UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ivo FUNDACIÓN INSTITUTO VALENCIANO DE ONCOLOGÍA

**DOCTORAL THESIS**

# Next-generation sequencing in the identification of biomarkers in cutaneous melanoma according to the etiopathogenic development pathway and their potential clinical relevance


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València, January 2022

*"La ciencia es la base del*
*desarrollo del país"*
Margarita Salas

*"Knowledge belongs to everyone*
*and all people should have*
*equal access to it"*
Alexandra Elbakyan

## Acknowledgements

Esta tesis doctoral es el resultado de años de trabajo y no habría sido posible sin el apoyo de muchas personas e instituciones.

Quiero agradecer a la Asociación Española Contra el Cáncer, y en especial a la Junta Provincial de València, por las becas que me han permitido a mí y a otras jóvenes investigadoras desarrollar nuestra tesis doctoral. Este agradecimiento se extiende a todas las personas que han participado en las carreras que a lo largo de toda la provincia de València han recaudado fondos para que estas ayudas fueran una realidad. Este esfuerzo nacido de la sociedad civil muestra el compromiso de ésta hacia la investigación. Un compromiso del que deben aprender las instituciones públicas para apostar de una manera constante y decidida por la ciencia y por sus profesionales

En este proceso me han guiado mis directores Eduardo y Zaida.

Gracias, Eduardo, por ser mi mentor. De ti he aprendido a hacer ciencia y a gestionarla siendo imaginativo cuando la situación lo requiere, además de valorar la importancia de dar a los pacientes la atención que merecen. Tener un director respetuoso que siempre me ha animado a llegar más lejos es algo que valoro enormemente y que muchas personas en mi situación querrían.

Gracias, Zaida, por ser una constante en este proceso. Durante 5 años me has enseñado a perseverar cuando los experimentos fallan y sobretodo has cuidado de mí. He sido testigo de los mejores y los peores

momentos a los que nos podemos enfrentar como personas, y en todos ellos has sido un ejemplo de dignidad y humanidad.

I would also like to thank Dr. Kumar and Dr. Virós, my supervisors in the rotations I have performed abroad, for their contributions in my formation. D'aquestes experiències m'emporte haver conegut gent bona que m'ha fet sentir a casa, i que ja forma part de la meua vida malgrat la distància.
Quiero acordarme también de mis compañeras becarias Elena, Mireia, Fernanda y Gabriela. Gracias por formar el mejor grupo de apoyo posible y por habernos mantenido a flote unas a otras.

Gracias a mis compañeros y compañeras del Laboratorio de Biología Molecular del IVO, desde José Antonio, que siempre ha tenido una palabra de ánimo, pasando por facultativos, técnicos (y Belén como la mejor "R pequeña"). Durante una tesis se cometen muchos errores, pero tener compañeras que siempre responden cuando pides ayuda marca la diferencia.

A mi servicio adoptivo de Derma, gracias por dejar a este "biólogo" intruso ocupar cada una de las consultas, seminarios y esquinas como despacho itinerante. En las últimas etapas de esta tesis os habéis convertido en mi segundo hogar. Mención especial a los resis. Gracias Fátima, Elisa y Edu por haberme acogido e integrado, casi hasta despertando mi interés por la dermatopatología.

Gracias a médicos, enfermeras, auxiliares y secretarias por cuidarme. Nuria, Paloma y Arantxa, habéis sido en muchas ocasiones la última línea de defensa antes del abismo, sacándome una sonrisa hasta en los momentos más duros.

En general, gracias al resto de servicios que han participado de alguna forma en este proyecto (por supuesto esto incluye a las chicas de la cafetería que tan bien nos tratan cada día). Formar parte del IVO estos años me ha ayudado a comprender que el aprecio que tiene la sociedad valenciana a esta institución radica en la dedicación y esfuerzo de trabajadores y trabajadoras, demostrando que todo lo que merece la pena se construye desde abajo.

Dejando lo más importante para el final quiero agradecer a mi familia, amigos y amigas, su apoyo a lo largo de estos años. Pese a no comprender siempre mi trabajo, no han dudado en acompañarme durante todo el proceso.

## Resum

El melanoma és el tipus de càncer de pell més mortífer i perillós, ja que fins els tumors de menor mida poden acabar generant metàstasi. Al llarg dels anys, s'ha tractat de classificar des del punt de vista clínic, epidemiològic i molecular. Les classificacions actuals utilitzen el nivell d'exposició solar i la localització tumoral per dividir en diferents grups als pacients de melanoma.

Al 1998, David Whiteman i col·laboradors proposaren un model de desenvolupament del melanoma que anomenaren "model de vies divergents". Aquest presentava dos vies per la melanomagènesi: una vinculada a la proliferació melanocítica (nevogènica) i l'altra relacionada amb l'exposició solar crònica (CSD). Malgrat aquest model fou corroborat des del punt de vista clínic i epidemiològic, encara no s'ha aportat una caracterització molecular en profunditat.

A nivell general s'havien identificat gens les mutacions dels quals eren rellevants per al desenvolupament del melanoma, com el gen *KIT*. Però, encara s'havia d'estudiar amb més cura la distribució d'estes mutacions entre els distints subgrups de melanoma, així com el seu possible valor pronòstic.

En aquesta tesi s'han emprat tècniques de seqüenciació -massiva i tradicional- per caracteritzar els perfils mutacionals de les dues poblacions proposades pel model de vies divergents, trobant diferències tant al nombre de mutacions com als gens afectats. També hem vist com els melanomes mutats en *KIT* semblen desenvolupar-se per una via independent de l'etiopatogènia coneguda, mancant l'estatus mutacional d'aquest gen de valor pronòstic per la supervivència dels pacients.

## Resumen

El melanoma es el tipo de cáncer de piel más mortífero y peligroso, ya que tumores de pequeño tamaño pueden generar metástasis. Hasta la fecha, se ha tratado de clasificar desde el punto de vista clínico, epidemiológico y molecular, empleándose actualmente el nivel de exposición solar y la localización del tumor como criterios principales para dividir en distintos grupos a los pacientes de melanoma.

En 1998, David Whiteman y colaboradores propusieron un "modelo de vías divergentes" para el desarrollo del melanoma. Este presentaba dos vías: una vinculada a la proliferación melanocítica (nevogénica) y otra relacionada con la exposición solar crónica (CSD). Corroborado desde el punto de vista clínico y epidemiológico, todavía no se ha aportado una caracterización molecular en profundidad.

A nivel general se habían identificado genes cuyas mutaciones eran relevantes para el desarrollo del melanoma, como por ejemplo *KIT*. Sin embargo, todavía se había de estudiar con más detalle la distribución de estas mutaciones entre los distintos subgrupos de melanoma, así como su posible valor pronóstico.

En esta tesis se han empleado técnicas de secuenciación – masiva y tradicional – para caracterizar los perfiles mutacionales de las poblaciones del modelo de vías divergentes. Encontramos diferencias tanto en el número de mutaciones como en los genes afectados. También hemos visto cómo los melanomas con mutaciones en *KIT* parecen desarrollarse por una vía independiente de la etiopatogenia conocida, careciendo el estatus mutacional de este gen de valor pronóstico para la supervivencia de los pacientes.

## Abstract

Melanoma is the deadliest and most dangerous type of skin cancer, given that a small tumor can spread and result in metastasis. Over the years, classifications have been made either from a clinical, epidemiological or molecular point of view. Current classifications use the degree of solar exposure and tumoral location to divide into different melanoma groups.

In 1998, David Whiteman and collaborators proposed the divergent pathway model for melanoma development. This presented two pathways to melanomagenesis: one related to melanocytic proliferation (nevogenic) and the other related to chronic sun exposure (CSD). Despite corroborations of this model from the clinic and epidemiology, it is yet to be molecularly characterized in depth.

At a general level, different genes had been identified with relevant mutations for the development of melanoma, as is the gene *KIT*. However, there was a lack of knowledge on how these mutations were distributed among different melanoma subgroups, as well as the potential prognostic value.

In this thesis we have implemented sequencing techniques – both massive and traditional – to characterize the mutational profile of the two populations proposed by the divergent pathways model. We found differences both in the number of mutations and in the genes carrying the mutations. We have also seen how melanomas harboring *KIT* mutations seem to develop in a way which is independent from the known etiology, and how the mutational status of this gene lacks prognostic value on the outcome of the patients.

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# 1. INTRODUCTION

## 1.1. Melanoma. Cell of origin and malignant transformation.

### *1.1.1. Melanocytes*

Melanocytes are the cells in charge of melanin production, a pigment found in human skin as well as in other vertebrates. They can be found mainly at the basal layer of the epidermis, but also in the hair bulb, eyes, ears, meninges and other mucosal epithelia. These cells derive from the neural crest, which is a group of highly migratory embryonic cells induced in the zone between the neural and non-neural ectoderm at the time of gastrulation. After a process of differentiation, the multipotent neural crest stem cells generate melanocyte precursors called melanoblasts. These will migrate, proliferate and differentiate *en route* to its eventual destinations in the basal epidermis or mucosal epithelia. The number of melanocytes remain relatively constant over life, with a low division rate of twice a year (1,2).

Melanin production is stimulated by the ultraviolet radiation (UVR)-induced DNA damage to keratinocytes. When such harm is detected, keratinocytes secrete alpha-melanocyte stimulating hormone (α-MSH) which will bind to the melanocortin 1 receptor (MC1R) and induce melanin production. Polymorphisms in *MC1R* gene, and to a lesser extent polymorphism in other genes coding proteins also involved in the synthesis of melanin (*ASIP*, *SCL45A2*, *TYR*, *TYRP1*), together with a series of enzymatic and non-enzymatic chemical reactions will determine the type of melanin produced from a common tyrosine

precursor. Pheomelanin is a reddish-yellow form of melanin which provides a low degree of protection from UVR, and is thought to have a genotoxic role in the cell because of its production of reactive oxygen species (ROS). Contrarily, eumelanin is a brownish-black from of melanin which provides a high degree of protection against UVR and balances the effect of pheomelanin (3–5).

Mature melanocytes produce melanin in lysosome-like structures called melanosomes. Once melanin is produced, melanosomes are transported along melanocyte microtubules up to the point where they are transferred to adjacent basal keratinocytes. There, they will distribute forming a layer that will protect the genetic material of keratinocytes from UVR (6).

The process by which melanocytes transform into malignant melanoma cells is not fully understood, since it involves several complex factors affecting different cellular pathways. However, melanoma cells will eventually acquire the common characteristics of cancer cells, including abnormal proliferation, immortality, and evasion of the immune system, among others.

### *1.1.2. Melanocytic nevi*

When a melanocyte acquires an initiating mutation – e. g. $BRAF_{V600E}$ - (7), it will undergo increased proliferation up to a point where it will enter a state similar to senescence. Once the melanocytic nevus is formed, a certain degree of proliferation will be kept, though balanced by attrition factors hence providing these melanocytic nevi with a stable size (1). The number of acquired nevi varies greatly among individuals

and over lifetime, depending on the genetic predisposition and the exposure to UVR, usually peaking during the third to fifth decades of life and regressing later on (8,9).

There are some nevi that display characteristics at either clinical or histopathological level that make them different from common melanocytic nevi., albeit do not constitute a precursor of melanoma. Atypical nevi are suggested clinically as a brown patch of 5 mm in diameter with variable pigmentation, asymmetry or irregular borders. From a histopathological point of view, dysplastic nevi show architectural disorder, and genetically, they would harbor a higher mutational burden than melanocytic nevi (1,10). Despite being considered as a precursor of melanoma in some studies, this is a matter of debate and most authors do not align with this hypothesis and, accordingly, there is no formal indication of surgical removal to prevent its progression to melanoma. Furthermore, these have been found to increase the risk melanoma (11).

### *1.1.3. Melanoma in situ*

Melanoma in situ refers to a proliferation of malignant melanocytes limited to the epidermis, thus not invading deeper layers of the skin, and with a radial growth pattern (12). Histologically, these are defined by poor circumscription, asymmetry, predominance of individual melanocytes over nests with confluent growth along the dermo-epidermal junction, effacement of rete ridges, and pagetoid scatter. Nests of atypical melanocytes with confluence, variability in shape and

size, and consumption of the epidermis; random distribution, and involvement of the adnexal epithelium can also be seen (13).

Having suffered from a melanoma in situ increases the risk of developing second primary tumors, specifically subsequent primary malignant melanoma. However, the characteristic superficial component of these tumors makes them little aggressive and, once excised, the life expectancy of the patients is the same as the general population (14,15).

### *1.1.4. Invasive melanoma*

The next step in malignancy for melanoma cells is gaining ability to leave the epidermal epithelium and enter the subjacent mesenchymal tissue of the dermis or mucosa. In such process, keratinocytes play a crucial role by secreting factors that help loosening the melanocyte-keratinocyte interaction. These will eventually downregulate the expression of cohesive proteins, contribute to the breakdown of the basement membrane, and induce signaling pathways that favor invasion (16). Also, since melanocytes derive from the neural crest, there is a certain expression of epithelial-mesenchymal transition (EMT) markers that would have remained active after the differentiation into melanocytes, and would contribute to invasion once malignant transformation occurs (17).

At this point, the risk of metastatic disease and death correlates mainly with the depth of invasion, known as Breslow thickness. Survival rates decrease and the clinical management of patients require treatments beyond surgical removal of the melanoma (18,19).

## 1.2. Risk factors for Melanoma

Melanoma is a complex disease resulting from the intervention of multiple factors. These factors can be classified into two main categories: environmental factors and host factors.

### *1.2.1. Environmental factors*

#### *1.2.1.1. Sun exposure*

Sun exposure is the major environmental cause of melanoma (20). Solar radiation comprises ultraviolet, visible, infrared, radio waves, X-rays, and gamma rays. Its ultraviolet spectrum, the spectrum that has been related to skin cancer development, is composed of three different types of radiation, classified according to their wavelength. UVA range is 315-400 nm and practically reaches the surface of the planet fully (95%); UVB range is 280-315 nm and only 5% passes through the ozone layer; UVC range is 100-280 nm and, despite being the most dangerous type, it hardly reaches the Earth's surface (21). Since both UVA and UVB hit our skin, it is difficult to establish the different contributions on the development of melanoma. Although recent studies suggested a more active role of UVB in the acceleration of melanomagenesis (22), it has been described that both types of radiations result in the mutational signature associated with UV-induced damage. This signature is based on cytosine to thymine (C>T) changes in dipyrimidine sites (23) though recent studies suggest that UVA could have a different mutational signature, showing this C>T changes in TCG sequence and a very low frequency of CC>TT

mutations, which is detected rarely but constantly in UVB range (24,25)

Individual sun exposure is commonly classified as intermittent- with short, intense sun exposure usually linked to recreational activities-, and chronic- with a continuous exposure linked to occupational habits over lifetime-. The intermittent pattern resulting in sunburns, especially during childhood, has been shown to increase melanoma risk. (26,27).

Regardless of growing knowledge on the influence of UVR in carcinogenesis, there has been a surge in the popularity of indoor tanning. These sunbeds irradiate UVA and, to a lesser extent UVB, thus increasing the risk of melanoma in a clientele of young people (28). A review of regulations and informative campaigns should be addressed to raise awareness on this issue and not limiting the efforts to the use of sun screen outdoors.

#### *1.2.1.2. Others*

There are other environmental factors that have been associated with melanoma risk. The evidence supporting them is diverse, and include retrospective and prospective studies though, in some cases, it can be difficult to make a thorough assessment on the potential threats of an agent. They are usually linked to occupational exposure: workers exposed to petroleum or mineral oils at the automobile industry; printers and lithographers; workers from electricity and electronic industries; workers exposed to polyvinyl chloride (PVC) and from the clothing industry; farmers and veterinarians exposed to certain chemicals found in the agriculture industry; and airplane pilots exposed

to ionizing radiation (29–32). These are only some examples of environmental factors beyond UVR.

### *1.2.2. Host factors*

#### *1.2.2.1. Family History*

A family history of melanoma constitutes a severe risk factor for melanoma development. Having a first-degree relative with melanoma is sufficient to double the risk of developing a melanoma. However, to be considered as familial melanoma, a family must have either 2 first-degree relatives or 3 or more relatives, depending on the incidence rates of the population where the family belongs, on the same side of the family diagnosed with melanoma (33). Familial melanomas are thought to account for 10% of all melanoma cases. Out of them, between 20 and 40% have an alteration in the *CDKN2A* gene, which is a cyclin-dependent kinase inhibitor that will be explained more extensively in the next chapter. Mutations in the *CDK4* gene are also linked to familial melanoma, at a much lesser degree. Other genes with heritable susceptibility include *ACD, BAP1, TERT, POT1, MITF, TERF2IP, TERT,* and *MC1R* (34,35).

#### *1.2.2.2. Phenotype*

Different phenotypic characteristics have been correlated with an increased risk of melanoma. The number of melanocytic nevi is one of the most studied and, as stated in previous sections, it depends also on the size, location, and type, being dysplastic/atypical nevi more relevant when assessing this risk. For instance, a recent meta-analysis showed

that people with more than 100 nevi are seven times more likely to develop a melanoma (11,36). The importance of number of common melanocytic nevi as a risk factor for melanoma development is due to the fact that they represent a clinical marker of a constitutive susceptibility to melanocytic proliferation, a trait that facilitates one of the steps of melanomagenesis (37).

Phenotypic traits include the degree of pigmentation, which affects skin, eye, and hair colors and can contribute to the development of melanoma (26). Numerous studies have found that people with fair skin, or with an inability to tan, are more at risk to eventually have a melanoma. Regarding the color of eyes and hair, while increasing the risk when light-colored, there is not a consensus on whether these represent an independent risk factor, or are linked to skin color (38,39).

## 1.3. Epidemiology. Melanoma over time.

The prevalence of melanoma varies around the world, given the role of UV radiation on its development and the different impact on populations depending on their skin pigmentation and latitude of their country (26,40). However, a common ground within European-descent populations has been the steady increase of melanoma incidence over the last decades, as shown by studies from research groups and health organizations. This has been due to unhealthy behaviors when it comes to occupational and recreational sun exposure, but also to improved diagnostic methods that allow for an early detection of melanoma (41–

43). In the case of Spain, for instance, the 2014 adjusted incidence reached 8.6 in males and 7.5 in females while in 1993 these parameters were 4.7 and 6.4, respectively (44).

Fortunately, mortality rates approach a plateau thanks to the novel treatments that have been incorporated in recent times. Nevertheless, this contention on mortality is very unequally distributed, and developing countries with low incidence rates still present high mortalities, calling on an advocation for a solidary democratization of the access to treatments.

Regarding the numbers of melanoma in 2020, Australia and New Zealand lead the incidence per 100.000 people, with northern European countries also having notable magnitudes while southern countries show a softer affectation.

To complete this contextualization, it is worth considering that despite being a small percentage of all cancers, melanoma incidence is expected to increase in the following decades within the range of 5-45% along with the world population. Predictions from the International Agency for Research on Cancer (IARC) state that national differences will continue among countries depending on the degree of pigmentation and latitude. This evolution, will surely increase the burden on the health systems around the globe, adding another incentive to boost prevention programs as well as investments on research projects (45).

## 1.4. Genetics and Melanoma

Mutations – nucleotide exchange alterations at the DNA level caused by extrinsic or intrinsic factors –, are the genetic basis of cancer (46). Throughout scientific history, the search for an explanation on disease appearance and heritability has been a popular goal. In the 1800s, Broca reported the heritability of some forms of cancer and Mendel established the laws of inheritance. In the early 1900s, Rous work suggested that tumorigenesis resulted from the effect of units smaller than cells, and Boveri and Morgan published their advances on the role of chromosomes (47–49). These were ultimately complemented by the description of the DNA structure derived from the work of Rosalind Franklin, and Watson and Crick (50,51).

The advent of sequencing technologies led to an increase in the knowledge of gene sequence and the identification of mutations contributing to the development of cancer. These findings generated a classification of genes based on whether a mutation caused an activation of the transforming capability (oncogenes) or an inactivation of the tumor-suppressor activity (tumor suppressor genes) (52).

With the expansion of sequencing technologies that came with Next-Generation Sequencing (NGS), the number of identified mutations grew exponentially (53). To help contextualize this immense variety of genes, a classification based on essential cellular functions was developed and termed, known as cancer hallmarks. These were: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing

angiogenesis, genome instability and mutation, resisting cell death, and sustaining proliferative signaling (54).

The study of melanoma genetics has evolved along with the capabilities of sequencing technology. Early discoveries were limited to specific mutations in single genes, while current NGS approaches allow mutational determinations in multiple genes, and even exomes or genomes (55,56). As a result, the crucial role of different genes on the development of melanoma was elucidated, showing an affectation on a wide variety of cellular functions and molecular pathways (57,58). The integration of mutational and expression information contributed to emerging molecular subtype classifications. The most popular one was proposed by The Cancer Genome Atlas (TCGA) group, sorting into 4 subtypes according to the presence of hotspot mutations in *BRAF, RAS* and *NF1* genes though practically it did not include acral nor mucosal melanomas (59,60).

In the following sections, a selection of genes involved in melanoma development will be briefly summarized, in the context of their role within the cell. It is worth noting that these genes mainly involve the MAPK and AKT pathways

### *1.4.1. Cell cycle progression, apoptosis, and senescence*

#### *1.4.1.1. TP53*

TP53 protein is encoded by the gene *TP53*, which is located in chromosome 17 and is considered to be the “guardian of the genome”. TP53 is activated in response to different types of cellular stress such

as DNA damage, hypoxia, replicative stress, oncogene expression, or metabolic dysfunction. When any of these conditions are detected, TP53 halts the cell cycle to either repair the DNA damage or induce apoptosis, though it can also mediate the induction of senescence or metabolic adaptations. This is done by the core of the protein, which is a DNA-binding domain that binds directly to the DNA thanks to a loop-sheet-helix motif stabilized by a zinc atom (61–63).

The relevance of *TP53* gene as a tumor suppressor is highlighted in human cancer, where it is one of the most commonly mutated genes among different cancer types. Melanoma is no exception and a prevalence of around 10% has been reported (59). These mutations are usually grouped at hotspots around residues involved in the contact with DNA or in securing the structure of the surface of DNA-binding (64).

To counter the effects of TP53-loss resulting from mutated *TP53*, numerous lines of research are being developed aimed at the pharmacological reactivation of mutant TP53. Some seek to produce small molecules that can bind and stabilize the active conformation of TP53 when mutations cause a conformational change that leads to a loss of function (62). In this case, the existence of a wide variety of mutations increases the challenge of finding drugs that can counter the effect of different mutations. Other efforts are directed at dealing with mutations that cause a stop codon. The use of aminoglycosides seems to induce read-through of nucleotide changes that would otherwise end up in a truncated protein (65).

#### *1.4.1.2. CDKN2A*

*CDKN2A* is a tumor suppressor gene located on chromosome 9 (66). It produces two different transcripts through the alternative splicing of exon 1. One transcript will generate the protein p16, which binds to CDK4 and CDK6 and blocks their catalytic site, hence preventing the activation of genes required for the progression from phase G1 to phase S of the cell cycle. The other transcript will generate the protein p14, which role is performed in a different pathway than p16. p14 binds to the protein HDM2, sequestering it in the nucleolus and thus, avoiding the ubiquitinization of p53 (67,68).

As a tumor suppressor gene, mutations affecting these proteins result in the enhancement of carcinogenesis. Such alterations are found in different types of cancer either at a somatic or germinal level. In melanoma, somatic mutations affecting *CDKN2A* are found in around 5% of the cases (59). Germline *CDKN2A* mutations, however, have been reported in around 20% of familial melanoma cases, and people carrying this mutation have a 65-fold increased risk of melanoma (69).

#### *1.4.1.3. CDK4 and RB1*

The gene *CDK4* is located in chromosome 12 and codifies for the protein CDK4. This is a cyclin-dependent serine/threonine kinase which role is to control cell cycle progression from G1 phase to S phase, with a high degree of homology with the protein CDK6 (70). CDK4 requires a two-step activation, first by the binding of a D-type cyclin, and then by a phosphorylation usually in the tyrosine residue at the position 172. Upon activation, CDK4 will phosphorylate RB1, which

is codified by *RB1* gene -located in chromosome 13- (71). RB1 is a so called "pocket protein", due to the shape of its structural domain, by which it binds to cellular factors such as the E2F family of transcription factors when phosphorylated. This results in the progression of the cell cycle, thus making RB1 a central player, acting as a switch of the restriction point (72,73).

Alterations within these genes, either in the form of amplifications or point mutations, can result in aberrant pathway activation. For example, a common mutation found in the residue 24 prevents the binding of p16, thus enhancing the kinase activity of CDK4 (74).

Given the consequences of a constitutive activation of this node as a result from genetic alterations, several efforts have been put into developing targeted inhibitors. Specifically, the latest generation aims at a competitive and reversible binding to the ATP pocket of inactive-CDK4 resulting in cell cycle arrest. These include Palbociclib, Ribociclib, and Abemaciclib, and have achieved a specificity towards CDK4 that reduce the toxicities derived from this kind of treatments. Also, new approaches are being explored to combine these inhibitors with immunotherapy treatments (75).

### *1.4.2. Chromatid segregation and genetic stability*

#### *1.4.2.1. PPP6C*

*PPP6C* gene is located in chromosome 9 and encodes for the catalytic subunit of PP6, a serine-threonine metallophosphatase that functions within heterotrimeric PP6 holoenzymes (76). The role of this protein is to dephosphorylate residues of other proteins that had been previously

phosphorylated by kinases. This is achieved through metal-activated water molecules (77). PP6 is involved in several cell functions such as the transition from G1 phase to S phase, the regulation of mitotic spindle formation and chromosome segregation, and also the repair mechanisms of DNA damage (78).

Given these roles, mutations in this gene have relevant repercussions, including oncogenic effects, as is the case of melanoma, where PPP6C has been found to be mutated in around 10% of the cases. Such mutations tend to be clustered within a region flanking the arginine codon at position 264 and affect a highly conserved region. These result in a defective interaction between the regulatory and the catalytic subunits, thus contributing to abnormal cell governing and melanoma development (79).

### *1.4.3. Chromatin remodeling and transcriptional control*

#### *1.4.3.1. ARID2*

The gene *ARID2* is located in chromosome 12 and encodes for the protein ARID2. It functions as a transcriptional co-activator by binding DNA without sequence specificity, and is a member of the SWI/SNF chromatin-remodeling complex (79,80). This complex works in an ATP-dependent manner and is involved in several biological processes such as transcriptional regulation, cell cycle modulation, embryonic development, and DNA damage repair (81).

One of the prominent roles of ARID2 is the negative regulation of cell-cycle progression and cellular proliferation via targeting the Rb pathway. Hence, alterations in this gene can enhance carcinogenesis as

is the case of melanoma, where mutations in *ARID2* can reach around 7% in prevalence (82).

These mutations seem not to be clustered in a hotspot but spread along the gene sequence. This suggests that there is not a critical domain within the protein, but a global loss-of-function when any of the domains is compromised. Furthermore, there are some lines of research that aim at developing anti-cancer therapies based on these chromatin-associated proteins (80).

### *1.4.4. Epigenetic regulation*

#### *1.4.4.1. IDH1*

*IDH1* is a gene located in chromosome 2 encoding the protein IDH1. This homodimeric enzyme is located in the cytosol and peroxisomes, and catalyzes the oxidative carboxylation of isocitrate to alpha-ketoglutarate (a-KG) to produce NADPH from NADP+. This function is required for a normal cellular metabolism and epigenetic regulation (83).

Mutations in this gene are found in several pathologies including melanoma, where it has been reported to have a mutation prevalence of 6% (84). Such mutations are common in a hotspot region around amino acid 132. This substitution at a key residue results in a neomorphic enzymatic activity, which converts the produced a-KG into D-enantiomer of 2-hydroxyglutarate (D2HG). This product is hardly cleansed, thus accumulating within tumor cells and causing the alteration of methylation patterns (85).

Given the creation of a tumor-specific neoantigen as a result of its mutation, and despite the role of IDH1 in carcinogenesis being still unclear, there are ongoing trials to explore the use of IDH vaccines in glioma patients (85,86).

### *1.4.5. Telomere maintenance*

#### *1.4.5.1. TERT promoter*

The gene *TERT* is located in chromosome 5 and encodes the catalytic subunit of telomerase, the protein in charge of elongating the telomeres. In most somatic cells, the transcription of this component is repressed, as it is only active in proliferative cells of self-renewing tissues. Telomere regulation is key to the normal functioning of the cell, and alterations within that pathway can contribute to carcinogenesis immortalizing neoplastic cells, which had a high proliferative rate (87). In the case of melanoma, and later on in other neoplasms, genetic alterations have been found in the promoter region of *TERT*. These were first reported as a high-penetrant disease-segregating causal germline C>T mutation in a melanoma family at -57bp, and then at a somatic level including changes at -124bp, -146bp, or tandem CC>TT at -138/-139bp and -124/-125bp from the ATG starting site. Such alterations resulted in the creation of a binding site for transcription machinery and thus enhanced the transcription of TERT (88,89).

Over the years, the knowledge on the role of *TERT* promoter mutations has been widened, and it is now reportedly relevant to assess aggressiveness and survival outcome of melanoma patients in

combination with other mutations in driver genes such as *BRAF* or *NRAS* (90–94).

### *1.4.6. Proliferation and survival of the cell*

#### *1.4.6.1. BRAF*

The *BRAF* gene located at the chromosome 7 encodes a serine/threonine protein kinase that takes part in the signal transduction pathway between growth stimuli and cellular response (Mitogen-activated protein kinase; MAPK pathway). In normal conditions, BRAF is phosphorylated by RAS resulting in a conformational change. In turn, the activated form of BRAF phosphorylates MEK, thus continuing the signal transduction (95). Mutations in this gene lead to a constitutive activation of the BRAF protein, hence promoting carcinogenesis. Around 50% of melanomas harbor a mutation in *BRAF*, being the most common the change from valine to glutamic acid in the codon 600 (V600E) (96). *BRAF* mutations is one of the *driver* mutations that require, though, additional genetic alterations for generating a melanoma, given that benign melanocytic nevi can also show a mutation in *BRAF* without an ulterior evolution to melanoma (97). The discovery of V600 mutation resulted in the development of the first targeted therapy for melanoma with BRAF inhibitors, such as vemurafenib, dabrafenib and encorafenib drugs, which started a new age in the treatment of melanoma patients (98,99).

#### *1.4.6.2. NRAS, KRAS, and HRAS*

The *RAS* family genes include *NRAS* (located in chromosome 1), *HRAS* (located in chromosome 11), and *KRAS* (located in chromosome 12). This family encode small GTPases which, as part of the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, participate in signal transduction. In normal conditions, RAS proteins are inactive when bound to a guanine diphosphate (GDP) molecule. This is exchanged for a guanine triphosphate (GTP) by guanine nucleotide exchange factors (GEFs), which lead to the activation of RAS proteins (100,101).

Mutations in this family of proteins are most common in codons 61, 12 and 13, though the frequency varies among the different isoforms. These result in a constitutive activation of the signal transduction, either due to the disruption of the GTPase activity, or to a decrease in the sensitivity to GTPase-accelerating proteins (102,103).

*NRAS* is mutated in up to 15% of melanomas, while *HRAS* and *KRAS* show alterations in a much lower frequency (2-3%) (59). In spite of being associated to more aggressive melanomas, today there are no targeted drugs for melanomas harboring *RAS* mutations, and the clinical approached is focused on the use of MEK inhibitors (104).

#### *1.4.6.3. ROS1*

The gene *ROS1* is located in chromosome 6 and encodes the protein ROS1. It belongs to a subfamily of tyrosine kinase insulin receptors which, upon activation, auto-phosphorylate specific tyrosine residues in their intracellular domain. This effect makes them available as

docking sites for downstream proteins, which are involved in the MAPK, PI3K-AKT, and JAK-STAT3 pathways (105,106).

Given its role, alterations in its regulation lead to the constitutive activation of several signaling pathways that promote carcinogenesis in different tumor types, including melanoma. These alterations include point mutations, but normally are genomic rearrangements resulting in fusions with different protein partners (107).

There are currently several available inhibitors targeting the two main conformations affecting the protein binding sites for crucial molecules, such as crizotinib, ceritinib, brigatinib, or repotrectinib (106).

#### *1.4.6.4. NF1*

*NF1* gene is located in the chromosome 17, and spans 300kb covering 60 exons. In addition to its large size, the presence of pseudogenes make this tumor suppressor a complex gene (108). It encodes the protein NF1, a large protein with several functional domains that primarily acts as a GTPase-activating protein. The role of NF1 is thus to negatively regulate RAS by catalyzing the exchange of GTP to GDP, turning RAS into its inactive form. *NF1* is partially regulated upstream by KIT, which strengthens its crucial role in the MAPK and PI3K signaling pathways (109).

In melanoma, as in other pathologies where *NF1* is involved, mutations lead to a continuously activated RAS, hence contributing to uncontrolled cell proliferation. It has been reported that around 15% of melanomas harbor a mutation in *NF1*, which makes this gene one of the most commonly mutated in this type of skin cancer. Also, a mutated

*NF1* seems to be linked to a higher tumor mutational burden and a worse prognosis for the patient. In that sense, targeted treatments for these patients are focused on MEK and EGFR inhibitors, while the potential of immunotherapy remains uncharted territory (108,110,111).

#### *1.4.6.5. GNAQ and GNA11*

*GNAQ* (located in chromosome 9) and *GNA11* (located in chromosome 19) encode for the alpha subunit of G-protein-coupled receptor proteins from a family of transmembrane receptors that participate in the signal transduction from the extracellular environment into the cell. Both genes have a 90% homology in their amino acid sequence. Upon ligand binding, these proteins suffer a conformational change becoming active and exchanging GDP for GTP thus triggering downstream signaling in the MAPK and PI3K pathways (112,113).

Mutations within these genes lead to their constitutive activation, either due to a loss in the degree of GTPase activity or due to the proteins being locked in their active state (114) .

In melanoma, these genes are mainly mutated in the uveal subtype, and in other nevus-associated pathologies such as blue nevi. However, recent studies are paying attention at their presence in non-uveal cutaneous melanoma as well (112,115). Their mutation prevalence is 2% in general melanoma, while in uveal melanoma it can reach the range of 30%. Current treatments for melanomas harboring mutations in *GNAQ* or *GNA11* are focused on the use of MEK inhibitors, while efforts are being made to develop drugs able to target specific domains of these proteins (114).

#### *1.4.6.6. MAP2K2*

The gene *MAP2K2*, located in chromosome 19, encodes for the protein MAP2K2- also known as MEK2-. This kinase participates in the signal transduction MAPK pathway downstream of RAF, where it selectively phosphorylates serine/threonine and tyrosine residues within the activation loop of ERK. It thus has a crucial role in cell survival, proliferation, differentiation and motility (116,117).

In melanoma, *MAP2K2* has a low mutational prevalence, but given the central role it plays in signal transduction, it has become a main target in the development of targeted treatments (118,119). The use of MEK inhibitors such as trametinib or selumetinib, which bind specifically to this protein blocking the accessibility of the residue S217 to RAF kinases, is part of the clinical guidelines when treating *RAS* mutated or *RAF* resistant tumors. They are also prescribed in combination with BRAF inhibitors to address specially aggressive melanomas (117).

#### *1.4.6.7. PIK3CA and PIK3R1*

The genes *PIK3CA* (located in chromosome 3) and *PIK3R1* (located in chromosome 5) encode for different elements of the PI3K protein kinase. *PIK3CA* codifies for the catalytic subunit p110a, while *PIK3R1* codifies for the regulatory subunit p85 (120). Upon activation, PI3K phosphorylates certain lipids in the plasma membrane, generating phosphatidylinositol-P2 ($PIP_2$) and phosphatidylinositol-P3 ($PIP_3$), which will mobilize some proteins to the inner surface of the plasma membrane. One of such proteins will be AKT, which participates in the

signal transduction pathway, hence giving the name to the PI3K-AKT pathway (121).

Despite the confronting roles of these two subunits- p110a being oncogenic and p85 being a tumor suppressor-, mutations in either of them have an impact in enhancing carcinogenesis, with their corresponding characteristics. Regardless of the low mutational prevalence of these genes in melanoma, they are relevant since targeting the PI3K-AKT pathway is being considered as an opportunity to overcome resistance to BRAF or MEK inhibitors in melanomas harboring mutations in *BRAF* (121–123).

#### *1.4.6.8. PTEN*

The gene *PTEN* (located in chromosome 10) encodes for the PTEN protein, a lipid and protein phosphatase that negatively regulates the PI3K pathway (124). The lipid phosphatase activity mediates the decrease in the levels of intracellular $PIP_3$ thus reducing downstream activity of AKT. The protein phosphatase activity leads to the inhibition of growth factor-stimulated MAPK signaling, as well as reducing the capacity of the cell to spread and migrate.

Given the role of tumor suppression that *PTEN* exerts, mutations within this gene lead to an uncontrolled growth of the cell and escape from apoptosis, enhancing carcinogenesis (125). Such mutations are found in melanoma in a frequency of around 10% (59).

Recent studies have shown that mutations in *PTEN* are associated with a worse prognosis of melanoma patients (126), and that the mutational

status for this gene has an impact when addressing melanomas with concurrent mutations in *BRAF* (127,128).

#### *1.4.6.9. RAC1*

*RAC1* is located in chromosome 7 and encodes a member of the RHO family of small GTPases. The RAC1 protein is inactive when bound to GDP and active when bound to GTP, in which case it promotes signal transduction for cell proliferation and survival by phosphorylation of RAF and MEK (129).

Mutations in this gene have been reported in different cancers including melanoma, in which the specific prevalence is 4%. The most common mutation affects the codon 29, causing the change of proline for serine (P29S). This results in a conformational change that, despite retaining the ability to hydrolyze GTP and GDP, favors a faster exchange of GDP for GTP, hence activating the pathway (130,131).

Notwithstanding the specifics for the role of this gene have been acquired recently, studies suggest that when found in combination with *BRAF* or *NF1* mutations, alterations in *RAC1* can result in a reduced survival of melanoma patients (132). In line of the difficulties associated to targeting a small GTPase like RAC1, due to the absence of clear pockets that might allow the docking of inhibitor molecules, therapeutic efforts are being directed elsewhere. The most promising hypothesis are towards the search of small molecules that could impede RAC1 mobilization to the membrane or prevent the binding of effector proteins (129).

#### *1.4.6.10. KIT*

The *KIT* gene located in chromosome 4 encodes for a receptor tyrosine kinase (RTK). Upon binding of ligand, activation occurs via dimerization of its monomers, hence triggering MAPK and PI3K-AKT signaling pathways (133,134).

Mutations in this gene lead to the activation of the receptor in the absence of a ligand, which results in uncontrolled growth, and have been reported in different tumors. There is a variety of possible mutations affecting the different domains of the receptor, but the most common are located in exon 11 (lysine-to-proline at codon 576; L576P) and exon 13 (methionine-to-glutamic acid at codon 642; K642E) (135).

In the case of melanoma, the prevalence of *KIT* mutations differs among different subtypes. According to the literature, these mutations are more common in acral and mucosal melanomas. However, a differential prevalence of *KIT* mutations has also been linked to CSD melanomas, though further research should be addressed in this line (136,137).

In terms of population, European-descendants show a slightly higher presence of *KIT* mutations compared to Asians, despite the higher proportion of mucosal and acral melanomas in Asia (137).

When it comes to the management of melanoma patients harboring mutations in *KIT,* the advent of new targeted drugs such as imatinib, dasatinib, nilotinib, or sunitinib, has improved their outcome (134). However, it is still unclear if *KIT* mutations directly influence the survival and thus, whether the mutational status might have prognostic value or not.

## 1.5. Developmental pathways of melanoma

So far, it has been explained how melanoma is originated from the abnormal growth of melanocytes, and how this malignant evolution is participated by environmental and host factors via genetic mutations. The combination of such circumstances give rise to different developmental pathways of melanoma, which will be addressed in this section.

### *1.5.1. Acral*

Melanomas occurring on acral skin -soles, palms, and nail unit-, conform the subtype of acral melanoma. These account for a small portion (4-6%) of the melanomas diagnosed in European-descendent populations, though they are more prevalent in other ethnicities (138). Acral melanomas tend to appear in older patients, and seem to be more frequent in those with a low number of atypical nevi and a low accumulation of solar damage, so this developmental pathway would be independent of both nevi-proneness and UVR-damage accumulation (139).

These melanomas usually fall under the specific histological subtype of acral lentiginous melanoma, but a few of them can show a superficial spreading melanoma pattern. Regarding the genetic background of acral melanomas, they are characterized by a lower prevalence of *BRAF* mutations when compared to non-acral cutaneous melanoma, while showing a higher prevalence of mutations in the receptor tyrosine kinase *KIT* (140,141).

In general terms, the prognosis of acral melanoma patients is worse than other subtypes. The higher prevalence in older patients, together with the occurrence in areas that are not revised often, and the resemblance of some acral melanomas to other benign entities, lead to a challenging and delayed diagnosis (142). Also, it is possible that the high presence of *KIT* mutations might contribute to a worse outcome.

### *1.5.2. Mucosal*

Mucosal melanomas are those arising in the mucosal membranes in the sinonasal or oral cavity, and the respiratory, gastrointestinal, and genitourinary tracts. These melanomas are rare, accounting for around 3.5% of all melanomas in European-descendent populations and present with a stable incidence, which is more frequent in females. The higher incidence found in Asian populations is likely due to the lower prevalence of cutaneous melanoma in Asian populations (143). The mucosal subtype is characterized by its aggressiveness and by having a worse prognosis than cutaneous melanoma. This is contributed by a late median onset age (70 years), a late diagnosis, the difficulty to reach the affected region, and also because of the richness in vascular and lymphatic supply on such areas (144).

The etiology of mucosal melanoma is still unclear, as its anatomic site is not influenced by UV light. This aligns with the findings on the lower mutational burden of mucosal melanomas. Moreover, these molecular studies found a lower prevalence of *BRAF* and *NRAS* mutations when compared to cutaneous melanomas. Contrarily, mucosal melanomas

seem to have a similar prevalence of mutations in *NF1*, and a higher frequency of mutations in *KIT* and *SF3B1* (133).

### *1.5.3. Uveal*

Uveal melanomas develop from melanocytes in the uvea, which includes the pigmented areas of the iris, ciliary body, and choroid. Occurrence of this type of melanoma is rare, representing around 5% of all diagnosed melanomas, and its incidence varies by region (145). Taking Europe as an example, there is an increasing gradient from southern countries like Spain (<2 cases per million population) to northern countries like Norway (>8 cases per million) (146).

The etiology of uveal melanoma remains uncertain. In fact, the latitude-related incidence questions the role of UVR as a driver for uveal melanoma (147). Since the cornea, lens, and vitreous act as a shield for UVR, this carcinogen agent would only reach the anterior parts of the eye, where just 10% of uveal melanomas arise. Other risk factors include light-colored eyes, melanocytoma, fair skin, and the BAP1-tumor predisposition syndrome (113).

Uveal melanomas have a distinctive molecular profile, with mutations in *GNAQ* and *GNA11* in around 50% of the cases, and a lower prevalence of *BRAF* and *NRAS* alterations than cutaneous melanoma (148). Other genes harboring mutations in uveal melanoma are *CYSLTR2, PLCB4, BAP1, SF3B1* and *EIF1AX* (113).

The onset of these tumors is usually between 50 and 70 years old with a challenging prognosis given the tendency to present hematogenous dissemination. Primary tumors can be treated with radiotherapy or

surgery, depending on the size (149). Despite efforts to maintain functional sight, in some cases an eventual enucleation is the only viable approach. Metastatic disease can be treated systemically, though surgery is an option when the metastases are developed in accessible organs such as the liver (150). Moreover, recent studies are exploring the potential of immunotherapy as treatment for uveal melanoma (151).

### *1.5.4. Divergent pathways for non-acral cutaneous melanomas*

The role of melanocytic proliferation and a long-term exposure to UVR as contributors to melanoma development is unquestionable (1,152,153). Back in 1998, David Whiteman and collaborators proposed a model for melanoma development that described at least two causal pathways (Figure 1) (154). One was associated with proneness to melanocytic proliferation -hereinafter nevogenic-, with little dependence on UVR. These melanomas would arise in younger individuals in areas which are intermittently exposed to solar radiation (e.g. trunk), and would be more likely to harbor mutations in *BRAF*. The other pathway would be associated with a cumulative solar damage -hereinafter CSD-, resulting in a later onset of the disease in patients with few melanocytic nevi. These melanomas would usually arise in chronically sun-exposed areas such as the head and neck, and have been suggested to associate with mutations in *KIT*. This divergent pathways model was further explored and proven clinical relevance (9,155–158). Despite efforts implemented at the mutational level (1,159), these have been mainly circumscribed to associations with said *BRAF, KIT,* or

*NRAS* mutations, so an in-depth molecular characterization of these groups is still missing.

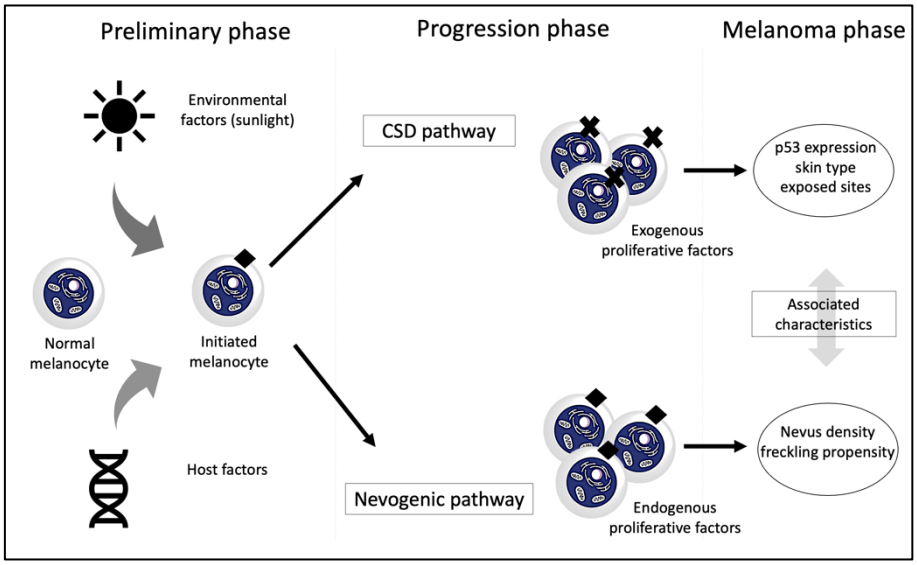


**Figure 1. Divergent pathways model.** This graphical representation of the model proposed by Whiteman shows how melanocytes are activated early in life and then will progress to melanoma in at least two different pathways depending on the influence of endogenous or exogenous factors.

# 2. JUSTIFICATION, HYPOTHESIS, AND OBJECTIVES

## 2.1. Justification

Over time, classifications of cutaneous melanoma have evolved based on consensus among dermatologists and pathologists, with the latest WHO classification using the degree of solar damage and location to group these tumors (160). In parallel, groundbreaking advances have been made in the field of molecular biology, especially regarding sequencing capabilities. These resulted in prodigious amounts of information that gave rise to additional molecular classifications of cutaneous melanoma based on mutational profiles (59).

In spite of these classifications carried within the nevogenic and CSD melanomas as described by Whiteman, these were scattered among different groups. Thus, it was not possible to establish the unbiased weight of UVR and melanocytic proliferation as primary contributors to melanoma development, especially their influence at the molecular level.

Hence, a mutational characterization of cutaneous melanoma as per the divergent pathways is still lacking, to corroborate whether there is a biological background supporting that model.

Complementarily, the prevalence of *KIT* mutations has been shown to be higher in acral and mucosal melanomas, but it is yet to be elucidated whether *KIT* mutations can be associated with a specific developmental pathway of melanoma, and the potential use of this mutational status as a prognosis marker.

## 2.2. Hypothesis

The developmental pathway of melanoma determines the genetic characteristics and clinical behavior of the tumor.

## 2.3. Objectives

The main objective was to characterize melanomas according to different developmental pathways.

The specific objectives were addressed in corresponding scientific publications, and included:

1. To assess the use of FFPE melanoma samples for next generation sequencing.
2. To establish the mutational prevalence for *ARID2, BRAF, CDK4, CDKN2A, GNA11, GNAQ, HRAS, IDH1, KIT, KRAS, MAP2K2, NF1, NRAS, PIK3CA, PIK3R1, PPP6C, PTEN, RAC1, RB1, ROS1, TP53,* and the promoter region of *TERT* in a series of cutaneous melanomas, evaluating the differences according to the etiopathogenic pathway of melanoma.
3. To characterize melanomas harboring mutations in *KIT*
4. To assess the prognostic value of *KIT* mutational status.

# 3. USE OF FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES FOR NGS

## 3.1. Overview

The design of our retrospective study included using tumor samples from biobanks. The most common option for the storage of biospecimens is formalin-fixation and paraffin-embedding (FFPE), which is convenient for economic and spatial reasons. However, the fixation process can cause chemical and physical changes in the genetic material (DNA fragmentation, abasic sites, deamination of cytosines) that may result in sequencing artifacts. Also, this damage is more likely to occur and accumulate over time.

Considering this, together with the precious value of the material coming from small sized tumors like melanoma, a method to elucidate which samples were worth undergoing NGS was needed. In this chapter, we display the technical work that we performed in this regard, which resulted in a scientific publication.

## 3.2. Reference and contribution of the candidate

Millán-Esteban D, Reyes-García D, García-Casado Z, Bañuls J, López-Guerrero JA, Requena C, Rodríguez-Hernández A, Traves V, Nagore E. Suitability of melanoma FFPE samples for NGS libraries: time and quality thresholds for downstream molecular tests. Biotechniques. 2018 Aug; 65 (2): 79-85. Doi: 10.2144/btn-2018-0016. PMID: 30091391.

The candidate participated in the study design, experimental procedures, data analysis, and writing of the manuscript.

## 3.3. Suitability of melanoma FFPE samples for NGS libraries: time and quality thresholds for downstream molecular tests

### *3.3.1. Introduction*

Formalin-fixed paraffin-embedded (FFPE) samples represent a valuable underexploited resource for large, retrospective and prospective studies with long-term clinical follow-up (161). The existence of FFPE samples collections worldwide, and their advantages such as easy handling, long-term inexpensive storage, suitability for immunohistochemical analyses, and low-cost of large-scale application, make this type of storage a relevant option (162).

The primary goal of formalin fixation is preserving cellular structure for histological examination and diagnosis, so some challenges arise regarding the suitability of genetic material extracted from FFPE samples for molecular testing (163).

Many factors during the fixation process influence nucleic acids conditions. Such as, at the pre-fixation step, the type and amount of tissue or the time elapsed from surgery to fixation. At the fixation step, this could be the type of fixative, temperature, pH or duration. At the post-fixation step, it could be the storage time and conditions (162).

The effects on the genetic material include cross-linking, which decreases the efficacy of posterior PCR (163). Hence, steps must be taken to improve the use of FFPE in molecular testing, especially those factors affecting downstream techniques, because this will permit the

reevaluation of stored FFPE samples independently of their origin (164).

Recently, one of the most commonly used downstream techniques is NGS, which allows sequencing multiple cancer-related genes in a high-throughput manner (165). Despite the tested use of FFPE tissue in NGS (166), further studies are required to check whether archival FFPE samples are suitable for NGS (167,168). A recent study found that FFPE samples stored for more than 7 years were not worth being included in NGS studies (169).

It would be helpful to have predictive quality indicators to know whether samples are suitable for NGS. This would avoid wasting precious samples that could undergo alternative molecular studies if not suitable for NGS, an as-yet expensive molecular approach.

In this context, there are parameters to elucidate the quality of the genetic material from FFPE samples. Among others, DNA integrity number (DIN) and RNA integrity number (RIN) from Agilent Technologies (CA, USA), in a range of 1 to 10, give information regarding the fragmentation of the DNA or RNA based on electrophoresis (170,171). Also, the quality control (QC) value from Illumina (CA, USA) enables the researcher to test the quality of the DNA based on a qPCR, giving values closer to zero when the quality is the highest (172,173).

Our aim was to evaluate the suitability of melanoma FFPE samples for an amplicon-based NGS custom panel analysis according to the storage time, type of sample, QC and DIN values.

### 3.3.2. Materials and Methods

#### 3.3.2.1. Sample collection

FFPE blocks came from the Biobank at the Instituto Valenciano de Oncología (Valencia, Spain). A cohort of 59 samples were analyzed including 37 primary melanoma tumors and 22 wide local excision tissues retrieved and stored at the Pathological Anatomy Department from January 2000 to April 2017. Therefore, the time elapsed from the surgery (time of storage) was up to 17 years and the distribution was made based on a CART analysis regarding the library functionality: 42.4% of the samples were referred to as "old" (>7 years) and 57.6% of the samples were referred to as "recent" (≤7 years). The time of sample fixation in formalin solution was estimated based on the date of the surgery when the sample was taken. Two categories were defined: <1 day (usually overnight fixation), when the day after the surgery was a work day and >1 day when it was a holiday (usually >1-2 days).

This study took place as part of a bigger project that had the approval of the Ethics Committee at the Instituto Valenciano de Oncología, and patients signed a voluntary cession of the samples to the Biobank.

The main outcome variable was the functionality of the sample, which was defined as the ability of a sample to construct an amplicon-based library with a length of 300-350 bp, visible as a single band on an electrophoresis test.

#### 3.3.2.2. FFPE processing

The Pathological Anatomy Department of our center had standardized protocol for the processing of FFPE samples. A first formalin fixation

of the samples was followed by the block preparation procedure. This was performed in the Excelsior ES automatic processor (ThermoScientific, CA, USA) and included a formalin fixation step (30 min), an increasing multiple-step dehydration (9 h 45 min), a triple clearing step with xylene (2h 15 min), and a final three-step embedding in paraffin wax (4 h). Then, blocks were kept at room temperature at the Biobank.

#### *3.3.2.3. DNA isolation and quantification*

From each FFPE block, a 3 µm section was used for hematoxylin and eosin (H&E) stain. Then, a pathologist evaluated and selected the area with tumor-enriched cells for macrodissection. Using the H&E slide as a reference, three 0.6 mm needle biopsies were taken from every primary tumor. For the wide local excision tissue, three 10 µm sections were cut and collected into 1.5 ml tube (Eppendorf).

DNA was extracted using the QIAamp® DNA Investigator kit (QIAGEN, Hilden, Germany), with the following modifications: given the toughness of the skin, we established an overnight incubation at 56ºC for the proteinase K to assure a complete digestion. Also, we introduced the optional carrier addition to maximize the extraction yield.

Quantification was obtained using Quant-iT™ PicoGreen™ dsDNA Assay kit (Invitrogen, MA, USA). All samples had a concentration above 2.5ng/µl and were accepted for the study.

#### *3.3.2.4. DNA reparation*

The NEBNext® FFPE Repair Mix (New England Biolabs, Hertfordshire, UK) kit was used to repair the C:G>T:A changes induced by nucleotide deamination, usually present in FFPE samples.

#### *3.3.2.5. Quality assessment tests*

Real-time PCR was performed using 1X Sybr Green PCR Master Mix (Applied Biosystems) and FFPE QC Kit (Illumina) following the manufacturer's instructions. Briefly, 2 µl of diluted DNA (1:100) was added to 8 µl of the mix containing SybrGreen and Illumina primers. All runs were processed in an ABI7500 Fast PCR system (Applied Biosystems) using the default run protocol: 50ºC/2 min – 95ºC/10 min – 40 cycles of 95ºC/30 s, 57ºC/30 s, 72ºC/30 s). All reactions were performed in triplicates. The resulting QC value was an indicator of the sample quality, with a lower value being the better quality indicator.

Gel electrophoresis was performed using Genomic DNA ScreenTape in a 4200 TapeStation (Agilent Technologies). Briefly, 1 µl of DNA and 3 µl of simple buffer were added to each well.

DIN value obtained was an indicator of the integrity of the DNA, thus a higher DIN value meant a better quality.

#### *3.3.2.6. Library construction*

Low input DNA libraries of the gene panel containing 21 melanoma-related genes were constructed according to the manufacturer's instructions using a custom GeneRead DNAseq Panel (QIAGEN). Shortly, DNA fragments were amplified in a multiplex PCR to obtain

a total of 633 amplicons of 200 bp in length (GeneRead DNAseq Panel PCR kit V2 Qiagen). At this point, a normalization step was included and 100 ng of each sample continued the process. The ends of the molecules were enzymatically repaired and universal adaptors were ligated, then unique combinations of MID adaptors were ligated (NEBNext Ultra DNA Library Prep Kit, New England Biolabs).

#### *3.3.2.7. Library functionality*

Final library size was checked with a bioanalyzer using D1000 DNA ScreenTape (Agilent Technologies). The final amplicon size including the MID adapters made an average of 350 bp. Thus, the presence of a single band in the range of 300-350 bp classified the library as functional.

#### *3.3.2.8. Statistical analysis*

The statistics were performed using IBM SPSS Statistics 20.0. Normal distribution of continuous variables was checked using the Kolmogorov-Smirnov test. Pearson test was used to study the correlation among parametric variables and Spearman test was used for non-parametric variables. A 1-factor ANOVA test was used to compare means of continuous variables and qualitative ones. Also, continuous variables were categorized with a CART analysis[(A)] using library functionality as a filter. Diagnostic parameters, including sensitivity, specificity, predicted positive and negative value, accuracy, and are under the curve from a ROC test were calculated to evaluate the capacity of each parameter or algorithm to predict library functionality.

### 3.3.3. Results

The study included a total of 59 unpaired FFPE samples corresponding to 37 melanoma primary tumors and 22 unmatched wide local excision tissues stored for a median of 5 years (range: 1-17 years). The characteristics of the samples are displayed in Table 1.

#### 3.3.3.1. Influence of date of surgery on library functionality

There was a great variability in library yield independent of the tissue origin or the time of storage, despite the existence of a normalization step to include 100 ng in the end reparation step. A 1-factor ANOVA test showed that the mean storage time was significantly lower in samples with functional library than in samples with non-functional library (4.93 vs. 9.49 years, respectively; $p<0.001$). The best cut-off that differentiated functional from non-functional samples was established at 7 years by CART analysis, and recent samples (≤7 years) showed a significantly higher percentage of library functionality than old samples (>7 tears; 70.6 vs. 20%; $p<0.001$) (Figure 2A). The fixation time did not influence the functionality (Table 1).

#### 3.3.3.2. QC and DIN values as quality predictor parameters

QC values were inversely correlated with time of storage ($r = -0.616$; $p<0.001$) (Figure 2B) and library functionality ($r_s = -0.334$; $p=0.009$). A 1-factor ANOVA test showed that QC means were statistically different between functional and non-functional samples (4.03 vs. 5.7; $p=0.002$).

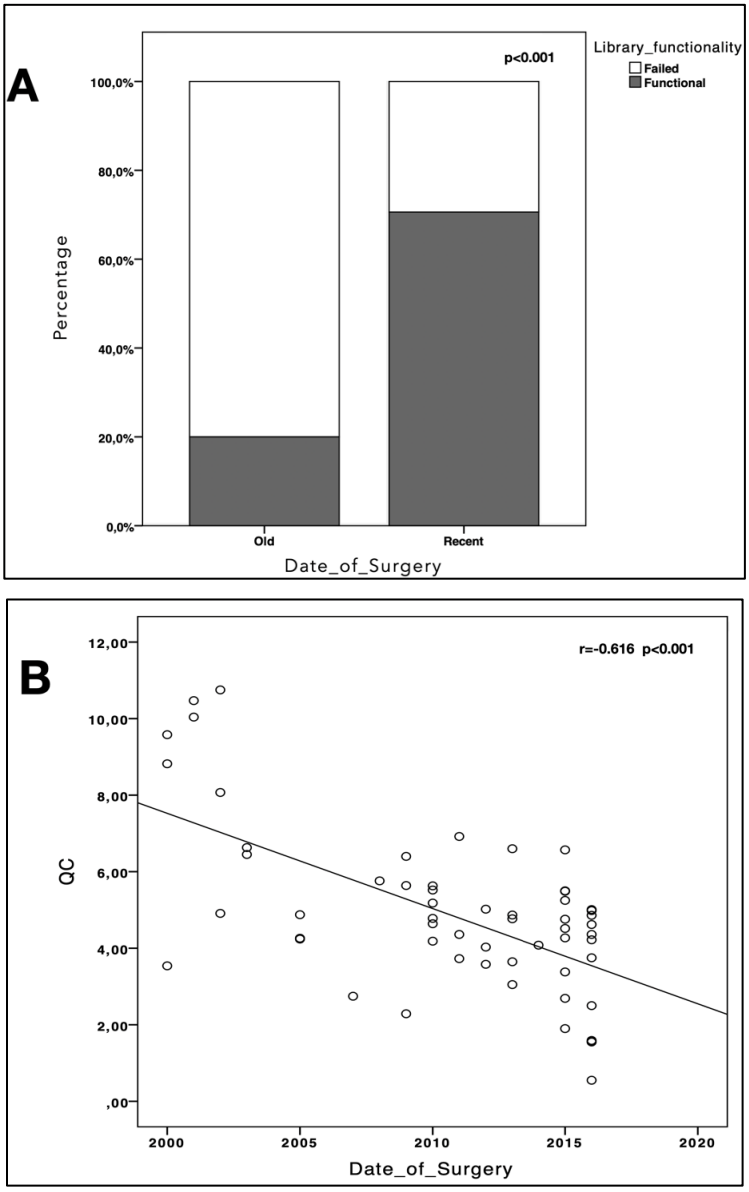


**Figure 2. Statistical analysis on FFPE samples.** Here are presented the graphical distribution of failed (white) and functional (gray) libraries for different variables (Date of Surgery, QC threshold, and DIN threshold) (A, C, E). Also, the correlations studied between QC and date of surgery (B), DIN and date of surgery (D), and QC and DIN (F).

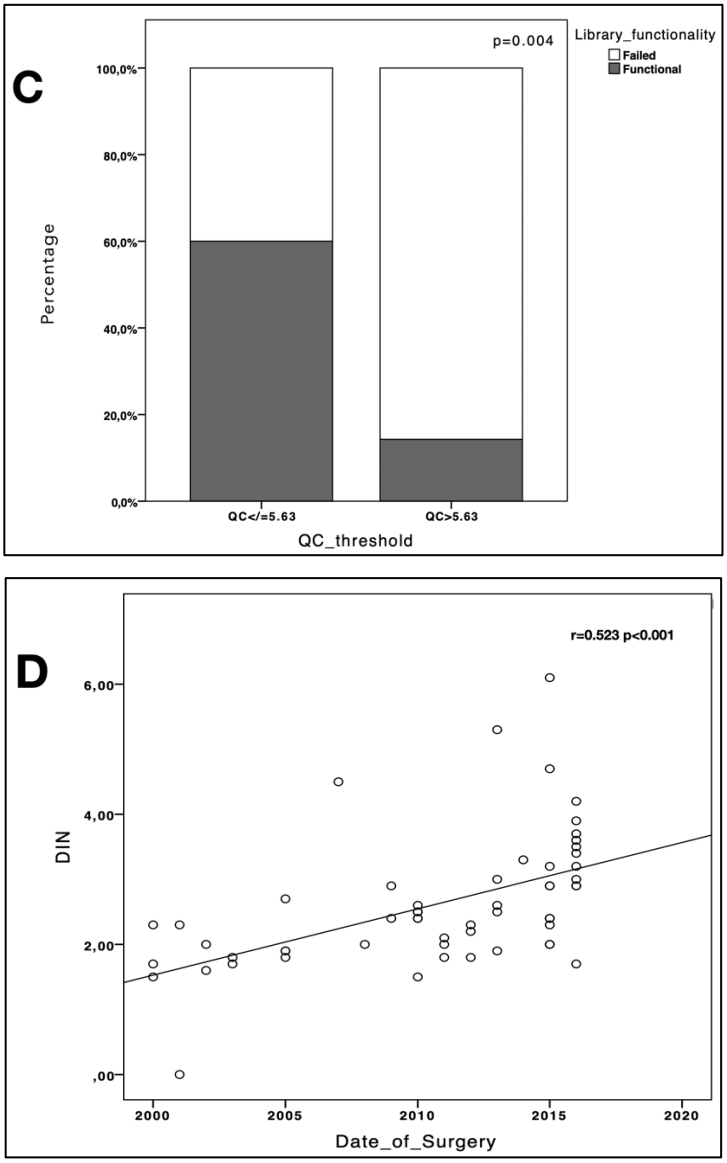


**Figure 2 (continued). Statistical analysis on FFPE samples.** Here are presented the graphical distribution of failed (white) and functional (gray) libraries for different variables (Date of Surgery, QC threshold, and DIN threshold) (A, C, E). Also, the correlations studied between QC and date of surgery (B), DIN and date of surgery (D), and QC and DIN (F).

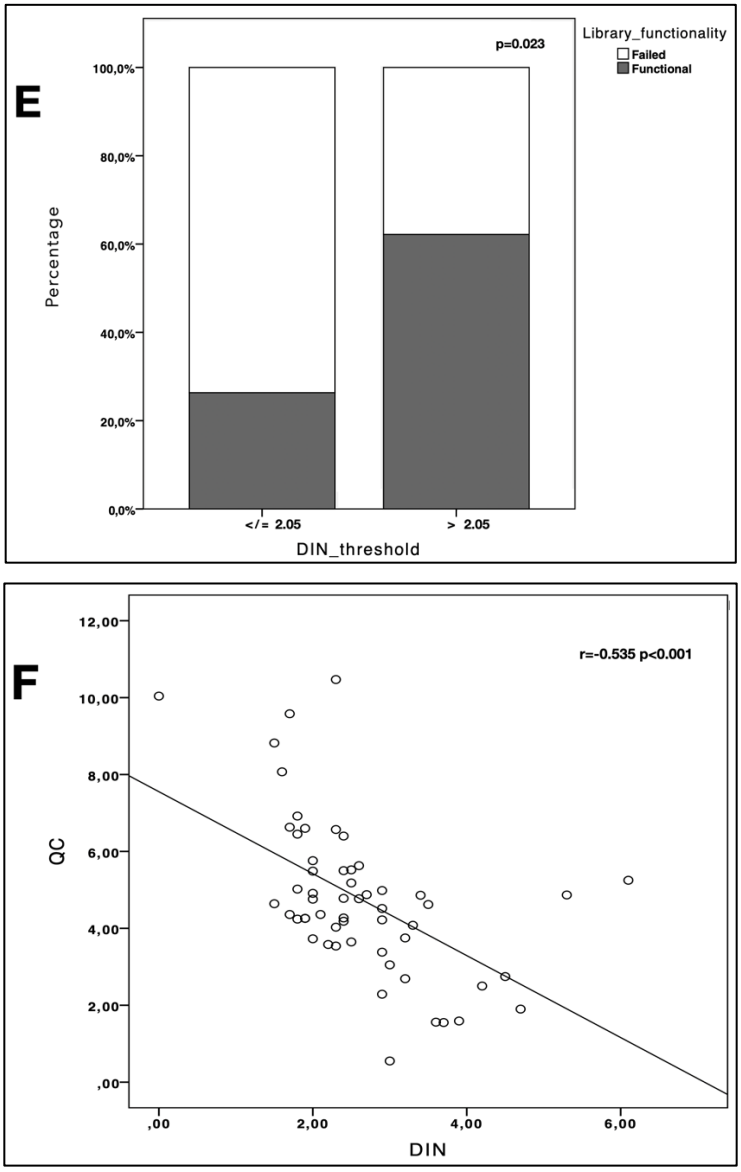


**Figure 2 (continued). Statistical analysis on FFPE samples.** Here are presented the graphical distribution of failed (white) and functional (gray) libraries for different variables (Date of Surgery, QC threshold, and DIN threshold) (A, C, E). Also, the correlations studied between QC and date of surgery (B), DIN and date of surgery (D), and QC and DIN (F).

According to the CART analysis, cut-off value for QC was established at 5.63 (93.1% of samples with a QC≤ 5.63 produced functional libraries compared with 6.9% of samples with a QC> 5.63; p=0.004) (Figure 2C).

**Table 1. Sample distribution according to final sample library functionality.**

| | Total | | Functional | | Non-Functional | | p-value |
|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | |
| **QC** | | | | | | | |
| QC ≤5.63 | 45 | 76.2 | 27 | 93.1 | 18 | 60.0 | 0.005 |
| QC>5.63 | 14 | 23.7 | 2 | 6.9 | 12 | 40.0 | |
| **DIN** | | | | | | | |
| DIN ≤2.05 | 19 | 33.9 | 5 | 17.9 | 14 | 50.0 | 0.023 |
| DIN>2.05 | 37 | 66.1 | 23 | 82.1 | 14 | 50.0 | |
| **Time of storage** | | | | | | | |
| Old | 25 | 51.0 | 5 | 17.2 | 20 | 66.7 | <0.001 |
| Recent | 34 | 57.6 | 24 | 82.8 | 10 | 33.3 | |
| **Fixation time** | | | | | | | |
| <1 day | 25 | 44.6 | 15 | 51.7 | 10 | 33.3 | 0.3 |
| ≥1 day | 11 | 19.6 | 5 | 17.2 | 6 | 20.0 | |
| N/A | 20 | 35.7 | 8 | 27.6 | 12 | 40.0 | |

p-value by Chi-square test. DIN: DNA Integrity Number; N: Number; QC: Quality Control

DIN values showed a direct correlation with the time of storage (r = 0.523; p<0.001) (Figure 2D), as well as with library functionality ($r_s$ = 0.319; p=0.016). We established a cut-off for DIN value at 2.05 based on CART analysis, and results showed that DIN values greater that 2.05 gave functional libraries in a higher proportion than those less than or equal to 2.05 (82.1% vs. 17.9%; p=0.023) (Figure 2E). As expected,

QC and DIN were inversely correlated ($r = -0.535$; $p<0.001$) (Figure 2F).

When looking at the possible differences between tumor tissue and wide local excision samples within old and recent groups, it was found that for the old cohort, lower QC values were more frequent in tumor samples (13/14; 92.9%) than in wide local excision samples (1/14; 7.1%; $p=0.009$). No difference was found for DIN or functionality in this group. For the recent cohort, functional libraries corresponded in a higher proportion to the wide local excision (15/24; 62.5%) rather than tumor samples (9/24; 37.5%). No difference was found for DIN or QC in this group.

#### *3.3.3.3. Convergence of parameters in a decision tree*

Time of storage, QC and DIN were simultaneously assessed by CART test to analyze their impact on the library functionality, and a decision tree was developed (Figure 3). The storage time was the parameter that better discriminated the library functionality. Samples stored for 7 years or less gave functional libraries in 70.6% of the cases. For samples stored for more than 7 years the value of QC in the first place, and of DIN in the second place, discriminated samples by their functionality. Thus, samples with QC less than or equal to 5.63 and DIN greater than 2.05, allowed identification of a group with 44% of functional libraries. The diagnostic parameters were evaluated for each variable individually and for the algorithm obtained by CART analysis. The latter was also evaluated only for the oldest samples (Table 2).

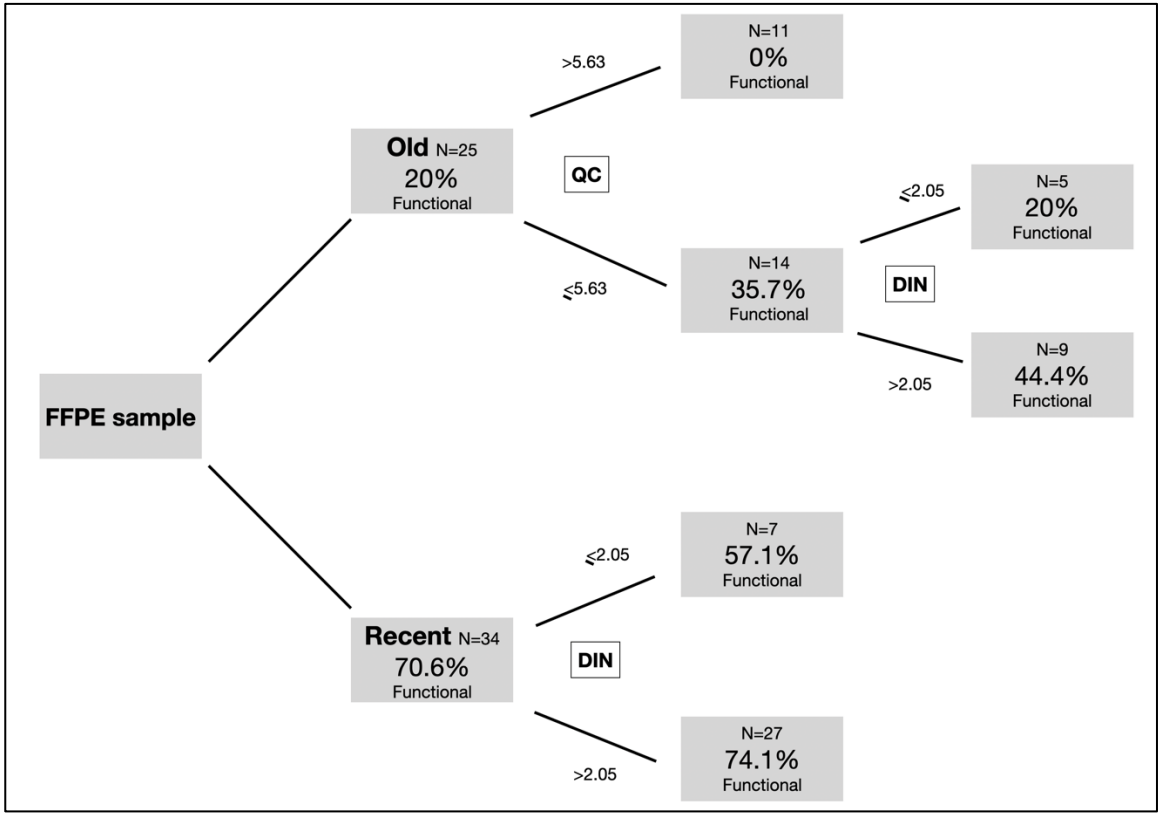


**Figure 3. Decision tree.** A flowchart to optimize the use of FFPE samples according to our results

**Table 2. Diagnostic test parameters for each variable and the algorithm obtained by CART analysis.**

| Variable | S (%) | SP (%) | PPV (%) | PNV (%) | A (%) | ROC (AUC) |
|---|---|---|---|---|---|---|
| QC | 80.8 | 68.2 | 60.0 | 85.7 | 72.9 | 0.33 |
| DIN | 70.3 | 66.1 | 62.2 | 73.7 | 68.0 | 0.66 |
| Time of storage | 77.9 | 73.1 | 70.6 | 80.0 | 75.3 | 0.75 |
| CART* | 91.3 | 72.9 | 65.1 | 93.8 | 79.5 | 0.73 |
| CART (for old samples)** | 87.7 | 62.8 | 44.4 | 93.8 | 69.1 | 0.78 |

"CART" includes the results for the diagnostic parameters of the proposed flowchart obtained by CART analysis.
*Positive for the test if the date of surgery < 7 years or if QC≤ 5.63 and DIN > 2.05.
**Only for old samples, with a date of surgery > 7 years. Positive if QC≤ 5.63 and DIN > 2.05.
A: Accuracy; PNV: Predicted negative values; PPV: Predicted positive values; QC: Quality control; S: Sensitivity; SP: Specificity.

The decision tree provided better values in the diagnostic parameters (sensitivity = 91.3%; specificity = 72.9%; predicted positive values = 65.1%; predicted negative values = 93.8%; accuracy = 79.5%; ROC area under the curve = 0.733) than each parameter individually. In addition, the decision tree restricted to the old samples also showed acceptable figures (ROC = 0.78).

### *3.3.4. Discussion*

There are several studies in the literature that have evaluated QC and DIN/RIN values prior to NGS (171). Yakovleva *et al.* established in 2017 a cut-off of RIN > 2.0 for using FFPE samples in downstream processes (170). Similarly, Bonfiglio *et al.* proposed in 2016 the use of

a DIN cut-off at 3.0 (173). All these previous studies agree with our findings, but all of them used FFPE samples that had been stored for a maximum of 6 years. Hence, we contribute valuable information regarding samples stored for longer periods, with a functional analysis on constructed amplicon-based libraries and not only on genomic DNA. These findings suggest that older FFPE samples should preferably be used for pathological analysis and molecular tests that do not require such quality starting material.

A flow-chart proposal was developed including all variables to determine the best approach when working with FFPE samples, taking into consideration the quality parameters QC and DIN as well as the time of storage (Figure 3). A deeper analysis of the diagnostic parameters of this proposal pointed out the utility of our approach given its accuracy of up to 79.5%. This could be translated into the practical work and suggests that, if willing to have a small percentage of compromise, all recent samples could be used. Then, only by using the QC value as a predictor parameter, those samples stored for >7 years could be included if their QC is ≤5.63 with an expected adequate functionality in 35.7% of samples. The use of the DIN value would increase the specificity in those studies where genetic material is precious and must be highly optimized and the expected adequate functionality could be increased up to 44.4% of the samples.

This study has demonstrated that QC (≤5.63) and DIN (>2.05) are able to discriminate between functional and non-functional samples beyond storage time, particularly for old samples. It showed that QC and DIN were appropriate quality parameters, for which values differed between

old and recent samples, and between functional and non-functional libraries. On the other hand, differences were found in QC between tumor samples and wide local excision samples, although they were not relevant for functionality in old samples. In recent samples, wide local excision samples worked better than tumors in terms of functionality, which might be explained by the role of melanin in tumor samples, which can lead to inhibition of PCR due to the association of remnants of pigment melanin with genomic DNA (174,175).

The strengths of this study include the performance over amplicon-based libraries instead of genomic DNA, as seen in previous studies. Also, the results can be extrapolated to different approaches. As a limitation, the modest size of the cohort should be highlighted, so future studies must corroborate these findings.

### *3.3.5. Conclusion*

In conclusion, the storage time was the most important variable that influenced sample viability for amplicon-based library construction. The addition of QC and DIN helped refine the rate of samples suitable for NGS and particularly to identify which ones within old samples could be used in this regard.

# 4. MOLECULAR CHARACTERIZATION OF THE DIVERGENT PATHWAYS

## 4.1. Overview

This chapter will address the main objective of this dissertation, which is the characterization at the molecular level of both nevogenic and CSD melanomas. As mentioned in previous sections, these two groups are included in current classifications of melanoma, but are not considered as independent entities.

To explore the role of UVR and melanocytic proliferation as melanoma drivers, this chapter will describe the clinical and molecular approach to study these two groups. Here we aim to confirm whether nevogenic and CSD melanomas constitute two different biological entities. To do so, it will be seen how the inclusion criteria were carefully established to get the purest cohort for each of the etiopathogenic pathways.

## 4.2. Reference and contribution of the candidate

Millán-Esteban, D.; Peña-Chilet, M.; García-Casado, Z.; Manrique-Silva, E.; Requena, C.; Bañuls, J.; López-Guerrero, J.A.; Rodríguez-Hernández, A.; Traves, V.; Dopazo, J.; Virós, A.; Kumar, R.; Nagore, E. Mutational Characterization of Cutaneous Melanoma Supports Divergent Pathways Model for Melanoma Development. Cancers 2021, 13,5219.https://doi.org/10.3390/cancers 13205219.

The candidate participated in the study design, experimental procedures, data analysis, and writing of the manuscript. Specifically, the mechanistic analysis of pathways was performed and led by Dr. María Peña-Chilet, an expert bioinformatician.

## 4.3. Mutational characterization of cutaneous melanoma supports divergent pathways model for melanoma development

### *4.3.1. Introduction*

The divergent pathways model suggested the clinical classification of cutaneous melanoma into two groups: one associated with melanocyte proliferation proneness (nevogenic) and the other with cumulative solar damage (CSD) (154). Both groups share an initiation step in which activation of melanocytes proceeds via exposure to ultraviolet radiation (UVR) early in life and host factors. Afterward, the progression towards melanoma diverges depending on exogenous and endogenous factors. Nevogenic melanomas arise in individuals constitutively predisposed to melanocytic proliferation, characterized by a high nevi count, with little involvement of acquired UVR damage. Those tumors appear in young/middle-aged people on intermittently sun-exposed areas like the trunk.

In contrast, CSD melanomas occur mainly in individuals with a low number of nevi, located on chronically sun-exposed skin like the head and neck, with solar elastosis on the healthy skin surrounding the melanoma. Those tumors emerge after a lifetime of cumulative sun exposure in older patients (176,177). Epidemiological studies have confirmed the divergent pathways hypothesis based on the distribution and number of nevi, UV-related skin damage, patient age at diagnosis and other clinical aspects (155,157,158,178). Furthermore, these two

populations would correspond to subgroups within the current WHO classification, which differentiates between high-CSD and low-CSD melanomas, but considering for the latter the proneness to melanocytic proliferation (160).

The differential molecular characterization of tumors from two etiopathogenic pathways, despite advanced sequencing initiative, has remained uninvestigated even two decades later and proven clinical relevance (179,180). The sequencing studies on cutaneous melanoma, in general, showed that the most prevalent mutations include those in *BRAF, TERT* promoter (*TERTp)*, *NRAS, NF1, ARID2,* and *TP53*. Based on the mutational pattern, cutaneous melanoma is classified into four molecular mutually exclusive subtypes. The four groups are based on mutations in *BRAF* ("BRAF+"), *NRAS/HRAS/KRAS* ("RAS+"), *NF1* ("NF1+"), or the absence of those three types of mutations, referred to as triple wild type ("3wt") (59,181,182).

The big genomic data repositories can foster models to predict relevant aspects of molecular and patient phenotypes. Such models, based on the molecular pathways, reveal relevant features of the disease. These novel tools allow prediction about the effects of alterations in the modelled system *in silico*, with potential new therapeutic targets and to predict the functional impact of loss-of-function (LoF) mutations on the different cell mechanisms in complex diseases (183–185).

This study sequenced tumors from cutaneous melanoma patients developed through two mutually exclusive routes to understand molecular differences and similarities using a custom gene panel covering most frequently altered genes. The data were analyzed using

comprehensive bioinformatics tools to characterize two seemingly different types of melanoma.

### *4.3.2. Material and Methods*

We designed a retrospective study using the mutational data obtained from next generation sequencing (NGS) and the information included in our melanoma databases. These contained prospectively collected data from all melanoma patients treated at the Instituto Valenciano de Oncología (IVO) since 2000 and the Hospital General Universitario de Alicante (HGUA) since 1995. Clinical, pathological, and epidemiological data assessed by expert dermatologists and pathologists were included (186). The study had the approval of the IVO ethics committee.

#### *4.3.2.1. Patient selection and classification*

Tumor samples were collected after informed consent and stored as formalin-fixed paraffin-embedded (FFPE) blocks at the corresponding Biobanks, after confirmation of melanoma diagnosis by a single pathologist per institution. Patients were classified based on the total number of melanocytic nevi and the histological presence/absence of solar elastosis in the healthy skin surrounding the melanoma. The latter was graded according to a previously described score (11 degrees; range: 0 to 3+) (187). We selected patients from the two mutually exclusive groups: nevogenic, characterized by the presence of more than 50 nevi and no solar elastosis; and CSD that included patients with less than 20 melanocytic nevi and moderate to severe solar elastosis.

#### *4.3.2.2. Sample preparation*

FFPE blocks were retrieved from the corresponding Biobanks, and glass slides were prepared for hematoxylin and eosin staining to guide the macrodissection of the tumor. Either three unstained sections of 10 µm thick tissue were manually scraped, or three 0.6 mm needle biopsies were taken from every sample to ensure a high tumor content, depending on tumor cellularity below or above 70%, respectively.

DNA extraction was performed using the QIAamp® DNA Investigator kit (QIAGEN, Hilden, Germany) with minor modifications. An overnight incubation step at 56ºC for the proteinase K was set to assure complete digestion of the skin, and an optional RNA carrier was added to maximize the extraction yield. Also, the NEBNext® FFPE Repair Mix (New England Biolabs, Hertfordshire, UK) was used to repair the DNA, hence minimizing sequencing artifacts due to C:G>T:A changes induced by nucleotide deamination, usually present in FFPE samples. DNA concentration was quantified using the Quant-iT™ PicoGreen™ dsDNA (ThermoFisher) fluorimetric assay, and those samples with >2.5ng/µL continued the process.

#### *4.3.2.3. Gene panel and library construction*

A Custom GeneRead™ DNAseq Targeted Panel V2 (QIAGEN®) was designed including coding regions for 21 genes involved in melanomagenesis: *ARID2, BRAF, CDK4, CDKN2A, GNA11, GNAQ, HRAS, IDH1, KIT, KRAS, MAP2K2, NF1, NRAS, PIK3CA, PIK3R1, PPP6C, PTEN, RAC1, RB1, ROS1,* and *TP53* (Table S1). The panel consisted of 633 amplicons distributed in 3 primer pools with an

average size of 200bp (range:120-275bp) and an average coverage of 98.2% (range: 75.3-100%). Barcoded libraries were generated from 7.5ng of DNA per primer pool according to the manufacturer's instructions, reducing the PCR volume to 12 µL to minimize sample usage. After purification with AMPure beads (Beckam Coulter, Brea, CA, USA), libraries were checked for appropriate size using Genomic DNA ScreenTape in a 4200 TapeStation (Agilent Technologies). The mutational status of the *TERT* promoter was determined by Sanger sequencing, as described previously (188).

#### *4.3.2.4. Next-generation sequencing*

Libraries were diluted to a final concentration of 13 pM and sequenced using v3-600 cycles plates on a MiSeq® sequencer (Illumina). Raw sequences from samples with coverage of 300X in ≥70% of the regions were filtered and processed. SNV and indel variants with a variant allele frequenct of >5% were annotated using VariantStudio 3.0 (Illumina) and Varsome (189) software. All pathogenic, likely pathogenic, and predicted pathogenic variants were visually checked with the Integrative Genome Viewer (IGV 2.3.32).

#### *4.3.2.5. Mechanistic analysis of pathways*

To evaluate the functional implications of the individual mutational profiles, gene expression data of skin normal tissue was downloaded from GTEx data portal (190). Using the normalized expression data from individuals, *in silico* knockdowns were simulated by multiplying the expression value by 0.01. Using KEGG signaling pathways

topology information (191), each signaling pathway was decomposed in its functional circuits as described elsewhere (192), and the activation levels of each circuit were obtained for each mutational profile using tissue expression values after applying a re-scaling transformation of the rank of the matrix to [0, 1]. An equal number of samples was randomly selected from GTEx skin tissue data to account for the different mutational profiles, obtaining a dataset of circuit activation levels from all samples, corresponding to each mutational profile.

Then, the differences in the circuits' activation levels between the groups (CSD vs. Nevogenic, CSD vs. normal tissue, and nevogenic vs. normal tissue) were evaluated. A linear model fit was performed and computed moderated t-statistics and log-odds of differential expression by empirical Bayes moderation using limma package from R/Bioconductor (193). All p-values were adjusted for multiple comparisons using Benjamini and Hochberg FDR method. To account for random sample selection, a bootstrap of 50 iterations was performed and combined the statistical results using Fisher's p-values combination method. We selected those circuits with an adjusted p-value $<0.05$, and with a level of concordance of Fold Change values of 70% (meaning that at least 70% of the bootstraps showed a level of concordance in the sign of the fold change), obtaining a list of differentially activated circuits characteristic of each group (Nevogenic and CSD).

These selected circuits were further annotated with the hallmarks of cancer using the Cancer Hallmarks Annotation Tool (CHAT), based in text-mining searching (194). For each circuit, only those hallmarks with

a score higher than the ninetieth percentile (0.18) were selected. To evaluate the impact over each hallmark of each group, the ratio of the number of significant circuits for each hallmark and the total of circuits annotated for each hallmark was calculated. Moreover, to evaluate the impact of the mutational profile over the whole pathway, a Fisher test was done to combine the individual values obtained from the independent circuits within the pathway, in order to obtain the overall level of dysregulation of the whole signaling pathway.

An univariate enrichment analysis was ultimately performed to elucidate whether a hallmark was significantly enriched in each group with respect to normal skin. The t-statistic and the adjusted p-values obtained from both nevogenic vs. normal skin, and CSD vs. normal skin limma models were taken to obtain a ranking of the circuits, together with the circuits annotated to hallmarks. Then, an analysis similar to a gene set enrichment analysis (gsea) was performed using Bioconductor msgsa R package to fit a logistic regression model relating the probability of circuits belonging to the functional hallmark set with the value of the ranking statistic.

#### *4.3.2.6. Statistical analysis*

Clinical variables and mutational status for the analyzed genes were categorized. A *Chi*-square test was applied to evaluate differences among the groups. Univariate and adjusted logistic regression models were used to establish the association between variables. A value of $p<0.05$ was set to define significance. The statistical analyses were

performed using IBM Corp. released 2011 (IBM SPSS Statistics for Macintosh, version 20.0. Armonk, NY: IBM Corp.).

### *4.3.3. Results*

#### *4.3.3.1. Mutational distribution among nevogenic and CSD melanomas*

A total of 119 primary melanomas provided informative sequences: 82 (68.9%) from the nevogenic group and 37 (31.1%) from the CSD group (Figure S1). The median age of the patients at diagnosis was 59 years that included 65 men (54.6%) and 54 women (45.4%). The nevogenic group included 42 (51.2%) men and 40 (48.8%) women whereas the CSD group included 23 862.2%) men and 14 (37.8%) women. A detailed description of demographic and clinicopathological characteristics of the cohort is displayed in Table 3.

**Table 3. Demographic and clinicopathological characteristics of the cohort.**

| Variable | | Total | | Nevogenic | | CSD | |
|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % |
| **Sex** | Male | 65 | 54.6 | 42 | 51.2 | 23 | 62.2 |
| | Female | 54 | 45.4 | 40 | 48.8 | 14 | 37.8 |
| **Phototype** | 1 | 2 | 1.7 | 2 | 2.4 | 0 | 0 |
| | 2 | 32 | 26.9 | 23 | 28.0 | 9 | 24.3 |
| | 3 | 68 | 57.1 | 46 | 56.1 | 22 | 59.5 |
| | 4 | 15 | 12.6 | 10 | 12.2 | 5 | 13.5 |
| | 5 | 1 | 0.8 | 0 | 0 | 1 | 2.7 |
| | Unknown | 1 | 0.8 | 1 | 1.2 | 0 | 0 |
| **Sunburns at the area of melanoma** | None | 12 | 26.7 | 8 | 27.6 | 4 | 25.0 |
| | Mild | 15 | 33.3 | 9 | 31.0 | 6 | 37.5 |
| | Severe | 16 | 35.6 | 10 | 34.5 | 6 | 37.5 |
| | N/A | 1 | 2.2 | 1 | 3.4 | 0 | 0 |
| | Unknown | 1 | 2.2 | 1 | 3.4 | 0 | 0 |

**Table 3 (continued). Demographic and clinicopathological characteristics of the cohort**

| Variable | | Total | | Nevogenic | | CSD | |
|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % |
| **Basal cell carcinoma** | No | 96 | 81.4 | 72 | 88.9 | 24 | 64.9 |
| | Yes | 22 | 18.6 | 9 | 11.1 | 13 | 35.1 |
| **Multiple melanoma** | No | 109 | 93.2 | 76 | 93.8 | 33 | 91.7 |
| | Yes | 8 | 6.8 | 5 | 6.2 | 3 | 8.3 |
| **Familial melanoma** | No | 102 | 87.2 | 67 | 82.7 | 35 | 94.6 |
| | Yes | 15 | 12.8 | 13 | 16.0 | 2 | 5.4 |
| **Anatomical location** | Head/neck | 30 | 25.2 | 2 | 2.4 | 28 | 75.7 |
| | Limb | 25 | 21.0 | 18 | 22.0 | 7 | 18.9 |
| | Trunk | 59 | 49.6 | 57 | 69.5 | 2 | 5.4 |
| | Acral | 4 | 3.4 | 4 | 4.9 | 0 | 0 |
| | Other | 1 | 0.8 | 1 | 1.2 | 0 | 0 |
| **Histological type** | LMM | 18 | 15.1 | 1 | 1.2 | 17 | 45.9 |
| | SSM | 73 | 61.3 | 60 | 73.2 | 13 | 35.1 |
| | NM | 15 | 12.6 | 11 | 13.4 | 4 | 10.8 |
| | ALM | 3 | 2.5 | 3 | 3.7 | 0 | 0 |
| | Desmoplastic | 2 | 1.7 | 2 | 2.4 | 0 | 0 |
| | Spitzoid | 2 | 1.7 | 2 | 2.4 | 0 | 0 |
| | Other | 6 | 5.0 | 3 | 3.7 | 3 | 8.1 |
| **Ulceration** | No | 99 | 83.2 | 70 | 85.4 | 29 | 78.4 |
| | Yes | 20 | 16.8 | 12 | 14.6 | 8 | 21.6 |
| **Sentinel node** | Negative | 19 | 67.9 | 14 | 82.4 | 5 | 45.5 |
| | Positive | 6 | 21.4 | 3 | 17.6 | 3 | 27.3 |
| | Unknown | 3 | 10.7 | 0 | 0 | 3 | 27.3 |
| **Age*** | <=59 | 59 | 50.0 | 58 | 71.6 | 1 | 2.7 |
| | >59 | 59 | 50.0 | 23 | 28.4 | 36 | 97.3 |
| **Breslow*** | <=1.08 | 59 | 50.0 | 48 | 59.3 | 11 | 29.7 |
| | >1.08 | 59 | 50.0 | 33 | 40.7 | 26 | 70.3 |

* Categorized by the median of the studied population

Overall, the most mutated genes/loci were *TERTp* (52.2%), *BRAF* (50.4%), *NF1* (16.8%), *NRAS* (13.4%), *ROS1* (11.8%), and *TP53* (10.9%); with the remaining genes investigated having a mutational frequency of <10% (Table 4). Of the total, 106 (89.1%) melanomas

were classified into the four major groups: 48/119 (40.3%) *"BRAF+"*, 15/119 (12.6%) *"RAS+"*, 10/119 (8.4%) *"NF1+"*, and 33/119 (27.7%) "3wt"; however, 13 (10.9%) patients due to mutations in overlapping genes eluded classification: 3/119 (2.5%) melanomas showed both a *BRAF* and *RAS* mutation, 9/119 (7.6%) showed both a *BRAF* and *NF1* mutation, and 1/119 (0.8%) showed a mutation in both *RAS* and *NF1* (Table 4). A graphical representation of the mutational concurrence can be found in Figure S2.

**Table 4. Mutational prevalence in our cohort and classification into molecular subtypes.**

| Gene | Mutation Prevalence | Gene | Mutational Prevalence |
|---|---|---|---|
| *TERTp* | 52.21 | *RB1* | 4.20 |
| *BRAF* | 50.42 | *PIK3R1* | 4.20 |
| *NF1* | 16.81 | *GNA11* | 4.20 |
| *NRAS* | 13.45 | *CDK4* | 3.36 |
| *ROS1* | 11.76 | *PPP6C* | 3.36 |
| *TP53* | 10.92 | *PTEN* | 2.52 |
| *ARID2* | 9.24 | *HRAS* | 1.68 |
| *CDKN2A* | 7.56 | *MAP2K2* | 1.68 |
| *RAC1* | 5.88 | *GNAQ* | 0.84 |
| *IDH1* | 5.04 | *KRAS* | 0.84 |
| *KIT* | 4.20 | *PIK3CA* | 0.84 |
| Molecular subgroup | % within cohort | Molecular subgroup | % within cohort |
| "*BRAF+*" | 40.3 | "*BRAF+RAS+*" | 2.5 |
| "*RAS+*" | 12.6 | "*BRAF+NF1+*" | 7.6 |
| "*NF1+*" | 8.4 | "*RAS+NF1+*" | 0.8 |
| "3wt" | 27.7 | | |

The nevogenic tumors had a higher frequency of *BRAF* mutations than the CSD melanomas, though the difference was not statistically

significant (46/82, 56.1% vs. 14/37, 37.8%; p=0.077). In contrast, CSD melanomas had a higher frequency of mutations than nevogenic melanomas in *NF1* (14/37, 37.8% vs. 6/82, 7.3%; p<0.001), *ROS1* (10/37, 27.0% vs. 4/82, 4.9%; p=0.001), *GNA11* (4/37, 10.8% vs. 1/82, 1.2%; p=0.032), and *RAC1* (6/37, 16.2% vs. 1/82, 1.2%; p=0.004) (Table 5; Figure 4; Table S2. The differences were further assessed by univariate logistic regression, and after adjustment, only mutations in *NF1* and *ROS1* remained independently associated with CSD melanomas (Table S3).

**Table 5. Prevalence of mutations according to etiopathogenic group and molecular subgroups.**

| Gene | Status | Total | | Nevogenic | | CSD | | p-value |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | |
| *TP53* | wt | 106 | 89.1 | 76 | 92.7 | 30 | 81.1 | 0.108 |
| | mutated | 13 | 10.9 | 6 | 7.3 | 7 | 18.9 | |
| *NF1* | wt | 99 | 83.2 | 76 | 92.7 | 23 | 62.2 | <0.001 |
| | mutated | 20 | 16.8 | 6 | 7.3 | 14 | 37.8 | |
| *BRAF* | wt | 59 | 49.6 | 36 | 43.9 | 23 | 62.2 | 0.077 |
| | mutated | 60 | 50.4 | 46 | 56.1 | 14 | 37.8 | |
| *ROS1* | wt | 105 | 88.2 | 78 | 95.1 | 27 | 73.0 | 0.001 |
| | mutated | 14 | 11.8 | 4 | 4.9 | 10 | 27.0 | |
| *NRAS* | wt | 103 | 86.6 | 70 | 85.4 | 33 | 89.2 | 0.773 |
| | mutated | 16 | 13.4 | 12 | 14.6 | 4 | 10.8 | |
| *CDK4* | wt | 115 | 96.6 | 79 | 96.3 | 36 | 97.3 | 1 |
| | mutated | 4 | 3.4 | 3 | 3.7 | 1 | 2.7 | |
| *ARID2* | wt | 108 | 90.8 | 77 | 93.9 | 31 | 83.8 | 0.094 |
| | mutated | 11 | 9.2 | 5 | 6.1 | 6 | 16.2 | |
| *CDKN2A* | wt | 110 | 92.4 | 76 | 92.7 | 34 | 91.9 | 1 |
| | mutated | 9 | 7.6 | 6 | 7.3 | 3 | 8.1 | |
| *KIT* | wt | 114 | 95.8 | 80 | 97.6 | 34 | 91.9 | 0.173 |
| | mutated | 5 | 4.2 | 2 | 2.4 | 3 | 8.2 | |

**Table 5 (continued). Prevalence of mutations according to etiopathogenic group and molecular subgroups.**

| Gene | Status | Total | | Nevogenic | | CSD | | p-value |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | |
| *RB1* | wt | 114 | 95.8 | 79 | 96.3 | 35 | 94.6 | 0.646 |
| | mutated | 5 | 4.2 | 3 | 3.7 | 2 | 5.4 | |
| *PPP6C* | wt | 115 | 06.6 | 79 | 96.3 | 36 | 97.3 | 1 |
| | mutated | 4 | 3.4 | 3 | 3.7 | 1 | 2.7 | |
| *PTEN* | wt | 116 | 97.5 | 80 | 97.6 | 36 | 97.3 | 1 |
| | mutated | 3 | 2.5 | 2 | 2.4 | 1 | 2.7 | |
| *IDH1* | wt | 113 | 95.0 | 78 | 95.1 | 35 | 94.6 | 1 |
| | mutated | 6 | 5.0 | 4 | 4.9 | 2 | 5.4 | |
| *GNA11* | wt | 114 | 95.8 | 81 | 98.8 | 33 | 89.2 | 0.032 |
| | mutated | 5 | 4.2 | 1 | 1.2 | 4 | 10.8 | |
| *GNAQ* | wt | 118 | 99.2 | 82 | 100.0 | 36 | 97.3 | 0.311 |
| | mutated | 1 | 0.8 | 0 | 0 | 1 | 2.7 | |
| *RAC1* | wt | 112 | 94.1 | 81 | 98.8 | 31 | 83.8 | 0.004 |
| | mutated | 7 | 5.9 | 1 | 1.2 | 6 | 16.2 | |
| *KRAS* | wt | 118 | 99.2 | 81 | 98.8 | 37 | 100.0 | 1 |
| | mutated | 1 | 0.8 | 1 | 1.2 | 0 | 0 | |
| *HRAS* | wt | 117 | 98.3 | 81 | 98.8 | 36 | 97.3 | 0.527 |
| | mutated | 2 | 1.7 | 1 | 1.2 | 1 | 2.7 | |
| *MAP2K2* | wt | 117 | 98.3 | 82 | 100.0 | 35 | 94.6 | 0.095 |
| | mutated | 2 | 1.7 | 0 | 0 | 2 | 5.4 | |
| *PIK3CA* | wt | 118 | 99.2 | 82 | 100.0 | 36 | 97.3 | 0.311 |
| | mutated | 1 | 0.8 | 0 | 0 | 1 | 2.7 | |
| *PIK3R1* | wt | 114 | 95.8 | 80 | 97.6 | 34 | 91.9 | 0.173 |
| | mutated | 5 | 4.2 | 2 | 2.4 | 3 | 8.1 | |
| *TERTp* | wt | 54 | 47.8 | 41 | 51.9 | 13 | 38.2 | 0.220 |
| | mutated | 59 | 52.2 | 38 | 48.1 | 21 | 61.8 | |
| Pathogenic mutations | <=2 | 74 | 62.2 | 55 | 67.1 | 19 | 51.4 | 0.108 |
| | >2 | 45 | 37.8 | 27 | 32.9 | 18 | 48.6 | |
| Mutational subtype* | "*BRAF*+" | 48 | 45.3 | 39 | 52.0 | 9 | 29.0 | <0.001 |
| | "*RAS*+" | 15 | 14.2 | 12 | 16.0 | 3 | 9.7 | |
| | "*NF1*+" | 10 | 9.4 | 1 | 1.3 | 9 | 29.0 | |
| | "3wt" | 33 | 32.4 | 23 | 30.7 | 10 | 32.3 | |

* 13 tumors showing concurrent mutations from different subtypes were excluded.

Mean comparison showed a statistically significant difference in the total number of pathogenic mutations between nevogenic and CSD melanomas (1.9 vs. 3.4; p=0.029), but no differences were found in the number of UV-induced mutations.

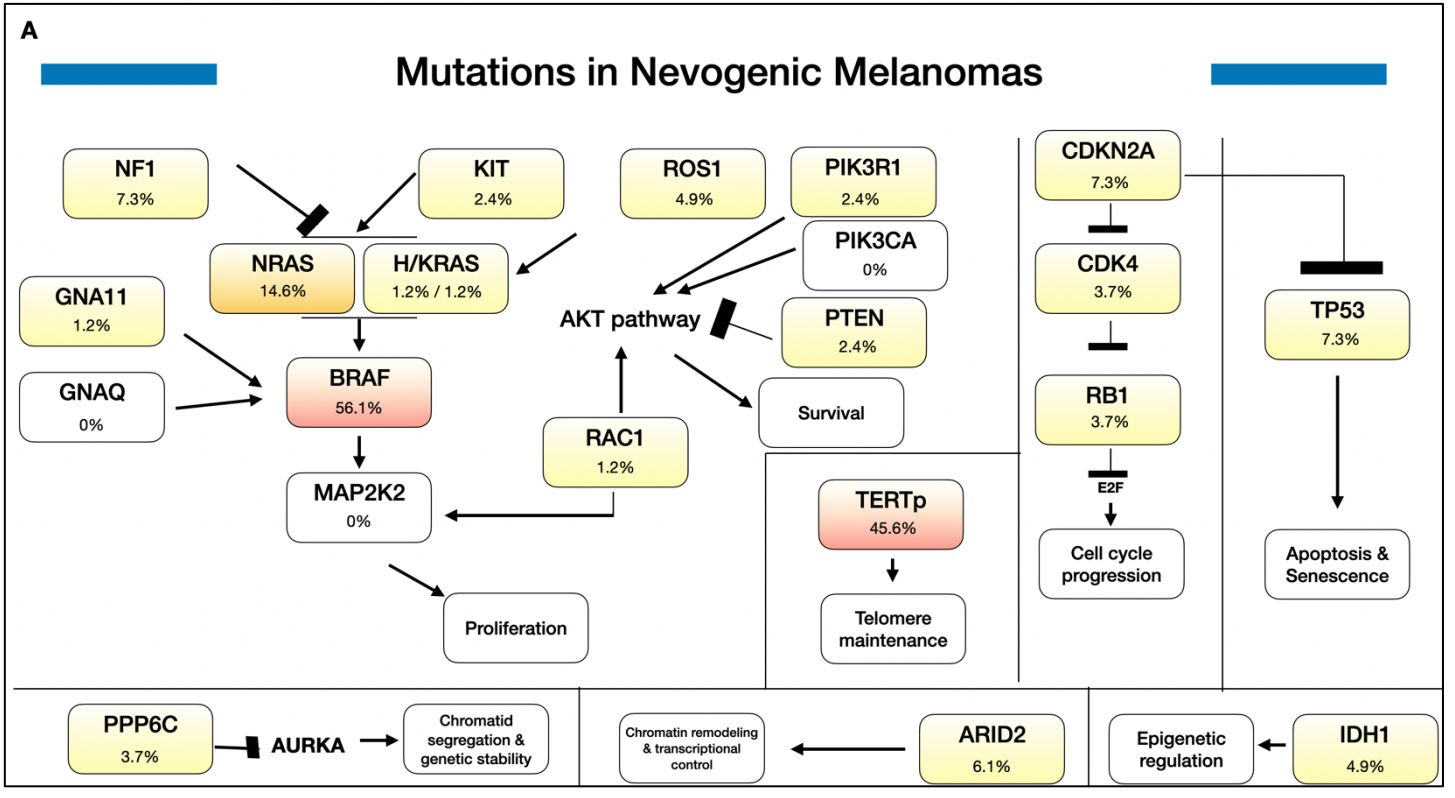


**Figure 4. Mutational prevalence and mutational association for etiopathogenic pathways.** Frequency of mutations in the different genes for the nevogenic group (A).

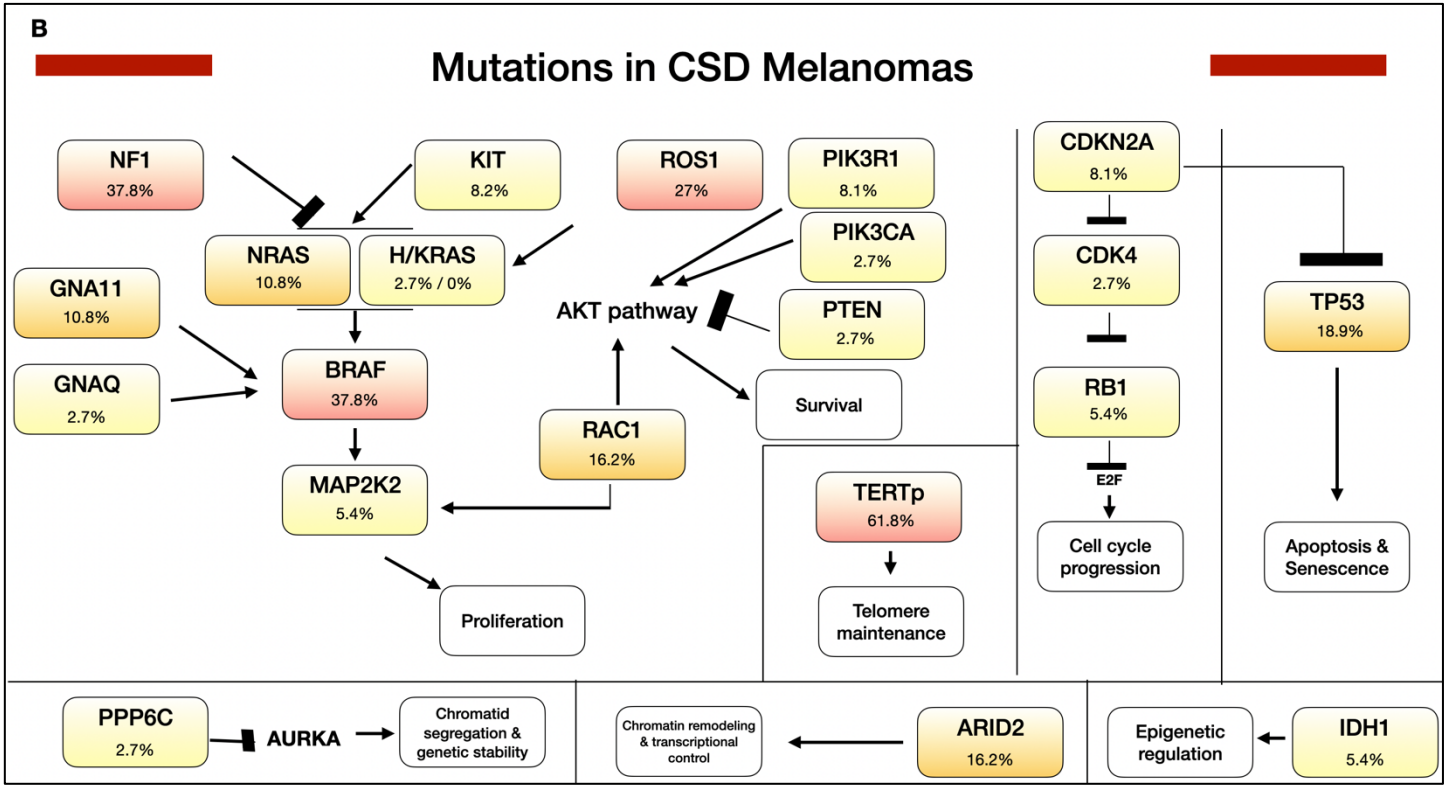


**Figure 4 (continued). Mutational prevalence and mutational association for etiopathogenic pathways.** Frequency of mutations in the different genes for the CSD group (B).

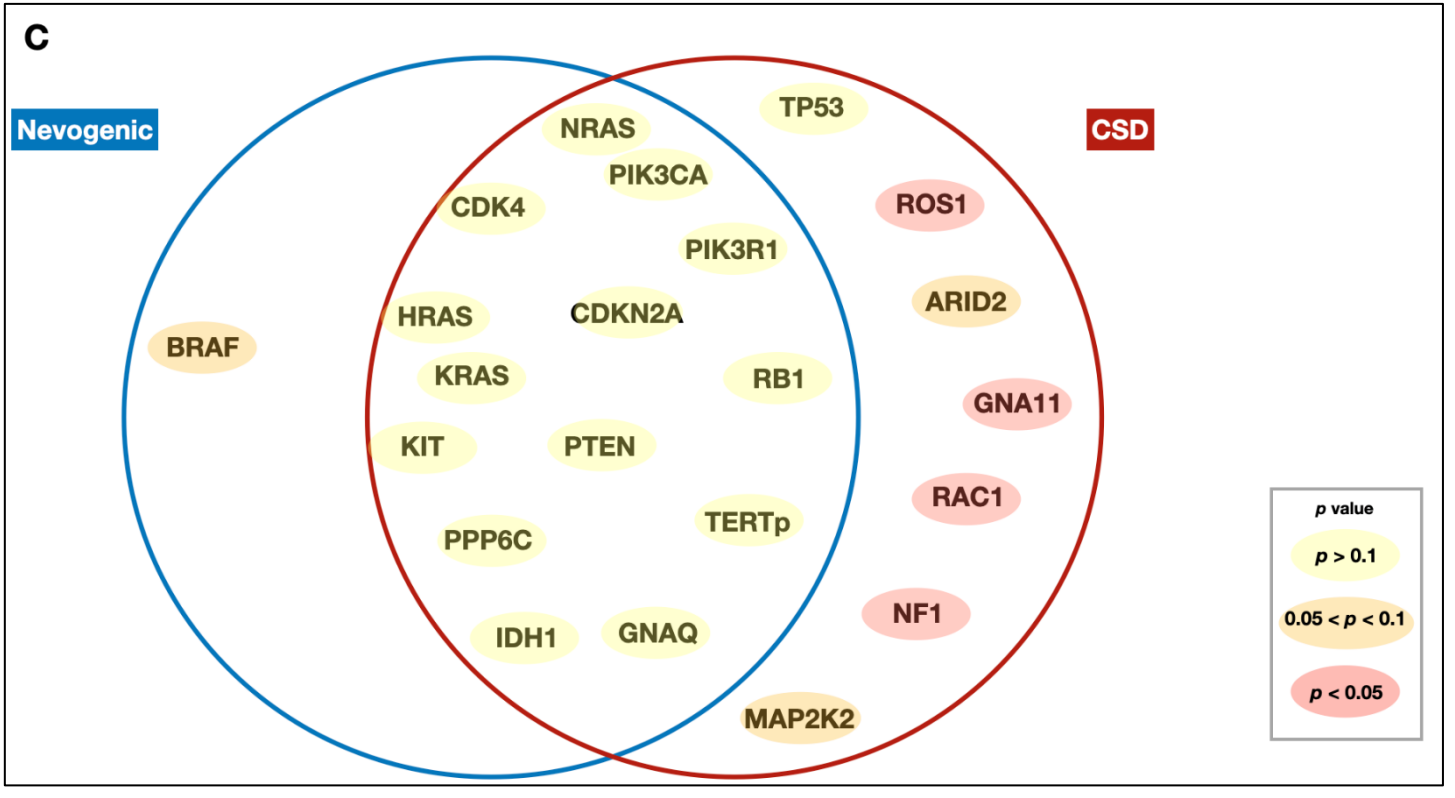


**Figure 4 (continued). Mutational prevalence an mutational association for etiopathogenic pathways.** Graphical representation of the association of mutations in the different genes with either group based on their p-value (C).

The distribution of the molecular subtypes among the described major etiopathogenic groups was the following: the *"BRAF+"* subtype was significantly associated with nevogenic melanomas (39/75, 52.0% vs. 9/31, 29.0%), while *"NF1+"* was related to CSD melanomas (1/37, 1.3% vs. 9/31, 29.0%) ($p<0.001$) (Table 5)..

#### *4.3.3.2. Mechanistic analysis of pathways*

The mechanistic analysis based on mutational profiles to predict the effect on normal skin showed that 67 circuits were significantly dysregulated in nevogenic melanomas (66 upregulated; 1 downregulated), and 122 circuits were dysregulated in CSD melanomas (109 upregulated; 13 downregulated). Fifty-one circuits were statistically significantly higher in CSD than in nevogenic melanomas (Figure S3). A radar plot was visualized with the altered pathways in the context of the annotated hallmarks of cancer (Figure 5). The plot showed that mutational profiles from CSD melanomas had a higher number of dysregulated circuits (counts; "Cs") annotated to hallmarks of cancer than those from nevogenic melanomas, especially when considering proliferative signaling (26 vs. 50 Cs), replicative immortality (11 vs. 15 Cs). The enrichment analysis based on simulated circuit activity data from normal skin showed that dysregulations in proliferative signaling and replicative immortality were statistically significant in nevogenic ($p=0.01$; $p=0.002$) and CSD ($p=0.0004$; $p=0.002$) melanomas compared to their corresponding normal skin (Table S4).

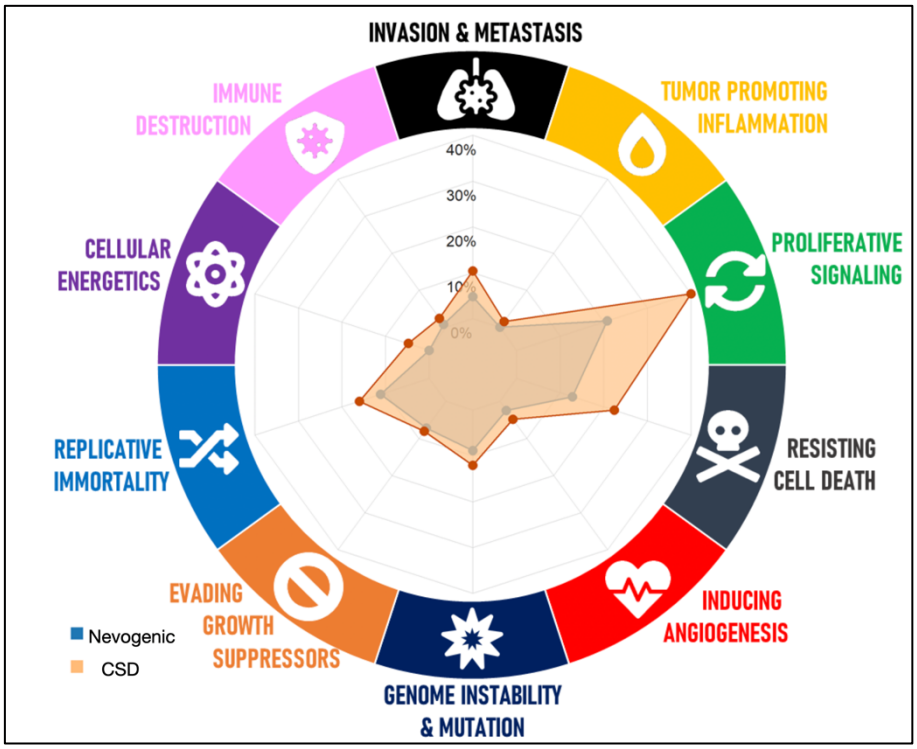


**Figure 5. Radar plot of cancer hallmarks.** This graphical representation shows the distribution of dysregulated circuits for each group. Percentages are used as an approximation to reflect the differences in the overall number of dysregulated circuits per hallmark found in each group.

### *4.3.4. Discussion*

Cutaneous melanoma is a complex disease sorted by different characteristics. However, a clinical classification represents a helpful approach given the disease's etiology, evolution, and mutational status. The divergent pathways model confirmed through clinical and epidemiological studies posits two different cutaneous melanoma groups (nevogenic and CSD). In this study, based on the molecular

characterization of the two divergent groups, we show a higher frequency of mutations in different genes in CSD melanomas than in nevogenic melanoma except for *BRAF* mutations.

Although UV-radiation is crucial to the initiation in both melanomas, the CSD type, predicated on chronic sun exposure leading to the accumulation of mutations, reflects the etiology through a typical corresponding mutational signature. The role of UVR on melanocyte proliferation and melanoma development involves direct and indirect mutagenesis processes, including the formation of photoproducts and free radicals resulting from the biochemical interaction of UVA and melanin (20). Chronic exposure to sun damage leads to multiple alterations affecting the cell's normal functioning and increases the chance of melanomagenesis. Several prominent genes mutated in CSD melanomas included *NF1, ROS1, GNA11,* and *RAC1*. *NF1* encodes a GTPase-activating protein that downregulates RAS activity, so loss-of-function mutations activate the MAPK pathway upstream of RAS. *ROS1*, a receptor tyrosine kinase of the insulin receptor family, is constitutively activated when mutated and also leads to the activation of the pathway; mutated *RAC1* increases the GDP/GTP nucleotide exchange rate; and *GNA11* is a subunit of a G protein-coupled receptor responsible for mediating GTP-binding and limiting the activation of the pathway and the activating mutations result in the constitutive activation of MAPK (195–199). Our findings align with the crucial role of the activated MAPK pathway in melanoma for uncontrolled cell proliferation. Based on our *in silico* simulation analyses using expression data from normal skin as a reference, the higher number of

dysregulated circuits within the proliferation pathway found in CSD melanomas could suggest a more relevant role of this pathway in carcinogenesis than in nevogenic melanomas. Also, this dysregulation was more significant in CSD than in nevogenic melanomas when compared with their corresponding normal tissue. However, a difference in the number of dysregulated circuits might not translate into actual differences in individual gene expression levels, so further studies on these two groups should be performed to elucidate whether the proliferation levels are more elevated in CSD than nevogenic melanomas or not[(B)].

Many studies have described that melanomas with a higher tumor mutation burden (TMB) would have a better outcome than those with a lower TMB (200,201). A higher tumor mutational burden leads to increased potential neoantigens and an improved response to immunotherapy (202–204). Even though our study did not assess TMB, the higher frequency of mutations in CSD melanomas indicates the trend. Our molecular characterization of CSD melanomas draws attention to the fact that there are genes specific to this group where mutations have not been yet explored as therapeutic targets. Given the revolution that targeted drugs constituted as inhibitor-based drugs against melanomas harboring mutations in *BRAF, MEK,* and *KIT* (55,205), studies like the present one contribute to the identification of potential lines of work aimed at improving the medical attention of these patients.

Alternatively, the development of melanoma in the absence of accumulated UVR in occasionally exposed anatomical sites remains

intriguing, and here we have shown how these nevogenic melanomas were associated with *BRAF* mutations. Multiple studies have shown this association in young nevus-prone patients with melanomas arising at intermittently exposed sites (206,207). However, this alone does not explain the development of melanoma since *BRAF* mutations have been widely reported in benign melanocytic nevi, which do not necessarily transform into melanoma (8,97,208). Additional contributing factors are reflected in the literature, with pigmented pheomelanin being extensively studied. Compared to eumelanin, pheomelanin has an inherent genotoxic effect via the production of reactive oxygen species (ROS) or consumption of antioxidants, enhancing carcinogenesis independently of UVR (3,4).

Moreover, some studies in rodents suggested additional factors that might contribute to melanomagenesis in similar conditions to nevogenic melanomas. For instance, *BRAF* mutations seem to enhance carcinogenesis resulting from UVB, meaning fewer exposures might be required for melanocyte progression into melanoma (24). Also, previous studies have suggested that susceptibility to UV might vary through the different sequence regions in the human genome, depending on nucleosome structure, bound transcription factors, or other factors (209,210). Hence, a more in-depth sequencing approach covering both coding and non-coding regions could be beneficial to elucidate the real prevalence of UV-signature mutations. Complementarily, germline alterations have not been checked in our study, and they could further explain the development of this group of

melanomas, with the missing impetus coming from normal germline variants in RNA-binding proteins or DNA repair genes (211).

The role of *TERT*p mutations in the development of melanoma has been widely studied since the stabilization of telomeres in cells is one of the hallmarks of cancer (212,213). In our study, CSD melanomas showed a higher prevalence of mutations within the promoter region of *TERT* albeit without statistical significance. The lack of association could be due to limited sample size since previous reports had suggested a UVR influence on these mutations, with *TERT*p alterations more frequent in CSD melanomas (188,214). The *TERT* promoter mutations in previous studies have been shown to associate with markers of poor prognosis, increased tumor growth, hematogenous dissemination, and define the subsets of melanoma patients with poor disease-free and disease-specific survival (92,215–217).

Finally, there are some limitations in the present study. The use of a custom panel instead of a whole-exome or a whole-genome approach results in some potentially relevant genes being left out (e. g. *MAP2K1, CTNNB1*) (218,219). Also, our analysis has focused on SNV and indels and did not include copy number variants, which are relevant as well when characterizing tumors (220).

### *4.3.5. Conclusion*

We present a detailed cohort of cutaneous melanoma patients classified into etiopathogenic groups showing distinct molecular profiles. These data provide further corroboration that nevogenic and CSD melanoma

subtypes, defined by the divergent pathway theory of melanoma, reflect the disease's specific biology.

# 5. CHARACTERIZATION OF MELANOMAS HARBORING MUTATIONS IN *KIT*

## 5.1. Overview

In previous chapters, we have gone through the relevance of the genes included in our study, and their association with either of the targeted groups. The knowledge on their role in melanomagenesis varies, with some studied in depth while others are yet to be defined more clearly.

In the case of the gene *KIT*, mutations have been usually associated with acral and mucosal melanomas, and an etiology related to UVR has been suggested.

Given the availability of clinical data from a large cohort of melanoma patients, together with the mutational status for *KIT*, we widened the scope of our study to assess the prevalence of *KIT* mutations in cutaneous melanoma in general, taking a special look at the possible etiology related to sun exposure. This resulted in the publication of an original paper.

## 5.2. References and contribution of the candidate

Millán-Esteban D, García-Casado Z, Manrique-Silva E, Virós A, Kumar R, Furney S, López-Guerrero JA, Requena C, Bañuls J, Traves V, Nagore E. Distribution and clinical role of *KIT* gene mutations in melanoma according to subtype: a study of 492 Spanish patients. Eur J Dermatol. 2021 Mar 1. Doi: 10.1684/ejd.2021.3971. PMID: 33648909.

The candidate participated in the study design, experimental procedures, data analysis, and writing of the manuscript. The bioinformatic analysis of the TCGA data was performed by Dr. Simon Furney. Due to space limitations with the journal, part of the methods

and results were placed as supplementary material. However, these have been put back as main text for the present chapter to include all the information.

## 5.3. Distribution and clinical role of *KIT* gene mutations in melanoma according to subtype: a study of 492 Spanish patients

### *5.3.1. Introduction*

Activating mutations in the gene encoding KIT receptor (*KIT*) lead to persistent upregulation of MAPK and PI3K signaling cascades without a ligand (221,222). Several studies have shown mutations in the gene mainly in cutaneous acral and mucosal melanomas in 15-30% of patients (223–225). According to a hypothesis related to a divergent pathway, melanomas can arise via a nevogenic pathway for which host factors are critical, and patients present with melanocytic instability. In such patients, melanomas are generally located at unexposed or intermittently sun-exposed areas of skin without chronic sun damage (CSD). Such lesions usually carry *BRAF* mutations. Alternately, the CSD pathway, through cumulative sun exposure, leads to melanomas at usually exposed body sites with a substantial degree of solar elastosis, which are characterized by a relatively increased proportion of *NRAS* and *KIT* mutations (136,137,154,156,226).

Only a few studies have investigated the prevalence of *KIT* mutations in non-acral cutaneous melanoma (59) as most studies have focused on acral and mucosal melanomas (227,228) (Table 6). The Cancer Genome Atlas network reported a complete genomic profile for cutaneous melanoma (59) that included mutational status of *KIT*. Mutations in *KIT* were only present in 12 (3.5%) of the melanomas. Besides, a transcriptomic classification of melanoma into three groups ("immune-high", "keratin-high", and "*MITF*-low") based on consensus hierarchical clustering analysis was proposed. Patient survival differed significantly among the groups but difference in prevalence based on mutational status had not been performed.

In this study, we performed a retrospective investigation on the mutational status of *KIT* gene in primary tumors from a large number of Spanish melanoma patients which included all melanoma subtypes. We determined the association between *KIT* mutations and various clinical variables including different tumor characteristics.

Our data show that the frequency of *KIT* mutations was low in non-acral melanoma and that, contrary to previously reported data, prevalence did not differ between CSD and non-CSD melanomas. Moreover, our data suggest the role of *KIT* mutations as an aggressiveness marker.

**Table 6. Prevalence of *KIT* mutations found in literature.**

| Country | *KIT*+ patients (n) | Total (n) | % | Type | Reference |
|---|---|---|---|---|---|
| China | 223 | 2793 | 7.98 | All types | (229) |
| Japan | 2 | 40 | 5.00 | All types | (230) |
| Korea | 4 | 47 | 8.51 | Acral | (231) |
| France | 0 | 20 | 0 | All types | (232) |
| China | 13 | 105 | 12.38 | All types | (233) |
| Brazil | 6 | 29 | 20.69 | ALM | (234) |
| USA | 5 | 15 | 33.33 | Mucosal | (235) |
| Turkey | 4 | 106 | 3.77 | All types | (236) |
| Japan | 22 | 171 | 12.87 | All types | (237) |
| Turkey | 4 | 106 | 3.77 | All types | (238) |
| Netherlands | 1 | 24 | 4.17 | Mucosal | (239) |
| Canada/Germany | 7 | 50 | 14.00 | Mucosal | (240) |
| Italy | 4 | 33 | 12.12 | All types | (241) |
| China | 9 | 39 | 23.08 | Acral | (242) |
| Korea | 17 | 202 | 8.42 | All types | (243) |
| France | 1 | 17 | 5.88 | Mucosal | (244) |
| Sweden | 2 | 56 | 3.57 | Mucosal | (245) |
| China | 0 | 20 | 0 | All types | (246) |
| USA | 9 | 79 | 11.39 | All types | (247) |
| Spain | 5 | 56 | 8.93 | All types | (248) |
| Japan | 3 | 79 | 3.80 | All types | (249) |
| China | 7 | 40 | 17.50 | Mucosal | (250) |
| USA | 42 | 162 | 25.93 | All types | (251) |
| China | 54 | 502 | 10.76 | All types | (252) |
| Japan | 1 | 12 | 8.33 | All types | (253) |
| Japan | 1 | 16 | 6.25 | Mucosal | (254) |
| USA | 12 | 189 | 6.35 | All types | (224) |
| Germany | 5 | 34 | 14.71 | Mucosal | (255) |
| USA | 15 | 101 | 14,85 | All types | (223) |
| Italy | 1 | 29 | 3.45 | All types | (256) |

**Table 6 (continued). Prevalence of *KIT* mutations found in literature.**

| Country | *KIT*+ patients (n) | Total (n) | % | Type | Reference |
|---|---|---|---|---|---|
| USA | 14 | 122 | 11.48 | Acral | (257) |
| USA | 1 | 16 | 6.25 | Mucosal | (258) |
| China | 15 | 65 | 23.08 | Mucosal | (259) |
| Australia | 3 | 27 | 11.11 | Mucosal | (260) |
| Mexico | 2 | 62 | 3.23 | Mucosal | (261) |
| Italy | 1 | 69 | 1.45 | All types | (262) |
| TCGA | 12 | 308 | 3.90 | All types | (59) |
| Total | 527 | 5782 | 9.11 | | |

### *5.3.2. Material and methods*

We designed a retrospective study using the melanoma database of the Instituto Valenciano de Oncología (IVO), which contains prospectively collected information from all melanoma patients treated at the institute, a tertiary referral oncology hospital in Spain. The data had been collected from January 2000, and included clinical, pathological and epidemiological information assessed by two expert dermatologists at first visit (263). The study had the approval of the Ethics Committee at the IVO and informed consent from patients.

#### *5.3.2.1. Sample recruitment and classification*

Melanoma patients included in the study were diagnosed between 2000 and 2018 at IVO. Tumor samples were collected and stored in the Biobank. Melanoma diagnosis was pathologically confirmed by a single pathologist. Patients were classified based on the

presence/absence of *KIT* mutation. Additionally, patients were classified according to the latest WHO classification of melanomas (non-CSD, CSD, acral, and mucosal) (264). In this classification, the difference of solar elastosis in the unaffected skin surrounding the melanoma.

#### *5.3.2.2. DNA extraction and mutation analysis of KIT*

DNA was extracted using QIAGEN® commercial kits (QIAamp DNA FFPE Tissue Kit® and QIAamp DNA Investigator Kit®). Exons 9, 11, 13, and 17 of the *KIT* were amplified using primers described in Table S5. PCR was carried using $MgCl_2$ 1.5mM; dNTPs 200 µM in Buffer II 1x; using primers 0.2-0.4 µM; and AmpliTaq Gold 1U/tube. The temperature program used for PCR was 95ºC – 6 min; 40 cycles of 94ºC – 45 sec., 56ºC – 1 min, 72ºC – 1 min; 72ºC – 10 min. Amplification products were purified and checked on an agarose gel (2%).

Sanger sequencing was performed using 10 pM of each primer and the ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems®. The sequencing conditions were: 96ºC – 1 min; 25 cycles of 96ºC – 10 sec., 50ºC – 5 sec., 60ºC – 4 min; 4ºC hold. Resulting sequencing products were purified via ethanol precipitation and sequenced according to established protocols in a Sanger sequencer (3031x Genetic Analyzer; Applied Biosystems®).

#### *5.3.2.3. The Cancer Genome Atlas data analysis*

For the TCGA study, data from the whole available set were downloaded the original publication (59). We established a *Signature*

*7* status, based on DNA damage due to UVR, for all patients. This signature was described based on trinucleotide combinations. Signature 7 was characterized, mostly but not exclusively, by C>T changes in dipyrimidine context at the first two nucleotides (229,230). For the TCGA mutation data signature analysis, the primary data were the somatic mutation calls from TCGA MAF (skcm_clean_pairs.aggergated.capture.tcga.uuid.somatic.maf) of the whole-exome sequences of tumors from the TCGA skin cutaneous melanoma (SKCM) cohort. Mutational signature analysis was conducted using the R package deconstructSigs (265) to estimate the proportion of Signature 7 mutations in each tumor genome (*https://cancer.sanger.ac.uk/cosmic/signatures_v2*). For a patient classified as Signature 7 positive had at least 25% of the mutations as per the signature (230). The expression cluster was also extracted for the TCGA cohort, in which tumors had been clustered in "immune-high", "keratin-high", and "*MITF*-low" (59).

#### *5.3.2.4. Statistical analysis*

Categorical variables included clinical characteristics and mutational status for *KIT* gene. A *Chi* square test was applied to study differences among the groups, using a threshold of $p<0.05$ to define statistical significance. First analysis included all selected samples, and the second analysis was carried out by excluding acral and mucosal subtypes (Table 7 and Table S6).

Melanoma-specific and overall survival were calculated using the Kaplan-Meier method and differences between the curves were tested by log-rank test. The statistical analyses were performed using IBM SPSS Statistics for Macintosh, version 20.0. (IBM Corp.; Armonk, NY, USA).

### *5.3.3. Results*

Tumors from 606 melanoma patients with recorded clinical variables were screened for *KIT* mutations. Eleven patients with unknown site of primary tumors were excluded from the analysis. We also excluded 90 patients with no record of peritumoral solar elastosis and 13 patients with rare histological subtypes. The remaining 492 primary tumors from the same number of patients were classified into four groups according to the latest WHO guidelines: non-CSD (384/492; 78.0%), CSD (64/492; 13.0%), acral (36/492; 7.3%) and mucosal (8/492; 1.6%) (Table 7).

**Table 7. Distribution of melanomas tested for *KIT* mutations according to clinical variables.**

| Variable | Total | | *KIT* mutational status | | | | p value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | n | % | n | % | n | % | |
| **WHO groups** | | | | | | | |
| Non-CSD | 384 | 78.0 | 373 | 97.1 | 11 | 2.9 | <0.001 |
| CSD | 64 | 13.0 | 61 | 95.3 | 3 | 4.7 | |
| Acral | 36 | 7.3 | 33 | 91.7 | 3 | 8.3 | |
| Mucosal | 8 | 1.6 | 4 | 50 | 4 | 50 | |
| **Sex** | | | | | | | |
| Men | 257 | 52.2 | 249 | 96.9 | 8 | 3.1 | 0.264 |
| Women | 235 | 47.8 | 222 | 94.5 | 13 | 5.5 | |

**Table 7 (continued). Distribution of melanomas tested for *KIT* mutations according to clinical variables.**

| Variable | Total | | *KIT* mutational status | | | | p value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | n | % | n | % | n | % | |
| **Sunburns in MM area** | | | | | | | |
| Absent | 193 | 40.7 | 180 | 93.3 | 13 | 6.7 | 0.017 |
| Weak/Moderate | 172 | 36.3 | 170 | 98.8 | 2 | 1.2 | |
| Severe | 109 | 23.0 | 106 | 97.2 | 3 | 2.8 | |
| **Past personal history of severe sunburns** | | | | | | | |
| ≤5 | 402 | 83.2 | 387 | 96.3 | 15 | 3.7 | 1 |
| >5 | 81 | 16.8 | 78 | 96.3 | 3 | 3.7 | |
| **Solar lentigos** | | | | | | | |
| No | 65 | 13.9 | 61 | 93.8 | 4 | 6.2 | 0.274 |
| Yes | 404 | 86.1 | 391 | 96.8 | 13 | 3.2 | |
| **Solar lentigos in MM area** | | | | | | | |
| No | 281 | 58.5 | 265 | 94.3 | 16 | 5.7 | 0.114 |
| Yes | 199 | 41.5 | 194 | 97.5 | 5 | 2.5 | |
| **Second tumor** | | | | | | | |
| No | 422 | 85.9 | 403 | 95.5 | 19 | 4.5 | 0.335 |
| Yes | 69 | 14.1 | 68 | 98.6 | 1 | 1.4 | |
| **History of non-melanoma skin cancer** | | | | | | | |
| No | 454 | 92.3 | 435 | 95.8 | 19 | 4.2 | 0.672 |
| Yes | 38 | 7.7 | 36 | 94.7 | 2 | 5.3 | |
| **Number of nevi** | | | | | | | |
| <20 | 318 | 66.1 | 301 | 94.7 | 17 | 5.3 | 0.027 |
| ≥20 | 163 | 33.9 | 161 | 98.8 | 2 | 1.2 | |
| **Multiple melanoma** | | | | | | | |
| No | 470 | 95.9 | 452 | 96.2 | 18 | 3.8 | 0.048 |
| Yes | 20 | 4.1 | 17 | 85.0 | 3 | 15.0 | |
| **Family history of melanoma** | | | | | | | |
| No | 458 | 93.7 | 439 | 95.9 | 19 | 4.1 | 0.635 |
| Yes | 31 | 6.3 | 29 | 93.5 | 2 | 6.5 | |

**Table 7 (continued). Distribution of melanomas tested for *KIT* mutations according to clinical variables.**

| Variable | Total | | *KIT* mutational status | | | | p value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | n | % | n | % | n | % | |
| **Family history of pancreatic cancer** | | | | | | | |
| No | 465 | 95.1 | 445 | 95.7 | 20 | 4.3 | 1 |
| Yes | 24 | 4.9 | 23 | 95.8 | 1 | 4.2 | |
| **Family history of cancer** | | | | | | | |
| No | 241 | 49.3 | 234 | 97.1 | 7 | 2.9 | 0.181 |
| Yes | 248 | 50.7 | 234 | 94.4 | 14 | 5.6 | |
| **Sun exposure pattern in MM area** | | | | | | | |
| Rare | 83 | 16.9 | 75 | 90.4 | 8 | 9.6 | 0.014 |
| Occasional | 317 | 64.4 | 309 | 97.5 | 8 | 2.5 | |
| Usual | 92 | 18.7 | 87 | 94.6 | 5 | 5.4 | |
| **Anatomical site of the primary** | | | | | | | |
| Head and neck | 93 | 18.9 | 91 | 97.8 | 2 | 2.2 | <0.001 |
| Upper Limbs | 67 | 13.6 | 65 | 97.0 | 2 | 3.0 | |
| Trunk | 182 | 37.0 | 179 | 98.4 | 3 | 1.6 | |
| Lower Limbs | 78 | 15.9 | 75 | 96.2 | 3 | 3.8 | |
| Acral | 64 | 13.0 | 57 | 89.1 | 7 | 10.9 | |
| Mucosal | 8 | 1.6 | 4 | 50.0 | 4 | 50.0 | |
| **Histological type** | | | | | | | |
| LMM | 27 | 5.5 | 27 | 100.0 | 0 | 0 | <0.001 |
| SSM | 305 | 62.0 | 294 | 96.4 | 11 | 3.6 | |
| NM | 119 | 24.2 | 116 | 97.5 | 3 | 2.5 | |
| ALM | 36 | 7.3 | 33 | 91.7 | 3 | 8.3 | |
| Others | 5 | 1.0 | 1 | 20.0 | 4 | 80.0 | |
| **Ulceration** | | | | | | | |
| Absence | 361 | 73.5 | 352 | 97.5 | 9 | 2.5 | 0.004 |
| Presence | 130 | 26.5 | 118 | 90.8 | 12 | 9.2 | |
| **Microscopic satellite** | | | | | | | |
| No | 466 | 95.1 | 446 | 95.7 | 20 | 4.3 | 1 |
| Yes | 24 | 4.9 | 23 | 95.8 | 1 | 4.2 | |

**Table 7 (continued). Distribution of melanomas tested for *KIT* mutations according to clinical variables.**

| Variable | Total | | *KIT* mutational status | | | | p value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | n | % | n | % | n | % | |
| **Vascular invasion** | | | | | | | |
| No | 475 | 97.5 | 456 | 96.0 | 19 | 4.0 | 0.09 |
| Yes | 12 | 2.5 | 10 | 83.3 | 2 | 16.7 | |
| **Associated nevus** | | | | | | | |
| No | 380 | 78.0 | 360 | 94.7 | 20 | 5.3 | 0.011 |
| Yes | 107 | 22.0 | 107 | 100.0 | 0 | 0 | |
| **CSD** | | | | | | | |
| Non-CSD | 428 | 87.0 | 410 | 95.8 | 18 | 4.2 | 0.745 |
| CSD | 64 | 13.0 | 61 | 95.3 | 3 | 4.7 | |
| **Stage** | | | | | | | |
| In situ | 19 | 3.9 | 19 | 100.0 | 0 | 0 | 0.389 |
| Localized | 341 | 69.3 | 329 | 96.5 | 12 | 3.5 | |
| Locoregional | 126 | 25.6 | 117 | 92.9 | 9 | 7.1 | |
| Distant | 5 | 1.0 | 5 | 100.0 | 0 | 0 | |
| Unknown | 1 | 0.2 | 1 | 100.0 | 0 | 0 | |
| ***BRAF*** | | | | | | | |
| wt | 280 | 57.3 | 259 | 92.5 | 21 | 7.5 | <0.001 |
| Mutated | 209 | 42.7 | 209 | 100.0 | 0 | 0 | |
| ***NRAS*** | | | | | | | |
| wt | 445 | 90.8 | 426 | 95.7 | 19 | 4.3 | 0.949 |
| Mutated | 43 | 8.8 | 41 | 95.3 | 2 | 4.7 | |
| Unknown | 2 | 0.4 | 2 | 100.0 | 0 | 0 | |
| **Breslow thickness** | | | | | | | |
| ≤ 2 mm | 286 | 60.5 | 277 | 96.9 | 9 | 3.1 | 0.111 |
| > 2 mm | 187 | 39.5 | 175 | 93.6 | 12 | 6.4 | |

CSD: cumulative solar damage; MM: malignant melanoma; LMM: lentigo maligna melanoma; SSM: superficial spreading melanoma; ALM: acral lentiginous melanoma

*KIT* mutations were present in 4.3% of all tumors (21/492). The highest frequency of *KIT* mutations was reported in mucosal melanoma (4/8, 50.0%), followed by acral (3/36, 8.3%), CSD (3/64, 4.7%), and non-

CSD melanomas (11/384, 2.9%). The difference in the distribution of *KIT* mutations in different melanoma subtypes was statistically significant (p<0.001). The distribution of *KIT* mutations in non-cutaneous and cutaneous melanoma is shown in Table 8 and Table S7.

**Table 8. Distribution of *KIT* mutations within our cohort.**

| Nº of cases | % | WHO classification | Exon | Nucleotide change* | Protein change |
|---|---|---|---|---|---|
| 5 | 23.8 | 1 mucosal, 2 non-CSD, 2 acral | 11 | c.1727T>C | p.(L576P) |
| 3 | 14.3 | 1 mucosal, 1 non-CSD, 1 CSD | 11 | c.1924A>G | p.(K642E) |
| 1 | 4.8 | Non-CSD | 11 | c.1676T>A | p.(V559D) |
| 1 | 4.8 | Non-CSD | 11 | c.1676T>C | p.(V559A) |
| 1 | 4.8 | Non-CSD | 11 | c.1660_1674del | p.(E554_K558del) |
| 1 | 4.8 | Non-CSD | 13 | c.1936_1937delTA | p.(Y646Pfs*3) |
| 1 | 4.8 | CSD | 11 | c.1735G>A | p.(D579N) |
| 1 | 4.8 | Acral | 11 | c.1729_1734dup | p.(P577_Y578dup) |
| 1 | 4.8 | Non-CSD | 11 | c.1655_1672del18 | p.(M552_W557del) |
| 1 | 4.8 | Mucosal | 9 | c.1463C>A | P.(T488K) |
| 1 | 4.8 | CSD | 11 | c.1732_1734delTAT | P.(Y578del) |
| 1 | 4.8 | Non-CSD | 17 | c.2458G>T | p.(D820Y) |
| 1 | 4.8 | Mucosal | 13 | c.1936T>G | p.(Y646D) |
| 1 | 4.8 | Non-CSD | 9 | c.1463C>T | p.T488M |
| 1 | 4.8 | Non-CSD | 9 | c.1427G>T; c.1430C>T | p.(S476I); p(S477F) |

*KIT* mutations were more frequent (p=0.017) in tumors at the sites without previous history of sunburns (13/193; 6.7%) than at the sites with either moderate or severe sunburns (5/281; 1.8%). Similarly, the frequency of mutations in melanomas at rarely exposed sites (8/83, 9.6%) was higher (p=0.014) than in tumors at usually or occasionally

exposed sites (13/409; 3.2%). The difference in the mutation frequency based on anatomical sites of primary tumors was also statistically significant ($p<0.001$). The frequency of the mutations in tumors with ulceration was 9.2% (12/130) and 2.5% (9/361) in tumors without ulceration ($p=0.004$). None of the nevus-associated melanomas had *KIT* mutation, and none of the melanomas harboring *BRAF* mutation carried a *KIT* mutation. No *KIT* mutations were detected in lentigo maligna melanoma (LMM) (Table 7).

We also analyzed the data after exclusion of patients with mucosal and acral melanomas (Table S6). The frequency of *KIT* mutation in all other tumors was 3.1% (14/448). We observed no statistically significant differences in mutation frequency in tumors from CSD sites (3/64; 4.7%) and non-CSD sites (11/384; 2.9%). The anatomical distribution of the *KIT* mutations was significantly different ($p<0.001$) (Table S6).

There was no statistically significant association between *KIT* mutations and family history of melanoma or other cancer type in either the entire set of patients or in patients without mucosal and acral melanomas. A trend was observed for an association between mutations in the *KIT* gene and vascular invasion and thicker melanomas (Table 7 and Table S6).

After a median follow-up of 72 months, 108 patients died, 70 of which were due to melanoma. The five-year estimated melanoma-specific and overall survival of patients with *KIT* mutated melanomas was 77.6% and 73.3%, and 82.0% and 87.5% for those without mutations, respectively. Neither comparison showed statistically significant differences (Figure S4).

The analysis of the TCGA data showed that the frequency of the "keratin-high" expression pattern tumors was higher in *KIT* mutated melanomas (9/12; 75%) than in wild type (83/304; 27.3%); the frequency of "immune" tumors was lower in mutated melanomas (2/12; 16.7%) than in wild type (163/304; 53.6%); and the frequency of "*MITF*-low" was lower in mutated melanomas (1/12; 8.3%) than in wild type (58/304; 19.1%) (p=0.004).

We also found a lower proportion of Signature 7 cases within mutated melanomas (7/12; 58.3%) than in *KIT* wild type (233/308; 75.6%) albeit without statistical significance (p=0.183)

### *5.3.4. Discussion*

In this study, based on the prevalence of *KIT* mutations in melanoma, a large patient set confirmed the high occurrence of such mutations in acral and mucosal melanomas. However, in CSD and non-CSD melanomas, we did not observe the previously reported difference in *KIT* mutation prevalence. This was further corroborated with TCGA data, given that tumors mutated for *KIT* did not associate with signature 7, which is attributed to UV exposure. The data also suggest that *KIT* mutations define aggressiveness in melanoma, which is consistent with TCGA data.

Our results concur with the prevalence of *KIT* mutations in previous studies, focused on European-descent populations as well as the TCGA population (59,224,238,239,256), but differ regarding the most prevalent mutated subtype (Table S8). However, it should be pointed

out that the number of acral and mucosal melanoma, which are generally associated with a high frequency of *KIT* mutations, was low in our dataset.

Our results differ from previous studies reporting a higher prevalence of *KIT* mutations in CSD melanomas (136,137). In fact, we found no significant difference in *KIT* mutation prevalence between CSD and non-CSD tumors. Clinically, melanomas developed on usually sun-exposed skin, and a past history of sunburns was not associated with *KIT* mutation. Interestingly, none of the LMM, the paradigmatic type of chronic sun exposure-associated melanoma, harbored *KIT* mutation. Thus, these findings strengthen the hypothesis that *KIT* mutations are acquired in a CSD-independent manner. Furthermore, we showed that most *KIT*-mutated melanomas appeared not to follow a nevogenic development pathway, given the lower number of nevi in melanoma patients with *KIT*-mutated melanomas, and that none of the nevus-associated melanomas presented *KIT* mutations. Hence, the development of *KIT* mutations would be independent of the common etiopathogenic pathways.

Also, *KIT* mutations have previously been associated with worse survival in patients with melanoma (252). In our cohort, survival was worse in patients with *KIT*-mutated melanomas but differences were not statistically significant, most likely due to the small number of *KIT*-mutated cases. However, our pathological results provide further evidence linking *KIT* mutation and aggressive melanomas due to the association with ulceration, vascular invasion and increased Breslow thickness in our patients.

This aggressiveness could be expected given the role of the *KIT* receptor in the cell. *KIT* mutations lead to the constitutive activation of the receptor, which in turn triggers both MAPK/ERK and PI3K/AKT signaling cascades (136). As a result of their activation, processes such as cell growth and proliferation are enhanced and pro-apoptotic signaling is reduced (134). Moreover, the fact that mutations affect different domains of the protein, both extracellular and intracellular, makes it more challenging to develop targeted therapies (222).

Our findings are consistent with the information published by the TCGA network. We performed analyses with available sequencing data to check associations with *KIT* mutation. Firstly, concerning the aggressive profile, *KIT* mutations were significantly over-represented in the "keratin-high" RNA expression group, which was associated with worst survival. Secondly, the poor association with sun exposure was supported by the fact that UV damage-associated signature 7 was less prevalent in *KIT*-mutated melanomas.

### *5.3.5. Conclusion*

In conclusion, we present the largest study to date on *KIT* mutations in melanoma patients in a Spanish population. Our results support the role of *KIT* mutations as an aggressiveness marker in melanoma patients and suggest that the pathogenesis of *KIT*-mutated melanomas is independent of the common etiopathogenic pathways (involved in the development of nevi and chronic sun damage).

# 6. ASSESSMENT OF THE PROGNOSTIC VALUE OF *KIT* MUTATIONAL STATUS

## 6.1. Overview

In this chapter, we will explore the prognostic value of *KIT* mutations in the survival of melanoma patients. This is thanks to the availability of both clinical data and mutational status for the gene *KIT* for a large cohort of patients. We will address whether the association of *KIT* mutations with an aggressive role suggested by our previous work results in a difference in the survival.

The work referred to in this chapter resulted in the publication of a letter to the editor.

## 6.2. Reference and contribution of the candidate

Millán-Esteban D, García-Casado Z, Manrique-Silva E, Kumar R, Nagore E. *KIT* mutational status does not constitute an independent prognostic marker in cutaneous melanoma. A study on 688 Spanish patients. Melanoma Res. 2021 Feb 1; 31 (1): 101-103. Doi: 10.1097/CMR.0000000000000712. PMID: 33351554.

The candidate participated in the study design, experimental procedures, data analysis, and writing of the manuscript.

## 6.3. *KIT* mutational status does not constitute an independent prognostic marker in cutaneous melanoma. A study on 688 Spanish patients

### *6.3.1. Introduction*

*KIT* mutations (*KIT*+) actívate both MAPK and PI3K/AKT pathways through ligand-independent receptor dimerization, and have specific inhibitors available. Those mutations are, however, only common in less frequent acral and mucosal subtypes of melanoma. Therefore, their prognostic value in cutaneous melanoma in general has remained less well studied (222,225). Limited studies have suggested a link between *KIT*+ and worse outcome in melanoma patients (243,252).

In this study, we aimed to determine the prognostic potential of *KIT*+ in cutaneous melanoma.

### *6.3.2. Materials and methods*

We selected a large cohort of 688 stage I-III Spanish cutaneous melanoma patients treated at the Fundación Instituto Valenciano de Oncología (FIVO) between 2000 and 2020. Patients were divided into two groups according to the presence or absence of *KIT* mutations in their primary melanoma (*KIT*+/*KIT*-wt).

Main outcome variables were disease-free survival (DFS), melanoma-specific survival (MSS), and overall survival (OS), in which the events were relapse, death due to melanoma, and death by any cause, respectively. Covariates were Breslow thickness (categorized by

median=1.34 mm), histological ulceration and stage at diagnosis [localized (I-II) vs. locoregional (III)].

Tumor DNA extraction and *KIT* determination, covering exons 9, 11, 13, and 17, were performed as described previously (266). Statistical methods were performed using SPSS 20.0 software, taking a p-value <0.05 to indicate statistical significance. Correlations of covariates with the presence or absence of *KIT* mutations were analyzed using two-sided Fisher exact test. Estimates of DFS, MSS, and OS were conducted by Kaplan-Meier method, testing differences between categories by log-rank test. Hazard ratios (HR) together with 95% confidence intervals (Cis) were calculated with Cox proportional hazard method.

### *6.3.3. Results*

Of the 688 melanoma tumors, we found that 3.6% were *KIT*+. Such mutations were more frequent in older (5.3%) than in younger patients (2.0%) (p=0.025); and in melanomas located in acral sites (13.6%) rather than in non-acral sites (2.3%) (p<0.001) (Table S9).

After a median follow-up of 65.2 months, 193 patients relapsed and 133 patients died, 87 of which were due to melanoma. No differences were found in OS nor MSS between *KIT*+ and *KIT*-wt patients, regardless of stage at diagnosis. DFS differed by *KIT* mutational status but only for localized disease, in which *KIT*+ were associated with a decreased survival (p=0.015) (Table 9; Figure 6).

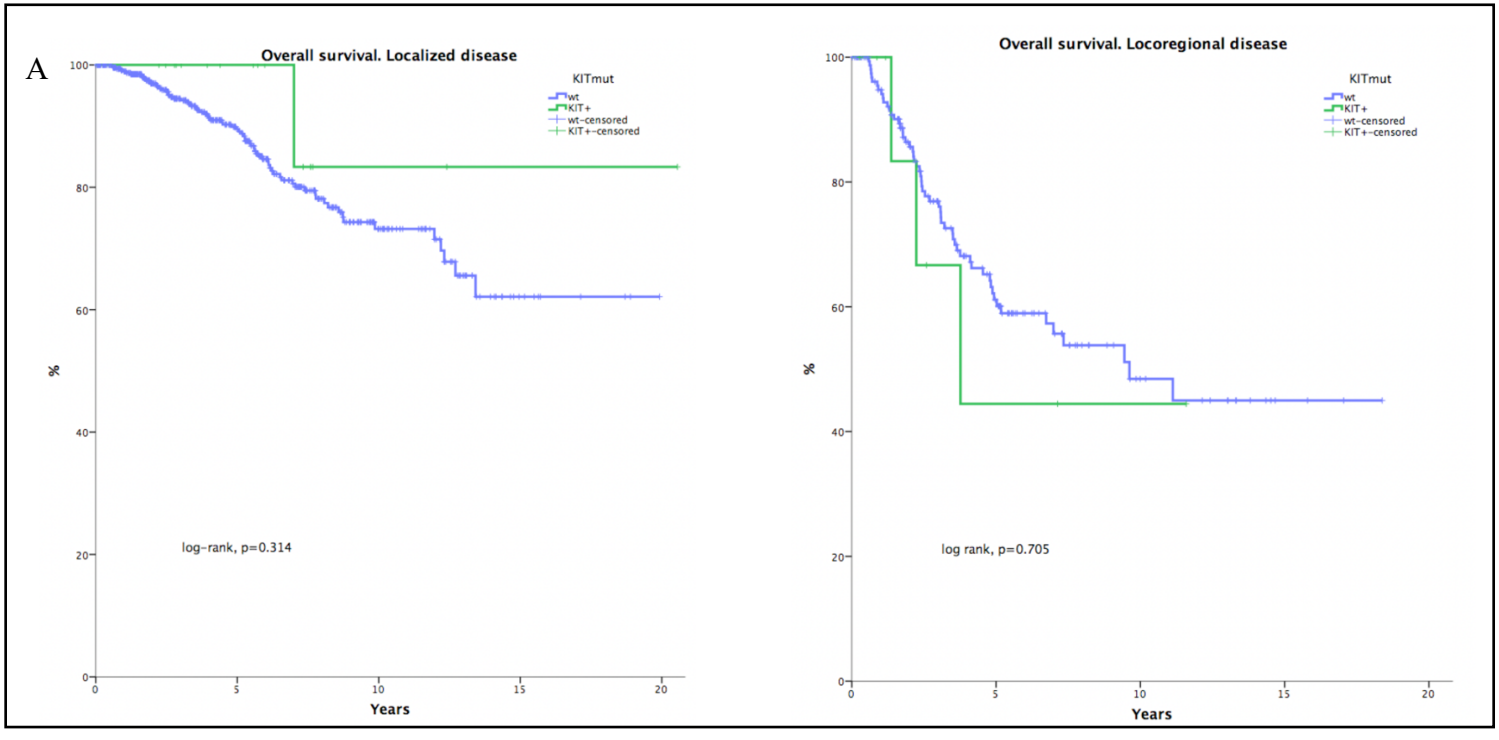


**Figure 6. Kaplan-Meier charts.** Overall survival for localized and locoregional disease (A).

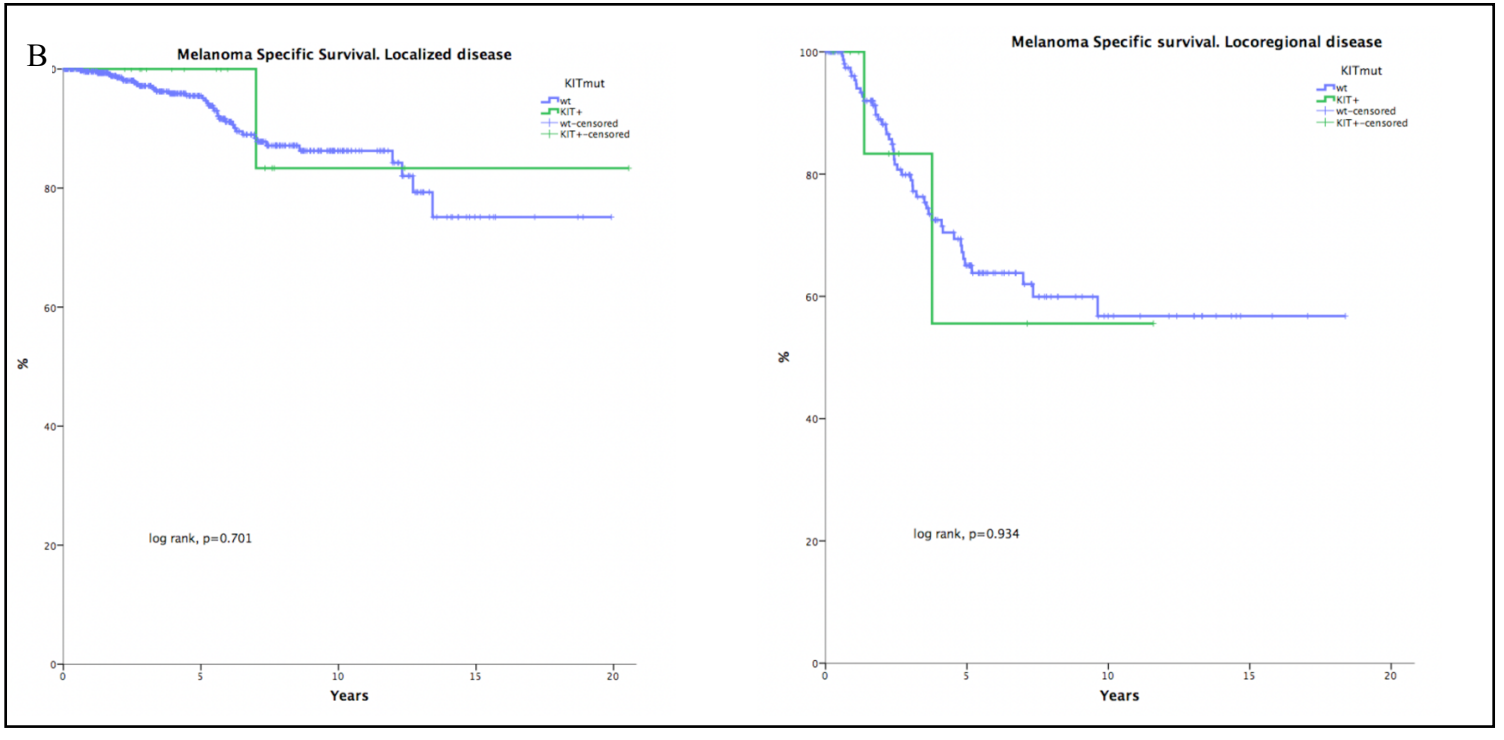


**Figure 6. (continued). Kaplan-Meier charts.** Melanoma specific survival for localized and locoregional disease (B).

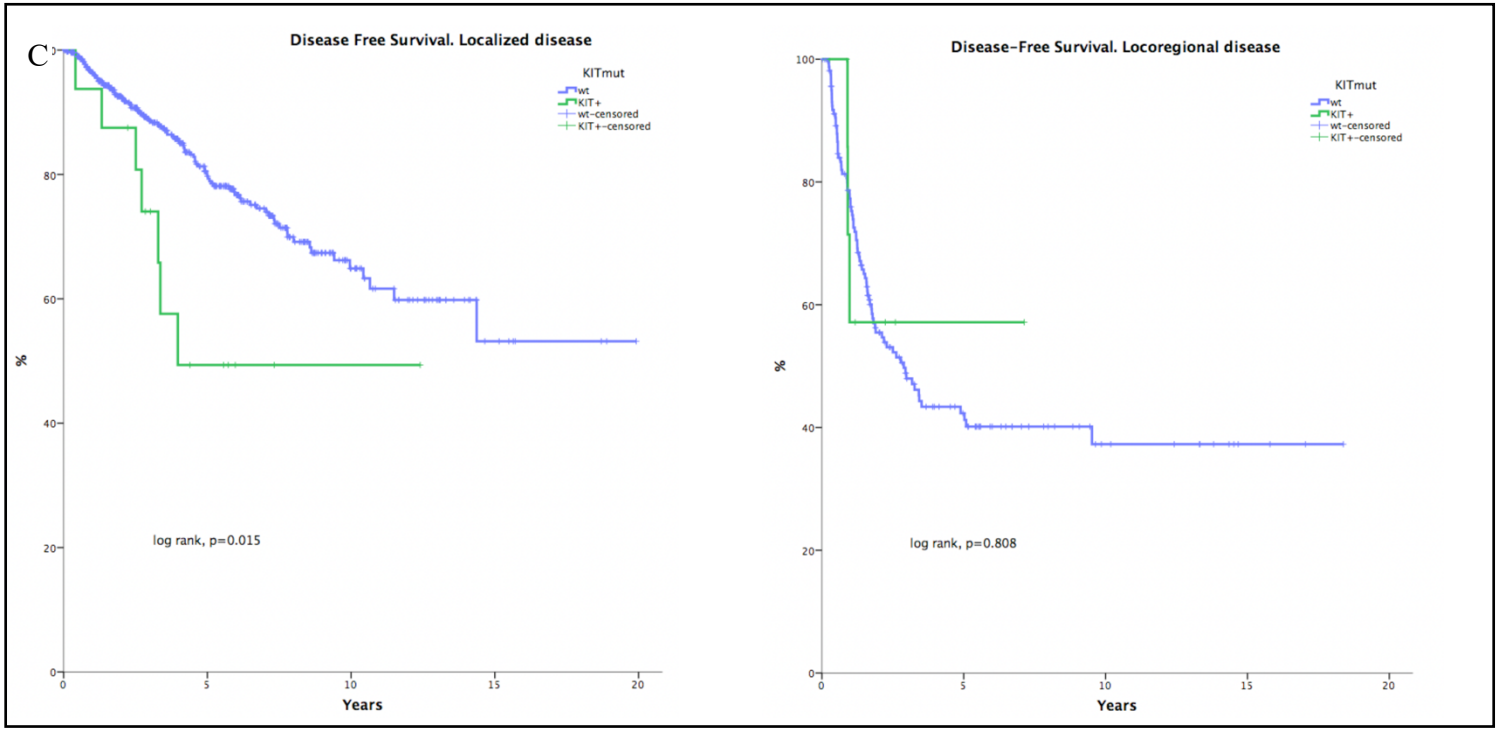


**Figure 6. (continued). Kaplan-Meier charts.** Disease-free survival for localized and locoregional disease (C).

**Table 9. *KIT* status univariate models for disease-free survival, overall survival and melanoma-specific survival.**

| | % Estimated 5-year survival | | 95% CI HR | | | p-value |
|---|---|---|---|---|---|---|
| | *KIT* wt | *KIT*+ | HR | Lower | Upper | |
| **Localized disease (stage I-II)** | | | | | | |
| DFS | 79.7 | 49.4 | 2.5 | 1.2 | 5.4 | 0.019 |
| OS | 89.5 | 100.0 | 0.4 | 0.1 | 2.7 | 0.333 |
| MSS | 95.5 | 100.0 | 0.7 | 0.1 | 5.0 | 0.703 |
| **Locoregional disease (stage III)** | | | | | | |
| DFS | 42.3 | 57.1 | 0.9 | 0.3 | 2.8 | 0.809 |
| OS | 61.1 | 44.4 | 1.3 | 0.4 | 4.0 | 0.705 |
| MSS | 65.1 | 55.6 | 1.1 | 0.3 | 4.4 | 0.934 |

CI: confidence interval; DFS: disease-free survival; HR: hazard ratio; MSS: melanoma-specific survival; OS: overall survival

However, after fully adjusted multivariate analyses, *KIT* mutational status was no longer associated with DFS (Table 10). By performing bivariate analyses, we identified that the prognostic value of *KIT* mutational status was dependent on its association with age, acral site, and ulceration; but not with Breslow thickness (Table S10).

**Table 10. Covariates COX at localized disease.**

| Variable | Univariate | | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|---|
| | 95% CI | | | | 95% CI | | | |
| | HR | Lower | Upper | p-value | HR | Lower | Upper | p-value |
| Sex | 0.9 | 0.6 | 1.3 | 0.553 | - | - | - | - |
| Age | 2.5 | 1.7 | 3.8 | <0.001 | 1.7 | 1.1 | 2.6 | 0.013 |
| Acral site | 2.2 | 1.3 | 3.5 | 0.002 | 1.2 | 0.7 | 2.1 | 0.537 |
| Breslow thickness | 3.4 | 2.3 | 5.0 | <0.001 | 2.2 | 1.4 | 3.5 | <0.001 |
| Ulceration | 4.4 | 2.9 | 6.5 | <0.001 | 2.5 | 1.6 | 4.0 | <0.001 |
| *KIT* mutation | 2.516 | 1.2 | 5.4 | 0.019 | 1.4 | 0.6 | 3.2 | 0.401 |

Dash indicates not included in multivariate analysis due to lack of significance in univariate analysis. CI: confidence interval; HR: hazard ratio.

### 6.3.4. Discussion

Most studies on *KIT*+ in melanoma have focused on acral and mucosal subtypes, and the few reporting on general cutaneous melanoma have suggested a link between such mutations and worse survival. However, those findings may be questioned given the lack of adjusted multivariate analyses to establish independent associations (252), or the short follow-up period (243). Our results are limited by the low prevalence of *KIT*+ in cutaneous melanoma, and much larger series are needed to obtain definite conclusions. However, here we showed how *KIT*+ are not related to aggressiveness markers in melanoma. The association found between *KIT*+ and worse DFS at localized stage was ruled out by adjusted analysis, and was shown to be mostly due to their high prevalence on acral melanoma (267,268).

### 6.3.5. Conclusion

We conclude that *KIT* mutational status is not an independent prognostic marker in cutaneous melanoma, but further research is needed to confirm this finding.

# 7. GENERAL DISCUSSION

Melanoma is a deadly form of skin cancer, which has been studied extensively over the years. From a clinical point of view, efforts have been put into defining different types of classifications according to various characteristics. The current one, established by the WHO in 2018, used the location and the amount of sun damage to sort into groups, and was claimed to be supported by the presence of characteristic common genetic alterations in each group.

The role of UVR exposure and melanocytic proliferation as key to melanoma developmental pathways was suggested by Whiteman back in 1998, being corroborated later on clinically and epidemiologically. Despite the outstanding breakthroughs in molecular biology -especially among sequencing techniques-, and the widely use of FFPE as a means of storage, a molecular basis for these two groups had been limited to single prevalent genes. Thus, a more in-depth characterization was due. The gene *KIT* is one of these relevant genes which mutations have been associated with some subtypes of melanoma -mainly acral and mucosal-, but few studies had checked its prevalence according to the most recent WHO classification. The elucidation of the possible impact on survival of *KIT* mutations might also help improving the assessment of these patients. The present thesis has contributed addressing these issues covering technical, molecular, and clinical aspects of melanoma research.

We showed that FFPE melanoma samples should be quality tested prior to their inclusion for NGS, especially when they have been archived for long periods of time. This study, which resulted in a scientific

publication, was designed after we faced complications during the construction of the amplicon libraries for the custom panel. This helped us sort which samples to include in the downstream molecular techniques and, even more importantly, which samples not to include. Since melanomas are usually small-sized tumors, the material available is precious and the risks should be minimized when using it. Though the inter-site variation must be considered when establishing thresholds in quality parameters, our findings constitute a procedure proposal for the management of FFPE samples in NGS.

Thenceforth, there has been a constant flow of publications in this line of work, proving the relevance of the issue. These agree on the impact of storage time for FFPE when it comes to obtaining useful genetic material, but differ on the time threshold to apply. It would depend on the downstream molecular technique to be used, since amplifications of certain gene fragments through basic PCR have been achieved using FFPE samples that had been stored for years up to decades later (269,270), but considerably shorter periods of storage have been suggested when aiming at performing NGS (271,272).

Also, a consensus is clear about the need to establish procedures – which echoes the results of our work –, that help identifying which FFPE samples can be used in NGS, via the implementation of quality control parameters (273,274). In any case, further efforts must be put in place to elucidate alternative fixatives that are less damaging to the genetic material, as well as repairing methods that enable the construction of NGS libraries starting from FFPE blocks (275,276).

The differential mutational profile of nevogenic and CSD melanomas that we have shown here, gives molecular support to the divergent pathways model proposed more than 20 years ago. Also, our findings on the distribution of *KIT* mutations among the WHO groups and its role as a prognostic factor, contribute to understand the process of melanoma development and management. The research papers derived from those chapters have been published recently, and no relevant publication has been reported since.

Our research showed that CSD melanomas harbor a higher number of mutations than in nevogenic melanomas, which is consistent with the etiology of this group (1), linked to a chronic exposure to UVR. Such mutations have been found more prevalently in CSD melanomas for genes *NF1, RAC1, GNA11,* and *ROS1* when compared to nevogenic melanomas, which are prominently mutated in the *BRAF* gene – as reported previously (277) – and whose development is thought to be potentially influenced by melanin subtypes. We also found a potential difference in the expression level, based on the results of the bioinformatics modeling, with a higher number of predicted dysregulated circuits in CSD than in nevogenic melanomas. This correlates with the differences found in the number of mutations between both groups, and points out the relevance of accumulated mutations as disruptor of cell functions. Thus, it is undoubted that expression dysregulation plays a crucial role in tumorigenesis, and melanoma is no exception (278,279). Hence, further efforts should be placed into confirming our findings. Also, the recent advent and accessibility to whole-exome, whole-genome, and RNASeq NGS lead

the way to what future studies in the field should aim at (280,281), in order to generate a wider and more robust characterization.

These prospects in technical availability could also help weighing in the role of specific genes in melanoma development and prognosis. In this thesis, we showed that melanomas harboring mutations in the gene *KIT* seemed to have an etiology independent of the common pathways, which contradicted some previous works that associated CSD melanomas with such mutations (136,137). Some studies suggested that mechanical stress could influence the development of acral melanomas, with weight-bearing areas such as the front or the heel being more likely to present a melanoma than the arch (282,283). Future larger studies with WES or WGS would provide corroboration to this debate, as well as confirming the alleged lack of prognostic value.

## 7.1. Strengths

There are a couple of points on which the strengths of this thesis rely. First of all, the clinical work that resulted in the creation of thorough databases at the Dermatology departments. Thanks to the efforts of the clinicians involved over the years, we have been able to access detailed clinical information that has been crucial to identify the individuals for the selected groups, as well to perform different analyses.

In addition, the fact that the sequencing experimental approach has been performed by the candidate from the DNA extraction up to the identification of variants, constitutes a high degree of robustness on the knowledge of every step of the process.

When externalized -as it was the case with the mechanistic analysis of pathways-, we entrusted collaborators with a great expertise in the procedures to be performed.

Finally, all the work presented here has resulted in several scientific publications which have been peer-reviewed, thus a critic view has already been applied.

## 7.2. Limitations

There are various limitations in the work that we have presented. The size of our cohort would be the main limitation to our study. Though the different size between nevogenic and CSD melanomas is a representation of the differences found in the population, the modest numbers can certainly limit our conclusions.

Also, the custom panel amplicon technology that was used for sequencing could be considered a limitation. Despite being appropriate when the study was designed, recent advances in NGS have opened the possibility to engage in exome-wide and genome-wide analyses that would provide much more information.

## 7.3. Future perspectives

Based on the findings collected in this thesis, we can point out what the next steps could be in this line of work. A wider study using whole-exome sequencing with a bigger cohort size would confirm and increase the scope of this molecular characterization.

Also, expression analyses would be interesting to empirically demonstrate if our findings in the bioinformatics model regarding the different expression between the groups are true.

Finally, since we have focused on nevogenic and CSD melanomas as described by Whiteman, there are other groups of melanoma patients that should be studied in equally depth, so that a complete molecular profile can be described in the search to provide a better care to people suffering from this disease.

# 8. CONCLUSIONS

1. Time of storage affects the viability of formalin-fixed paraffin-embedded samples for next generation sequencing. Quality parameters should be used to ensure functionality.

2. Nevogenic and CSD melanomas display specific molecular profiles, corroborating that they constitute different biological entities. Nevogenic melanomas harbored less mutations – mainly in the *BRAF* gene –, while CSD melanomas harbored a higher number of mutations – associated with the genes *NF1, RAC1, ROS1,* and *GNA11*.

3. Melanomas harboring mutations in *KIT* are most common in acral and mucosal locations but their development is independent of the common etiopathogenic pathways.

4. *KIT* mutational status is not an independent prognostic marker in cutaneous melanoma.

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# 10. ANNEXES

**Notes:**

A: CART analysis.

Classification and regression tree (CART) analysis is a statistical method that is commonly used in clinical research. In this technique, several input variables are assessed to elucidate their impact on the outcome variable. Its methodology consists in a progressive binary split of the data set into progressively smaller groups, using in each step the variable that maximizes the homogeneity of the two resulting groups with respect to the output variable (284).

B: Generalization of the mechanistic analysis' results.

The bioinformatics approach performed for the in silico pathway analysis provides information that can be useful to interpret the impact of mutations in different cellular circuits. The field of bioinformatics has evolved greatly in the past decades, and its tools are used widely to predict and model the effect of genetic alterations. In this case, a robust software employed in previous publications has been used to measure the dysregulation of cellular circuits as a result of the mutations found in our cohort. Nevertheless, this is still a model and future expression analysis should be performed to confirm whether this is actually happening in melanoma cells.

**Table S1. Genes included in the amplicon panel in detail.**

| Gene | Chromosome | Location GRCh37 | Nº of target regions | Nº of target bases | Nº of bases covered | % Bases covered | Nº of amplicons | Size minimum | Size average | Size maximum |
|---|---|---|---|---|---|---|---|---|---|---|
| ***ARID2*** | 12 | 46123609 | 21 | 5,928 | 5,928 | 100.0 | 57 | 157 | 208 | 225 |
| ***BRAF*** | 7 | 140415817 | 20 | 2,860 | 2,846 | 99.5 | 36 | 130 | 199 | 225 |
| ***CDK4*** | 12 | 58142297 | 7 | 1,052 | 1,052 | 100.0 | 12 | 131 | 186 | 225 |
| ***CDKN2A*** | 9 | 21968217 | 6 | 1,184 | 892 | 75.3 | 11 | 120 | 185 | 244 |
| ***GNA11*** | 19 | 3094639 | 7 | 1,220 | 1,064 | 87.2 | 14 | 156 | 202 | 234 |
| ***GNAQ*** | 9 | 80336228 | 7 | 1,220 | 1,064 | 87.2 | 13 | 188 | 214 | 225 |
| ***HRAS*** | 11 | 532578 | 5 | 780 | 780 | 100.0 | 11 | 129 | 193 | 255 |
| ***IDH1*** | 2 | 209101792 | 8 | 1,405 | 1,405 | 100.0 | 18 | 145 | 200 | 225 |
| ***KIT*** | 4 | 55524171 | 21 | 3,354 | 3,354 | 100.0 | 41 | 142 | 200 | 225 |
| ***KRAS*** | 12 | 25362718 | 5 | 787 | 787 | 100.0 | 11 | 130 | 180 | 224 |
| ***MAP2K2*** | 19 | 4090585 | 11 | 1,423 | 1,242 | 87.3 | 19 | 135 | 192 | 275 |
| ***NF1*** | 17 | 29422317 | 60 | 9,900 | 9,900 | 100.0 | 115 | 130 | 204 | 240 |

**Table S1 (continued). Genes included in the amplicon panel.**

| Gene | Chromosome | Location GRCh37 | Nº of target regions | Nº of target bases | Nº of bases covered | % Bases covered | Nº of amplicons | Size minimum | Size average | Size maximum |
|---|---|---|---|---|---|---|---|---|---|---|
| ***NRAS*** | 1 | 115251145 | 4 | 650 | 650 | 100.0 | 7 | 174 | 207 | 224 |
| ***PIK3CA*** | 3 | 178916603 | 20 | 3,607 | 3,546 | 98.3 | 46 | 147 | 204 | 264 |
| ***PIK3R1*** | 5 | 67522493 | 17 | 2,637 | 2,637 | 100.0 | 28 | 130 | 201 | 225 |
| ***PPP6C*** | 9 | 127911941 | 8 | 1,189 | 1,189 | 100.0 | 14 | 134 | 191 | 225 |
| ***PTEN*** | 10 | 89624216 | 9 | 1,392 | 1,375 | 98,8 | 17 | 141 | 196 | 237 |
| ***RAC1*** | 7 | 6414356 | 7 | 776 | 721 | 92.9 | 10 | 130 | 186 | 225 |
| ***RB1*** | 13 | 48878038 | 27 | 3,327 | 3,307 | 99.4 | 45 | 130 | 200 | 268 |
| ***ROS1*** | 6 | 117609644 | 43 | 7,904 | 7,904 | 100.0 | 88 | 136 | 203 | 225 |
| ***TP53*** | 17 | 7572916 | 12 | 1,503 | 1,503 | 100.0 | 20 | 130 | 180 | 225 |
| **TOTAL** | | | 325 | 54,098 | 53,146 | 98.2 | 633 | 120 | 200 | 275 |

**Table S2. Mutation Heatmap.** Graphical description of mutational distribution among the different genes for both groups, together with the total and group prevalence in percentage. Each column represents one sample. Cells are colored green when mutated, grey when wild-type, and white when non-informative.

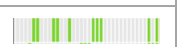
| Gene | Total | | Nevogenic | Gene | CSD | |
|---|---|---|---|---|---|---|
| BRAF | 50.4 | 56.1 |  | BRAF | 37.8 |  |
| TERTp | 52.2 | 48.1 |  | TERTp | 61.7 |  |
| NF1 | 16.8 | 7.3 |  | NF1 | 37.8 |  |
| NRAS | 13.4 | 14.6 |  | NRAS | 10.8 |  |
| ROS1 | 11.8 | 4.9 |  | ROS1 | 27.0 |  |
| TP53 | 10.9 | 7.3 |  | TP53 | 18.9 |  |
| ARID2 | 9.24 | 6.1 |  | ARID2 | 16.2 |  |
| CDKN2A | 7.56 | 7.3 |  | CDKN2A | 8.1 |  |
| RAC1 | 5.88 | 1.2 |  | RAC1 | 16.2 |  |
| IDH1 | 5.04 | 4.9 |  | IDH1 | 5.4 |  |
| KIT | 4.2 | 2.4 |  | KIT | 8.1 |  |

**Table S2 (continued). Mutation Heatmap.**

| Gene | Total | | Nevogenic | Gene | CSD | |
|---|---|---|---|---|---|---|
| RB1 | 4.2 | 3.7 |  | RB1 | 5.4 |  |
| GNA11 | 4.2 | 1.2 |  | GNA11 | 10.8 |  |
| PIK3R1 | 4.2 | 2.4 |  | PIK3R1 | 8.1 |  |
| CDK4 | 3.4 | 3.7 |  | CDK4 | 2.7 |  |
| PPP6C | 3.4 | 3.7 |  | PPP6C | 2.7 |  |
| PTEN | 2.5 | 2.4 |  | PTEN | 2.7 |  |
| HRAS | 1.7 | 1.2 |  | HRAS | 2.7 |  |
| MAP2K2 | 1.7 | 0 |  | MAP2K2 | 5.4 |  |
| GNAQ | 0.8 | 0 |  | GNAQ | 2.7 |  |
| KRAS | 0.8 | 1.2 |  | KRAS | 0 |  |
| PIK3CA | 0.8 | 0 |  | PIK3CA | 2.7 |  |

**Table S3. Genes Odds Ratio (OR).** Genes OR to be associated with CSD (Nevogenic is reference).

| Variable | Univariate | | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI | | p value | OR | 95% CI | | p value |
| | | Lower | Upper | | | Lower | Upper | |
| **TP53+** | 3.0 | 0.9 | 9.5 | 0.069 | - | - | - | - |
| **NF1+** | 7.7 | 2.7 | 22.3 | <0.001 | 5.654 | 1.765 | 18.118 | 0.004 |
| **BRAF+** | 0.5 | 0.2 | 1.1 | 0.067 | - | - | - | - |
| **ROS1+** | 7.2 | 2.1 | 24.9 | 0.002 | 5.658 | 1.433 | 22.346 | 0.013 |
| **NRAS+** | 0.7 | 0.2 | 2.4 | 0.573 | - | - | - | - |
| **CDK4+** | 0.7 | 0.1 | 7.3 | 0.790 | - | - | - | - |
| **ARID2+** | 3.0 | 0.8 | 10.5 | 0.089 | - | - | - | - |
| **CDKN2A+** | 1.1 | 0.3 | 4.7 | 0.880 | - | - | - | - |
| **KIT+** | 3.5 | 0.6 | 22.1 | 0.178 | - | - | - | - |
| **RB1+** | 1.5 | 0.2 | 9.4 | 0.662 | - | - | - | - |
| **PPP6C+** | 0.7 | 0.1 | 7.3 | 0.790 | - | - | - | - |
| **PTEN+** | 1.1 | 0.1 | 12.7 | 0.932 | - | - | - | - |
| **IDH1+** | 1.1 | 0.2 | 6.4 | 0.903 | - | - | - | - |
| **GNA11+** | 9.8 | 1.1 | 91.2 | 0.045 | 7.097 | 0.615 | 81.911 | 0.116 |
| **GNAQ+** | - | - | - | - | - | - | - | - |
| **RAC1+** | 15.7 | 1.8 | 135.5 | 0.012 | 7.519 | 0.673 | 83.950 | 0.101 |
| **KRAS+** | - | - | - | - | - | - | - | - |
| **HRAS+** | 2.3 | 0.1 | 37.0 | 0.570 | - | - | - | - |
| **MAP2K2+** | - | - | - | - | - | - | - | - |
| **PIK3CA+** | - | - | - | - | - | - | - | - |
| **PIK3R1+** | 3.5 | 0.6 | 22.1 | 0.178 | - | - | - | - |
| **TERTp+** | 1.7 | 0.8 | 4.0 | 0.184 | - | - | - | - |

**Table S4. Enrichment analysis table.** Here we can see for both groups whether a hallmark is statistically significantly enriched in melanoma with respect to normal skin.

| Cancer Hallmark | p value | |
|---|---|---|
| | Nevogenic | CSD |
| **Cellular Energetics** | 0.1795 | 0.1720 |
| **Evading Growth Suppressors** | 0.1353 | 0.2803 |
| **Genome Instability and Mutation** | 0.2756 | 0.4863 |
| **Immune Destruction** | 0.6243 | 0.5718 |
| **Inducing Angiogenesis** | 0.2487 | 0.4201 |
| **Invasion and Metastasis** | 0.5365 | 0.8989 |
| **Replicative Immortality** | 0.0026 | 0.0002 |
| **Resisting Cell Death** | 0.3328 | 0.4693 |
| **Sustaining Proliferative Signaling** | 0.0103 | 0.0004 |
| **Tumor Promoting Inflammation** | 0.5929 | 0.2058 |

**Table S5. *KIT* primer sequences.**

| Exon | Primer | Primer sequence |
|---|---|---|
| **9** | Forward | CTTCCCTTTAGATGCTCTGCTTCTG |
| | Reverse | CAGAGCCTAAACATCCCCTTAAATTGG |
| **11** | Forward | CTCTCTCCAGAGTGCTCTAATGAC |
| | Reverse | GTTCCTTAAAGTCACTGTTATCTCTACC |
| **13** | Forward | GACATCAGTTTGCCAGTTGTGC |
| | Reverse | CCAAGCAGTTTATAATCTTAGCATTGCC |
| **17** | Forward | AAATGGTTTTCTTTTCTCCTCCAACC |
| | Reverse | TCCTTTGCAGGACTGTCAAGC |

**Table S6. Distribution of CSD and non-CSD melanomas tested for *KIT* among clinical variables.**

| Variable | Total | | *KIT* | | | | p-value |
|---|---|---|---|---|---|---|---|
| | | | **Wild type** | | **Mutated** | | |
| | N | % | N | % | N | % | |
| **Sex** | | | | | | | |
| Men | 240 | 53.6 | 234 | 97.5 | 6 | 2.5 | 0.430 |
| Women | 208 | 46.4 | 200 | 96.2 | 8 | 3.8 | |
| **Sunburns in MM area** | | | | | | | |
| Absent | 155 | 35.7 | 148 | 95.5 | 7 | 4.5 | 0.186 |
| Weak/moderate | 170 | 39.2 | 168 | 98.8 | 2 | 1.2 | |
| **Past personal history of severe sunburns** | | | | | | | |
| ≤ 5 | 365 | 82.8 | 355 | 97.3 | 10 | 2.7 | 0.476 |
| > 5 | 76 | 17.2 | 73 | 96.1 | 3 | 3.9 | |
| **Solar lentigos** | | | | | | | |
| No | 55 | 12.9 | 55 | 100.0 | 0 | 0 | 0.378 |
| Yes | 373 | 87.1 | 361 | 96.8 | 12 | 3.2 | |
| **Solar lentigos at MM area** | | | | | | | |
| No | 239 | 54.7 | 230 | 96.2 | 9 | 3.8 | 0.589 |
| Yes | 198 | 45.3 | 193 | 97.5 | 5 | 2.5 | |
| **Second tumor** | | | | | | | |
| No | 383 | 85.5 | 370 | 96.6 | 13 | 3.4 | 0.703 |
| Yes | 65 | 14.5 | 64 | 98.5 | 1 | 1.5 | |
| | | | | | | | |
| **Personal history of non-melanoma skin cancer** | | | | | | | |
| No | 412 | 92.0 | 400 | 97.1 | 12 | 2.9 | 0.312 |
| Yes | 36 | 8.0 | 34 | 94.4 | 2 | 5.6 | |
| Number of nevi | | | | | | | |
| < 20 | 282 | 63.9 | 270 | 95.7 | 12 | 4.3 | 0.097 |
| ≥ 20 | 159 | 36.1 | 157 | 98.7 | 2 | 1.3 | |

**Table S6 (continued). Distribution of CSD and non-CSD melanomas tested for *KIT* among clinical variables**

| Variable | Total | | *KIT* | | | | p-value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | N | % | N | % | N | % | |
| **Multiple melanoma** | | | | | | | |
| No | 428 | 96.0 | 416 | 97.2 | 12 | 2.8 | 0.105 |
| Yes | 18 | 4.0 | 16 | 88.9 | 2 | 11.1 | |
| **Family history of melanoma** | | | | | | | |
| No | 414 | 93.0 | 402 | 97.1 | 12 | 2.9 | 0.254 |
| Yes | 31 | 7.0 | 29 | 93.5 | 2 | 6.5 | |
| **Family history of pancreatic cancer** | | | | | | | |
| No | 422 | 94.8 | 409 | 96.9 | 13 | 3.1 | 0.530 |
| Yes | 23 | 5.2 | 22 | 95.7 | 1 | 4.3 | |
| **Family history of cancer** | | | | | | | |
| No | 223 | 50.1 | 218 | 97.8 | 5 | 2.2 | 0.293 |
| Yes | 222 | 49.9 | 213 | 95.9 | 9 | 4.1 | |
| **Sun exposure pattern in MM area** | | | | | | | |
| Rarely | 49 | 10.9 | 47 | 95.9 | 2 | 4.1 | 0.578 |
| Occasionaly | 312 | 69.6 | 304 | 97.4 | 8 | 2.6 | |
| Usually | 87 | 19.4 | 83 | 95.4 | 4 | 4.6 | |
| **Anatomic site of the primary** | | | | | | | |
| Head and neck | 93 | 22.2 | 91 | 97.8 | 2 | 2.2 | <0.001 |
| Upper limbs | 66 | 15.8 | 65 | 98.5 | 1 | 1.5 | |
| Trunk | 182 | 43.4 | 179 | 98.4 | 3 | 1.6 | |
| Lower limbs | 78 | 18.6 | 75 | 96.2 | 3 | 3.8 | |
| **Histological type** | | | | | | | |
| LMM | 27 | 6.0 | 27 | 100.0 | 0 | 0 | 0.539 |
| SSM | 304 | 67.9 | 293 | 96.4 | 11 | 3.6 | |
| NM | 117 | 26.1 | 114 | 97.4 | 3 | 2.6 | |
| **Ulceration** | | | | | | | |
| Absence | 340 | 76.1 | 331 | 97.4 | 9 | 2.6 | 0.339 |
| Presence | 107 | 23.9 | 102 | 95.3 | 5 | 4.7 | |

**Table S6 (continued). Distribution of CSD and non-CSD melanomas tested for *KIT* among clinical variables.**

| Variable | Total | | *KIT* | | | | p-value |
|---|---|---|---|---|---|---|---|
| | | | Wild type | | Mutated | | |
| | N | % | N | % | N | % | |
| **Microscopic satellite** | | | | | | | |
| No | 424 | 94.9 | 410 | 96.7 | 14 | 3.3 | 1 |
| Yes | 23 | 5.1 | 23 | 100.0 | 0 | 0 | |
| **Vascular invasion** | | | | | | | |
| No | 433 | 97.5 | 420 | 97.0 | 13 | 3.0 | 0.300 |
| Yes | 11 | 2.5 | 10 | 90.9 | 1 | 9.1 | |
| **Associated nevus** | | | | | | | |
| No | 338 | 76.3 | 325 | 96.2 | 13 | 3.8 | 0.045 |
| Yes | 105 | 23.7 | 105 | 100.0 | 0 | 0 | |
| **CSD** | | | | | | | |
| Non-CSD | 384 | 85.7 | 373 | 97.1 | 11 | 2.9 | 0.434 |
| CSD | 64 | 14.3 | 61 | 95.3 | 3 | 4.7 | |
| **Stage** | | | | | | | |
| In situ | 12 | 2.7 | 12 | 100.0 | 0 | 0 | 0.961 |
| Localized | 319 | 71.2 | 309 | 96.9 | 10 | 3.1 | |
| Locoregional | 112 | 25.0 | 108 | 96.4 | 4 | 3.6 | |
| Distant | 4 | 0.9 | 4 | 100.0 | 0 | 0 | |
| Unknown | 1 | 0.2 | 1 | 100.0 | 0 | 0 | |
| **BRAF** | | | | | | | |
| wt | 242 | 54.4 | 228 | 94.2 | 14 | 5.8 | <0.001 |
| Mutated | 203 | 45.6 | 203 | 100.0 | 0 | 0 | |
| **NRAS** | | | | | | | |
| wt | 407 | 91.3 | 394 | 96.8 | 13 | 3.2 | 0.955 |
| Mutated | 37 | 8.3 | 36 | 97.3 | 1 | 2.7 | |
| Unknown | 2 | 0.4 | 2 | 100.0 | 0 | 0 | |
| **Breslow thickness** | | | | | | | |
| ≤ 2 mm | 269 | 61.7 | 261 | 97.0 | 8 | 3.0 | 0.783 |
| > 2 mm | 167 | 38.3 | 161 | 96.4 | 6 | 3.6 | |

**Table S7. Detailed information on *KIT* mutated cases within our cohort.**

| ID | Age | Sex | WHO Classification | Localization | Histological Type | Ulceration | Breslow | *KIT* status (NM_000222.2) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Exon | Nucleotide change | Protein change |
| 80 | 42 | Male | Non-CSD | Back | NM | Yes | 3.3 | 11 | c.1660_11674del | p.(E554_K558del) |
| 117 | 49 | Male | Mucosal | Glans penis | Mucosal | Yes | 9.0 | 11 | c.1727T>C | p.(L576P) |
| 157 | 59 | Female | Non-CSD | Sole | NM | Yes | 5.5 | 11 | c.1727T>C | p.(L756P) |
| 417 | 61 | Male | Acral | Sole | ALM | Yes | 12.0 | 11 | c.1727T>C | p.(L756P) |
| 740 | 60 | Female | Non-CSD | Foot dorsum | SSM | No | 1.2 | 13 | c.1936_1937delTA | p.(Y646Pfs*3) |
| 1494 | 61 | Male | Non-CSD | Left palm | SSM | Yes | 5.0 | 11 | c.1924A>G | p.(K642E) |
| 1663 | 53 | Female | CSD | Left temple | SSM | No | 0.28 | 11 | c.1735G>A | p.(D579N) |
| 1776 | 88 | Male | CSD | Left forearm | SSM | No | 1.10 | 11 | c.1924A>G | p.(K642E) |
| 1817 | 48 | Female | Acral | Subungual left thumb | ALM | Yes | 5.5 | 11 | c.1729_1734dup | p.(P577_Y578dup) |
| 1868 | 79 | Female | Non-CSD | Left calf | SSM | No | 1.75 | 11 | c.1727T>C | p.(L576P) |
| 1948 | 35 | Female | Non-CSD | Right thigh | SSM | No | 1.0 | 11 | c.1676T>A | p.(V559D) |
| 2056 | 84 | Female | Acral | Subungual left thumb | ALM | Yes | 1.16 | 11 | c.1727T>C | p.(L756P) |
| 2062 | 38 | Female | Non-CSD | Arch of the foot | SSM | No | 0.7 | 11 | c.1655_1672del18 | p.(M552_W557del) |
| 2081 | 73 | Female | Mucosal | Perianal | Mucosal | Yes | 5.8 | 9 | c.1463C>A | p.(T488K) |
| 2112 | 53 | Female | CSD | Thorax | SSM | Yes | 0.81 | 11 | c.1732_1734delTAT | p.(Y578del) |
| 2131 | 61 | Male | Non-CSD | Sole | SSM | Yes | 4.9 | 17 | c.2458G>T | p.(D820Y) |
| 2167 | 73 | Female | Mucosal | Vulvar | Unclassified | Yes | 8.0 | 13 | c.1936T>G | p.(Y646D) |

**Table S7 (continued). Detailed information on *KIT* mutated cases within our cohort.**

| ID | Age | Sex | WHO classification | Localization | Histological Type | Ulceration | Breslow | Exon | *KIT* status (NM_000222.2) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Nucleotide change | Protein change |
| 2403 | 82 | Male | Non-CSD | Scalp (bald) | SSM | No | 2.45 | 9 | c.1463C>T | p.(T488M) |
| 2434 | 75 | Male | Non-CSD | Back | SSM | No | 0.4 | 11 | c.1676T>C | p.(V559A) |
| 2451 | 76 | Female | Non-CSD | Leg | NM | No | 6.9 | 9 | c.1427G>T;<br>c.1430C>T | p.(S476I);<br>p.(S477F) |
| 2482 | 86 | Female | Mucosal | Vulvar | Mucosal | Yes | 23.0 | 11 | c.1924A>G | p.(K642E) |

**Table S8. *KIT* mutation prevalence according to subtype and race.**

| Variable | N mutated | N wild-type | Percentage |
|---|---|---|---|
| **Type of melanoma** | | | |
| Cutaneous | 432 | 4815 | 8.23 |
| Mucosal | 50 | 422 | 10.59 |
| Acral | 33 | 237 | 12.22 |
| **Race distribution** | | | |
| *European-descent* | 143 | 1274 | 10.09 |
| Cutaneous | 97 | 891 | 9.82 |
| Mucosal | 27 | 301 | 8.23 |
| Acral | 20 | 151 | 11.70 |
| *Asian* | 371 | 4131 | 8.24 |
| Cutaneous | 335 | 3924 | 7.87 |
| Mucosal | 23 | 121 | 15.97 |
| Acral | 13 | 86 | 13.13 |

*TCGA not included

**Table S9. Fisher exact test.** This compares covariates between the two different groups of *KIT* mutational status.

| | Total | | *KIT*-wt | | *KIT*+ | | p-value |
|---|---|---|---|---|---|---|---|
| | N | % | N | % | *N* | % | |
| **Age** | | | | | | | |
| ≤ 58 | 347 | 50.4 | 340 | 98.0 | 7 | 2.0 | 0.025 |
| > 58 | 341 | 49.6 | 323 | 94.7 | 18 | 5.3 | |
| **Sex** | | | | | | | |
| Male | 367 | 53.3 | 357 | 97.3 | 10 | 2.7 | 0.221 |
| Female | 321 | 46.7 | 306 | 95.3 | 15 | 4.7 | |
| **Anatomic site of the primary** | | | | | | | |
| Head and neck | 140 | 20.3 | 136 | 97.1 | 4 | 2.9 | <0.001 |
| Upper limbs | 89 | 12.9 | 85 | 95.5 | 4 | 4.5 | |
| Trunk | 263 | 38.2 | 260 | 98.9 | 3 | 1.1 | |
| Lower limbs | 115 | 16.7 | 112 | 97.4 | 3 | 2.6 | |
| Acral | 81 | 11.8 | 70 | 86.4 | 11 | 13.6 | |
| **Anatomic site (non-acral vs. acral)** | | | | | | | |
| Non-acral | 607 | 88.2 | 593 | 97.7 | 14 | 2.3 | <0.001 |
| Acral | 81 | 11.8 | 70 | 86.4 | 11 | 13.6 | |
| **Histological type** | | | | | | | |
| LMM | 53 | 7.7 | 50 | 94.3 | 3 | 5.7 | 0.008 |
| SSM | 388 | 56.4 | 376 | 96.9 | 12 | 3.1 | |
| NM | 162 | 23.5 | 158 | 97.5 | 4 | 2.5 | |
| ALM | 48 | 7.0 | 42 | 87.5 | 6 | 12.5 | |
| Other | 37 | 5.4 | 37 | 100.0 | 0 | 0 | |
| **Breslow thickness** | | | | | | | |
| ≤ 1.34 | 344 | 50.0 | 333 | 96.8 | 16 | 3.2 | 0.684 |
| > 1.34 | 344 | 50.0 | 330 | 95.9 | 14 | 4.1 | |
| **Ulceration** | | | | | | | |
| Absence | 506 | 74.0 | 490 | 96.8 | 16 | 3.2 | 0.250 |
| Presence | 178 | 26.0 | 169 | 94.9 | 9 | 5.1 | |
| **Stage at diagnosis** | | | | | | | |
| Localized | 514 | 74.7 | 498 | 96.9 | 16 | 3.1 | 0.240 |
| Locoregional | 174 | 25.3 | 165 | 94.8 | 9 | 5.2 | |

**Table S10. Two by two multivariate analysis.** Multivariate COX analysis to determine the prognostic value of *KIT* mutational status.

| Variable | HR | 95% CI | | p-value |
|---|---|---|---|---|
| | | Lower | Upper | |
| Age | 2.5 | 1.6 | 3.7 | <0.001 |
| *KIT* mutation | 2.1 | 1.0 | 4.5 | 0.063 |
| | | | | |
| Acral site | 2.0 | 1.2 | 3.2 | 0.009 |
| *KIT* mutation | 1.9 | 0.8 | 4.2 | 0.133 |
| | | | | |
| Ulceration | 4.2 | 2.8 | 6.3 | <0.001 |
| *KIT* mutation | 1.6 | 0.7 | 3.5 | 0.233 |
| | | | | |
| Breslow thickness | 3.3 | 2.2 | 5.0 | <0.001 |
| *KIT* mutation | 2.4 | 1.1 | 5.2 | 0.026 |

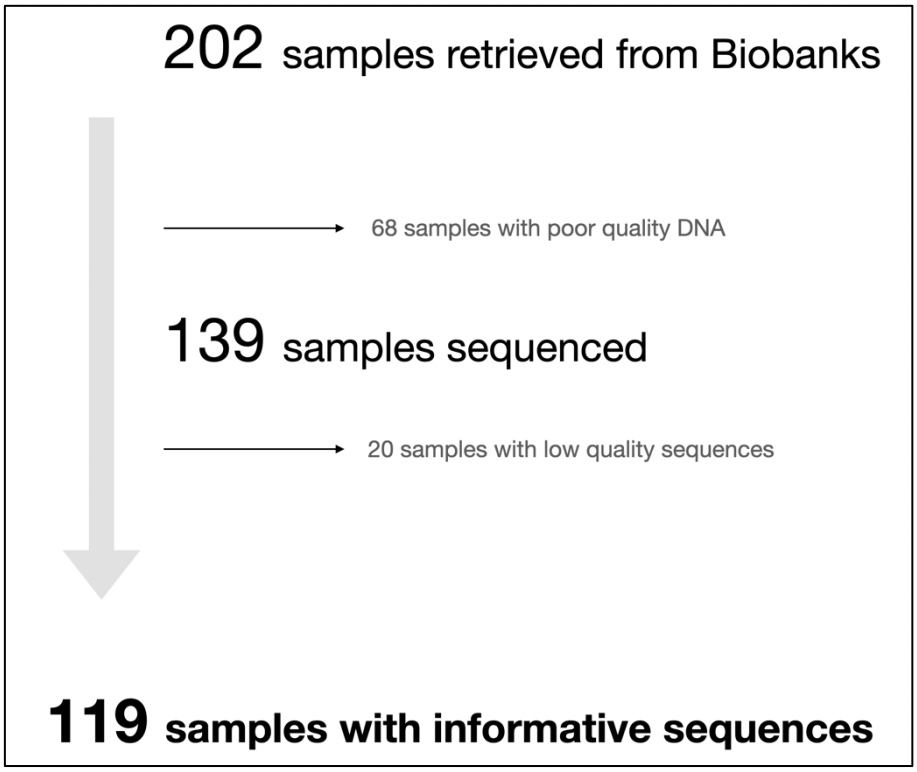



**Figure S1. Sample flowchart.** Here we can see the evolution in the number of samples after each step in the process.

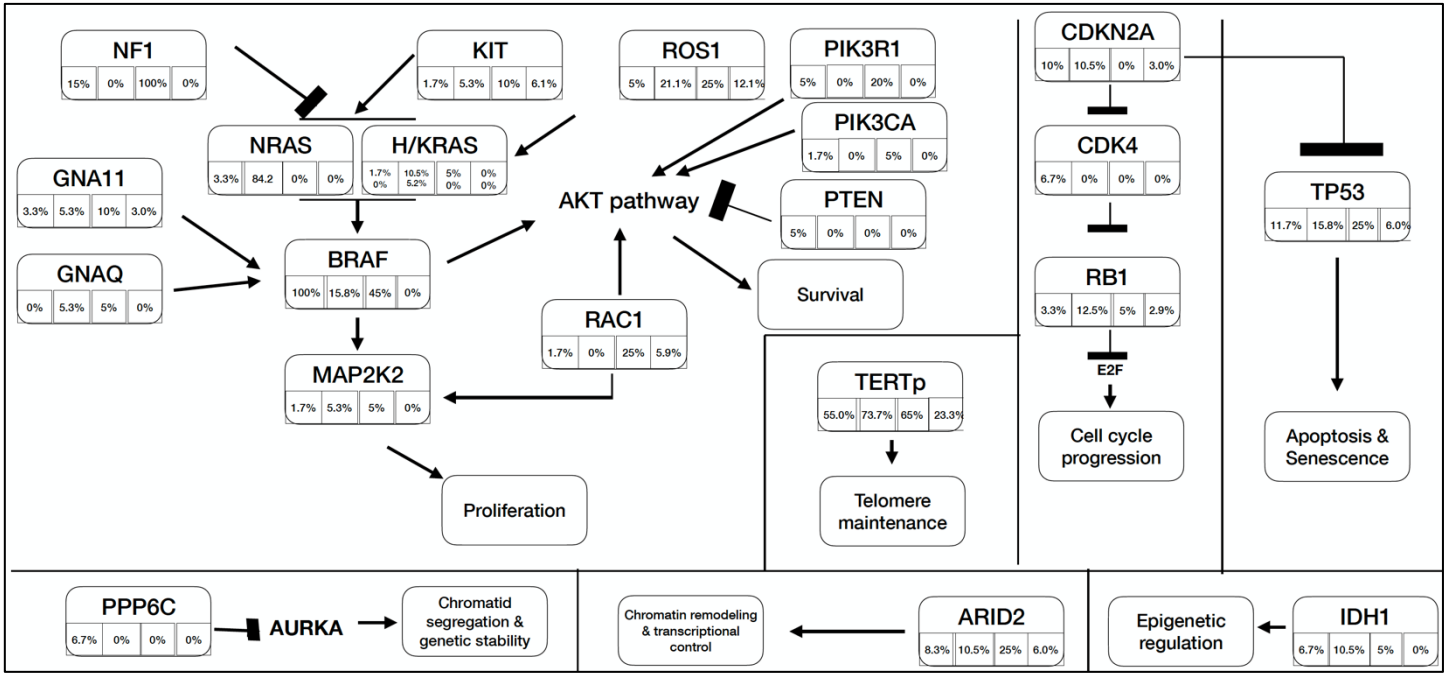



**Figure S2. Molecular subtype distribution.** Here we see the mutational prevalence of each gene for the non-exclusive four molecular subtypes BRAF+, RAS+, NF1+, 3wt. The lines indicate the downstream direction in each pathway: a normal arrow means activation, while a bar means inactivation.

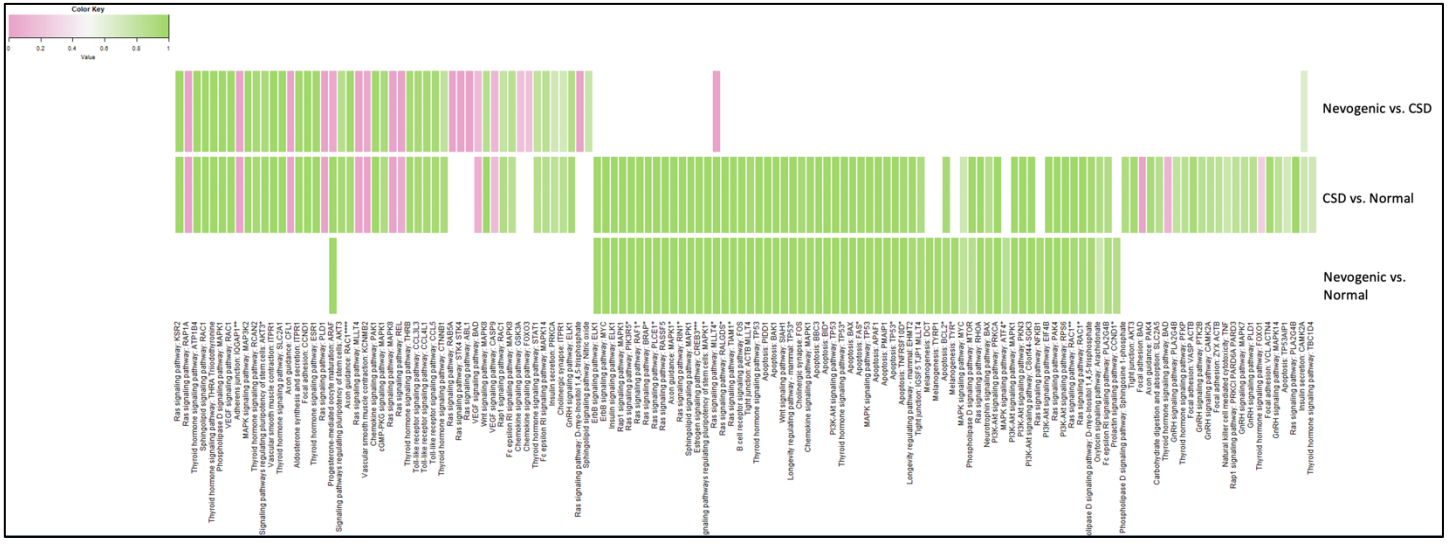



**Figure S3. Heatmap of dysregulated circuits.** This graphical representation shows with a color key whether certain circuits are infra-expressed (pink) or over-expressed (green) with respect to their reference. The lower row compares nevogenic melanomas vs. normal skin tissue; the middle one compares CSD melanomas vs. normal skin tissue; and the upper one compares nevogenic melanomas vs. CSD melanomas. We can see how CSD melanomas have more dysregulated circuits, which are mostly over-expressed.

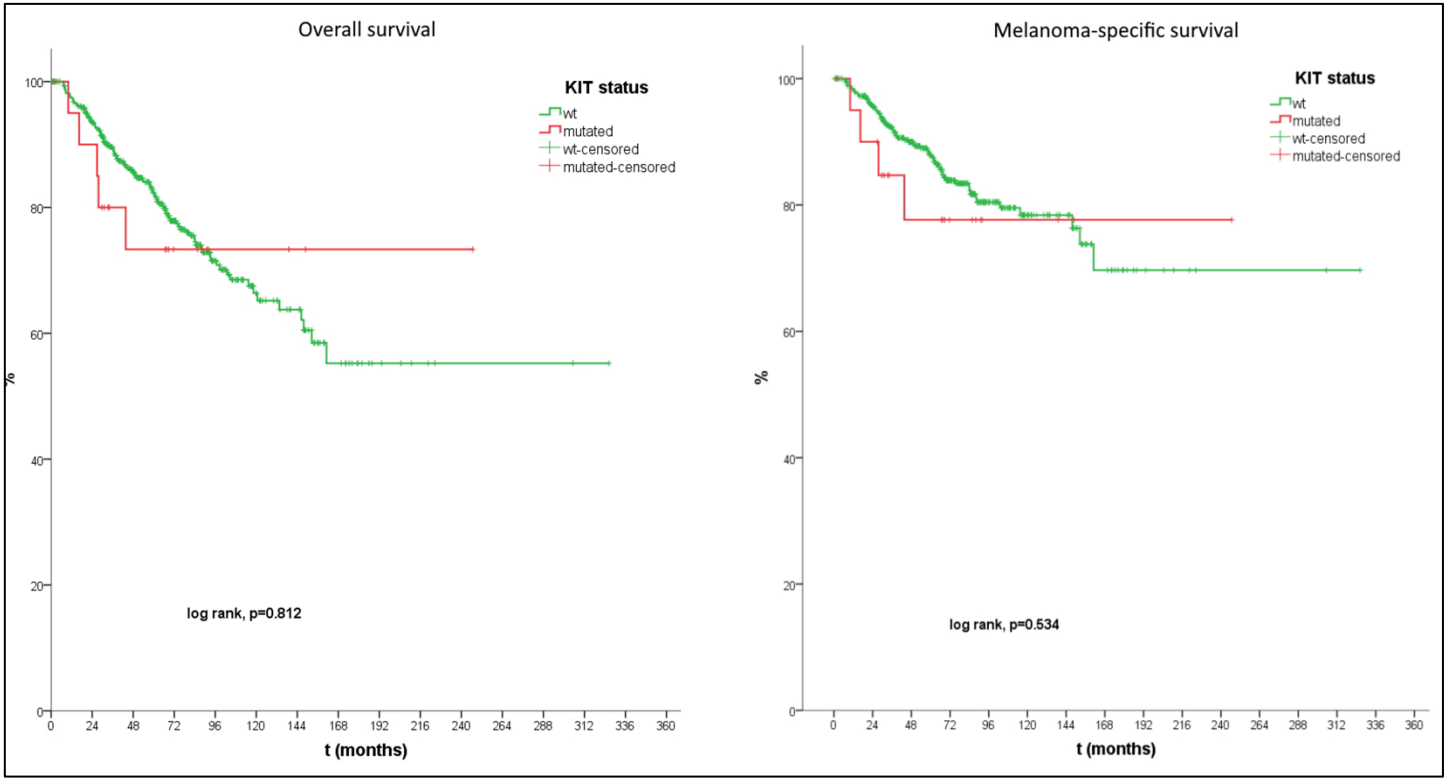



**Figure S4. Kaplan-Meier curves for *KIT* status.** Graphical representation of the overall and melanoma-specific survival for patients showing a mutation in *KIT* vs. wild-type patients.