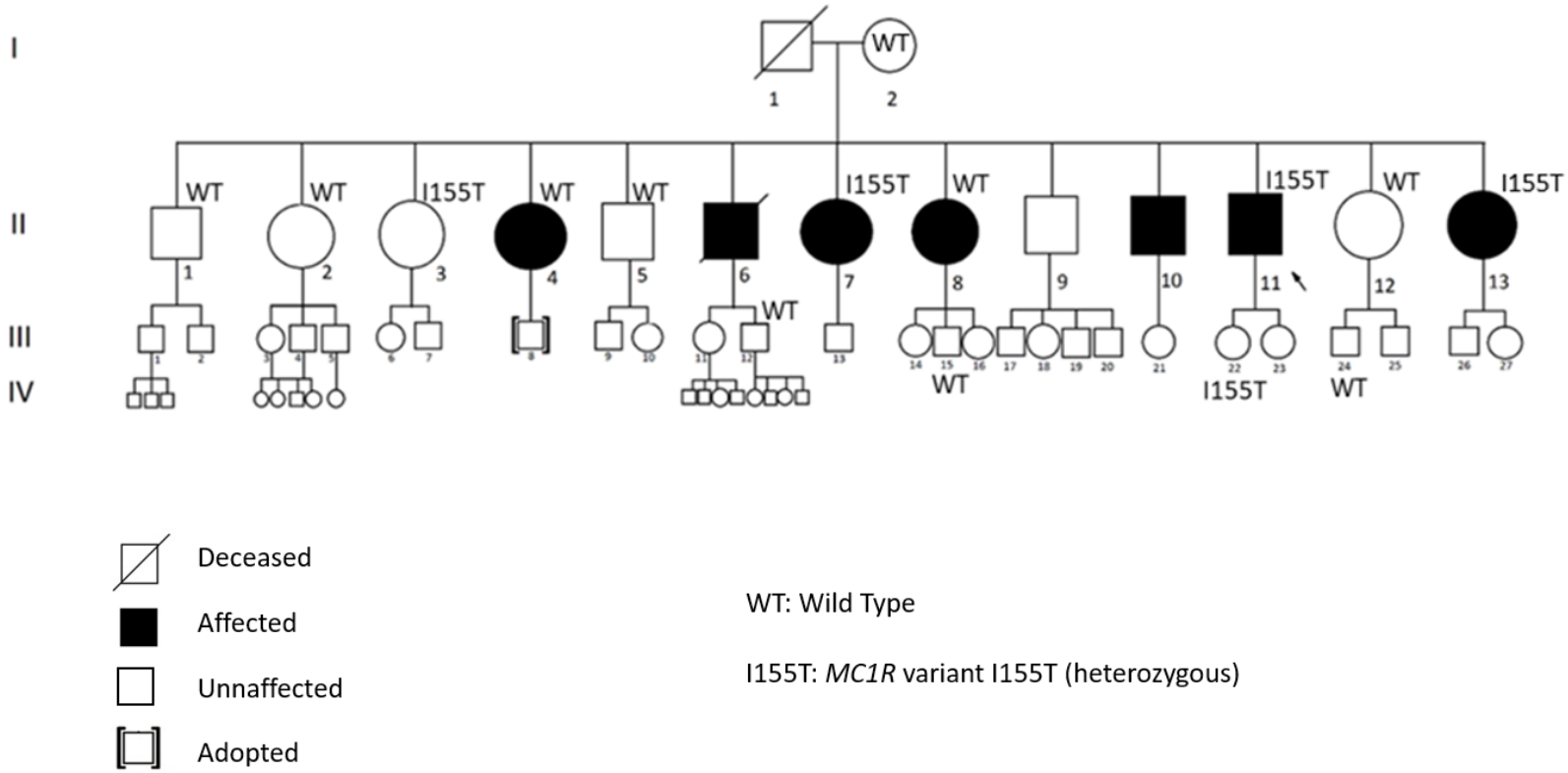


Figure 21. Pedigree of family A with *MC1R* genotypes for better evaluation.

Table 17. Age of first melanoma, number of melanomas, tumor Breslows and number of carcinomas in comparison to the *MC1R* status in affected XP individuals.

Individual (XP-C homozigous)	**Age of first melanoma**	**Number of melanomas**	**Tumor Brelows**	**Carcinomas**	***MC1R* status**
II.4	45	3	*In situ*	0	WT
II.7	33	10	*In situ*	2 BCC	I155T
II.8	35	7	Range from *in situ* to 0.24 mm	3 BCC	WT
II.11	28	24	Range from *in situ* to 2.0 mm	2 BCC 1 Basosquamous	I1557
II.13	22	7	Range from *in situ* to 2.3 mm	1 BCC	I155T

Table 15. *MC1R* results for all olther familial melanoma subjects of family B, C and D.

Subject	Phenotype	Skin Photo type	Age of onset	Breslow thickness of tumors	Tumor location	*MC1R* variants
Family B II.1	Affected sibling, 01 MM	II	53	0.8mm	Limb (arm)	p.Ile155Thr (155T) p.Thr314Thr (T314T)
Family B II. 3	Affected sibling, 01 MM	II	55	0.65mm	Trunk (back)	p.Ile155Thr (155T) p.Thr314Thr (T314T)
Family C I.1	Affected father, 01 MM	II	>50	1.0mm	Trunk (shoulder)	p.Arg163Gln (R163Q) p.Thr314Thr (T314T)
Family C II.1	Affected son, 01 MM	II	40	0.7mm	Trunk (back)	p.Arg163Gln (R163Q)
Family D II.2	Affected sibling, 01 MM	II	48	0.3mm	Neck	None
Family D II.3	Affected sibling, 01 MM	II	35	*In situ*	Trunk (back)	p.Asp294His (D294H)

Within the familial melanoma group, although no mutations in the *CDKN2A* gene were detected as described in section 4.1, nonsynonymous variants in *MC1R* were found in 72,7% (8/11) of affected individuals, of whom 50% (4/8) showed only one variant (either Ile155Thr, Arg163Gln or Arg294His, all non-synonymous) and half showed two variants but in all these double-mutant cases, the second variant was a synonymous amino acid change (Thr314Thr). In the non-affected child carrier of Ile155Thr, the second variant was also a synonymous amino acid change (Leu106Leu).

Table 16. *MC1R* results of all multiple primary melanoma subjects.

Subject	Phenotype	Photo type	Age of onset	Breslow thickness of tumors	Tumor location	*MC1R* variants
Individual E	03 MPM Atypical Nevi Syndrome	II	17 (17-23)	All *in situ*	Trunk (3)	p.Arg163Gln (R163Q) (*CDKN2A*-mutation carrier)
Individual F	07 MPM Atypical Nevi	II	18 (18-28)	0.6 mm; *in situ;* 0.28 mm; *in situ; in situ;* 0.55 mm; i*n situ* (Median Breslow thickness 0.20 mm)	Trunk (6) and limbs (1)	p.Gln23Ter (G23X) p.Val60Leu (V60L)
Individual G	03 MPM	III	53 (53-57)	2.15 mm; *in situ* and 0.38 mm (Median Breslow thickness 0.84 mm)	Trunk (3)	p.Val60Leu (V60L) p.Arg142His (R142H)
Individual H	02 MPM	III	59 (59-60)	0.6 mm and 0.9 mm (Median Breslow thickness 0.75 mm)	Trunk (2)	None
Individual I	06 MM	II	45 (45-57)	All *in situ*	Head (1), limbs (1), trunk (4)	None
Individual J	06 MM	II	35 (?) (35-84)	1.2mm; 3.0mm; 8.0 mm and *in situ*	Head (1), limbs (2), trunk (2)	p.Asp294His (D294H) (homozygosis)
Individual K	03 MPM	III	63 (63-65)	All *in situ*	Head (1), neck (1), trunk (1)	None
Individual L	03 MPM	II	48 (48-58)	0.3mm and 2 *in situ* (Median Breslow thickness 0.1 mm)	Trunk (2)	p.Ile155Thr (I155T)

MM: Malignant Melanoma; MPM: Multiple primary melanoma.

Within the MPM group, where the frequency of mutated *CDKN2A* was 12,5% (one in eight), the frequency of non-wild type *MC1R* was high (62,5%) in line with the findings of (Bassoli *et al.*, 2013) reporting a prevalence of 70,3% of *MC1R* variant-carrier (and a much higher frequency of 40,7% of *CDKN2A*-mutated individuals) among 27 MPM Italian patients (Bassoli *et al.*, 2013). Among MPM individuals studied herein with *MC1R* variants, we found the presence of only one *MC1R* variant in 37,5% (3/8), the presence of two nonsynonymous variants in 25% (2/8) and no patient had the presence of three variants. Bassoli *et al*. (2013) found that MPM patients carrying at least one *MC1R* variant were more often younger than 40 years old at the time of first melanoma diagnosis and with heavy sun-damage compared to wild-type gene carriers. Indeed, all (3/3) MPM individuals who had melanoma before the age of 40 carry at least one nonsynonymous *MC1R* variant but sun-damage was more prevalent among wild-type individuals (data not shown), which could be partially attributable to the older ages of the non-carriers in our subset of patients. Two patients presented variants considered RHC variants by some authors (R142H and I155T). Only one patient had one of the classic RHC variants (D294H) and she was homozygous. Of note, she presented the worse disease course of all MPM patients, having multiple, amelanotic and deeper melanomas, including metastasis.

Table 20. *MC1R* results of sporadic melanoma patients with main clinical characteristics.

	Patient ID	**Age of MM**	**Skin Photo type**	**Breslow thickness**	**History of sunburns**	**Other skin cancers**	**Number and type of *MC1R* variants**
Sporadic MM before 50 years (13)	MFA 43	46	III	0.75 mm	Intense unprotected exposition	None	None
	MFA 49	29	IV	*In situ*	Yes, blistering	None	p.Thr314Thr (T314T)
	MFA 55	35	III	5.0 mm with inguinal metastasis	None	None	**p.Arg160Trp (R160W)** p.Arg163Gln (R1163Q)
	MFA 57	31	III	*In situ*	Yes (one episode)	None	None
	MFA 60	20	II	*In situ*	Yes (few)	None	**p.Asp294His (D294H)**
	MFA 61	19	III	1.8 mm with metastasis	None	None	p.Arg163Gln (R1163Q)
	MFA 64	37	III	*In situ*	None	None	None
	MFA 65	22	II	*In situ*	Yes (few)	None Red hair	p.Met203Thr (M203T) p.Thr314Thr (T314T)
	MFA 66	42	IV	*In situ*	Yes	Few atypical nevi	p.Thr314Thr (T314T)

	Patient ID	Age of MM	Skin Photo type	Breslow thickness	History of sunburns	Other skin cancers	Number and type of *MC1R* variants
Sporadic MM before 50 years (13)	MFA 67	37	III	0.7 mm	Yes, blistering	None	p.Asp184His (D184H)
	MFA 70	28	III	0.74 mm	Yes (many)	None	**p.Asp294His (D294H)** p.Thr314Thr (T314T)
	MFA 63	30	II	*In situ*	Yes, blistering	None	None
	MFA 24	45	III	0.85 mm with CNS metastasis	Intense unprotected exposition	None (GCMN)	p.Ile155Thr (I155T)
Sporadic MM after 50 years (12)	MFA 42	52	III	1.2 mm	Intense unprotected exposition	BCC face	None
	MFA 44	50	IV	0.99 mm	Intense unprotected exposition	BCC face	p.Arg142His (R142H) **p.Asp294His (D294H)**
	MFA 45 jupira	62	IV	3.2 mm	Intense unprotected exposition	BCC shoulder	**p.Asp294His (D294H)**
	MFA 46	79	IV	*In situ*	Intense unprotected exposition	None	p.Thr314Thr (T314T)
	MFA 47	52	IV	0.43 mm	Intense unprotected exposition	None	None

	Patient ID	Age of MM	Skin Photo type	Breslow thickness	History of sunburns	Other skin cancers	Number and type of *MC1R* variants
Sporadic MM after 50 years (12	MFA48	57	III	*In situ*	Yes, blistering	BCC face; genital SCC	**p.Arg151Cys (R151C)**
	MFA51	66	II	*In situ*	Intense unprotected exposition	BCC face	None
	MFA53	53	II	1.2mm	Intense unprotected exposition	BCC face	p.Val60Leu (V60L) **p.Arg151Cys (R151C)**
	MFA56	61	III	0.4mm	Yes (few)	BCC face	None
	MFA58	55	III	0.95mm	None	None	p.Val60Leu
	MFA59	50	III	*In situ*	Yes	Adenoid carcinoma	p.Thr314Thr p.Val60Leu p.Val92Met
	MFA4	60	III	1.9mm	None	None (GCMN)	p.Ile155Thr (I155T)

*Sunburns are considered severe erythema, discomfort and/or blistering after sun exposure. Few: Less than three episodes in lifetime; Many: Three or more episodes in lifetime.
BCC: basal cell carcinoma; CNS: central nervous system; GCMN: giant congenital melanocytic nevus; MM: malignant melanoma; SCC: squamous cell carcinoma.

To further investigate the role of *MC1R* variants in melanoma, its association with sporadic and/or hereditary melanomas and phenotype, we analyzed 13 patients who have had only one superficial spreading melanoma (SSM) before age 50 years (10 of whom had MM before 40) without family history of melanoma (mean age at diagnosis 32.38 years – range 19 to 46) and 12 patients who have had only one SSM after the age of 50 (mean age at diagnosis 58.08 – range 50 to 79) without family history of melanoma and the results are shown in table 20.

Mean Breslow thickness in the melanoma group under the age of 50 years was 0.75mm (range from *in situ* to 5.0 mm) and in the group above 50 years of age was 0.85 mm (range from *in situ* to 3.2 mm.

MC1R nonsynonymous variants were found in 56% (14/25) of sporadic melanoma patients. Regarding the number of variants, wild-type or only synonymous variants is present in 46.1% (6/13) in the under 50 group and in 41.6% (5/12) in the above 50 group.

In order to better evaluate the impact of variants in terms of age of onset, we performed the statistical analysis between the ten individuals who had CM before the age of 40 versus the 12 individuals who had it after the age of 50. The total number of variants among the two groups (8/10 in the < 40 vs 8/12 >50) were not statistically significantly ($p = 0.47$). Other comparisons between the two groups, taking into consideration amount of non-synonimous variants, presence of RHC variants or pathogenic variants, are shown in table 21 and none was statiscally significant. Of the individuals who had sporadic melanoma (n=25) diagnosed before the age of 40 years (10 individuals), 80% (6/10 individuals) carried at least one *MC1R* non-synonimous sequence variant compared with 66.6% (7/12) of individuals in whom melanoma was diagnosed after age 50 years (12 individuals), suggesting *MC1R* might have an impact in age of diagnosis, although it was not statistically significant, probably because of the small sample size ($p = 0.97$). It is interesting to note that among the seven R variants carriers, 42.8%

had thick melanomas (>1mm Breslow). Of note, as for the MPM group, the most severe disease presentation in this group is also a carrier of a RHC classic variant (R160W).

Other skin cancers were absent in the melanoma group under the age of 50, while it was common in the melanoma group above 50 years (58.3% prevalence).

Table 21. Comparison between number and types of *MC1R* variants among sporadic melanoma patients stratified by age of onset (before 40 and after 50 years of age).

Variables	<40 years (n=10)	>50 years (n=12)	Proportion diference	95% CI for diference proportion	P value
At least 1 variant (any)	8 (80%)	8 (66.6%)	0,133	(-0,231; 0,497)	0,473*
At least 1 non-synonimous variant	6 (60%)	7/12 (58.3%)	0,017	(-0,396; 0,429)	0,937*
Only 1 non-synonimous variant	5 (50%)	4 (33.3%)	0,17	(-0,242; 0,576)	0,424*
2 non-synonimous variants	1 (10%)	3 (25%)	-0,15	(-0,458; 0,158)	0,594**
3 non-synonimous variants	0	0	-	-	-
No variants	4 (40%)	5 (41.6%)	-0,016	(-0,429; 0,396)	0,937*
RHC variants	3 (30%)	4 (33.3%)	-0,033	(-0,423; 0,356)	0,867*
Pathogenic variants (R160W, D294H, M203T?, I155T, D184H?, R142H, R151C)	5 (50%)	5 (41.6%)	0,083	(-0,334; 0,500)	0,695*

Chi-Square Test; Fisher´s Test

When all 44 melanomas cases (including familial, multiple primary and sporadic melanomas) are stratified by age of onset, 18 had CM before the age of 40 and 19 had CM after the age of 50. When the types of variants are analyzed, rather than the number of variants, in the group of patients with melanoma before 40 years, five (27.7%) are RHC carriers (D294H and R160W) and other 3 are rare variants carriers whose clinical significance is not yet so clear (Q23X, D184H ans M203T), resulting in a total of 44.4% (8/18) of pathogenic variant carriers (excluding I155T). In the group with late onset melanoma (after the age of 50), only three are RHC variants (D294H and R151C) carriers (16.6%) and another one has a rare pathogenic

variant (R142H, considered RHC by some authors (Ghiorzo *et al*., 2012; Duffy *et al*, 2019)), resulting in a 21.05% (4/19) of pathogenic variant carriers (excluding I155T), p = 0,170. Taking into consideration the variant I155T as pathogenic, the group of melanomas before the age of 40 shows pathogenic variants in 61.1% (11/18), in contrast to 42.1% (8/19) in the group of melanomas after the age of 50, p = 0,239.

4.4.1 Q23X Nonsense variant

This sequence change results in a premature translational stop signal at codon 23 (p.Gln23*). This truncated protein would express only the first 23 amino acids (Figure 21), corresponding to the extracellular portion of the receptor. This small fragment is, evidently, devoid of any MC1R receptor function, being a loss of function mutation.

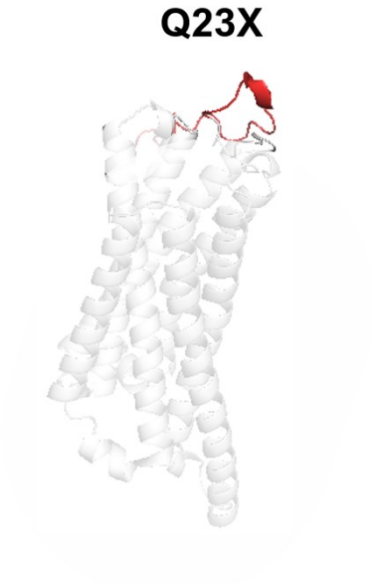


Figure 22. Tridimensional model of MC1R in its truncated form Q23X, built with RaptorX (Xu 2019) and visualized in Pymol Software. In red is represented the only effectively expressed part of the molecule.

Only one patient harbors this variant, who is thirty years old, has had seven thin melanomas at the itme of reporting, the first at age 18. Phenotypically, he has no red hair, but fair skin and light hair (phototype II) and multiple atypical nevi. This variant was not found

among controls. Its frequency in GNOMAD is 0.0003039 and in the Brazilian ABraOM is 0.000854.

Since it is a nonsense mutation right at the beginning of the coding region, the premature termination of protein synthesis at amino acid 23 yield a nonfunctional protein (which normally has 317 amino acids).

4.4.2 I155T Missense variant

Sequencing of the *MC1R* gene showed the missense variation p.Ile155Thr (c.464T>C), or I155T, in three of five affected members of family A (representative electropherograms are shown in figure 22), a prevalence of 60% in affected individuals of this family. DNA sequencing from this family demonstrated that one descendent (III.22) of an affected member (II.11), who is now 12 years old and clinically not affected also harbor this variant. Of the five non-affected siblings (individuals II.1, II.2, II.3, II.5 and II.12) who participated in the study, four do not harbor this variant, as well as the mother. One non-affected sister harbors this variant (II.3).

The I155T variant was also found in both siblings of family B and was not present in families C and D. This variant was also detected in one multiple primary melanoma patient. Among the sporadic melanoma patients, it was found in two patients. The overall frequency of this variant among the 44 melanoma patients studied was 18.2% (8 patients).

None of the 102 controls carried this genotyped variant (data not shown).

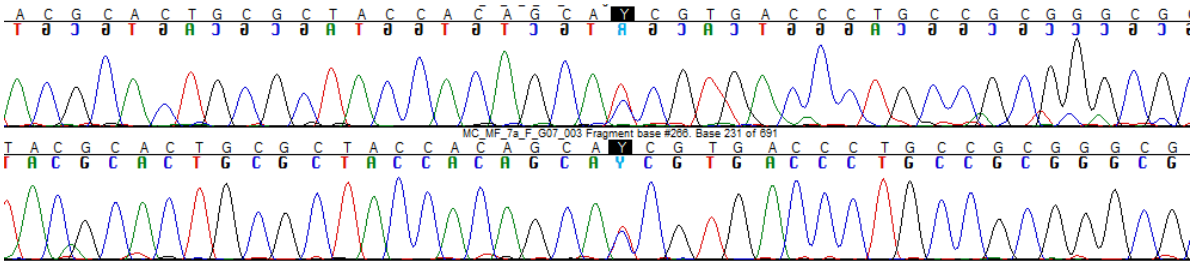


Figure 23. Representative electropherograms of two affected members of family A showing the variant I155T in *MC1R* in heterozygosis, validated by Sanger sequencing.

This variant is registered by rs1110400. According to ClinVar, this variant has "conflicting interpretations of pathogenicity", being described as benign, likely benign, pathogenic or even uncertain significance by multiple evidence submitted (data available at https://www.ncbi.nlm.nih.gov/clinvar/variation/239154/). The I155T variant was observed in 1,05% (90/8598) alleles from individuals of European American ancestry in the NHLBI Exome Sequencing Project (Auer *et al*., 2016). It is important to point out that in this large study there was no specific data on melanoma background of these patients. The frequency of this variant in GnomAD is 0.005640 and the frequency of this variant in the Brazilian Genomic Variants Database is 0.005551 (13 of 2342 alleles).

In family A, I155T seems to correlate to a worse prognosis among the carriers, given that carriers tend to have larger number of tumors, melanoma with deeper Breslow indexes, higher rates of invasive melanomas and earlier age of onset, as demonstrated in table 20.

Table 22. Phenotypic comparison between carriers and non-carriers of I155T in *MC1R* gene among affected members of family A.

	155T carrier (3)	Non-carrier of I155T (2)
Mean actual age of individuals	46.6	56
Mean age of onset of melanoma	27.6	40
Mean Breslow thickness of melanomas	0.17 mm	0.017 mm
Number of invasive tumors (> 1mm Breslow)	3	0
Mean number of tumors per person	14	5

The variant was analyzed by ten different mutation prediction tools and eight of them suggested it has significant negative effects on the protein, one of them suggested it has a medium effect and only one of them suggested the amino acid change could be neutral, as shown in table 23.

Table 23. Mutation prediction algorithms consulted for the variant I155T and the predicted consequence in the protein.

Software	Result
FATHMM	PASSENGER/OTHER (5.26)
Mutation Assessor	MEDIUM (3.165)
PANTHER	PROBABLY DAMAGING (preservation time: 455 / Pdel 0.57)
PhdSNP	DISEASE (0.709)
SNPs&Go	DISEASE (0.833)
Mutation Taster	DISEASE CAUSING (amino acid sequence changed). heterozygous in TGP or ExAC; known disease mutation at this position (HGMD CM005390); protein features (might be) affected; splice site changes)
PROVEAN	DELETERIOUS (-4.634)
SNAP2	Effect Score: 90 (EFFECT)
SIFT	AFFECT PROTEIN FUNCTION (0.00)
PolyPhen	PROBABLY DAMAGING (0.986) sensitivity: 0.74; specificity: 0.96

Protein structure homology-modelling server Swiss Model (https://swissmodel.expasy.org/) demonstrated that this amino acid substitution occurs in the second intracellular loop (il2) of the *MC1R* receptor (figure 23), a highly conserved region in vertebrates, as well as in the five human melanocortin receptor subtypes. Moreover, it has been documented before that mutations within intracellular loops of G-protein-couple receptor (GPCR) family proteins can in fact modify its function (Yang and Tao, 2020; Banerjee and Mahale, 2015).

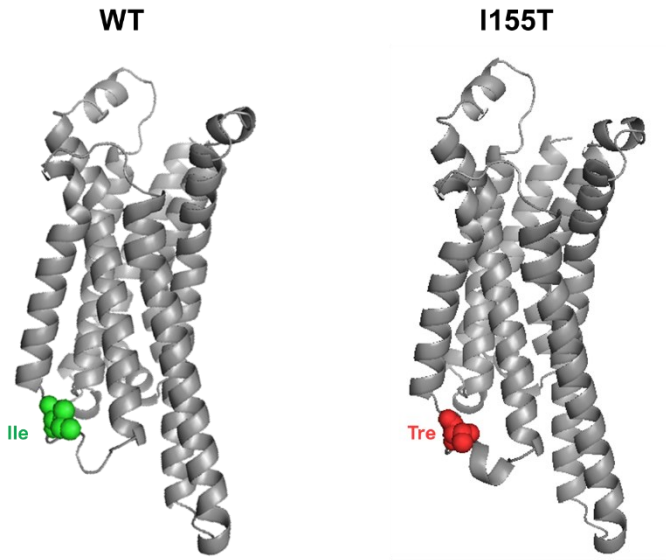


Figure 24. Swiss model protein structure homology-model represents the I155T located in the second loop in the MC1R molecule (https://swissmodel.expasy.org/).

4.4.3 M203T *MC1R* novel missense variant

One novel missense variant was detected in a 26-year-old female who has had one *in situ* melanoma at age 22 on the clavicle. She is a redhead individual with type II phototype, a few melanocytic nevi (rare, atypical) and history of a few sunburns without blistering. It is a missense variant, c.608T>C, leading to a substitution of a Methione by a Threonine in codon 203, has the rs769723368 and is not reported in ClinVar. There is no scientific information about this variant in literature up to the moment. Its frequency in GnomAD is 0.000004 and it is not described in the Brazilian Genomic Variants database (http://abraom.ib.usp.br/search.php). This variant was not found in 130 control samples tested.

The variant was analyzed by ten different mutation prediction tools and six of them suggested it has significant negative effects on the protein, one of them suggested it has a medium effect and three of them suggested the amino acid change could be neutral, as shown in table 24.

Table 24. Mutation prediction algorithms consulted for the variant M203T and the predicted consequence in the protein.

Software	Result
FATHMM	PASSENGER/OTHER (3.89)
Mutation Assessor	MEDIUM (3.255)
PANTHER	PROBABLY DAMAGING (preservation time: 455 / Pdel 0.57)
PhdSNP	DISEASE (0.786)
SNPs&Go	NEUTRAL (0.379)
Mutation Taster	DISEASE CAUSING (amino acid sequence changed; protein features (might be) affected; splice site changes)
PROVEAN	DELETERIOUS (-2.550)
SNAP2	Effect Score: 24 (EFFECT)
SIFT	TOLERATED (0.29)
PolyPhen	PROBABLY DAMAGING (0.999) sensitivity: 0.14; specificity: 0.99

We suggest this variant might be included as a R variant (as it was found in an individual with red hair) and considered to increase risk of early onset melanoma.

In the current study, only three red-headed individuals were enrolled, given that it is indeed a rare phenotype among Brazilians, and we found a low frequency of the "classical" RHC variants. The association with RHC phenotype was more relevant only to D294H variant. We showed a high number of patients with the I155T variant and it did not correlate to the RHC phenotype at all, we suggest it should not be considered a RHC variant. The same occurred for R142H variant, but in a much lower number and therefore weaker suggestion, as shown in table 25. The novel variant M203T was found in a red hair individual and, therefore, we suggest it might be considered a novel RHC variant.

Table 17. Phenotype of red hair in carriers of RHC classic variants, variants considered RHC by some authors and novel possible RHC variant described in this study.

	MC1R Variant	Number of patients	Reh Hair Phenotype
"Classic" RHC variants	R151C	2	0%
	R160W	1	0%
	D294H	6	33.3%
Considered RHC variants by some authors	I155T	8	0%
	R142H	2	0%
Not described/not classified	M203T	1	100%

4.5 Summary of *CDKN2A* and *MC1R* variants results through Sanger sequencing among all patients

Table 18. List of variants found in *CDKN2A* and *MC1R*, their available relevant data and phenotype of cases.

Gene[1]	**Description and consequence**	**Type**	**DbSNP[3]**	**gnomAD[4]**	**SIFT[6]**	**Polyphen[7]**	**Clin Var[8]**	**Frequency in cases (44 total) and controls, phenotype**
CDKN2A	c.325G>C; p.Ala109Pro (p16INK4a protein) and c.368G>C; p.Cys123Ser (p14ARF protein)	missense; missense	rs372481694	0.00002	T	PD	UNC	2.3% 1 MPM patient with FAMMM (Absent in 102 controls)
CDKN2A	c.442G>A; p.Ala148Thr (p16INK4a protein) and c. 289G>A; p.Aala97Thr (p14ARF protein)	missense; missense	rs3731249	0.0289	T	B	B	11.3% 5 melanoma patients (1 MPM, 2 over 50 and 2 under 50) (Present in 1.9% of controls)
MC1R	c.67C>T; p.Gln23Ter	nonsense	rs201533137	0.0003039	-	-	LP	2.3% 1 MPM patient with early age of onset (16) and a total of 7 melanomas (Absent in controls)

Gene[1]	Description and consequence	Type	DbSNP[3]	gnomAD[4]	SIFT[6]	Polyphen[7]	Clin Var[8]	Frequency in cases (44 total) and controls, phenotype
MC1R	c.178G>T; p.Val60Leu	missense	rs1805005	0.06974	D	PD	B	9.0% Two MPM patients, two sporadic melanoma patients (Present in 2.5% of controls)
MC1R	c.274G>A; p.Val92Met	missense	rs2228479	0.07634	T	B	B	2.3% One sporadic melanoma patient under 50 (Absent in controls)
MC1R	c.318G>A; p.Leu106Leu	missense	rs3212364	0.002736	-	-	B	2.3% One non-affected Family A offspring (Present in 1.2% of controls)
MC1R	c.425G>A; p.Arg142His	missense	rs11547464	0.005323	D	PD	B/LB	4.5% One MPM patient without red-hair phenotype and one patient with MM above 50 years (Absent in controls)
MC1R	c. 451C>T; p.Arg151Cys	missense	rs1805007	0.044158	D	D	CONF	4.5% One MPM without red-hair phenotype and one patient with MM above 50 years (Present in 1.5% of controls)
MC1R	c.464T>C; p.Ile155T	missense	rs1110400	0.005640	D	PD	UNC	18.2% Five familial melanoma patients from two different families and one MPM patient (Absent in controls)

Gene[1]	Description and consequence	Type	DbSNP[3]	gnomAD[4]	SIFT[6]	Polyphen[7]	Clin Var[8]	Frequency in cases (44 total) and controls, phenotype
MC1R	c.478C>T; p.Arg160Trp	missense	rs1805008	0.047166	D	D	CONF	2.3% One sporadic MM patient with systemic metastasis and early age of onset (35) (Absent in controls)
MC1R	c.488G>A; p.Arg163Gln	missense	rs885479	0.151540	T	B	B	11.3% Two familial melanoma patients, one MPM (harboring a *CDKN2A*), two sporadic MM patients with onset before 40 (Present in 6.1% of controls)
MC1R	c.550G>A; p.Asp184His	missense	rs530102853	0.00003	T	B	UNC	2.3% One sporadic MM with early age of onset (37) (Absent in controls)
MC1R	c. 608T>C p.Met203Thr	missense	rs769723368	0.000004	D	PD	Not Reported	2.3% One sporadic MM with RHC phenotype and early age of onset (22) (Absent in controls)

Gene[1]	Description and consequence	Type	DbSNP[3]	gnomAD[4]	SIFT[6]	Polyphen[7]	Clin Var[8]	Frequency in cases (44 total) and controls, phenotype
MC1R	c.880G>C; p.Asp294His	missense	rs1805009	0.00972	D	PD	UNC	13.6% One familial melanoma patient (age of onset 35 years), one MPM patient, two sporadic melanoma patients >50, two sporadic melanoma patients <50 Two of these six have RHC phenotype (33.3%) (Absent in controls)
MC1R	c.942A>G; p.Thr314Thr	missense	rs2228478	0.142913	-	-	B	22.7% Four familial melanoma patients, two sporadic melanoma patient > 50, four sporadic melanoma patients < 50 (Present in 21.5% of controls)

[1] Gene reference: CDKN2A (NM_058195.4); MC1R (NM_002386.4).
[2] ACMG: American College of Medical Genetics and Genomics; [3] dbSNP: Single nucleotide polymorphism database; [4] gnomAD: genome aggregation database; [5] CADD: combined annotation-dependent depletion; [6] SIFT: sorting intolerant from tolerant; [7] PolyPhen: polymorphism phenotyping; 8 Clin Var (ClinVar aggregates information about genomic variation and its relationship to human health). Abbreviations: B: benign; BCC: basal cell carcinoma; CDKN2A: cyclin dependent kinase inhibitor 2A; CM: cutaneous melanoma; CONF: conflicting interpretations of pathogenicity; D: deleterious; LB: likely benign; LP: likely pathogenic; MC1R: Melanocortin 1 receptor; MPM: multiple primary melanoma; P: pathogenic; T: tolerant; UNC: uncertain; VUS: variant of unknown significance.

4.6 Sun exposure

Significant sun exposure is an important risk factor for melanoma; therefore, we compared the history of sun exposure among individuals who had one sporadic melanoma before the age of 40 years (*n*=10), with those diagnosed after the age of 50 years (*n*=12). Results are shown in table 27.

No sun exposure history was reported in 30% of the group under 40 yrs. and in 16.7% in the group above 50 years; intense unprotected sun exposure throuout life without sunburns was reported in 0% of the group under 40 and in 58.3% of the group above 50 years. Furthermore, one or more episodes of sunburns without blistering were present in 40% of the under 40's group whilst it was described in 16.7% of the group above 50 years. History of blistering due to sun exposure is present in 30% of the group under 40 yrs. and 8.3% in individuals of the group above 50 yrs old (data in table 27). In summary, sunburns with or without blistering are significantly more frequent (p = 0.01) in the group who had melanoma at an early age, corroborating once more the importance of UV radiation in tumor development.

Table 27. Sun exposure history among sporadic melanoma patients.

	Sun exposure history			
	None	Intense unprotected without sunburns	Sunburn(s) without blistering	Blistering sunburn(s)
Melanoma < 40 years of age	(3/10) 30%	0 (0,0%)	(4/10) 40%	(3/10) 30%
Melanoma > 50 years of age	(2/12) 16.7%	(7/12) 58.3%	(2/12) 16.7%	(1/12) 8.3%

P = 0,010. Chi-square Test with Simulation of Monte Carlo.

5. DISCUSSION

In the present work we studied families with Familial Cutaneous Melanomas as well as patients with Multiple Primary Melanoma (MPM) in Brazilian individuals from the state of Minas Gerais who present features suggestive of an inherited predisposition to cutaneous melanomas. In addition, sporadic melanoma cases were also studied. This investigation involved complete clinical investigation as well as genetic analyses.

Diagnosis of an atypical presentation of XP was possible due to WES. The prevalence of seven affected individuals among 13 siblings (58.3%) is unexpectedly high for an autosomal recessive disease. The discrepancy between the overall number of melanomas (53) compared to the overall number os carcinomas (nine) is striking. Unlike this family, XP usually is a syndrome in which melanoma is not the most common, first, or predominant presentation, not being, therefore, considered a familial melanoma syndrome, as proposed by Abdo *et al*. (2020). Furthermore, clinical typical features of XP allowing its clinical suspicion was not seen among affected individuals, such as dramatic pigmentary skin changes from childhood, ophtalmologic involvement, early age of carcinomas onset and low life expectancy. The mutation found in *XPC* is rare and has never been associated with a clinical phenotype on the literature. XP members presenting simultaneously I155T *MC1R* variant tend to show a worse melanoma scenario, but the possible relation between XP and *MC1R* variants modifying its phenotype is still scarse.

Familial cases of melanoma, especially when inheritance is very likely as in family A, are important object of genetic study because of its rarity and because there is still significant lack of scientific knowledge about germline mutation-driver tumorigenesis in hereditary melanoma. Other high penetrance disease-causing genes might not been discovered yet, even if lately, it is becoming increasingly clear that multiple independent causal variants may contribute to disease susceptibility in most melanoma families (Barrett *et al*., 2015). A more comprehensive understanding of these variants and genes, such as *MC1R*, is important to

clarify melanoma development, to stratify high-risk patients in a future of personalized Medicine and to facilitate genetic targets in the future. As the costs for genomic sequencing continue to decrease, genetic testing is shortly becoming a reality and the true implications of each genetic alteration must be extensively evaluated. Identification of patients with a germline mutation predisposing to cancer enables genetic counseling, testing of family members, behavioral changes and appropriate surveillance, reducing morbidity and mortality in these patients (Toussi *et al*., 2019).

5.1 *XPC* mutation in family A

To date, more than 100 pathogenic variants have been reported in *XPC*. Of these, 36% are small deletions, 22% are nonsense, and 14% are splicing-related (Human Genome Mutation Database). Missense variants and gross deletions, as described here, are less common at 8% and 6%, respectively (Macke *et al*., 2020).

The mutation p.Arg307Trp (c.919C>T) is not described among the 84 variants of *XPC* gene in the Global Variome shared LOVD (accessed on June 21st, available in https://databases.lovd.nl/shared/variants/XPC?search_var_status=%3D%22Marked%22%7C%3D%22Public%22).

Individuals in the XP-C complementation group typically present with skin cancer even earlier than other subtypes (Bradford *et al*., 2011). Variants in *XPC* generally result in early onset skin cancer in childhood; the median age of onset for nonmelanoma skin cancer in Xeroderma Pigmentosum patients of all sub types is 9 years of age, and median age of first melanoma is 22 yrs. (Macke *et al.,* 2020). This was not observed among our XP-C patients, in which the first skin cancer (melanoma) occurred at age 22 in individual II.13. The mean age of onset of tumors in this family is 33.8 (range from 22 to 47).

Skin tumors are more often detected in areas exposed to sunlight. Up to 80% of them are located on the face (Santiago *et al*., 2020), consistent with our findings (75.4% are in the head and neck region).

It has been demonstrated that XP-C, XP-E and XP-V groups have no abnormal phototest or sunburn reactions; this could be explained by the preservation of transcription-coupled DNA repairs (Meneses *et al*., 2015). Patients with XP-C often fail to carry out photoprotection in early childhood because they do not experience exaggerated sunburn (Masaki *et al*., 2018). This is exactly what is reported by the family members studied; they do not tend to burn easily when sun exposed, they do not have a recollection of many blistering despite having a long history of excessive unprotected sun exposure. The first symptoms in XP-C patients are photo distributed lentigines around the age of 2 years. They have a later age of XP diagnosis and therefore accumulate more photo damage, leading to an earlier age and more quantity of skin cancer (Fassihi *et al.*, 2016). On the contrary, relevant pigmentary changes are referred by the patients only in adolescence.

XP-C patients are particularly susceptible to ocular problems relative to their skin changes compared with the other groups (Espi *et al*., 2018). Interestingly, no relevant ophthalmologic condition has been diagnosed in the affected family members.

It has been suggested that patients with mutations in complementation group C may be particularly prone to malignant melanoma (Lynch, 1984; Li *et al.*, 1993 and Fassihi *et al.*, 2016), which is extensively supported by our findings (53 MM, 8 BCC and 1 basosquamous carcinoma in total). Melanomas on the scalp, despite a thick head of hair, like occurred in individuals II.10 and II.11, has been described in two XP-C brothers harboring a homozygous missense p.Tyr585Cys mutation (Fassihi *et al.*, 2016).

In a study of 17 XP patients from Nepal, no patients with XP-C had neurological symptoms, contrasting with major cutaneous lesions in most of them. In that same study, *MC1R*

genotype was also evaluated in affected individuals, and it was shown that those carrying *MC1R* variant R163Q (n=9), in comparison to the non-carriers (n=8), tend to have a younger age at first carcinoma (median: 8 vs 11 years) and to be diagnosed with more numerous cutaneous carcinomas (median number: 4 vs. 1.5), P value 0.2 (Espi *et al.*, 2018). We found not only an association of carcinomas to *MC1R* I155T genotype, but most importantly, association of this variant to earlier age of melanoma onset, greater number of melanomas per person, deeper Breslows and higher chances of invasive tumors among XP patients.

A large study of 44 families with a total of 64 XP-C patients from Tunisia showed that patients presented moderate sunburn reactions, polymorphic pigmented macules, hypopigmented macules usually appeared before the hyperpigmented macules, photophobia was present in all patients and neurological disorders, or psychomotor development delay were absent from all patients (Jerbi *et al*., 2016). Sixty-seven percent of patients developed their first skin cancer at a median age of 8 years. About 47% developed BCC and SCC together. The proportion of patients who developed malignant melanoma (MM) was 23%, with a mean age of 13 years. Most of the melanomas were lentigo maligna (9/12). This group also suggested that XP-related individuals are likely more susceptible to developing internal cancer (leiomyosarcoma, thyroid nodules, leukemia). Among the XP-C individuals studied, no other malignancy has been found.

Other cases of XP-C with late onset of tumors, late diagnosis and a phenotype of multiple melanomas have been described. An atypical presentation of XP-C with multiple primary melanomas and unusual late onset (diagnosed at the age of 42) was described by Meneses *et al*. (2015). Subsequently, a patient with a homozygous missense variant (p.Tyr585Cys) in *XPC* who did not develop their first melanoma until age 28, and who displayed almost no pigmentary changes was reported (Fassihi *et al*., 2016). Recently, a case of late XP-C diagnosis through WES was published; the patient having had more than 10

melanomas, the first one at age 36 (Macke *et al*., 2020). Similarly to our cases, this patient reported no unusual sun sensitivity even with extensive sun exposure, presented freckling of sun-exposed areas, multiple atypical nevi and no basal cell or squamous cell carcinomas prior to the multiple melanomas.

Missense mutations in the *XPC* gene may allow partial functionality of DNA repair capacity, thus explaining late onset of skin lesions and less typical phenotype in these individuals. These data suggest that hypomorphic alleles can lead to an atypical late-onset presentation of XP, group C (Macke *et al*., 2020).

In Brazil, a similar case os late clinical diagnosis is reported. The patient was diagnosed at age 45, having positive family history, skin facial lesions since age 13 with multiple BCCs and SCCs, along with one melanoma. No genotyping was performed, and the patient died at age 62 years old. The largest cohort of Brazilian patients with clinical diagnosis of XP (a total of 32 individuals from 27 families) revealed a high frequency (17/27) of *XPC* variants among Brazilians (Santiago *et al*., 2020). In this study, skin hyperpigmented macules and lentiginous lesions were the first XP signs (average age: 2.5 years old) and the mean age of the first skin biopsy was 8.8 years. All cases presented mild-to-severe ocular deficits. A total of 431 basal cell carcinomas (BCC), 136 squamous cell carcinomas (SCC) and 84 melanoma skin tumors were detected in 20 of 32 patients, consistent with the XP tumor spectrum and opposite to what we found in the family studied (a total of 8 BCC, 1 SCC and 53 melanomas).

Patients with XP-C developed first symptoms on average age of 1.6 years and first biopsy occurred on an average age of 5.5 years (Santiago *et al*., 2020). These findings differ greatly from our patients, whose first symptoms of skin pigmentary changes were only noted in adolescence.

5.2 *CDKN2A* mutation found in a Multiple Primary Melanoma patient

A single case of MPM harbored the p.A109P in *CDKN2A*. *CDKN2A* associated melanomas tend to express a similar histologic phenotype of dense pigmentation, high pagetoid scatter and a non-spindle cell morphology in the vertical growth phase (Sargen *et al*., 2015). In addition, that they present at a younger age of diagnosis (median age 38 years versus 46 years), display higher number of multiple primary melanomas and superficial spreading melanomas (SSM) when compared to wild-type/nonpathogenic mutation carriers (Taylor *et al.*, 2016). Indeed, these characteristics are similar to those expressed in the three melanomas in the A109P *CDKN2A* carrying case reported herein, both histologically and the early age at diagnosis of the first CM (18 years of age).

The incidence of germline *CDKN2A* mutations in the general population is very low (Aoude *et al*., 2015).

The variant p.Ala109Pro/c.325G>C found in exon 2 can affect both proteins encoded by the same gene (p16 and p14). This sequence change replaces alanine with proline at codon 109 of the CDKN2A (p16INK4a) protein (p.Ala109Pro). The alanine residue is highly conserved and there is a small physicochemical difference between alanine and proline. Alternatively, this sequence change replaces cysteine with serine at codon 123 of the CDKN2A (p14ARF) protein (p.Cys123Ser). The cysteine residue is moderately conserved and there is a moderate physicochemical difference between cysteine and serine. Of the subset of missense mutations affecting both p16 and p14, p16 seems to be the primary target of inactivation, as p14 is not always functionally impaired (McKenzie *et al*., 2010). Yet, this variant has also been assigned a VUS status by three independent providers (Invitae; Accession: SCV000545545.6; Color Health, Inc, Accession: SCV000911405.2; Ambry Genetics, Accession: SCV000213894.5) and they stated that the available evidence was currently insufficient to determine the role of this variant in disease. This variant (p.Ala109Pro;

rs372481694) has been reported in population databases (https://www.ncbi.nlm.nih.gov/clinvar/variation/127525/, accessed on 9th March 2021). ClinVar (Variation ID: 127525) reports a frequency of 1.7e-05 in 236,600 control chromosomes (gnomAD) and has not been described in the Brazilian Genomic Variants database (ABraOM).

Assessing pathogenicity of nonsynonymous mutations is critical to evaluate melanoma risk in carriers. Pathogenicity of a variant is defined by direct evidence, i.e., for its segregation with disease and its absence in control DNA samples. Other option would be with indirect lines of evidence such as critical amino acid changes, occurrence in a highly conserved/relevant protein domain, functional assays and *in silico* analysis. To try and classify variants, multiple assays based on the known p16INK4a properties have been developed, i.e., p16INK4a subcellular distribution, CDK4/6 binding affinity, cyclin D-CDK4/6 inhibitory activity, cell cycle inhibition, senescence-inducing activity, and/or intracellular oxidative stress regulation. There have been discrepancies in the functional assessment of the same p16INK4a variant of uncertain significance (VUS) by different methods (Scaini *et al*., 2014) and there are no reports of functional data regarding this variant, probably because of its rarity.

The variant A109P was assigned a likely damaging score by most prediction algorithms (PROVEAN, PolyPhen2 and CADD; deleterious, possibly damaging and deleterious, respectively); only one algorithm (SIFT) indicated that it could be tolerated. Although these predictions are estimates, it has been shown that protein damage prediction algorithms for missense variants are 65-80% accurate when examining known disease variants (Nykamp *et al*., 2017). PolyPhen2 and SIFT are better at predicting loss of function than gain of function mutations (Flanagan *et al*., 2010).

3D modelling of p14ARF is not easily obtained through softwares Swiss-Model and RaptorX, but they do demonstrate that the amino acid change is located in the protein core,

having the potential to destabilize the whole protein conformation, especially if cysteine forms disulfide bonds with other cysteines in the protein. Indeed, according to softwares for predicting disulfide bonds in proteins (Cyscon, available in http://www.csbio.sjtu.edu.cn/bioinf/Cyscon/ and DiANNA, available in http://clavius.bc.edu/~clotelab/DiANNA/), it is likely that C123 bonds to C100 (Ferre and Clote, 2005; Yang *et al*., 2015), as shown in figure 25. This further suggests the clinical relevance of this variant, as the function both tumor suppressor proteins encoded by the mutant *CDKN2A* could be impaired.

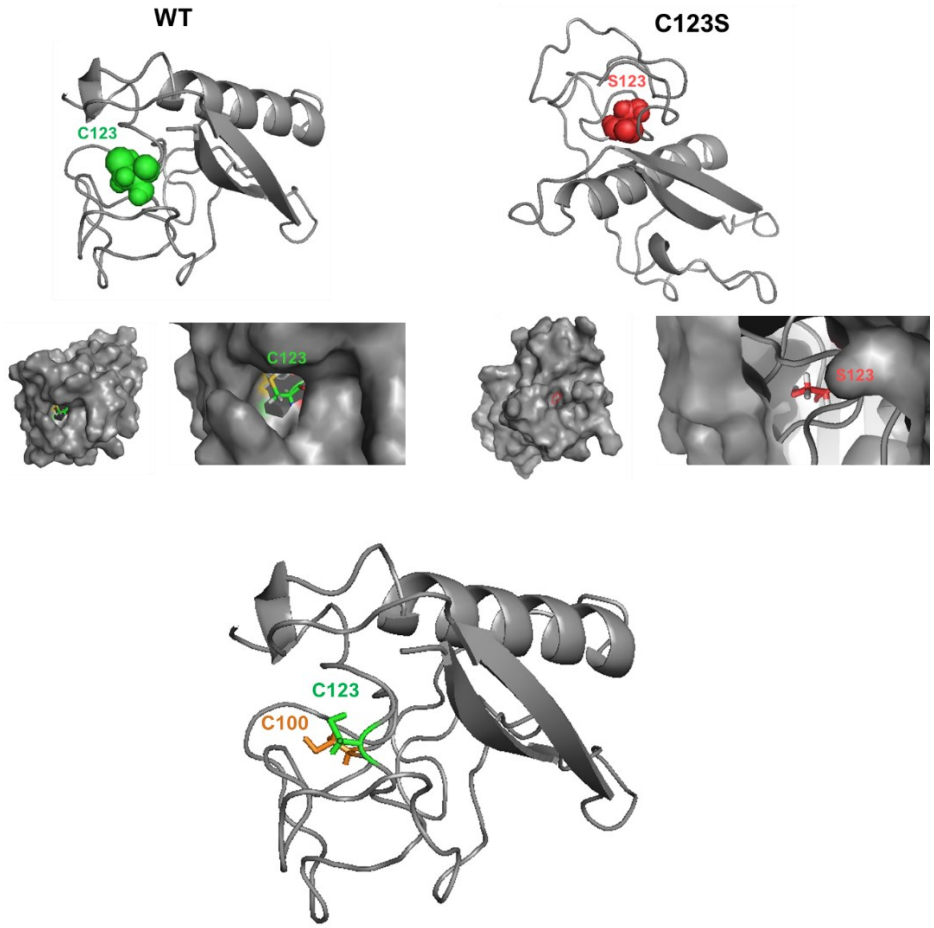


Figure 25. Incomplete 3D model of p14ARF protein showing the wild-type structure with cysteine in position 123 in green, in contrast to the mutated protein (C123S), showing a serine in red in this position. Figure below shows the likely disulfide bonds made between C123 and C100 due to their proximity, which would be compromised in the amino acid change. Source: http://www.csbio.sjtu.edu.cn/bioinf/Cyscon/; http://clavius.bc.edu/~clotelab/DiANNA/; https://swissmodel.expasy.org/).

One of the most famous *CDKN2A* disease-associated mutation is the p.G101W which is located in the third ankyrin motif in close proximity to amino acid change we found in p16, as demonstrated in the 3D model comparing the wild-type with the A109P and with the G101W structures (figure 26).

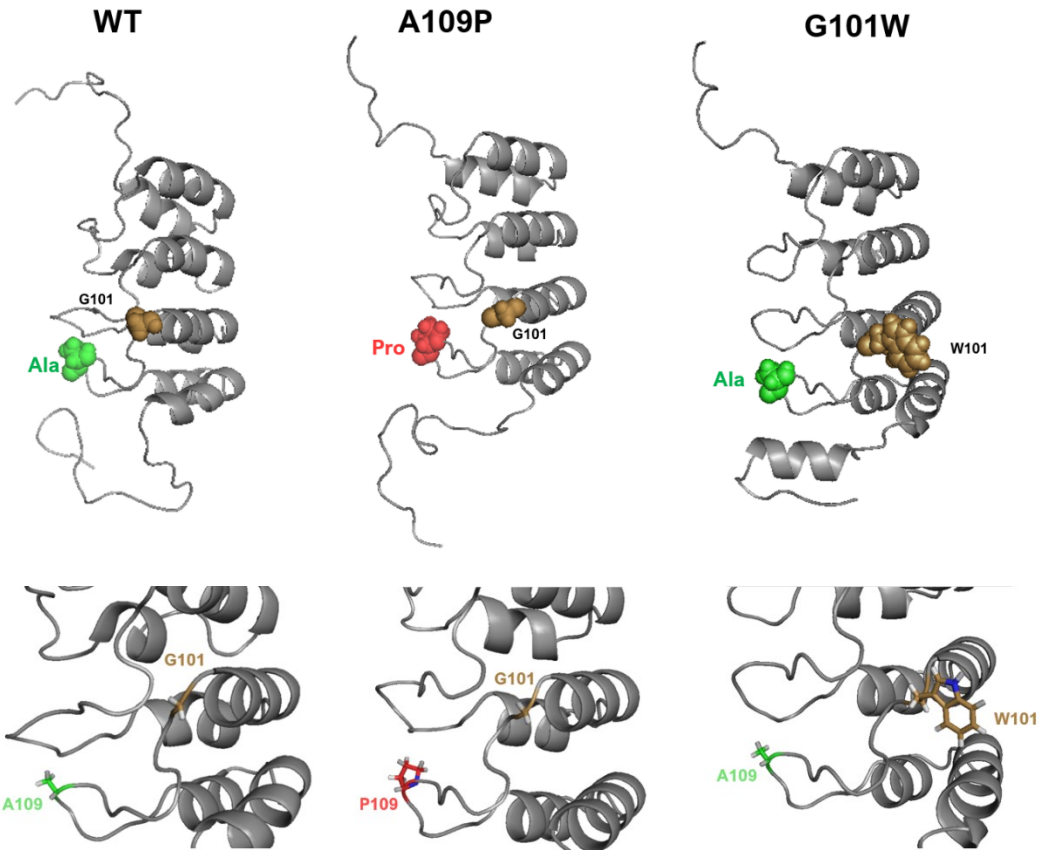


Figure 26. Comparison between the 3D structure models of p16(INK4) wild-type (left) and variants A109P (middle) and G101W (right) performed with RaptorX and visualized in PyMol. In green, the wild-type alanine residue in position 109 and in red, the mutated proline in a loop, in the vicinity of the disease-associated mutation G101W, which is represented in golden color.

Based on the above depicted protein folding model, the p.A109P amino acid change, which occurs in a loop between the third and fourth ankyrin motifs, does not lead to major deleterious conformational protein changes, even though the proline could cause greater inflexibility to the structure. The p.G101W change causes a more visible impairment of the cleft between the third and fourth motifs, known to be an important site for the CDK4/6 interaction. The loop regions of p16(INK4) do not have a clear role that enables correlation of

aminoi acid alterations in these positions to functional protein effects. Structural modelling can provide important insights about disease mechanisms; however, these theoretical data lack experimental evidence to support it. They can be more implicated in transcription effects, RNA stability or even post-translational changes (Holland *et al*., 1999). Of note, three p16(INK4) variants (T77P, R80P and R99P) where the original amino acid was substituted by a proline, as in the A109P, the mutants presented lower capacity of interaction with CDK4, but they were not predicted as relevant in bioinformatic tools, suggesting there might be a bias in the interpretation of proline substitutions in these programs (Kannengiesse *et al*., 2009).

5.2.1 *MC1R* variant found in *CDKN2A* mutation carrier

There is evidence that penetrance of *CDKN2A* mutations is modified by factors such as pigmentation characteristics, nevus phenotypes and *MC1R* variants (Demenais *et al*., 2010). In patients harboring *CDKN2A* sequence variants, those who co- carry any of the four most frequent *MC1R* variants (V60L, V92M, R151C and R160W) showed a significant increased melanoma risk is noted (Demenais *et al*., 2010). In addition, the higher the number of *MC1R* variants in CDKN2A PSV carriers, the higher the risk of melanoma (Demenais *et al*., 2010), a finding based on more than 800 CM cases and confirmed previous studies (Goldstein *et al*., 2005; Raimondi *et al*., 2008). In the current study, the presence of at least one *MC1R* variant in a *CDKN2A* mutated patient's DNA was associated with a three-fold increase in melanoma risk. The presence of at least one *MC1R* variant is also associated with an eartlier age at diagnosis of melanoma in families with *CDKN2A* mutations (Cuéllar *et al*., 2009).

The patient identified herein as a carrier of *CDKN2A* PSV (a MPM individual with first melanoma at age 18, clinical features shown in table 5, on page 69) also co-carried a previously reported missense *MC1R* variant, c.488G>A (p.Arg163Gln, rs 885479, protein change R163Q)

classified as benign according to ACMG guidelines by ClinVar (Richards *et al*., 2015). This variant was considered a rare nonsynonymous variant (present in less than 5%) in a large GenoMel study encompassing 815 *CDKN2A* mutation carriers that identified 33 variants (23 nonsynonymous amino acid changes) (Demenais *et al*., 2010). This variant, R163Q, is one of the designated "r" allele since it has a relatively weak association with RHC (**R**ed **H**air **C**olor) phenotype, clinical feature that our patient does not display; furthermore, it was significantly associated with melanoma development in a previously published meta-analysis data (Raimondi *et al*., 2008). Interestingly, the R163Q variant was associated to lentigo malignat melanoma in a Mediterranean population, independently of phenotype features, further supporting the notion that *MC1R* variants could increase melanoma risk due to their impact in pathways other than pigmentation (Puig-Butillé *et al*., 2013).

5.2.2 *CDKN2A* mutations and dermoscopy

Recent studies reported an association between dermoscopic features of multiple melanomas and genetic signature (Cuéllar *et al*., 2009; Bassoli *et al*., 2013; De Giorgi *et al*., 2015; Grazziotin *et al*., 2016). It has been proposed that all *CDKN2A* associated melanomas have a unique dermoscopical pattern of their melanocytic tumors (De Giorgi *et al*., 2015). *CDKN2A* mutation carriers tend to develop hypopigmented melanocyte lesions; structureless areas were more often seen in *CDKN2A* mutants with two RHC-MC1R variants, whereas streaks and pigmented networks were more frequently seen in individuals without any RHC-*MC1R* variant. In the present study, the patient harboring both *CDKN2A* and *MC1R* Arg163Gln (not an RHC-MC1R variant) indeed presents dermoscopically similar lesions, characterized by a not very dense pigmented network, some with shiny white central streaks, as shown in figure 27.

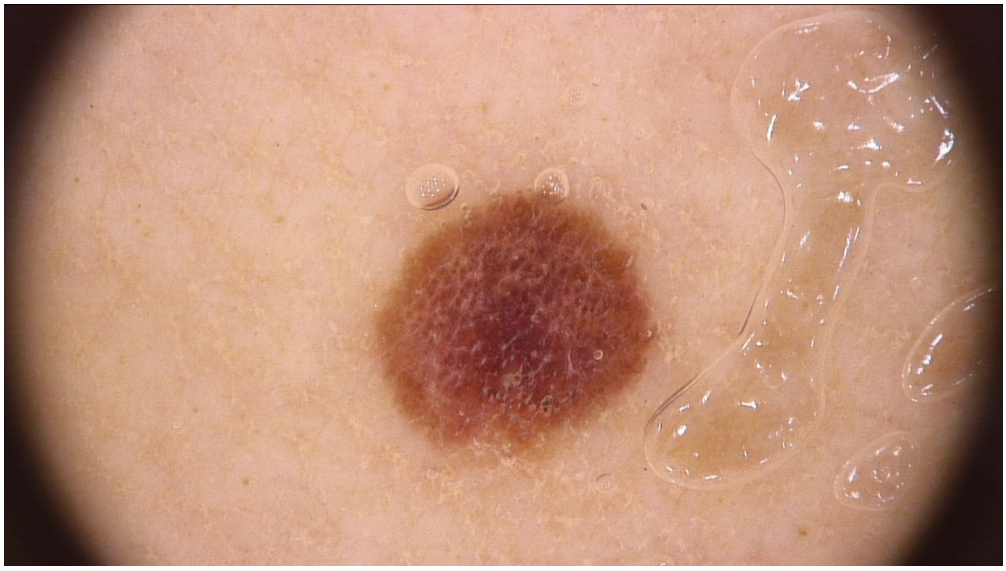


Figure 27. Dermoscopy of an *in-situ* melanoma of the *CDKN2A*-mutated patient, showing pigmented network with shiny white central streaks.

5.2.3 *CDKN2A* final considerations

In vitro studies provided a molecular basis for the link between *CDKN2A* and *MC1R*, showing that the increased expression of p16INK4a after exposure to UV radiation is potentiated by αMSH through its binding to *MC1R* (Pavey and Gabrielli, 2002). In addition, it has been suggested that *MC1R* polymorphisms not only interact with *CDKN2A* mutations to modulate melanoma risk but also contribute to a less suspicious clinical and dermoscopic appearance of melanomas (Longo *et al*., 2020).

Loss of wild-type *CDKN2A* has been shown to be an early event in the development of melanoma in Familial Atypical Multiple Mole Melanoma syndrome (FAMMM syndrome) (Chistodoulou *et al*., 2020). Biallelic *CDKN2A* loss is the most common genetic alteration distinguishing melanocytic nevi from invasive melanomas. In carriers of germline *CDKN2A* mutations, the wild-type allele is functionally inactivated in melanoma by a second somatic event, commonly through deletion. *CDKN2A* loss-of-heterozygosity (LOH) has been previously demonstrated in primary and metastatic melanomas of patients with FAMMM

syndrome. The timing of wild-type CDKN2A inactivation in hereditary melanoma development triggered by germline *CDKN2A* mutation is unknown. It is plausible that melanocytic nevi harboring subclones of cells with *CDKN2A* LOH might be at a higher risk to develop into melanoma (Chistodoulou *et al*., 2020). Unfortunately, LOH could not be studied in our patient.

In contrast to Helgadottir *et al*. (2020) who reported a worse survival of *CDKN2A* mutation carriers, Dalmasso *et al*. (2020) showed that *CDKN2A* mutation carriers had no difference in survival outcomes. One explanation for this difference in survival could be due to the fact that patients carrying germline mutations might be offered and adhere to a more stringent follow up regime than non carrier cases.

Genetic testing and counselling for *CDKN2A* improves sun-protective and surveillance behaviour without negative psychological sequelae (McMeniman *et al*., 2019). Therefore, routine germline testing could be incorporated into future iterations of the staging for melanoma to improve prognosis (Aoude *et al*., 2020).

5.3 *MC1R* variants

We found a total of 13 variants, eleven nonsynonymous variants in *MC1R* among the 44 cases studied, including a novel and some very rare variants, and significantly higher frequency of variants in cases compared to controls (see page 99, table 15, in the results section).

Some variants (D84E, R151C, R160W, D294H) that are consistently associated with a phenotype of red hair, light skin, poor tanning ability, and heavy freckling have been named RHC (**R**ed **H**air **C**olor) variants or "R" alleles. The less frequent R142H is also considered "R" variants by some authors (Duffy *et al*., 2019; Ghiorzo *et al*., 2012; Cust *et al*., 2013). The rare

I155T has been considered “R” variant by some authors (Ghiorzo *et al*., 2012; Raimondi *et al*., 2016) and “r” variant by others (Duffy *et al*., 2019). The other variants (V60L, V92M and R163Q) that have a weaker, or no association, with red hair color are called non-RHC (NRHC) variants or “r” alleles.

Both “R” and “r” alleles have been associated to melanoma (OR=2.66 [2.20-3.23] and 1.51 [1.32-1.73]) and with similar population attributable risk (15.8% and 16.6%) (Hu *et al*., 2014). Therefore, a novel class of *D* variants (D= Damaging) has been proposed by Hu *et al*. (2014) as important melanoma risk factors, independently of its association with red hair.

In a study of 2,160 patients, no significant associations were discovered between *MC1R* variants and histopathologic variables (Toussi *et al*., 2020).

In a previous meta-analysis of melanoma case-control studies it was shown that most *MC1R* variants, except the most frequent NRHC variants, V60L and V92M, were associated with a statistically significantly increased melanoma risk (Raimondi *et al*., 2008). This study showed a positive association between the two variants (I155T and R163Q) with melanoma but no association with red hair phenotype, despite the I155T variant being considered RHC variant by some authors. This is consistent with the phenotype of all studied patients harboring these two variants in our study (non-red hair fair skinned individuals). These results suggest that, for these variants, increased melanoma risk could be due to nonpigmentary pathways (Raimondi *et al*., 2008).

MC1R’s role in photoprotection goes beyond simple promoting eumelanogenesis. The association between *MC1R* and skin cancer could result from inflammatory or immune mechanisms influencing tumorigenesis, since it has been previously shown that αMSH has immunomodulatory and anti-inflammatory functions and MC1R is also expressed in immune cells (Tagliabue *et al*., 2018; Haddadeen *et al*., 2015). *MC1R* mediates UV radiation-induced epidermal thicknening as another photoprotective mechanism and variants have been

associated to photoageing. *MC1R* variants have been shown to reduce capacity for DNA repair in vitro (Haddadeen *et al*., 2015). Other hypothetical mechanisms for *MC1R* carcinogenesis is the modulation of melanocyte growth, development and differentiation, and an increased DNA damage possibly associated with reactive oxygen species production (Rouzaud *et al*., 2005).

5.3.1 *MC1R* variants detected in the current study and melanoma risk

Melanoma risk has been reportedly influenced not only by RHC variants, but also by certain NRHC variants indicating that this gene plays a role in melanomagenesis beyond pigmentation (Goldstein *et al*., 2005; Raimondi *et al*., 2008; Demenais *et al*., 2010; Cust *et al*., 2013; Haddadeen *et al*., 2015). Melanoma risk increases as the number of *MC1R* variations increase (Raimondi *et al*., 2008; Demenais *et al*., 2010). Some of these variants have been shown to cause partial loss of the receptor's signaling ability, making it unable to stimulate cyclic adenosine monophosphate (cAMP) production as strongly as the wild-type receptor in response to a-MSH stimulation (Raimondi *et al*., 2008).

A large meta-analysis reported a 'per person' odds ratio of 2.44 [95% CI 1.72-3.45] for R variants and 1.89 [95% CI 4.8-10] for r variants in developing melanoma (Williams *et al.*, 2011). These results are comparable with several other melanoma risk factors, including family history and phenotypic characteristics, although disease risks associated with common or atypical moles are stronger and range up to 10-fold (Kanetsky & Hay, 2018).

A large cohort of 5,160 melanoma patients demonstrated that 66% were carriers of *MC1R* variants and 28% of the melanomas were attributable to *MC1R* gene variants (Pasquali *et al*., 2015). It is estimated that *MC1R* variants confer a 1.5- to 4-fold increased risk of melanoma, and a 3- to 4-fold risk of thick melanomas. (Kanetsky and Hay, 2018).

In Mediterranean populations, the number of variants found of *MC1R* was 21 in Spain, 16 in France, 26-29 in Italy and 18 in Greece (Fernandez *et al*., 2007).

5.3.1.1 Variants associated with a significant melanoma risk

p.Gln23Ter (c.67C>T, rs201533137); Q23X

This rare nonsense variation was detected in a non redhead multiple primary melanoma patient (individual F), who has had seven melanomas at the time of reporting. He also harbors the common so-called "benign" missense variation p.Val60Leu. Q23X results in a premature translational stop signal in *MC1R* and is expected to create a truncated MC1R protein, causing loss of normal protein function. Yet, this variant has conflicting interpretations of pathogenicity, either VUS or likely pathogenic, in a report by McKenzie *et al*. (2003) who described this variant in two redhead Jamaican individuals who also harbored a second *MC1R* variant (R151C, which is considered a R variant) (McKenzie *et al.*, 2003). In addition, the Q23X variant was described in one patient among individuals at high risk for pancreatic cancer (Smith *et al.*, 2016); For the case reported related to pancreatic cancer, dditional clinical details and family history of this patient were not provided. The Q23X variant is present in 119/23452 alleles from individuals of African background (Lek *et al*., 2016) and the global minor allele frequency is 0.00140 (T). This variant is present in population databases (ExAC 0.00051) and it was not present in 102 control individuals we tested. Another possible mechanism for this variant's pathogenicity (besides generating a truncated protein), is the notion that mutations in the extracellular domains of the *MC1R*, such as C35Y and V38M, might induce a conformational change of the receptor impairing ligand-receptor interaction rather than directly participate in the binding to the ligand (Fargnoli *et al*., 2003).

p.Ile155Thr (c.464T>C, rs1110400); I155T

The I155T variant was previously reported in only one of 40 familial melanoma cases from Poland and, therefore, was deemed a very rare variant in that population (Dębniak *et al*., 2006). This variant was found in 1.5% of melanoma patients in a large French case control study that included 1,131 patients and in that population this variant was associated with melanoma risk almost at the risk attributed to the R alleles (Hu *et al*., 2014).

In a case-control Italian study, the presence of *MC1R* variation (two or more variants or at least one RHC variant, including I155T and R142H), number of nevi (10 or more) and childhood sunburn doubled MM risk in *CDKN2A*-negative individuals (Ghiorzo *et al.*, 2012). In the same study, it was suggested that *MC1R* plays a role in melanoma development in *CDKN2A*-negative cases both via pigmentary and non-pigmentary pathways.

In a study that used 11 computational tools based on different approaches to predict the damage-associated nonsynonymous single nucleotide polymorphisms (SNPs) on the coding region of *MC1R*, it was shown that the R variants D84E, R142H, R151C, I155T, R160W and D294H were classified as damaging by the majority of the tools while the R variants V60L, V92M and R163Q have been predicted as neutral by most of the algorithms. The I155T was predicted having damaging effect in 9/11 tested algorithms (Hepp *et al*., 2015) (figure 26).

Variants R160W, D294H, V60L, V92M, I155T and R163Q were individually associated with MM risk and carrying two non-synonymous variants was associated with much higher risk of MM in a Spanish population case-control study (odds ratio: 10.44, 95% confidence interval = 4.48-24.33, $p = 5x10^{-8}$) (Fernandez *et al*., 2007).

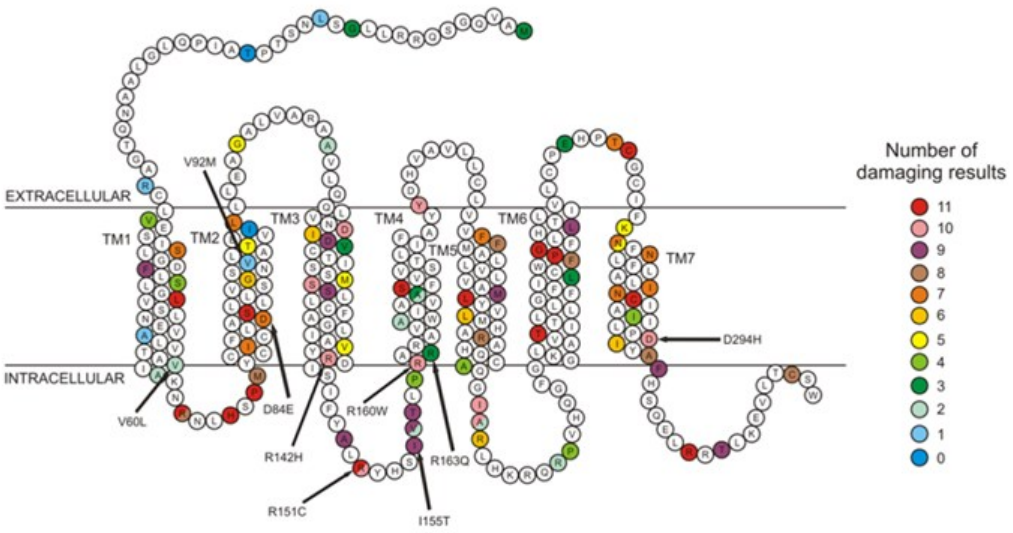


Figure 8. Two-dimensional structure of the MC1R protein and the 92 nonsynonymous SNP analyzed through 11 computational tools. The colors represent the count of damage as shown in the legend. The RHC associated mutations are indicated by the arrows. (Adapted from Hepp *et al*., 2015).

The I155T variant is a non-conservative amino acid substitution, which is likely to impact secondary protein structure as these residues differ in polarity, charge, size and/or other properties. At the position of this change, amino acids with similar properties to Isoleucine are conserved across species. Functional studies demonstrate that the I155T reduces cell surface expression of the MC1R protein and causes a loss of cAMP signaling, as well as it showed a dominant negative effect on wild-type receptor cell surface expression (Beaumont *et al.*, 2007).

The 155T occurs in the second intracellular loop. Two important known loss-of-function variants of *MC1R* gene, R151 and R160W, associated with RHC phenotype and increased skin cancer risk, are also located in the MC1R second intracellular loop (Sanchez-Laorden *et al*., 2009). It has been demonstrated in functional assay that the loss-of-function R151C and R160W MC1R alleles are export-deficient forms retained within the endoplasmic reticulum and the proximal Golgi, respectively. The same group also reported that two other mutations in the second intracellular loop, T157A and R162P, in the vicinity of I155T, did not display functional coupling to cAMP synthesis or detectable radioligand binding, consistent with extensive intracellular retention of MC1R (Sanchez-Laorden *et al*., 2009). The same

group (Sanchez-Laorden *et al*. 2006), demonstrated that aberrant processing with intracellular retention is the most likely cause of loss-of-function for many mutants, and concluded that mutations in virtually any region of the heptahelical protein, including its extracellular N terminus, a transmembrane fragment, intracellular loops or carboxyl terminal cytosolic extension, are likely to compromise normal MC1R processing. Beaumont *et al*. (2005) also demonstrated that alterations in amino acids of the intracellular loops, including I155T, compromised the export of MC1R to the membrane, resulting in less expression of this protein on the cell surface, even though the total cellular expression levels were normal. The I155T, was associated with a cell expression reduction of ~ 85% compared wih the wild-type clones. The variants R151C and the R160W seem to be in an important region to the MC1R phosphorylation by PKA and PKC, probably related to the intracellular signaling of the receptor to the surface, as well as initiation of other pathways (Garcia-Borron *et al*., 2005; Sanchez-Laorden *et al*., 2009). I155T is located between these two sites and, due to the physical-chemical character of the amino acid substitution (an apolar isoleucine by a polar threonine), might as well compromise this phosphorylation (Ibarolla-Vilava *et al*., 2014). Phosphorylation of intracellular loops is also relevant to the termination of GPCRs signaling, through binding to arrestin proteins that recognize a pattern of phosphorylated sites (Yang *et al*., 2017). Although this process is better described for the third intracellular loop, there might be a role for the second loop (Tobin, 2009) and, therefore, I155T could compromise MC1R function by this mechanism as well.

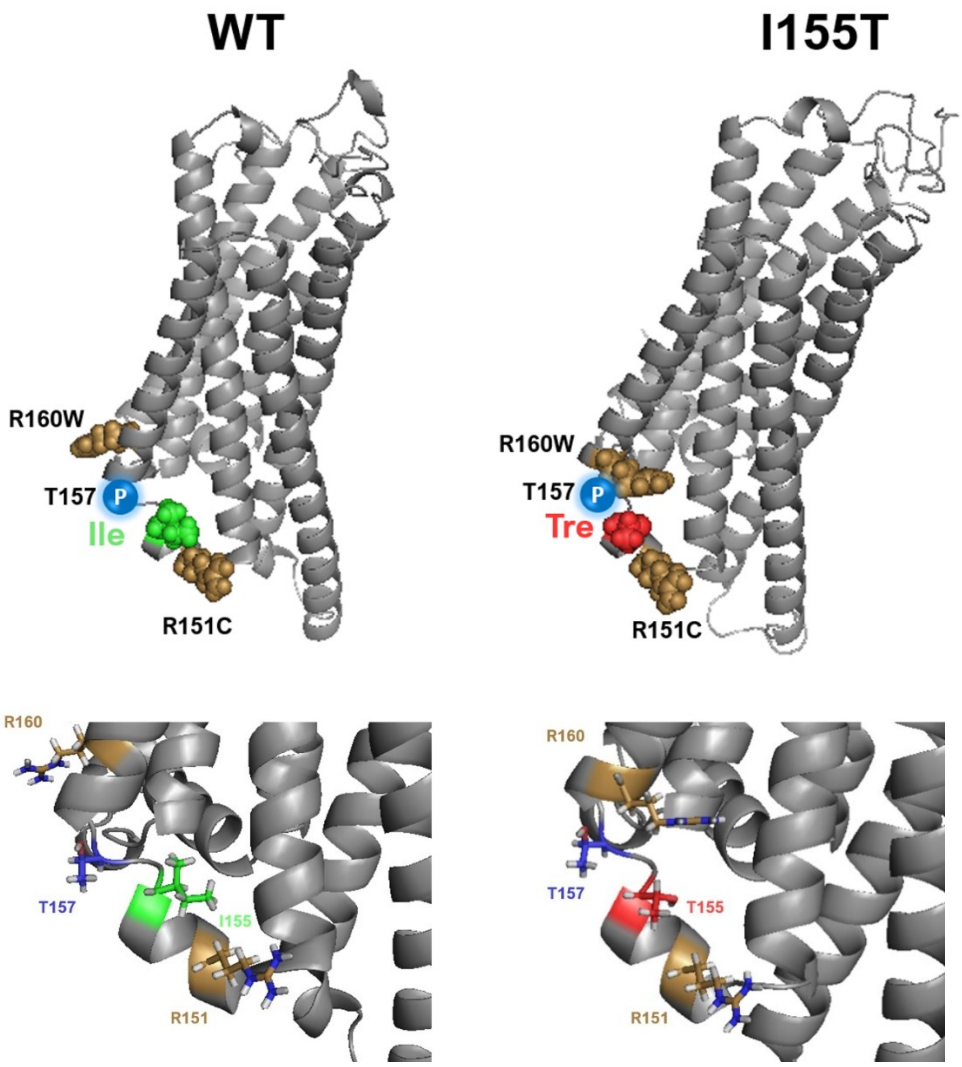


Figure 9. MC1R three-dimensional model. Representation of three nonsynonymous variants found in the Brazilian population. Classic RHC variants R151C and R160W are colored in golden, I155T is colored in green with the original amino acid (isoleucine) in the wild-type and the substituted amino acid is colored in red (threonine). Two figures below are magnification of receptor's second loop, where the three variants are collocated. The T157 residue is colored in blue, the phosphorylation site of the receptor.

p.Asp184His (c.550G>C, rs530102853); D184H

This rare missense variant was reported in a phototype II patient who has had one melanoma at the age 37 years (0.7 mm Breslow). The aspartic acid residue is weakly evolutionarily conserved and there is a moderate physicochemical difference between aspartic acid and histidine (Nykamp *et al*., 2017). The frequency of this variant in GnomAD is 0.00003. This variant has been reported in the literature in individuals affected with chronic sun-induced melanoma (Landi *et al*., 2006). Predictive algorithms (SIFT, PolyPhen-2, Align-GVGD) all

suggest that this variant is likely to be tolerated, but these predictions have not been confirmed by functional analyses. A Latvian study analyzed the receptor function in Val188Ile, a variant in a close position to the Asp184His, which might result in similar effects, and found that it behaves as wild type variant (Ozola *et al*., 2013). It is, therefore, currently classified as VUS.

p.Met203Thr (c.608T>C, rs769723368); M203T

This novel variant is located in the fifth transmembrane helix (TM5). Apparently, the amino acid change does not cause significant visible modifications as shown by the three-dimensional model in figure 28. However, the threonine in the mutant structure is more apolar than the naïve methionine in MC1R.

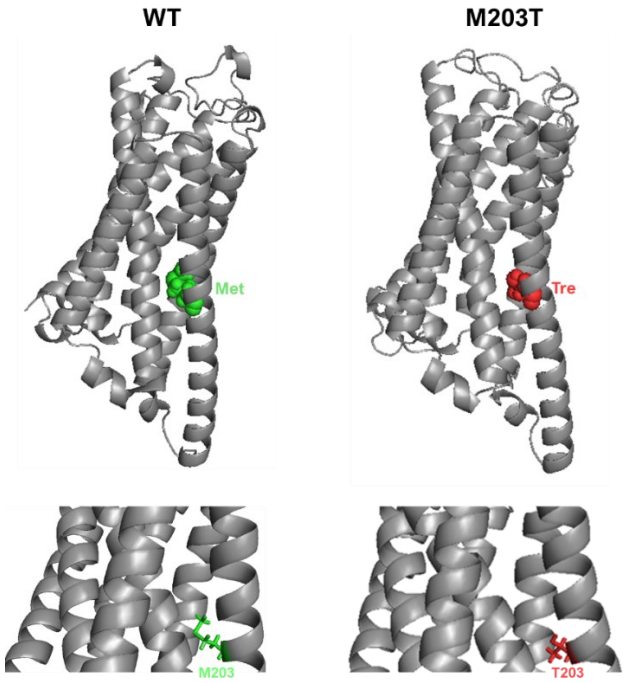


Figure 30. MC1R three-dimensional model of wild type (left) and mutated M203T (right). The original methionine is colored in green, and the mutated threonine is colored in red, showing that the amino acid change takes place in an inner part of the fifth transmembrane domain. Two figures below are magnification of the receptor's inner core.

The common structure of GPCRs consists in a conserved sctructural nucleous, formed by the four first transmembrane domains (TM1-TM4) and an active dynamic domain formed by the other three (TM5-TM7) (Schwartz *et al*., 2006). TM5 probably is enrolled in the

interface between the two areas, together with TM3 nad TM4, and would be implicated in the receptor stabilization for the translational of the signal. Mutations in these regions could compromise MC1R stability, but functional studies have not confirmed this notion (Roth *et al*., 2008). It has been shown that 63% of damaging variants are located in four domains (intracellular 2 and transmembrane 2, 5 and 7), whereas only 18% of non-damaging variants are located in these domains ($p < 0.0001$) (Hu *et al*., 2014).

p.Asp294His (c.880G>C, rs185009); D294H

It is considered a RHC variant. It was present in six patients in the current study, although only two presented the phenotype described above. The only amelanotic melanoma among the patients studied was in a D294H homozygous patient. This variant is a non-conservative amino acid substitution which is likely to impair secondary protein structure as these residues differ in polarity, charge, and size. This substitution occurs at a position that is conserved across species. The variant has been previously reported in association with RHC phenotype but also an increased risk of photoaging and congenital melanocytic nevi (Kinsler *et al*., 2012; Puig-Butillé *et al*., 2013; Ibarrola-Villava *et al*., 2014). Not all cases who carried this variant in the current study displayed all these phenotypiuc characteristics. *In vitro* assays of this variant showed that it could alter melanoma cell growth and adhesion to extracellular matrix (Robinson and Healy, 2002). The hormone αMSH increased intracellular cAMP in wild-type transfected melanoma cells but this response was compromised in the D294H variant transfected clones. In growth inhibition experiments, αMSH significantly reduced growth of wild type MC1R transfected cells but had no effect on cells transfected with variant MC1R. In addition, binding to fibronectin was significantly reduced by αMSH in the wild type transfectants whereas this was not seen in the variant transfected clones; binding to laminin was not affected by aMSH in this cell line. Functional characterization of the D294H variant

showed decreased ability to stimulate cAMP production, and altered cell surface expression of the MC1R protein (Schiöth *et al*., 1999; Sánchez-Laorden *et al*., 2009). These results provide evidence for differences in melanoma cell behavior secondary to *MC1R* variants, and reinforce an alternative non-pigmentary mechanism whereby *MC1R* variants could modify melanoma susceptibility or progression (Robinson and Healy, 2002). In summary, this variant is not expected to cause highly penetrant Mendelian disease but the D294H variant seems to be an established risk factor for melanoma.

5.3.2 *MC1R* variants and non-melanoma skin cancers

Germline *MC1R* variants are associated with melanoma and keratinocyte-derived skin cancers, even after controlling for skin type/hair colour (Haddadeen *et al*., 2015). The association between other skin cancers and *MC1R* has previously been studied and subjects carrying at least one *MC1R* variant (more consistent significant results obtained for the variants V60L, D84E, V92M, R151C, R160W, R163Q and D294H) showed an increased risk of non-melanoma skin cancer as well, overall, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (Tagliabue *et al*., 2015). Our study showed similar results, 4 of the 6 described variants found (V60L, R151C, R163Q and D294H), which are in bold in table 25, were significantly associated with non-melanoma skin cancer. Although no association was seen to variants R160W and V92L, this could be due to the fact that only one patient has each of these variants. A significant association of variants I155T (37.5%) and R142H (100%) with BCC/SCC was also found, although in the latter, the amount of patients harboring this variant was small (2).

Table 19. Correlation between the presence and amount of non-melanoma skin cancer in carriers of nonsynonymous *MC1R* variants. Variants in bold have been previously associated to increased risk of these cancers.

Nonsynonymous Variant	Number of patients	Number of patients with BCC/SCC (%)	Total number of tumors
Q23X	1	No	0
V60L	**5**	**2 (40%)**	**3**
V92M	1	0	0
R142H	2	2 (100%)	3
R151C	**2**	**2 (100%)**	**3**
I155T	8	3 (37.5%)	6
R160W	1	0	0
R163Q	**5**	**3 (60%)**	**4**
D184H	1	0	0
M203T	0	0	0
D294H	**6**	**3 (50%)**	**>13**

5.3.3 *MC1R* and other genetic alterations in familial MM and MPM cases from Brazil

The largest study that focused on hereditary melanoma in the Brazilian population included 64 familial cases and 60 MPM (Sá *et al*., 2018). The authors identified the following phenotypic proband characteristics: mean age at diagnosis 50 years, skin phototype I/II in 89.5%, sunburn during childhood in 85.5%, total body nevi ≥ 50 in 56.5%, Breslow thickness ≤1mm in 70.2%, tumors located on the trunk in 53.2% and SSM in 70.2% (Sá *et al*., 2018). However, no molecular data was reported about these patients.

A study from Southern Brazil (encompassing19 familial melanoma and/or multiple primary melanoma) analyzed the effect of four SNPs associated with gene pigmentation on melanoma risk (*TYR*, *HERC2*, *SLC24A5*, *SLC45A2*), but excluded *MC1R* (Reis *et al.*, 2020). In addition, another recent study aimed to identify SNVs on pigmented-related genes with importance in melanoma risk and clinical aspects of 103 sporadic cutaneous melanoma patients, demonstrated that inherited abnormalities in *ADCY3*, *CREB1*, and *MITF*

pigmentation-related genes, not only can increase the risk of cutaneous melanoma, but also influenced CM patients' clinicopathological features, but no data about the *MC1R* status of participants were provided (Lourenço *et al*., 2020).

Thirty-three cases of familial melanoma and/or multiple primary melanomas were genotyped in patients from Southern Brazil (Grazziotin *et al*., 2012) and no mutations in *CDKN2A* or *CDK4* were detected. In this group, at least one infrequent variant in the *MC1R* gene was present in 72.8% of cases and the 12 variants were different from the ones detected in our study. One explanation for this differnce in mutation spectrum could be the different ethnic-genetic makeup of the Brazilian population that may also be determined by geographical regions within the country. The *CDKN2A* variant A148T (or G442A) was found in 9.7% of samples of CM cases from Southern Brazil (Grazziotin *et al*., 2012). This polymorphism was significantly more frequent in patients with melanoma than in controls (12.6% versus 3.9%, P = 0.009) in southern Brazil, especially those with European ancestry (Bakos *et al*., 2011). This polymorphism was detected in 5/44 (11.3%) CM affected individuals studied herein and in only 2/102 controls – 1,9%) (P-value 0,026; CI [-0.0035-0.1916]).

de Avila *et al*. (2014) studied the *CDKN2A* status in the largest cohort of Brazilian melanoma prone-patients (n=59) and showed that only 8/59 (13.6%) of genotyped cases harbored a mutation in this gene. This rate is lower compared with other international studies, and may result from the small sample size, the inclusion of families with only two cases of CM instead of three and, in addition, the inclusion of multiple primary melanoma patients without family history (de Avila *et al*., 2014).

It has been reported that childhood sunburns are present in 85.5% of hereditary melanoma patients in Brazil (Sa *et al*., 2018). Furthermore, photoprotection measures are not a common practice among Brazilians, including University students (Dallazem *et al*., 2019). We found an important history of sun exposure in 95.3% of familial and multiple primary

melanoma patients, including blistering in 23% and 62.5% of familial and MPM patients, respectively. These may contribute to the low incidence of disease-driver mutations in the individuals we here investigated.

The occurrence of melanoma among family members, especially in a tropical country like Brazil can be secondary not only to genotypic variations, but also to other shared risk factors, such as similar environmental behaviors (family members tend to a sun exposure routine from an early age and the practice of using sunscreen or other protective measures tend to be similar), as well as the relatives tend to have similar phenotypes regarding skin pigmentation, ability to tan and amount of nevus. In patients with familial melanoma who have similar phenotype, low penetrance genes (i.e. *MC1R*) (Menin *et al.*, 2011), acting in conjunction with environmental factors, like sun exposure may have an important role. In contrast, incomplete gene penetrance could be accountable in melanoma-free mutation carriers. Kanetsky *et al*. (2010) estimated that around 15% of melanomas are attributable to genetic effects of the four RHC variants of *MC1R* (C252A, R151C, R160W, D294H). In individuals presenting protection phenotype, such as dark eyes and dark hair it ranged from 33% to 42%. *MC1R* variants explained about 21% of familial aggregation of melanoma in a case-control-family study from Australia with 567 cases (Cust *et al*., 2012). In this study, it was shown that *MC1R* variants are an important determinant of early-onset melanoma and this association appeared to be mediated through pigmentary and non-pigmentary pathways.

One study analyzed *CDKN2A* and *MC1R* in Latin American patients with familial and sporadic multiple primary melanomas, including 117 Brazilian patients, and compared their data with those of patients from Spain, trying to establish a guideline basis for melanoma genetic counseling in Latin America (Puig *et al.*, 2016). That study included a total of 186 patients from Latin America, including Argentina (*n*=10), Brazil (*n*=117), Chile (*n*=28), Mexico (*n*=6) and Uruguay (*n*=25) and reported a 24% mutation rate in *CDKN2A* in Latin

American patients compared with 14% in Spanish patients (n=904) (Puig *et al.*, 2016)); a much higher percentage than we found in the present study (5.2%). Among MPM in Latin America, they also reported a 10% *CDKN2A* mutations, approximately equal to our findings (12.5%) albeit the mutation described herein is novel. Latin American patients with melanoma carrying a *CDKN2A* mutation had an increased number of affected patients in the family and an earlier age at diagnosis, but the number of personal primary melanomas did not reach statistical significance (Puig *et al.*, 2016)). Latin American patients had higher prevalence of *MC1R* variants (80.5%) than the Spanish population (67.9%), with similar R variants frequency. The variant I155T, common among MPM/familiar melanoma patients in our study (31.5%), was detected in only two of the Brazilian patients (1.7%), one patient from Chile and nine patients from Spain (from a total of 904 studied) in the report by Puig *et al*. (2016).

5.3.4 *MC1R* final considerations

Polygenic predisposition, coupled with high UV exposure and phenotypic features, may combine to cause melanoma. Multiple loci are associated with melanoma risk, including many low-penetrance loci, which may have a cumulatively significant risk (McMeniman *et al*., 2020). Inherited variation at *MC1R* is a robust marker for increased risk of melanoma, even among individuals with "sun-resistant" phenotypes (Kanetsky and Hay, 2018).

Attributable risk refers to the level of disease reduction possible through the "removal" of a particular exposure. The attributable risk assumed across all nine most common MC1R variants (V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q and D294H) is reportedly ~ 40% (Pasquali *et al*., 2015; Kanetsky & Hay, 2018). These variants range in frequency from about 0.5% to 11% in the general population and harboring one of these variants is a robust marker for increased melanoma risk. Therefore, *MC1R* is being considered

an ideal genetic marker for CM risk in the general population. Namely, this CM risk screening tool could shape physician-patient communication to motivate melanoma primary and secondary prevention behaviors (Kanetsky & Hay, 2018). Multiple studies are being conducted nowadays worldwide to assess the comprehensibility and acceptability of *MC1R* test information and public acceptance. In the rapidly evolving field of public health genomics, in the future, *MC1R* may be able to be combined with other known melanoma risk factors in order to provide individuals with more precise risk levels than are currently available (Kanetsky & Hay, 2018). If studies show that *MC1R* genetic risk screening in the general population proves to be effective for motivating behavioral change, a significant proportion of melanomas may be detected at its earliest stage with extremely high curative rates and, also, a large number of MM may be prevented altogether.

Evidence of different levels of association between *MC1R* variants and melanoma prompted several authors to suggest the inclusion of *MC1R* genotype in a melanoma risk prediction model (Whiteman & Green, 2005; Han *et al*., 2006; Penn *et al*., 2014; Tagliabue *et al*., 2018; Kanetsky & Hay, 2018).

Future development of compounds to correct defective MC1R responses secondary to *MC1R* variants could result in photoprotective benefits for fair-skinned individuals and reduce their overall skin cancer risk (Haddadeen *et al*., 2015).

In the present study, we showed that even in a highly genetically heterogeneous population like Brazilians, predominantly composed by dark-skinned individuals, the so called "redhead gene" *MC1R* plays an important role in melanoma development. *MC1R* genotype may be a better predictor of early-onset melanoma risk than pigmentation status (Haddadeen *et al*., 2015) and our findings support this. Even variants consistently associated to red hair phenotype (RHC), rare among the general Brazilian population, are relatively common in our

melanoma population (20.45%). Future precision risk assessment of melanoma is a reality that may be available in the near future and *MC1R* status may be an important genetic marker.

6. CONCLUSIONS

In the present study, we focused on defining the molecular basis of familial/inherited melanoma in Brazilian patients. Applying Whole-Exome Sequencing (WES) to a family of thirteen siblings of which seven were affected by multiple melanomas revealed germline alterations in several genes (*ABCA4, ANKRD27, CDC27, GAMT, ELOVL5* and *OR4C3* were not associated with the disease). Sequencing *XPC* in 15 members of this family showed that the living parent (mother) and non-affected individuals are heterozygous for the mutation identified. One unaffected sibling is wild type. All five affected individuals are homozygous. Variant genotyping in the family demonstrated its co-segregation with the disease phenotype and confirmed the diagnosis of Xeroderma Pigmentosum group C in this family, in spite of atypical clinical presentation (late onset of skin changes and tumors, only modest pigmentary skin lesions, absence of atrophy or telangiectasia, absence of ophthalmic involvement, extraordinary number of melanomas compared to the number of carcinomas, higher rate of affected members than expected in an autosomal recessive Mendelian disease and higher life expectancy in comparison to XP patients). The identification of this mutation further contributes to the genotype-phenotype correlation of *XPC*-associated XP. The study expands the knowledge of the mutational spectrum of *XPC* and is valuable for genetic counseling of affected individuals and their families. This finding has important curation/clinical consequences as it leads to reclassification of p.Arg307Trp (c.919C>T) from "not reported in ClinVar" to a pathogenic variant (Richards *et al*., 2015). The siblings present phenotypic heterogeneity, despite having the same causative mutation, probably due to additional genomic changes such as *MC1R* variant I155T, diverse levels of UV-radiation exposure throughout life and, possibly, different expression of protein isoforms. These findings should be future addressed by evaluating the ability of repair in these cells.

Brazil lacks population-based studies that assess the epidemiological situation and the real impact of XP (Schelini *et al*., 2019). Health-care planning for patients with XP requires

multidisciplinary follow-up for early detection of neoplasms, genetic and family counseling, and emphasis on education and sun protection, which is still the best form of cancer prevention. Wearing sunglasses, hats, sunscreen, and avoiding daytime outdoor activities are the measures available so far and are challenging in a country like Brazil. Early diagnosis, even prenatal diagnosis, leading to a more awareness social behavior and regular medical appointments is the best way to improve quality of life and expand life expectancy in these patients.

A germline variant in *CDKN2A* was detected in only one of the 19 affected familial/MPM patients studied, a patient with MPM – a rate of 5.2%. This variant (A109P) previously assigned "variant of uncertain significance" status may clinically be associated with a phenotype of multiple primary melanomas, multiple atypical nevi (FAMMM syndrome) and early age at diagnosis of tumors in individuals harboring this genotype. The A148T, polymorphism previously associated with a higher risk of cutaneous melanoma in Southern Brazil, was identified in 11.36% (5 of 44) of all melanoma patients studied, in contrast to 1.9% in controls (p = 0,026; CI [-0.0035-0.1916]), signifying that it may also contribute to CM risk.

Nonsynonymous variants in *MC1R* are significantly more frequent among melanoma patients than in controls (61.4% vs 13.7%), consistent with this gene being a well-established melanoma susceptibility gene (P<0,001; 95% CI: (0,32; 0,63)). There is a higher proportion of *MC1R* nonsynonymous variants carriers in patients who have positive family history or more than one melanoma (13/19) in comparison to patients who have had only one melanoma throughout life and no family members affected (14/25) (68.42% versus 56%) (P= .394; 95% CI: -0,161; 0,409).

The present study suggests that the incidence of variant I155T in *MC1R* gene in Brazilian patients with multiple melanoma and familial melanoma is higher (31,5% - 6 patients of 19) than in other melanoma population studies, like Spain (1%) (Puig *et al*., 2016) or USA (3.2%) (White *et al*., 2019) and not so common among sporadic melanoma patients (8%). The

overall frequency of this variant among the 44 melanoma patients is still higher (18.2%) than in all population studies suggesting there might exist a founder effect in the region of Minas Gerais. This variant also seems to be associated to increased number of melanomas, earlier age of melanoma onset and deeper melanomas in XP-C individuals. *MC1R* variant Q23X is rare and associated with a phenotype of multiple primary melanomas and early age of onset, and it is not associated with red hair phenotype, in contrast with previously published single report of this variant.

A novel variant in *MC1R* is described (Met203Thr), associated with early age of onset of melanoma (22 years old) and red hair phenotype, suggesting that it should be added to the RHC variants group.

Three of the four classic RHC variants were found in this study, R151C, R160W, D294H respectively in 4,5%, 2,3% and 13,6% of patients. In these patients, melanoma before the age of 40 occurred in 55,5% and Breslow higher than 1 mm in 44,4%, reaffirming the worse prognosis in these carriers. Variants 155T and R142H, considered as RHC variants by some authors, according to our findings, do not have association with the red hair phenotype and should not be considered "automatic" RHC variants.

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8. APPENDIX

Appendix 1

Questionário

Projeto Melanoma Familial

Nome do paciente: _____ __

CPF: _____ _____

Data de nascimento: _____ Telefone de contato: _____

Paciente acompanhado por: _____

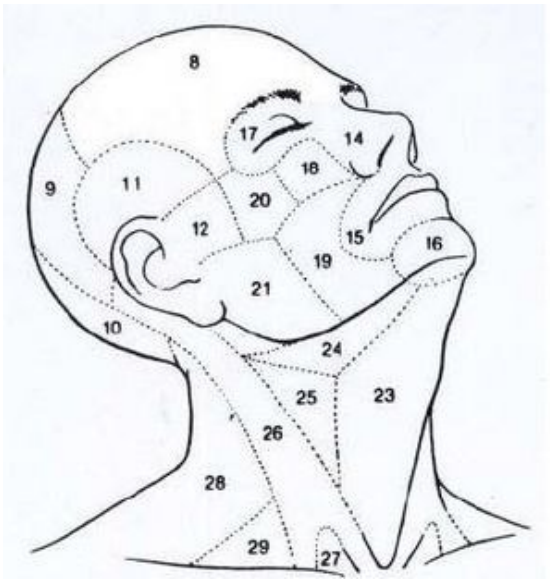
Sangue coletado dia: _____ Código do paciente: MFA_____ Lab nº _____

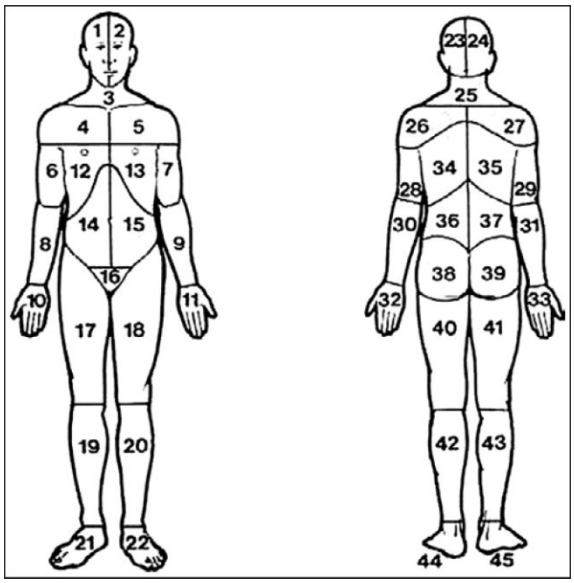
Paciente acometido clinicamente: □ Sim □ Não – Parentesco com o acometido:_____

Fototipo:_____

Mutação detectada: □Não □ Sim / Qual:_____

Melanoma (s)





Quantidade:_____

Idade (s) do diagnóstico: _____

Tipo(s): _____

Local (is): _____

Breslow(s): _____

Linfonodo sentinela: □ Não realizado □ Realizado, negativo □ Realizado, positivo:_____

Metástases: □Não □ Sim/ Locais: _____

Tratamento realizado: □ Apenas cirúrgico □ Outros: _____

Comorbidades

Atuais: _____ Pregressas: _____

Uso de medicamentos: _____

Número de nevos:

_____ poucos e típicos; _____ poucos mas atípicos; _____ > ou = 50 nevos

---

História oncológica

Algum outro câncer? □ Não

□ De pele → Tipo (s): _____

Local: _____

Mesma região do melanoma: □ Sim □ Não Número:_____

□ Outro órgão → Local: _____

Tratamento realizado: _____

História familiar Oncológica

□ Melanoma / Quem e quantos cada um: _____

□Pele NÃO-MELANOMA □Mama □ Pâncreas □SNC □Outro órgão / Qual (is): _____

Já usou algum imonussupressor: □ Não □ Sim/ Quais: _____

Tabagismo: □ SIM □ NÃO □ PREGRESSO DURAÇAO: _____

Queimadura solar prévia: □ Nega quaisquer excessos

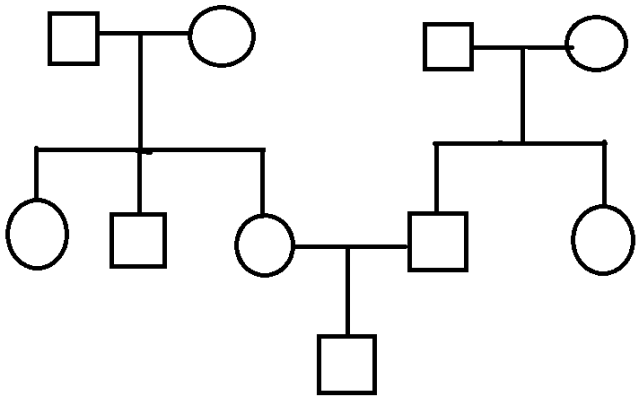
□ Bolhas □ Insolação (eritema intenso sem bolhas) □ Somente exposição intensa desprotegida

---

Heredograma

Filhos: □ Não □ Sim / Quantos _____

Examinados: □ Clinicamente □ Molecularmente □ Não examinados – Motivo: _____



Laudos Histopatológicos

Oficial/original:

_____

Lab/patologista:_____

Revisão:

_____

Observações pertinentes:

Appendix 2

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

N° Registro COEP: ____CAAE-0472.0.203.240-11____

Título do Projeto: "Melanoma Familial: Correlação entre genótipo e fenótipo"

O senhor (a) está sendo convidado (a) a participar, como voluntário, em uma pesquisa de título: "Melanoma Familial: Correlação entre genótipo e fenótipo". O documento abaixo contém todas as informações necessárias sobre a pesquisa que está sendo realizada. Sua colaboração neste estudo é muito importante, mas a decisão de participar deve ser sua. Para tanto, leia com cuidado as informações abaixo e não se apresse em decidir. Se você não concordar em participar ou quiser desistir em qualquer momento, isso não causará nenhum prejuízo a você. Se você concordar em participar basta preencher os seus dados e assinar a declaração concordando com a pesquisa. Se você tiver alguma dúvida pode esclarecê-la com o responsável pela pesquisa, Dra. Franciele Antonieta Bianchi Leidenz. Obrigada.

Objetivo do estudo

Este projeto está sendo proposto porque há pouco conhecimento acerca dos aspectos moleculares dos melanomas familiais, principalmente no Brasil. O encontro de mais de um membro de uma mesma família apresentando melanomas cutâneos significa, provavelmente, que há uma mutação no DNA desses indivíduos e essa alteração poderá passar de geração em

geração, causando elevada morbidade nos acometidos. Identificando tal alteração, esperamos contribuir para um melhor entendimento da doença e para o adequado acompanhamento e abordagem dos acometidos. O objetivo desse trabalho é estudar, tendo como base os indivíduos da família, os aspectos genéticos (moleculares) que envolvem o surgimento dos tumores nessa população. Pretendemos também, através de acompanhamento clínico, observar o comportamento de tais tumores conforme a mutação apresentada e, ainda, propiciar um adequado aconselhamento genético para todos os indivíduos da família.

Procedimentos

Serão incluídos nesta pesquisa tanto indivíduos portadores de melanomas, quanto alguns outros membros da família que podem carrear as alterações moleculares, mesmo que não acometidos pela doença, desde que concordem e assinem o termo de consentimento livre e esclarecido. São incluídos também pacientes portadores de melanoma sem histórico familiar desta doença, a fim de que possamos averiguar eventuais divergências nos achados moleculares entre o grupo dos pacientes com e sem história familiar de melanoma ou de melanoma múltiplo primário. Não serão administrados aos pacientes quaisquer medicamentos. O principal inconveniente aos quais os pacientes serão submetidos será a coleta de sangue total (10 ml). O desconforto associado à coleta de sangue é o habitual de um exame de sangue de rotina. A coleta será realizada com material estéril, descartável e por pessoas treinadas. O material colhido será devidamente etiquetado e utilizado somente para os propósitos dessa pesquisa, não havendo quaisquer custos para o paciente. As informações obtidas serão objeto de estrita confidencialidade e não envolvem custos ou pagamento de qualquer espécie.

Pesquisadores

A equipe de pesquisadores é composta pelos seguintes profissionais: Dr. Luiz Armando Cunha De Marco (coordenador da pesquisa), Dra. Franciele Antonieta Bianchi Leidenz e Dra. Flávia Vasquez Bittencourt. Todos os membros são médicos, pesquisadores e ligados formalmente à Universidade Federal de Minas Gerais, onde os estudos se desenvolverão.

Confidencialidade

Todos os dados dessa pesquisa serão mantidos em sigilo e apenas a equipe de pesquisadores terá acesso a eles. Cada participante receberá um código garantindo confidencialidade de sua identidade. Caso o (a) senhor (a) queira, terá acesso aos resultados individuais.

Benefícios e riscos

Esta pesquisa não oferece qualquer risco adicional ao participante, exceto aqueles inerentes à própria coleta de sangue total. As informações obtidas nos auxiliarão a compreender os mecanismos genéticos envolvidos no surgimento e nas manifestações clínicas dos melanomas familiares.

Não haverá qualquer tipo de despesa ao participante, no que tange a materiais ou testes. Ao assinar esse termo de consentimento o senhor (a) não está abrindo mão de seus direitos legais.

Através deste documento fica assegurado o direito ao Sr(a) _____ que terá todos

os esclarecimentos relativos à pesquisa garantidos, incluindo os métodos utilizados. A partir do momento que o paciente participante da pesquisa não desejar mais fazer parte da pesquisa, é reservado o direito de se retirar, estando livre de sofrer quaisquer penalidades ou danos. Se no transcorrer da pesquisa surgir alguma dúvida, poderá o participante procurar um dos pesquisadores: Dr. Luiz Armando Cunha De Marco, Dra. Flávia Vasquez Bittencourt e Dra. Franciele Antonieta Bianchi Leidenz, no tel. 3409-9134 (UFMG) ou no tel. (031) 996351770.

Eu, _____,

paciente voluntário, dou consentimento livre e esclarecido, para que se façam os testes necessários a esta pesquisa e posterior uso e publicação dos dados nos relatórios finais e conclusivos, a fim de que estes sirvam para beneficiar a ciência e a humanidade.

Declaro que recebi cópia do presente Termo de Consentimento.

Belo Horizonte, _____de _____de 20_____

Assinatura do participante ou responsável legal

_____

Assinatura do pesquisador

_____

Franciele Antonieta Bianchi Leidenz

Caso o Sr.(a) queira se informar sobre o projeto, independentemente da equipe do Coordenador, favor contatar:

COEP (Comitê de Ética em Pesquisa da UFMG)

Universidade Federal de Minas Gerais

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