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Genetic susceptibility and driver genes in cutaneous melanoma

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ABSTRACT

Project 1: "Intra-patient heterogeneity of *BRAF* and *NRAS* molecular alterations in primary melanoma and metastases"

Introduction. Melanoma represents approximately 2% of all cutaneous malignant neoplasias causing 1% of cancer-related deaths. The mitogen-activated protein kinase (MAPK) cascade is the most relevant molecular pathway implicated in melanoma pathogenesis with mutations in *BRAF, NRAS* and c-*KIT* considered as driver events. The identification of specific pathways involved in the pathogenesis of melanoma lead to a classification in molecular subtypes that has a key role for the therapeutic approach and patient management.

Objectives. This study aimed to evaluate the intra-patient molecular heterogeneity between primary melanoma and related metastases and to compare the consistency of mutational findings obtained by molecular and immunohistochemical (IHC) analyses.

Methods. Overall, 69 formalin-fixed paraffin-embedded (FFPE) tissues (30 primary melanomas and 39 related metastatic lesions) belonging to 30 melanoma patients were retrieved at the Dermatology Unit, University of L'Aquila, Italy. We investigated codon 600 in exon 15 of *BRAF* by competitive allele-specific TaqMan PCR, exon 2 of *NRAS*, and exons 11, 13 and 17 of c-*KIT* by Sanger sequencing. *c*-KIT copy number was assessed by quantitative real-time PCR. The presence of $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutants and c-KIT expression were evaluated by IHC using the following antibodies: $BRAF^{V600E}$ VE1 clone (Spring Bioscience), $NRAS^{Q61R}$ SP174 clone (Spring Bioscience), and CD117/c-Kit polyclonal (Spring Bioscience). Cohen's k coefficient test was used to measure the agreement between molecular and IHC methods in determining *BRAF* and *NRAS* mutational status. Statistical analysis was performed using the statistical package SPSS 17.0 (SPSS Incorporated, Chicago, USA).

Results. According to molecular analysis, $BRAF^{V600}$ mutations were observed in 47.8% of samples, whereas $NRAS^{Q61}$ mutations were detected in 23.2%. Only one missense mutation was found in the *c-KIT* gene. c-*KIT* gene amplification was found in 4.3% of cases. Intra-patient heterogeneity between primary melanoma and related metastases was observed for *BRAF* and *NRAS* genes in 13.3% of patients each. The

only mutated patient for the *c-KIT* gene showed a discordant status between primary melanoma and related metastases. Considering the global mutational profile of *BRAF*/*NRAS*/*c-KIT* genes, intra-patient heterogeneity was detected in 7 of 30 patients (23.3%). Following IHC analysis, 37.7% of samples were positive for anti-BRAF^{V600E} VE1 immunostaining, while 14.5% of specimens were positive for anti-NRAS^{Q61R} SP174 staining. Comparison between results achieved from molecular methods and IHC showed a discrepancy of 5.1% for *BRAF*^{V600E} mutation and 1.6% of *NRAS*^{Q61R}. Cohen's k coefficient was of 0.90 ($p<0.001$).

Conclusions. Our findings confirm that intra-patient heterogeneity between primary melanoma and related metastases is relevant and independent from the methodological approach, thus supporting the polyclonal model of melanoma progression. In addition, molecular methods and IHC provided highly consistent results in the detection of *BRAF*^{V600E} and *NRAS*^{Q61R} mutations supporting IHC as a rapid and cost-effective screening method in melanoma although a combined approach is necessary in cases with negative or doubtful immunostaining.

Project 2: "Genetic susceptibility to familial melanoma in Mediterranean populations"

Introduction. Approximately 5–12% of melanoma cases occur in a familial setting. *CDKN2A* and *CDK4* are well-known high-risk melanoma susceptibility genes. The introduction of NGS methodologies led to the identification of new melanoma susceptibility genes implicated in melanoma development including *BAP1*, *POT1*, *ACD*, *TERF2IP* and the promoter region of *TERT*. In addition to high-risk genes, intermediate-penetrance genes, such as *MC1R* and *MITF*, have been demonstrated to be candidate genes associated to melanoma predisposition.

Objectives. The project aimed to evaluate melanoma genetic predisposition in Mediterranean populations, that have specific epidemiological and clinical characteristics and have been poorly characterized in previous genetic studies worldwide.

Methods. Melanoma families were recruited from Melanostrum consortium centres in Spain (Barcelona and Valencia), Italy (Cesena, Genoa, L'Aquila, Padua and Rome) and Greece (Athens). Inclusion criteria were as follows: presence of 1st or 2nd degree relatives affected by cutaneous melanoma and information on genetic data for high-risk susceptibility genes *CDKN2A* and *CDK4*. For each family, epidemiological and clinical data of affected patients and unaffected relatives were collected. Data were recorded in a single database, comprehensive of family characteristics and molecular features, which was created through a harmonized design. Genetic data for high-risk genes *(CDKN2A, CDK4, POT1*, *ACD*, *BAP1*, *TERF2IP,* promoter region of *TERT)* and an intermediate-risk gene (*MC1R*) were provided by each centre. *CDKN2A* variants were categorized as class 1-2, (Benign/Likely benign), class 3 (Variant of Uncertain Significance, VUS) and class 4-5 (Likely pathogenic/Pathogenic), according to Varsome tool. Chi square test or Fisher's exact test were used, as appropriate, to test for the significance of mutation frequency according to family characteristics. Logistic regression and decision tree classification were used to perform the multivariate analysis. Data were analyzed using the SPSS statistical package (IBM) version 25.0. The statistical significance was considered at $p < 0.05$.

Results. Overall, 852 families with a total of 1365 affected patients and 2123 unaffected members, enrolled at MelaNostrum centres were included in the study: 520

were from Spain (61.0%), 318 were from Italy (37.3%) and 14 from Greece (1.6%). Frequency of *CDKN2A* variants in the families was 14.5%, with differences among centres; in detail, 9.1% in Italy, 14.6% in Spain and 50% in Greece. *CDKN2A* variants classified as "Likely pathogenic/Pathogenic" were 74.2%, "VUS" were 10.5% and "Benign/Likely benign" variants were 15.3%. Among "Likely pathogenic/Pathogenic" variants, the most frequent variant was G101W detected in 27.4% of families, followed by R24P found in 7.2% of families. G101W was identified in Italy (20.7%) and in Spain (31.8%) but not in Greece, while R24P was specific of Greek families (85.7%). The presence of *CDKN2A* variants was significantly associated with the number of affected members in the family ($p<0.0001$), the presence of multiple primary melanomas in affected members ($p<0.0001$) and familial history of pancreatic cancer ($p<0.0001$). Regression analysis, stratified by country, identified different algorithms of classification across the populations. In Italy, the number of affected members followed by family history of pancreatic cancer can be used to predict the major risk of being a carrier of *CDKN2A* variants, whereas in Spain the algorithm includes first the evaluation of multiple primary melanomas, then the presence of pancreatic cancer in the family and, finally, the number of affected members. No mutations were found in the *CDK4* gene and in the promotor region of *TERT* gene, whereas 0.2% of families carried *BAP1* mutations. The frequency of variants in *POT1*, *ACD* e *TERF2IP* genes was 3.9%, 1.7% and 3.7% respectively. We found a significant association between the presence of variants in the *POT1* gene and a high Breslow thickness, whereas *TERF2IP* mutations were associated with the development of renal carcinoma in the family. Regarding intermediate-penetrance genes, frequency of *MC1R* variants among affected individuals was 67.3%; of this, 52.1% of patients presented RHC variants which were associated with a fair phototype.

Conclusions. Our findings confirm the key role of *CDKN2A* variants and the rarity of other high-risk genes mutations in melanoma predisposition of Mediterranean populations. A high number of affected members within the family, the presence of multiple melanoma cases and pancreatic cancer cases are strong predictive factors for *CDKN2A* mutations. Moreover, our results support the importance of the genetic screening of susceptibility genes in familial melanoma patients and identified an algorithm of clinical factors for genetic testing within Mediterranean populations.

Chapter 1: Melanoma

1.1 Introduction

The first description of melanoma appeared in the writings of Hyppocrates in the 5th century B.C. and the earliest physical evidence of melanoma came from the diffuse melanotic metastases found in the skeletons of Pre-Columbian mummies in Perù [1] in the 4th century. Later, between 1650-1790, the European medical literature referred to "fatal black tumors with metastases and black fluid in the body" and the first surgical removal of a melanoma has been attributed to the Scottish surgeon John Hunter in 1787. Afterwards, in 1804, Rene Laennec (the inventor of the stethoscope) was the first to coin the term "*melanose*" to describe this kind of tumor (Figure 1) [2].

Melanoma is considered one of the most aggressive cancers and represents an increasing phenomenon over the last decades [3]. It arises from the malignant transformation of melanocytes, the melanin-producing cells of the skin, eye, mucosal epithelia, and meninges that are responsible for pigmentation and photo-protection [4]. In most cases, melanoma develops on clinically normal skin and appears as a pigmented neoformation, that may spread in the horizontal and vertical directions, with propensity to metastasize; or it could have origin from a congenital or acquired nevus which may play the role of a precursor [5].

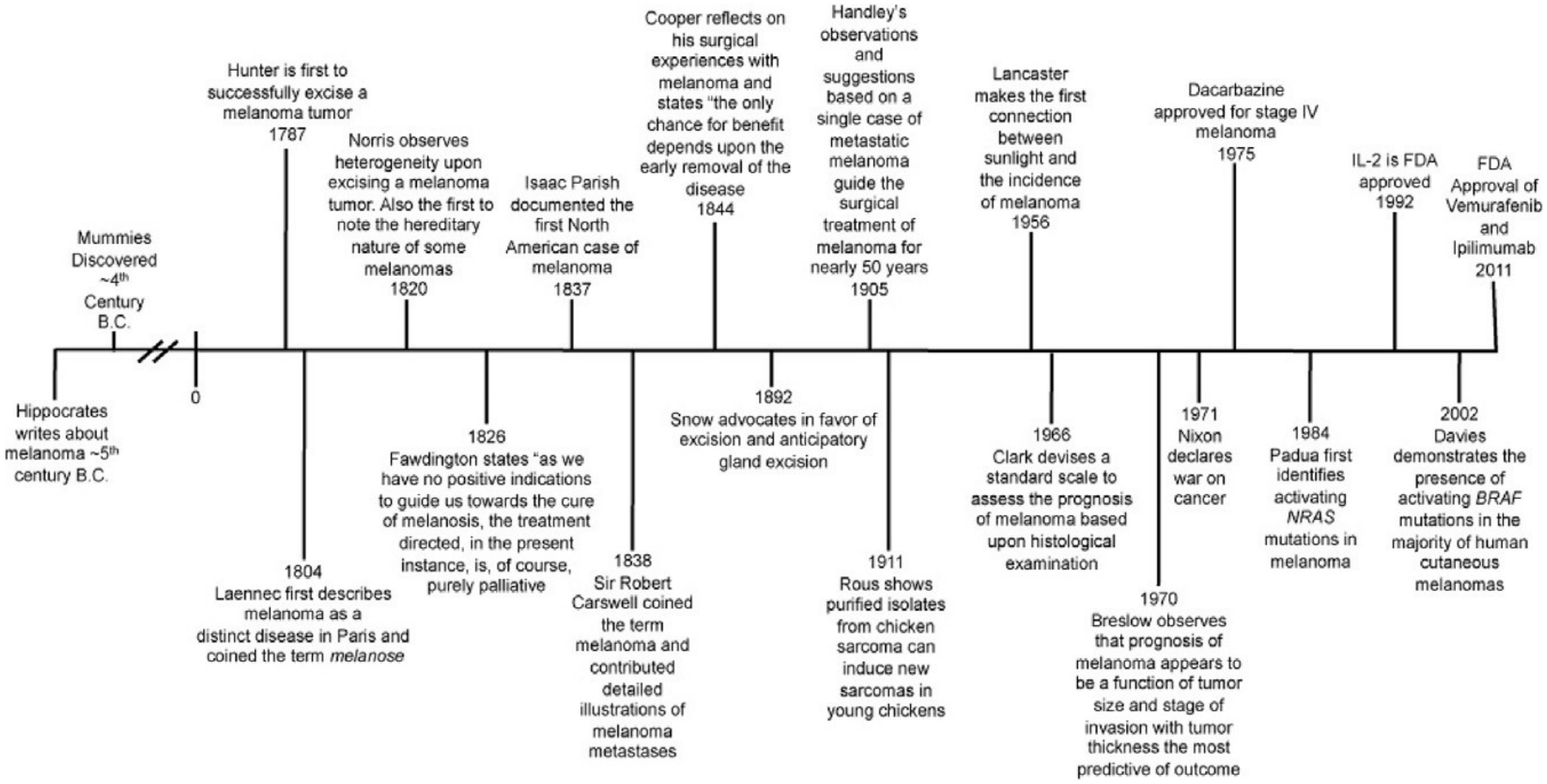


Figure 1. Timeline of melanoma history [2].

1.2 Epidemiology

Cutaneous melanoma represents approximately 2% of all skin cancers and causes about 1% of oncological-related deaths. Overall, 85% of the diagnosed melanomas occur in Oceania, North America and Europe, with an increasing incidence rate especially in Caucasian populations [6]. According to 2018 IARC data, Australia has the highest age-standardised melanoma incidence rate (33.6 per 100,000 cases) and mortality rate (3.2 per 100,000) in the world [7].

In the United States the incidence rate of melanoma has increased over the past 30 years, because of a variety of factors, such as exposure to ultraviolet (UV) radiation and increased surveillance [8]. In Europe, a recent study on incidence trends of melanoma in the period between 1995-2012 pointed out a statistically significant increment both in invasive (+4.0% in men, +3.0% in women) and *in situ* cases (+7.7% in men, +6.2% in women) [9].

Melanoma is more often diagnosed in young adults with an average age at diagnosis of 57 years. The incidence of diagnosed melanoma raises linearly between 25-50 years; however, it differs by age as well as sex. From age 25 to 40, women are more likely to develop melanoma than men, but after 75 years, men have a 3-fold increased risk of developing melanoma compared to women. Overall, men are 1.5 times more likely to develop melanoma than women [10]. The incidence of melanoma varies based on race, with a lifetime risk of 2.6% for Caucasians, 0.58% for Hispanics and 0.1% for Africans (Figure 2) [11].

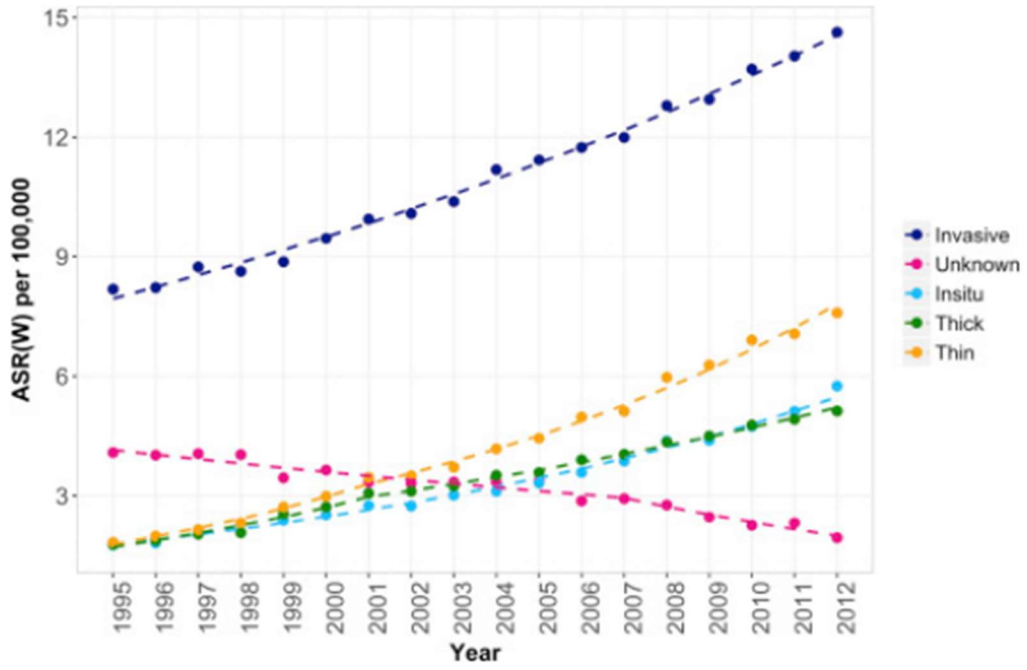


Figure 2. *The incidence rate trend of melanoma cases from 1995 to 2012* [12].

According to AIRTUM, about 12.300 new cases of cutaneous melanoma had been diagnosed in Italy in 2019. Melanoma represents 9% of youthful tumors in men and 7% in women. The risk to develop melanoma during lifetime is about 1:66 in men and 1:85 in women. Moreover, there is a geographic variability in the incidence of cutaneous melanoma with a decrescent trend from the North to the South; indeed, the incidence is higher in the North (21.8% in men and 18% in women) and in Central Italy (24.9% in men and 19% in women) than in the South (12.4% in men and 10.6% in women) [13].

1.3 Risk factors

The development of melanoma is due to a complex interaction among exogenous, endogenous and genetic factors. Among environmental factors, both prolonged intermittent and chronic UV exposure and sunburns, in particular in childhood, are well established risk factors for melanoma development.

Several studies reported that the place of birth and the first decade of life spent in countries with high solar irradiation (Australia and USA) as well as an early life in a high UV environment increase the risk of melanoma [14].

Multiple studies have shown that many years of occupational sun exposure (>20 years) lead to an increased risk of developing melanoma [15]. Exposure to UV radiation can also occur during medical treatment. Psoralen and ultraviolet A (PUVA) is used as therapy for psoriasis, eczema, and vitiligo and is associated with an increased development of melanoma and nonmelanoma skin cancers [16].

Regarding endogenous factors, the number of melanocytic nevi is the most important risk factor. The presence of dysplastic nevi has been associated with a 1.5- to 10-fold increased risk of developing melanoma [17]. The risk is higher also based on the number of dysplastic nevi; 1.5 times higher in patients with 11 to 25 nevi, 4.4 in patients with 26-50 nevi, 5.4 in patients with 51-100 nevi and 9.8 in cases with more than 100 nevi [10].

Two major melanoma risk factors are family and/or a personal history of melanoma. Approximately 5-12% of melanomas are diagnosed in a familial context. Individuals with at least one first-degree relative with melanoma have an approximately 2-fold increased risk of developing the disease and the risk increases with the number of affected first-degree relatives [18].

Two main genes have been previously associated to melanoma predisposition: cyclin-dependent kinase inhibitor 2A *(CDKN2A)* and cyclin-dependent kinase 4 (*CDK4).* The *CDKN2A* gene acts as a tumor suppressor gene and plays a crucial role in cell cycle regulation and senescence. The *CDK4* is an oncogene and is involved in cell cycle regulation. Mutations of these two genes confer susceptibility to familial melanoma.

Additional endogenous risk factors are fair skin (skin type I and II) and a red hair color phenotype (blond and red hair, blue eyes, freckles, photosensitivity). The red hair/fair skin phenotypes are associated with variants in the melanocortin-1 receptor (*MC1R*), which regulates the production of pheomelanin and eumelanin [19]. Further studies identified two additional genes, the telomerase reverse transcriptase (*TERT)* promoter and protection of telomeres 1 (*POT1)* genes, both involved in the telomere maintenance pathway, as associated to melanoma susceptibility (Figure 3) [20].

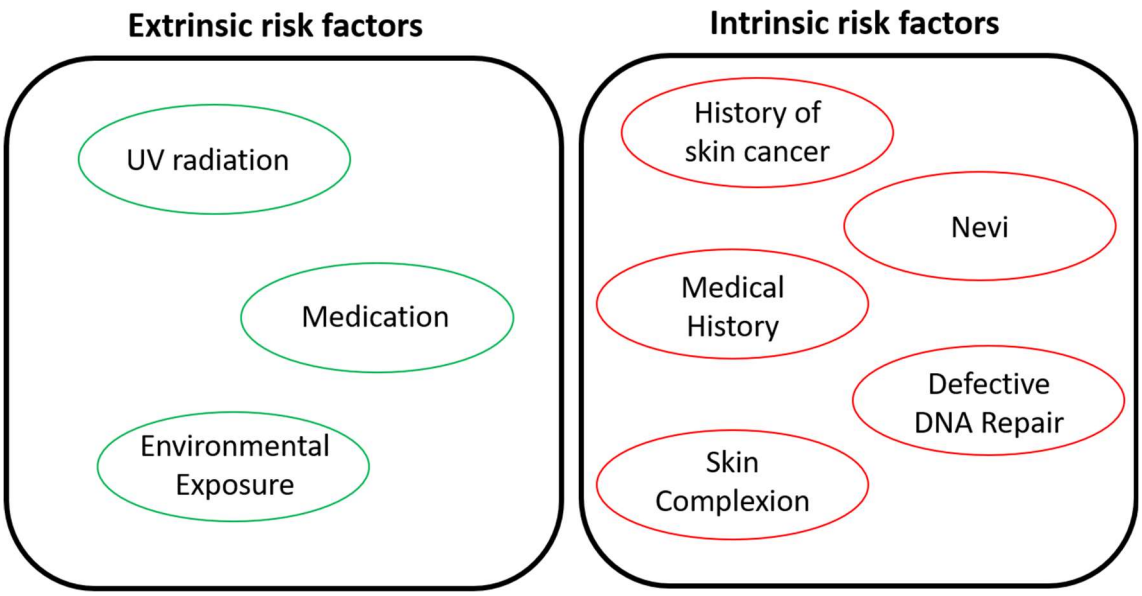


Figure 3. *Risk factors involved in the pathogenesis of melanoma.*

1.4 Diagnosis

1.4.1 Clinical diagnosis

Currently, the gold standard for melanoma diagnosis continues to be histopathology, considered the primary tool for classification, in conjunction with clinical features. The clinicopathological classification was proposed by McGovern [21] and Clark et al. [22] and led to the recognition that melanomas evolve through stages of tumour progression.

Early melanomas are recognised as spreading patch-like lesions in the epidermis and superficial dermis and are considered to be in radial growth phase (RGP), because they expand in horizontal direction. RGP melanomas have a good prognosis until deeper invasion of dermis occurs with formation of a tumour mass, defining the vertical growth phase (VGP) [23].

In 2014, Bastian et al. proposed a multidimensional classification for melanocytic lesions based on the role of UV radiation, cell or tissue of origin and recurrent genomic alterations [24]. Melanomas were divided between melanomas arising in sun-exposed skin and melanomas arising at sun-shielded sites or without known etiological associations with UV exposure. The first group is associated with varying degrees of chronic solar damage (CSD) and is classified as low-CSD and high-CSD melanomas.

Low-CSD melanomas, including superficial spreading melanoma (SSM) (Figure 4) and low-CSD nodular melanoma (NM), are associated with intermittent sun exposure in relatively young age individuals with a low to moderate CSD in affected skin [25].

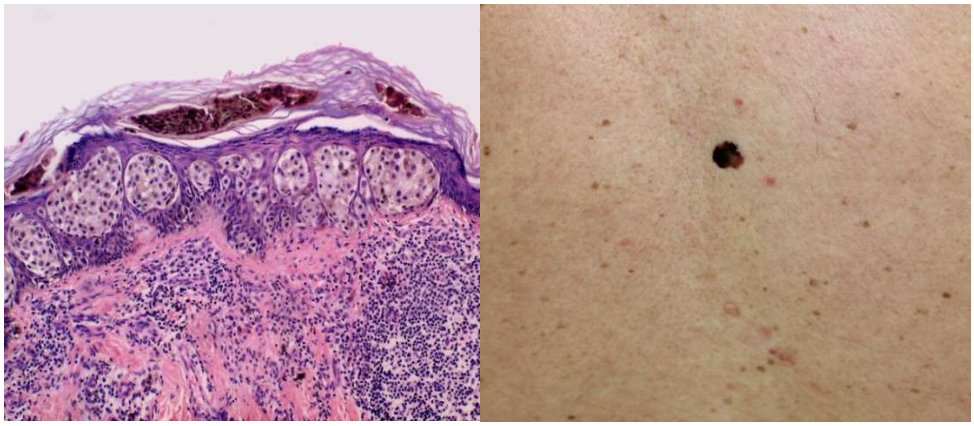


Figure 4. *Superficial spreading melanoma*

Instead, high-CSD melanomas, including lentigo malignant melanoma (LMM) (figure 5) and high-CSD nodular melanoma, are associated with chronic sun exposure in older age individuals, freckles and skin type I/II as risk factors [26].

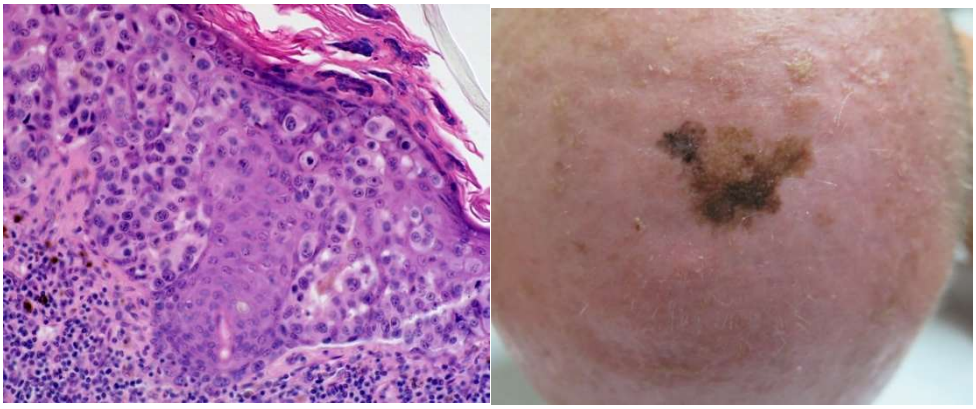


Figure 5. *Lentigo maligna melanoma.*

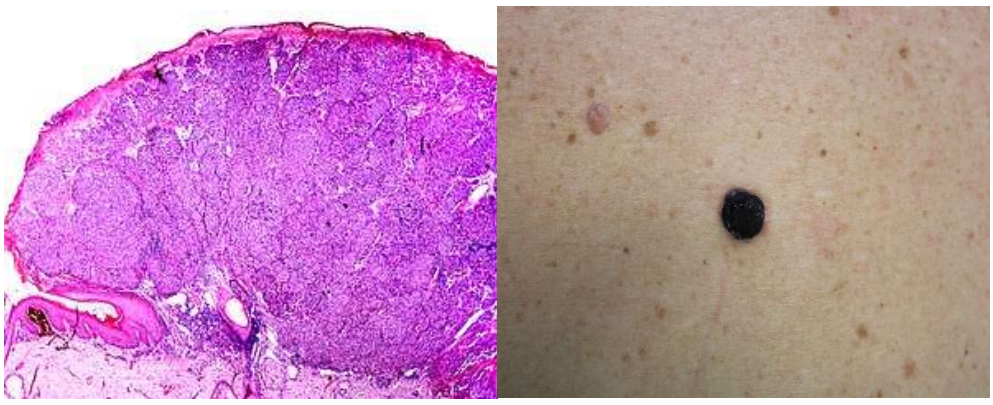


Figure 6. *Nodular melanoma*

Less common is desmoplastic melanoma, a rare tumor with a VGP that may arise in a RGP of lentigo maligna melanoma, associated with a high level of CSD, or less frequently in acral or mucosal melanomas, not associated with CSD.

Melanomas arising at sun-shielded sites or without known etiological associations with UV exposure arise in skin with or without CSD. This group includes Spitz melanoma that occurs in young individuals and is usually less aggressive in terms of lethal behavior and melanoma arising in congenital or in blue naevi whose risk factors are unknown, even if there is no apparent etiological relationship with sun exposure [23].

Moreover, acral melanoma (AM) (Figure 7) (including nodular melanoma in acral sites), mucosal and uveal melanomas are associated with this group. Acral melanomas typically begins as *in situ* melanoma and there is no identified relationship with sun exposure [27] [28], such as in mucosal melanoma in which CSD is typically absent. Uveal melanoma evidences a low mutation burden with no clear UV radiation signature.

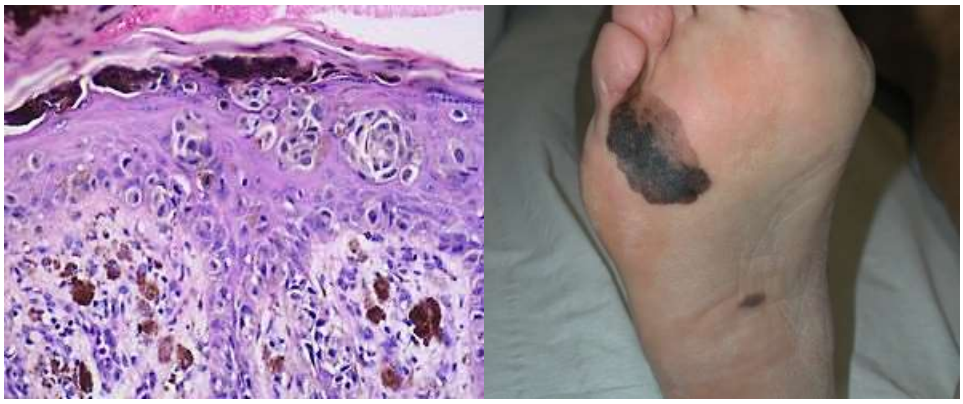


Figure 7. *Acral melanoma.*

1.4.2 Staging of melanoma

The prognosis of melanoma can be assessed in terms of prognostic attributes, defined by the American Joint Committee on Cancer (AJCC).

The AJCC Staging Manual (8^{th} edition) includes key features, as Breslow thickness, ulceration, involvement of lymph nodes, presence of microsatellite, satellite and/or in-transit metastases and the presence of distant metastatic disease.

The TNM Classification of Malignant Tumors (TNM) is a globally recognised standard for classifying cancers in order to define the stage, the major determinant for an appropriate treatment and prognosis [29].

Localized primary melanomas constitute TNM stage I and II; regional metastasis defines stage III and distant metastasis stage IV. Stage 0 represents *in situ* melanoma, which generally has no capability to metastasize [30].

1.5 Genetics in melanoma

One of the most significant discover in the understanding of cancer development was the knowledge that tumors arise following the acquisition of genetic mutations. These findings had shown skin cancers as the most mutated cancers in humans [31] [32]. Molecular pathways involved in somatic melanomas towards metastatic disease will be discussed in chapter 2.

Although the major of cutaneous melanomas was diagnosed as sporadic cases, approximatively 5-12% of melanomas occur in a familial context, in which heritable germline mutations conveyed increased risk of melanoma development [33]. Molecular pathways involved in familial melanoma will be discussed in chapter 3.

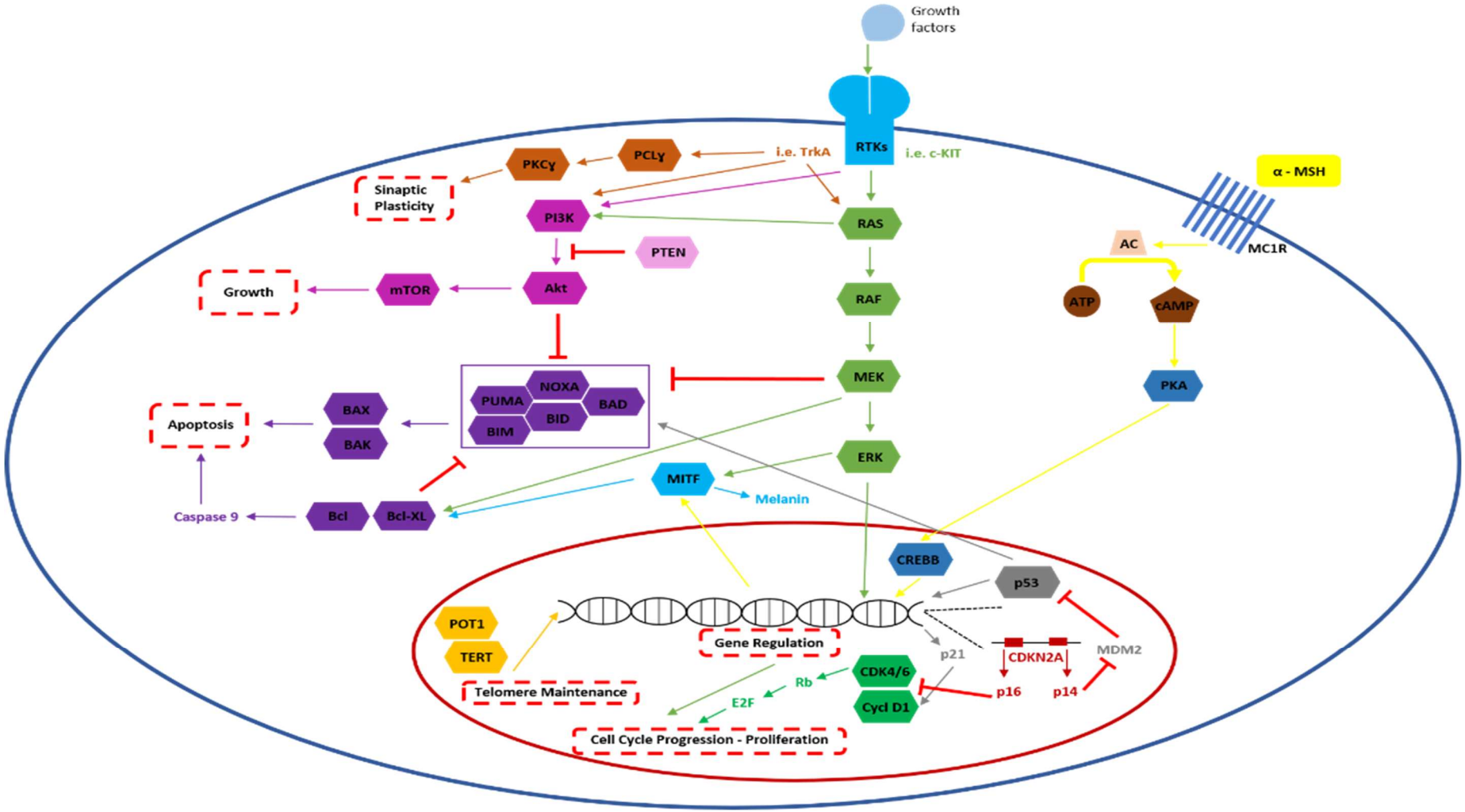


Figure 8. *Overview of molecular pathways involved in the pathogenesis of melanoma.*

1.6 Treatment of melanoma

Great advances in the management of melanoma from the 1800s until now were performed. To date, surgical treatment remains the cornerstone therapy but since 2011, multiple new treatment options including immune checkpoint inhibitors and molecular inhibitors of the MAPK pathway have improved the treatment outcomes and long-term survival of patients with unresectable advanced or metastatic melanoma [34].

The cytokine interferon alpha-2b (IFNa-2b) was the first immunotherapy approved for the treatment of melanoma. With long-term follow-up, interferon has failed to maintain statistically significant improvements in OS, and, with the introduction of immune checkpoint inhibitors and targeted therapies, use of interferon become less prominent in recent years [35]. Then, the cytokine interleukin-2 (IL-2) also has been used for melanoma treatment, but IL-2 therapy has been used primarily in the setting of metastatic disease.

The first of the immune checkpoint inhibitors to be approved by the the U.S. Food and Drug Administration (FDA) was the cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) blocking antibody Ipilimumab. Although the efficacy results are significant, the adverse event rate associated with ipilimumab therapy and the emergence of programmed cell death protein 1 (PD-1) blocking antibodies, named Pembrolizumab and Nivolumab, have greatly decreased utilization of ipilimumab although it remains a recommended therapy [36].

The first targeted therapy demonstrating substantial efficacy against melanoma was Vemurafenib, an adenosine triphosphate competitive BRAF inhibitor. In the phase III clinical trial, Vemurafenib conferred a survival advantage compared to dacarbazine chemotherapy in patients with *BRAF* ^{V600E}-mutant melanomas, with an overall response rate of 48% [37]. Dabrafenib, another BRAF inhibitor, provides similar clinical benefit. Vemurafenib and Dabrafenib were approved by the FDA for treatment of advanced *BRAF*-mutant melanoma in 2011 and 2013, respectively [38]. The combination of a BRAF and a MEK inhibitor, such as Dabrafenib and Trametinib or Vemurafenib and cobimetinib, increase the clinical benefit with fewer toxicities relative to single-agent treatment [39]. Recently, FDA and EMA approved encorafenib and binimetinib as further treatment combination for *BRAF*-mutated patients. The combination of BRAF and MEK

inhibitors provides some delay in disease progression, but most patients still relapse within a year. Efforts to improve durability of responses will likely include other drug regimens [40].

Melanomas with acquired resistance to targeted therapies show evidence of branched evolution and a high degree of genomic heterogeneity, even within single tumor sites. Comprehensively cataloging the landscape of BRAF inhibitor resistance will enable more accurate prediction of drug response patterns and guide future therapeutic strategies.

1.7 Aims and structure of the thesis

The aim of the present Ph.D. thesis was to investigate the molecular pathogenesis of cutaneous melanoma, at the germinal and somatic level in familial and sporadic melanoma, respectively.

The work was divided into two projects exploring the intra-patient heterogeneity of molecular alterations in primary melanomas and related metastases (Chapter 2) and the genetic susceptibility to familial melanoma in Mediterranean populations (Chapter 3).

Chapter 2

Project 1: "Intra-patient heterogeneity of *BRAF* and *NRAS* molecular alterations in primary melanoma and metastases"

2.1 Introduction

The most relevant molecular pathway implicated in melanoma pathogenesis is the mitogen-activated protein kinase (MAPK) cascade, which is dysregulated in approximately 80% of melanomas [41], leading to constitutive cell growth and proliferation. Within this pathway, mutations in BRAF proto-oncogene (*BRAF),* NRAS proto-oncogene (*NRAS)* and KIT proto-oncogene (c-*KIT)* are considered driver events and have a strong clinical relevance for melanoma treatment [42] [43] [44].

In melanocytes, *BRAF* induces the activation of MEK kinase, which in turn activates ERK, the final effector of the MAPK cascade (Figure 8). *BRAF* is mutated in about 50% of melanomas and the most prevalent mutation (about 80% of cases) is represented by the replacement of glutamic acid with valine at codon 600 ($BRAF^{V600E}$) [45]. The $BRAF^{V600E}$ variant, as the remaining mutations in the BRAF kinase domain, induces continuous stimulation of cell proliferation and tumor growth through activating phosphorylation of ERK. Oncogenic *NRAS* mutations are found in 15%-25% of cutaneous melanomas and are usually detected at codon 61, mainly with a glutamine to arginine/lysine/leucine substitution (Q61R/K/L). Finally, about 3% of all melanomas carry somatic mutations in exons 11 (L576P) and 13 (K642E) of the c-*KIT* gene, but additional *c-KIT* aberrations might include mutations in exon 17 and gene amplifications [3] [42].

Mutations in *BRAF* can already occur in nevi, but generally they lead to senescence because cells are unable to cope with the activation stress. Mutational UV signature is implicated in mutagenesis of superficial melanocytes of naevus as the predominant pathogenic mechanism that drives the progression to melanoma, typically via melanoma *in situ* as an intermediate step [46].

In situ melanoma develop from multiple pathogenic alterations accumulated over a long period of time. Whereas most mutations affecting the MAPK pathway occur in a mutually exclusive pattern, neurofibromin 1 (*NF1*) mutations commonly co-occur with other alterations in the MAPK cascade. *In situ* melanomas can persist for many years before becoming invasive, indicating that invasive growth requires additional genetic alterations [47] [48].

Once melanoma cells leave the epidermis and enter the subjacent mesenchymal tissue, melanoma becomes invasive and acquires other molecular alterations. *TERT* mutations are accumulated during earlier stages of progression, followed by a high frequency of inactivation of *CDKN2A*. In addition, mutations affecting members of the SWI/SNF chromatin remodelling complex, in particular AT-rich interaction domain 2 (*ARID2)* emerge at the transition to invasive melanoma [49].

Then melanoma cells can disseminate beyond the local site of the primary tumor and colonize other tissues. Mutational studies on tumor protein p53 (*TP53)* gene in primary melanomas showed lower mutation frequencies than reported in melanoma metastasis, indicating that mutations in this gene may arise later during the metastatic progression. Similarly, phosphatase and tensin homolog (*PTEN)* mutations are more frequent in melanoma metastasis and probably origin during the late stage of melanoma [41].

The second pathway depending on RAS for cell growth regulation is represented by the signal transduction PI3K-AKT-mTOR cascade. Under physiological conditions, intracellular levels of PIP2 and PIP3 phosphoinositols are increased by activation of PI3K and reduced by the activity of the phosphatase PTEN protein. High PIP3 levels sequentially activate downstream AKT (mainly, AKT3 in melanoma) and its substrate mTOR, modulating the synthesis of proteins involved in cell growth and survival as well as in apoptosis [50]. The activation of AKT: (a) promotes cell proliferation and (b) inhibits apoptosis by inactivation of many proapoptotic proteins, such as BCL-2 antagonist of cell death (BAD) (Figure 8). In summary, the combined effect of PTEN inactivation and PI3K–AKT stimulation results in an aberrant growth of neoplastic cells, with acquisition of resistance to apoptosis.

The identification of specific pathways involved in the pathogenesis of melanoma lead to a classification of molecular subtypes characterized by different features, such as

onset or progression of the disease. Molecular categorization of those subtypes became a very important tool for a more correct and precise therapeutic protocol [51].

The *BRAF* subtype is characterized by presence of *BRAF* mutations and could be associated with inactivation of *TP53*, especially in advanced lesions. Mutations of *BRAF* could be also associated with activation of PI3K-AKT-mTOR pathway and inactivation of PTEN and with alterations of p16*CDKN2A*-*CDK4*-RB pathway and/or amplification of *CDK4* gene. The *NRAS* subtype presents *NRAS* mutations, with eventual activation of AKT. The third subtype is characterized by *NF1* gene mutations occasionally concurrently with other RASopathy gene alterations. Finally, the triple wild-type subtype as a heterogeneous subgroup characterized by a lack of hot-spot *BRAF, N/H/K-RAS*, or *NF1* mutations showing mutations of *c-KIT* gene and gene amplification, with or without an increased level of protein expression [52].

2.1.1 Genetic heterogeneity in melanoma

The differences in genetic alterations between the primary tumor and metastatic lesions and among metastases constitute intra-patient intertumor heterogeneity.

Despite the monoclonal origin of most cancers, new clones arise during tumor progression which is due to the continuous acquisition of mutations. The coexistence of distinct subclones creates a complex network of interaction which affects cancer progression [53].

Mutational concordance between primary melanoma and related metastases is a critical aspect that has become even more relevant with the introduction of therapies targeting specific mutations of driver genes [54]. Intra-patient molecular heterogeneity between primary and metastatic lesions may exist, and changes in mutational pattern might occur during progression [53].

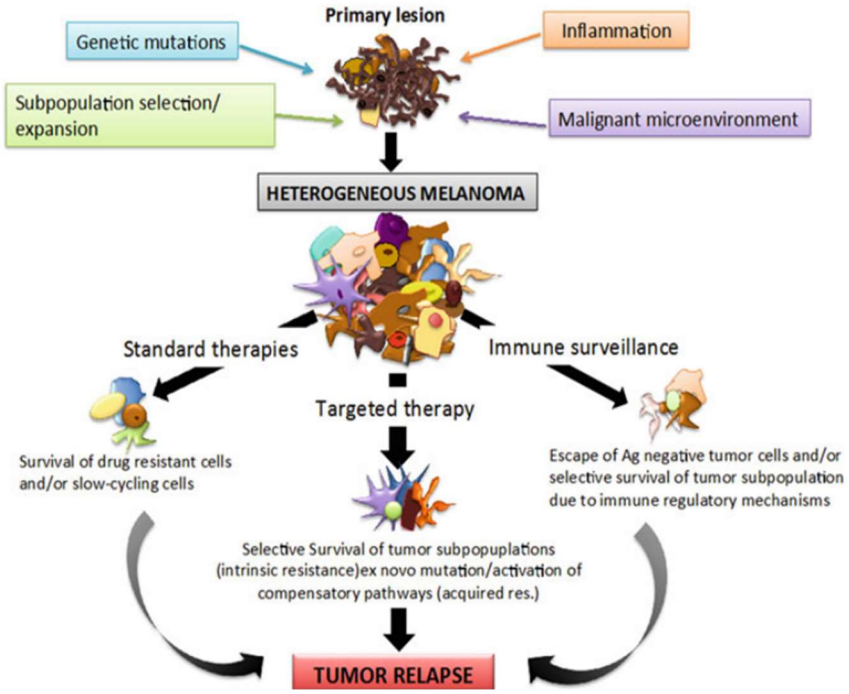


Figure 9. *Melanoma heterogeneity and clinical implications* [46].

Taking into consideration the complexity of different pathways as a unique functional network, the reasons because alterations referring to different genes can coexist in melanoma are better understood. For this reason, a melanogenesis model, named "linear", has been developed based on the progressive accumulation of molecular alterations.

In conclusion, a more detailed knowledge of molecular targets leads to the identification of genetic markers useful for an accurate prognosis and a more personalized therapy in melanoma patients.

2.2 Aim

We screened the mutational status of *BRAF*, *NRAS* and *c-KIT* genes in metastatic melanoma patients in order to evaluate the intra-patient molecular heterogeneity between primary tumor and related metastases and to compare the consistency of mutational findings obtained by molecular and immunohistochemical analyses.

2.3 Materials and Methods

A. Patients' and tumor samples

Patients with a confirmed diagnosis of metastatic melanoma were enrolled between January 2012 and December 2018 at the Department of Dermatology, University of L'Aquila, Italy. For each patient, tissues from primary melanoma and at least one metastasis were retrieved.

Hematoxylin-eosin-stained sections of primary and metastatic tissues were reviewed by pathologists to confirm the diagnosis. The following clinicopathological features of primary tumors and/or metastasis were collected: anatomical location, histopathological variant, Breslow thickness, presence of ulceration, number of mitosis/mm^2, presence of melanoma-associated nevus and chronic solar damage (histologically defined on the base of solar elastosis degree). Approval for this study was obtained from the Ethics Committee of the ASL-01 Avezzano, Sulmona, L'Aquila (protocol number 0012038/11).

B. Molecular analysis

Somatic DNA was extracted from 5 formalin-fixed paraffin-embedded (FFPE) tissue sections (each of 10-micron in thickness) obtained from tissue block by microdissection of marked tumor-rich areas from primary melanoma and metastases using a QIAmp Micro tissue kit (Qiagen, Hilden, Germany).

We investigated codon 600 in exon 15 of *BRAF* by competitive allele-specific TaqMan PCR (castPCR Technology), exon 2 of *NRAS*, and exons 11, 13 and 17 of c-*KIT* by Sanger sequencing. PCR amplification of the regions of interest was performed in a Simply-Amp PCR-System (Thermo-Fisher, Foster City, USA) using primers listed in Table 1.

Table 1. *List of primers used for molecular experiments.*

Target gene	Primer ID	Sequence (5' > 3')	Tm [°C]	Reference
***BRAF* exon 15**	forward	CTTCATGAAGACCTCACAGT	54.2	[54]
	reverse	CTTTCTAGTAACTCAGCAGC		
***NRAS* exon 2**	forward	CCCCTTACCCTCCACACC	57.0	[55]
	reverse	TGGCAAATACACAGAGGAAGC		
***c-KIT* exon 11**	forward	CCAGAGTGCTCTAATGACTGA	57.0	This study
	reverse	GTGACATGGAAAGCCCCTG		
***c-KIT* exon 13**	forward	ATCAGTTTGCCAGTTGTGCT	54.4	
	reverse	CAGCTTGGACACGGCTTTA		
***c-KIT* exon 17**	forward	GGTTTTCTTTTCTCCTCCAAC	55.0	
	reverse	GCAGGACTGTCAAGCAGAGAAT		
GAPDH	forward	CTGCACCACCAACTGCTTAG	58.5	
	reverse	GCAGTGATGGCATGGACTGT		

Tm: Melting Temperature; *BRAF:* BRAF proto-oncogene; *NRAS:* NRAS proto-oncogene; *c-KIT*: KIT proto-oncogene; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase

For competitive allele-specific TaqMan assay, PCRs containing 20 ng of DNA, 1X TaqMan Mutation Detection Assays (assay Hs00000111_mu for $BRAF^{V600E}$, Hs000000002_rm for $BRAF^{V600K}$), 1X TaqMan Genotyping Master Mix (Thermo Fisher), and water to reach the final volume of 20 µL were performed in 96-well plates with the use of the standard TaqMan protocol on 7500 Fast Real Time-PCR System (Thermo Fisher). Positive and negative controls were used for experiments of mutation detection.

The variants were detected using the Applied Biosystems Mutation Detection Software (Thermo Fisher). The difference between the C_T value of the mutant allele assay and the C_T of the gene reference assay was calculated for all mutant allele assays run on the sample. This ΔC_T value represents the quality of the specific mutant allele detected within the sample. This ΔC_T is used to determine the sample mutation status by comparison to a predetermined detection ΔC_T cut-off value.

PCR experiments were performed with 10 µl of HotStartTaq® Plus Master Mix (QIAgen) in a 20 µl volume, containing also of 0.3 µM of each primer, water up to the volume and <200 ng of DNA template. Water controls and positive controls were run in parallel with DNA samples. The thermal cycling conditions were as follows: 5 min at 95°C, followed by 45 cycles of 94°C for 40 s, Tm for 30 s and 72°C for 40s, and in the end 10 min at 72 °C. Amplicons were sequenced on 3500 Genetic-Analyzer (Thermo-Fisher, Foster City, USA).

c-KIT copy number was assessed by quantitative real-time PCR for exon 13 sequence and compared with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal control. Briefly, PCR reactions were performed by using PowerUP™ SYBR™ Green Master Mix (Thermo-Fisher), with a 20 µL total volume and 50 ng of genomic on a 7500 Fast Real Time-PCR System. The primers for c-KIT exon 13 and GAPDH are listed in Table 1. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Each gene was analyzed in triplicate. Samples that did not amplify by 35 cycles were considered to be of insufficient quality and excluded from the analysis. For each sample, ΔCt for c-KIT vs GAPDH was calculated as ΔCt=Ct (KIT)–Ct (GAPDH) and then was calibrated to individual reference genomic DNAs from 3 normal skin tissue samples and then confirmed to a commercial human genomic reference DNA (Thermo-Fisher). Relative copy number evaluation was performed by the comparative *2*-ΔΔCt method and converted to absolute copy numbers by assigning a value of 2 to the reference DNA.

C. Immunohistochemistry

IHC was performed on FFPE tissue sections of 4 µm thickness, obtained from the same tissue block for molecular experiments. The presence of $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutants and c-KIT expression was evaluated using the following monoclonal antibodies: $BRAF^{V600E}$ VE1 clone (Spring Bioscience, Pleasetown, USA), $NRAS^{Q61R}$ SP174 clone (Spring Bioscience), and CD117/c-Kit polyclonal (Spring Bioscience) at a dilution of 1:30, 1:80 and 1:100, respectively. Sections were freshly cut, dried at 60°C for 30 min, deparaffinized and rehydrated. Immunoreactions were performed on Ventana BenchMark GX automatic immune stainer (Ventana Medical Systems Inc., Tucson, USA) using the Ultra View Universal Alkalin Phosphatase Red Detection Kit, as previously reported (Massi et al, 2015). No chromogen was detected when primary antibody was omitted. Positive controls were mounted on each section subjected to immunostaining. Negative controls were included in each run.

Cytoplasmic staining of monoclonal $BRAF^{V600E}$ VE1 and $NRAS^{Q61R}$ SP174 clones and the polyclonal antibody CD117/c-Kit in melanoma cells was considered as positive or negative, according to previously published criteria [55] [56]. In detail, positive staining for $BRAF^{V600E}$ VE1 and $NRAS^{Q61R}$ SP174 was classified as

homogeneous (staining in >95% of cells) or heterogeneous (staining in <95% of cells) based on the percentage of cytoplasmic staining of melanoma cells. Negative staining was defined either as absence of any cytoplasmic labeling or staining of single interspersed melanoma cells (<10%). Intensity of staining was graded as weak, moderate or strong [55]. CD117/c-Kit staining was assessed on the percentage of stained cells and strength of staining: 0, no staining; 1+, weak staining in isolated groups of melanoma cells; 2+, weak and widespread staining in <50% of melanoma cells; 3+, moderate staining in 50-75% of melanoma cells; 4+, strong staining in >75% of melanoma cells. Staining was classified as positive in the presence of moderate/strong membranous and cytoplasmic staining (3+/4+) and as negative if there was absence of cytoplasmic staining and if staining was weak and widespread (0/1+/2+).

D. Statistical analysis

For statistical analysis, categorical variables for primary melanoma were grouped as follows: sex, age at diagnosis, AJCC stage 8th edition, anatomical site (head/neck, trunk, extremities, acral), histopathological subtype (SSM, NM), chronic solar damage (CSD or no CSD, NCSD) [57], presence of ulceration (yes, no), number of mitosis/mm^2 and presence of melanoma-associated nevus (yes, no). Synchronicity (defined as a metastasis diagnosed at the same time of the primary melanoma) and anatomical site (skin, lymph node, visceral, brain) were recorded for metastases.

Semi-quantitative data (age at diagnosis, Breslow thickness) were analyzed by means of Student's t-test or by medians with Mann-Whitney test. Univariate analysis by Chi-square test or by Fisher's exact test was used to test the significance of mutation frequency according to clinicopathological characteristics of melanoma patients and tumors. Molecular findings were used as the gold standard for statistical analysis. Cohen's k coefficient test was used to measure the agreement between molecular and IHC methods in determining *BRAF* and *NRAS* mutational status. Samples harboring the *BRAF*^{V600K} mutation according to molecular analysis, that were wild type on IHC VE1 staining, were not considered for Cohen's k analysis. P values less than 0.05 were considered statistically significant. Statistical analysis was performed using the statistical package SPSS 17.0 (SPSS Incorporated, Chicago).

2.4 Results

A. Patients' and tumor samples

Overall, we collected a total of 69 tumor tissues (30 primary melanomas and 39 related metastatic lesions) from 30 advanced melanoma patients (25 stage III and 5 stage IV). Enrolled patients included 17 males and 13 females, with a median age at first diagnosis of 65 years (range 25-84 years). In detail, 23 patients were diagnosed with 1 metastasis, 5 with 2 metastases and 2 with 3 metastases. Demographic and clinical characteristics of patients and tumor tissues are reported in Table 2.

Twelve primary melanomas (12/30, 40.0%) were located on the extremities, 10 on the trunk (10/30, 33.3%), 5 (5/30, 16.7%) on the head/neck region and 3 (3/30, 10.0%) on acral sites. The majority of tumors were of the nodular histological subtype (23/30, 76.7%) followed by superficial spreading melanoma (7/30, 23.3%); no acral lentiginous nor lentigo maligna melanomas were diagnosed in our series of patients. Median Breslow thickness was 4.2 mm (range from 0.6 to 30 mm). A pre-existing melanocytic nevus was associated in 36.7% (11/30) of primary melanomas.

Most metastatic tissues were collected from lymph nodes (23/39, 59.0%), followed by skin (11/39, 28.2%), brain (2/39, 5.1%), colon (1/39, 2.6%), liver (1/39, 2.6%) and parathyroid gland (1/39, 2.6%). Metastases were synchronous in 17 (17/30, 56.7%) patients and metachronous in 9 (9/30, 30.0%); 4 (4/30, 13.3%) patients had both synchronous and metachronous metastases. Median time to first metastasis was 3 months (range 0-51 months).

Table 2. *Demographic and clinical characteristics of melanoma patients and histopathological features of tumors and metastasis.*

		No. of samples
Characteristics of patients		***N = 30 (%)***
Sex	*Males*	*17 (56.7)*
	Females	*13 (43.3)*
Age	*Median age (range)*	*65 (25-84)*
No. of metastases	*2*	*5 (16.6)*
	3	*2 (6.7)*
Synchronous/metachronous	*Synchronous*	*17 (56.7)*
	Metachronous	*9 (30.0)*
Stage	*III*	*25 (83.3)*
	IV	*5 (16.7)*
Characteristics of primary melanoma		***N=30 (%)***
Breslow thickness	*Median value (range)*	*4.2 (0.6-30)*
Histopathological subtype	*SSM*	*7 (23.3)*
	NM	*23 (76.7)*
Anatomical site	*Head/neck*	*5 (16.7)*
	Trunk	*10 (33.3)*
	Extremities	*12 (40.0)*
	Acral	*3 (10.0)*
Associated Nevus	*Yes*	*11 (36.7)*
	No	*19 (63.3)*
Ulceration	*Yes*	*20 (66.7)*
	No	*10 (33.3)*
Solar Elastosis	*Yes*	*18 (60.0)*
	No	*12 (40.0)*
Characteristics of metastases		***N=39 (%)***
Anatomical site	*Lymph nodes*	*23 (59.0)*
	Skin	*11 (28.2)*
	Brain	*2 (5.1)*
	Visceral	*3 (7.7)*

SSM: superficial spreading melanoma; NM: nodular melanoma; [a]Stage III patients included 20 patients with nodal metastases and 5 patients with in-transit metastases without nodal involvement.

B. Molecular analysis

The distribution of *BRAF*, *NRAS* and *c-KIT* mutations in all analyzed tissues is shown in Table 3. *BRAF* mutations at codon 600 were detected in 33 of 69 (47.8%) tumor tissues, with 23 harboring the $BRAF^{V600E}$ (23/33, 69.7%) and 10 the $BRAF^{V600K}$ (10/33, 30.3%) mutation. Distribution of *BRAF* mutations was similar between primary melanomas (14/30, 46.7%) and metastatic samples (19/39, 48.7%) (p=0.42). Sixteen of

69 tumor tissues (16/69, 23.2%) carried *NRAS* mutations at codon 61 with the following genotypes: $NRAS^{Q61R}$ (8/16, 50.0%), $NRAS^{Q61L}$ (7/16, 43.7%), and $NRAS^{Q61K}$ (1/16, 6.3%). Among the 16 *NRAS* mutated tumors, 6 were primary melanomas (6/30, 20.0%) and 10 (10/39, 25.6%) metastatic samples (p=0.61). Finally, only one missense *c-KIT* mutation, L802F mutation in exon 17, was detected in 1 (1/69, 1.4%) primary melanoma diagnosed on the upper extremity. A mutation in at least one of the 3 genes was present in 21 of 30 (70.0%) primary melanomas and 29 of 39 (74.3%) metastatic samples, for a total of 72.5% tissues (50/69). All tissues were mutually exclusive for *BRAF*, *NRAS* and *c-KIT* mutations (Table 4). *c-KIT* gene amplification was carried out in 65/69 (94.2%) samples with 4 samples missing due to lack of DNA. An increased copy number (≥ 3 copies) was identified in 3 (3/69 4.3%) samples, with two of them being primary melanomas with a high level of CSD. All cases with *c-KIT* amplification carried concomitant $BRAF^{V600}$ mutation ($BRAF^{V600K}$ in 2 cases and $BRAF^{V600E}$ in 1 case).

A significant association between mutational status and clinical characteristics of the primary melanoma was observed only for median age at melanoma onset (Table 4). Patients with $BRAF^{V600}$ mutated melanomas were younger than those with *BRAF* wild-type melanomas (57 years vs 74 years, p<0.01) while *NRAS* mutations were more prevalent in older patients (77 years vs 60 years, p<0.01). A higher trend of *NRAS* mutations were found in primary melanomas showing CSD as evaluated by histological solar elastosis (p=0.06).

An intra-patient concordance of *BRAF* mutational status between primary melanoma and related metastases was detected in 86.7% (26/30) of patients, with 9 (9/26, 34.7%) concordant for $BRAF^{V600E}$, 3 (3/26, %) for $BRAF^{V600K}$, and 14 (14/26, 53.8%) for the wild-type genotype (Table 3 and Figure 10). Intra-patient *BRAF* molecular heterogeneity was observed in 13.3% (4/30) of patients. Notably, all patients (7/30, 23.3%) with multiple metastases showed a consistent *BRAF* mutational status between primary melanoma and all analyzed metastatic tissues. Concerning *NRAS* mutational profile, a concordant *NRAS* mutational status was observed in 26 of 30 (86.7%) patients, having the majority of them (21/30, 70.0%) a wild-type genotype (Figure 10). Among the 4 discordant cases (4/30, 13.3%), 3 (3/4, 75%) showed wild-type primaries and mutated metastases with the following genotypes Q61R, Q61L and Q61K (Table 3). Concordance rate of *BRAF* and *NRAS* mutational status did not differ by sex, timing of metastases appearance and metastatic site (Table 5).

Regarding the single patient mutated for *c-KIT*, a discordant mutational status was observed between his primary and the metastatic lesion.

Considering the overall somatic profile of all *BRAF/NRAS/c-KIT* genes, intra-patient heterogeneity was present in 23.3% (7/30) of patients (6 with 1 metastasis and 1 with 2 metastases). Discordance rates were not associated to sex, synchronicity, and anatomical sites of metastasis (Table 5).

Table 3. *Summary of mutational patterns in primary and metastatic melanoma samples according to immunohistochemical and molecular analyses.*

Pt	Primary Melanoma								Metastasis no. 1								Metastasis no. 2							
	Age (yr)	Site	IHC* data			Molecular data			Site	IHC* data			Molecular data				Site	IHC* data			Molecular data			
			BRAF	NRAS	c-KIT	*BRAF*	*NRAS*	*c-KIT*		BRAF	NRAS	c-KIT	*BRAF*	*NRAS*	*c-KIT*			BRAF	NRAS	c-KIT	*BRAF*	*NRAS*	*c-KIT*	
1	79	T	wt	wt	wt	wt	Q61L	wt	L	wt	wt	m, het	wt	Q61L	wt		SK	wt	wt	wt	wt	Q61L	wt	
2	81	A	wt	wt	s, hom	wt	wt	wt	L	wt	wt	wt	wt	wt	wt									
3[a]	56	A	wt	wt	wt	wt	wt	wt	SK	wt	wt	wt	wt	wt	wt		L	wt	wt	wt	wt	wt	wt	
4	73	E	s, hom	wt	wt	V600E	wt	wt	SK	s, hom	wt	wt	V600E	wt	wt									
5	54	T	m, het	wt	wt	wt	wt	wt	S	wt	wt	wt	V600K	wt	wt									
6	69	E	wt	w, het	wt	wt	wt	wt	L	wt	wt	wt	wt	wt	wt									
7	82	E	wt	s, hom	wt	wt	Q61R	wt	L	wt	s, hom	wt	wt	Q61R	wt									
8	80	T	wt	wt	wt	wt	Q61L	wt	L	wt	wt	wt	wt	Q61L	wt									
9	65	T	s, het	wt	wt	V600E	wt	wt	L	w, het	wt	wt	V600E	wt	wt									
10	70	E	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt		B	s, hom	wt	m, het	V600E	wt	amp	
11	57	E	s, hom	wt	wt	wt	wt	wt	L	m, het	wt	wt	wt	wt	wt									
12	70	H	wt	wt	wt	V600K	wt	wt	SK	wt	wt	wt	V600K	wt	wt		L	wt	wt	wt	V600K	wt	wt	
13	57	T	s, hom	wt	wt	V600E	wt	wt	SK	s, hom	wt	wt	V600E	wt	wt									
14[b]	25	E	s, het	wt	m, het	V600E	wt	wt	L	s, hom	wt	m, het	V600E	wt	wt		L	s, hom	wt	wt	V600E	wt	wt	
15	65	T	s, hom	wt	wt	V600E	wt	wt	L	w, het	wt	wt	V600E	wt	wt									
16	56	T	wt	wt	m, het	V600K	wt	amp	L	wt	wt	wt	wt	Q61L	wt									
17	45	E	wt	m, hom	wt	wt	wt	wt	L	wt	s, het	wt	wt	Q61R	wt		SK	wt	s, het	wt	wt	Q61R	wt	
18	35	E	s, hom	wt	wt	V600E	wt	wt	L	s, het	wt	m, het	V600E	wt	wt									
19	52	H	s, hom	wt	m, het	V600E	wt	wt	V	wt	wt	m, het	wt	wt	wt									
20	63	E	wt	wt	wt	wt	wt	L802F	L	wt	wt	wt	wt	wt	wt									
21	58	H	wt	wt	m, het	V600K	wt	wt	SK	wt	wt	wt	V600K	wt	wt		V	wt	wt	wt	V600K	wt	wt	
22	76	A	wt	wt	m, het	wt	wt	wt	L	wt	wt	m, het	wt	wt	wt									
23	66	E	wt	wt	wt	V600K	wt	amp	B	wt	wt	wt	V600K	wt	wt									
24	84	E	wt	wt	wt	wt	wt	wt	L	wt	wt	wt	wt	Q61L	wt									
25	76	H	wt	wt	s, hom	wt	wt	wt	SK	wt	wt	s, hom	wt	wt	wt									
26	69	T	wt	wt	wt	wt	Q61K	wt	L	m, het	wt	wt	V600E	wt	wt									
27	49	T	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt									
28	44	T	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt									
29	74	E	wt	s, hom	m, het	wt	Q61R	wt	SK	wt	s, het	wt	wt	Q61R	wt									
30	75	H	wt	s, hom	m, het	wt	Q61R	wt	V	wt	s, het	wt	wt	Q61R	wt									

A, acral site; amp, amplification (gene copy number ≥3); B, brain, E, extremities, H, head and neck; S, skin; V, visceral; IHC, immunohistochemistry; L, Lymph node; SK, skin; wt, wild type. [a]This patient also developed a third nodal metastasis that was wild type for *BRAF* and *NRAS* both at IHC and molecular analysis. [b]This patient also developed a third skin metastasis that carried the $BRAF^{V600E}$ mutation both at IHC and molecular analysis. *Staining intensity (s, strong; m, moderate; w, weak) and distribution (het, heterogeneous; hom, homogeneous) are indicated for IHC positive cases.

Table 4. *Frequency of BRAF and NRAS mutations according to clinical and histopathological characteristics of patients and tumours.*

			BRAF			***NRAS***		
		All samples	*Wild type*	*Mutated*	*p*	*Wild type*	*Mutated*	*p*
Characteristics of patients		**N=30**	**N=16**	**N=14**		**N=24**	**N=6**	
Sex	Males	17 (56.7)	7 (43.7)	10 (71.4)	0.16	14 (58.3)	3 (50.0)	0.71
	Females	13 (43.3)	9 (56.3)	4 (28.6)		10 (41.7)	3 (50.0)	
Age at diagnosis	Median age (range)	65 (25-84)	74.5 (45-84)	57.5 (25-73)	**<0.01**	60.5 (25-84)	77 (69-82)	**<0.01**
Characteristics of primary tumors		**N=30**	**N=16**	**N=14**		**N=24**	**N=6**	
Breslow thickness (mm)	Median (range)	4.2 (0.6-30)	3.8 (1.2-20.0)	4.9 (0.6-30.0)	0.91	5.7 (0.6-30)	2.7 (1.2-19)	0.23
Anatomical site	Head/neck	5 (16.7)	2 (12.5)	3 (21.4)	0.28	4 (16.7)	1 (16.7)	0.69
	Trunk	10 (33.3)	4 (25)	6 (42.9)		7 (29.2)	3 (50.0)	
	Extremities	12 (40.0)	7 (43.7)	5 (35.7)		10 (41.6)	2 (33.3)	
	Acral	3 (10.0)	3 (18.8)	0		3 (12.5)	0	
Solar Elastosis	No	12 (40.0)	6 (37.5)	6 (42.9)	0.94	12 (50.0)	0	0.06
	Yes	18 (60.0)	10 (62.5)	8 (57.1)		12 (50.0)	6 (100.0)	
Nevus-associated melanoma	No	19 (63.3)	11 (68.8)	8 (57.1)	0.71	16 (66.7)	3 (50.0)	0.64
	Yes	11 (36.7)	5 (31.2)	6 (42.9)		8 (33.3)	3 (50.0)	
Histological subtype	SSM	7 (23.3)	2 (12.5)	5 (35.7)	0.20	5 (20.8)	2 (33.3)	0.60
	NM	23 (76.7)	14 (87.5)	9 (64.3)		19 (79.2)	4 (66.7)	
Ulceration	No	10 (33.3)	6 (37.5)	4 (28.6)	0.71	9 (37.5)	1 (16.7)	0.52
	Yes	20 (66.7)	10 (62.5)	10 (71.4)		15 (62.5)	5 (83.3)	
No. mitosis	Median (range)	5 (0-24)	5.0 (0-24)	5.5 (0-23)	0.89	5.0 (0-23)	5.0 (1-24)	0.73
Characteristics of metastasis		**N=39**	**N=20**	**N=19**		**N=29**	**N=10**	
Anatomical site	Brain	2 (5.1)	0	2 (10.5)	0.42	2 (6.9)	0	0.85
	Lymph node	23 (59.0)	13 (65.0)	10 (52.6)		17 (58.6)	6 (60.0)	
	Skin	11 (28.2)	5 (25.0)	6 (31.6)		8 (27.6)	3 (30.0)	
	Visceral	3 (7.7)	2 (10.0)	1 (5.3)		2 (6.9)	1 (10.0)	

BRAF: BRAF proto-oncogene; *NRAS:* NRAS proto-oncogene; SSM: superficial spreading melanoma; NM: nodular melanoma.

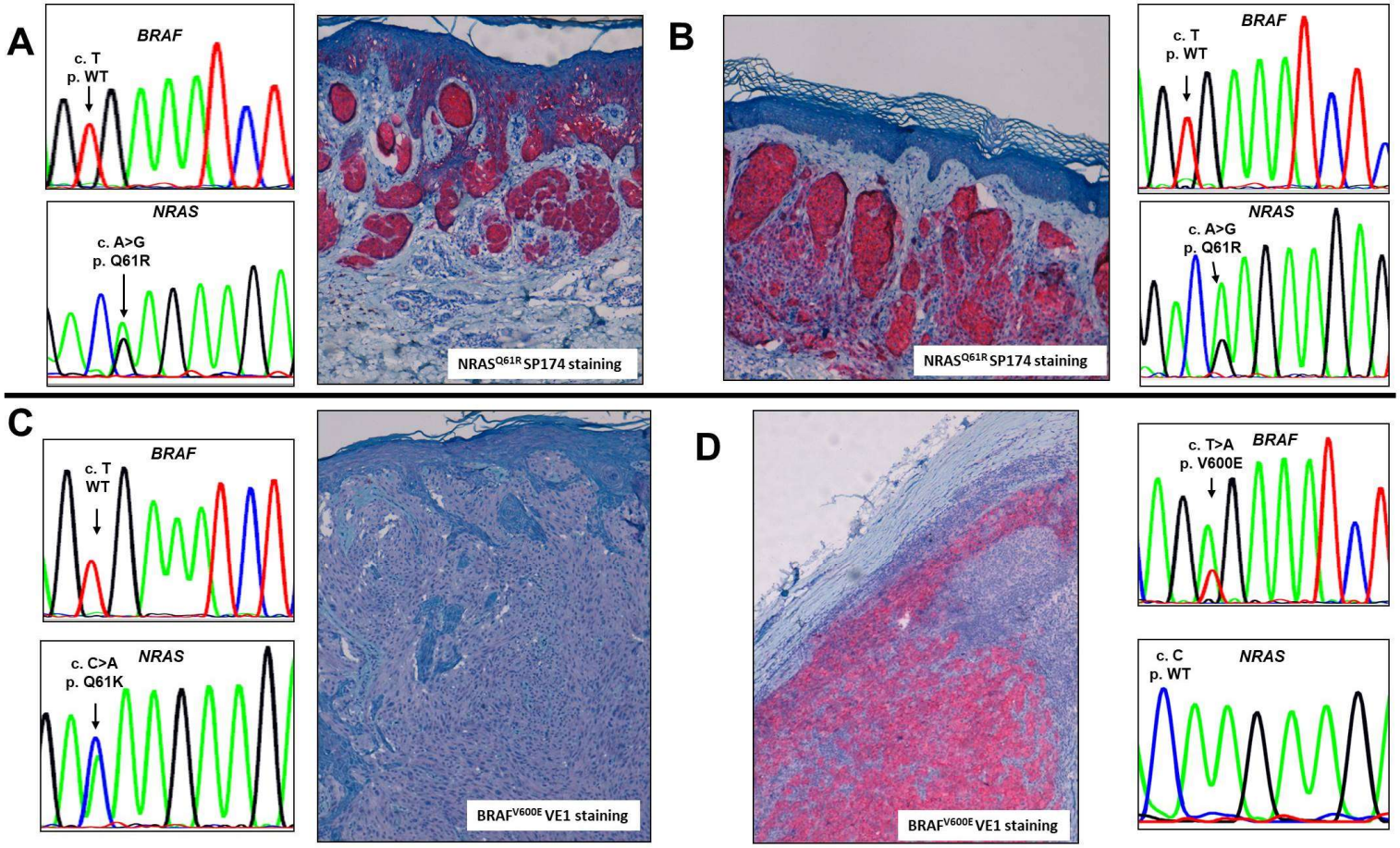


Figure 10. Illustrative cases of BRAF and NRAS mutational status in primary melanoma and related metastasis by sequencing and IHC. *(A-B). Intra-patient NRAS concordance. Primary melanoma (A) and cutaneous metastasis (B): positive $NRAS^{Q61R}$ SP174 immunostaining and $NRAS^{Q61R}$ mutation sequencing in both lesions. Magnification ×10. (C-D). Intra-patient BRAF and NRAS heterogeneity. (C) Primary melanoma: negative $BRAF^{V600E}$ VE1 immunostaining and BRAF wild type on mutation sequencing. Identification of the $NRAS^{Q61K}$ mutation. (D) Metastatic lymph node: positive $BRAF^{V600E}$ VE1 immunostaining and $BRAF^{V600E}$ mutation sequencing. Magnification ×10.*

Table 5. *Association between concordance of BRAF or NRAS mutational profiles with clinical characteristics of patients and metastases.*

	All patients	***BRAF* status**			***NRAS* status**		
		Discordance	*Concordance*	*p*	*Discordance*	*Concordance*	*p*
Characteristics of patients	**N=30**	**N=4**	**N=26**		**N=4**	**N=26**	
Sex							
M	17 (56.7)	2 (50.0)	15 (57.7)	0.77	3 (75)	14 (53.8)	0.43
F	13 (43.3)	2 (50.0)	11 (42.3)		1 (25)	12 (46.2)	
Synchronous primary/metastases							
No	9 (30)	2 (50.0)	7 (27.0)	0.48	0	9 (34.6)	0.18
Yes	17 (56.6)	2 (50.0)	15 (57.7)		3 (75)	14 (53.8)	
Characteristics of metastases	**N=39**	**N=4**	**N=35**		**N=5**	**N=34**	
Anatomical location of metastases							
Lymph Nodes	23 (59)	2 (50)	21 (60)	0.56	4 (80)	19 (56)	0.73
Skin	11 (28.2)	1 (25)	10 (28.6)		1 (20)	10 (29.4)	
Visceral	3 (7.7)	1 (25)	2 (5.7)		0	3 (8.8)	
Brain	2 (5.1)	0	2 (5.7)		0	2 (5.8)	

BRAF: BRAF proto-oncogene; *NRAS:* NRAS proto-oncogene: M: male; F: female

C. Immunohistochemistry

A total of 26 (26/69, 37.7%) samples showed positive immunostaining with anti-BRAF^{V600E} VE1 antibody with homogenous staining in 19 of 26 (73.1%) tissues. Staining intensity was strong in 22 tumors (22/26, 84.6%), moderate in 3 (11.5%) and week in 1 (1/26, 3.9%). Overall, primary melanomas (12/30, 40.0%) and metastatic lesions (14/39, 35.9%) showed similar frequency of *BRAF*^{V600E} staining (p=0.46). Regarding *NRAS*, 10 tumor tissues (10/69, 14.5%) were positive for *NRAS*^{Q61R} SP174 immunostaining and 59 were negative (59/69, 85.5%). Staining intensity was strong in 8 tumors (8/10, 80.0%), moderate or weak in 1 (1/10, 10.0%) each. No significant difference was observed in the frequency of *NRAS*^{Q61R} positive staining between the groups of primary melanomas (5/30, 16.7%) and metastatic lesions (5/39, 12.8%) (p=0.45).

All 11 melanomas arising in association with a melanocytic nevus showed concordance for *BRAF* and *NRAS* between melanoma cells and nevus cells, with 5 of them (5/11, 45.4%) showing a positive immunostaining for *BRAF*^{V600E} and 3 (3/11, 27.3%) for NRAS^{Q61R}; the remaining cases were negative for both mutations (Figure 13).

Sixteen tumor tissues (16/69, 23.2%) showed positive CD117/c-KIT expression, with 13 cases presenting a moderate and 3 cases a strong immunostaining. A trend for a higher prevalence of positivity was observed in the group of primary melanomas (9/30, 30.0%) than in metastatic samples (7/39, 17.9%), although it was not statistically significant (p=0.06).

Considering the mutational findings obtained by IHC, the intra-patient *BRAF*^{V600E} concordance between primary lesions and related metastases was present in 27/30 (90.0%) patients, including 10 (10/30, 33.3%) with *BRAF*^{V600E} positive lesions and 17 (17/30, 56.7%) negative. Intra-patient BRAF^{V600E} heterogeneity was observed in 10.0% (3/30) of patients (Figure 13). For NRAS^{Q61R}, the majority of patients (29/30, 96.6%) showed an intra-patient concordant immunostaining between the primary lesion and related metastases, being 4 (4/30, 13.3%) consistent for *NRAS*^{Q61R} positivity and 25 (25/30, 83.3%) for negative staining. A discrepant NRAS^{Q61R} staining was observed in 1 patient (3.3%). Finally, intra-patient concordance of CD117/c-KIT expression between primary lesions and related metastases was present in 22/30 (73.3%) patients, including 3 (3/30, 10.0%) with positive and 19 (18/30, 63.3%) with negative tissues (Table 4). Three patients with multiple metastases showed heterogeneity among their tumor tissues.

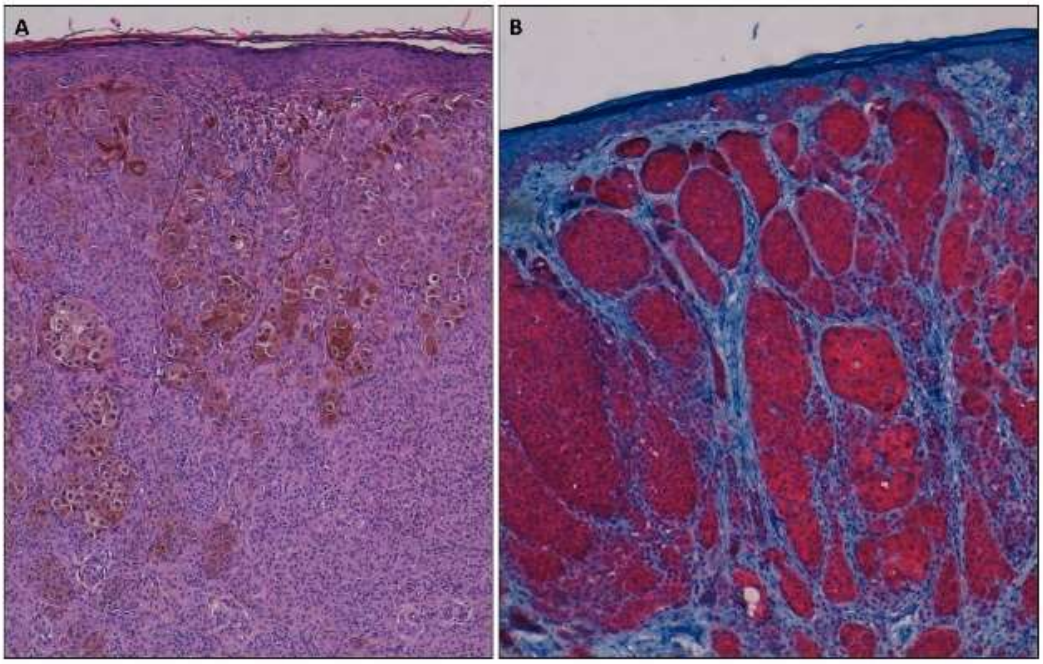


Figure 11. BRAFV600E VE1 staining in nevus-associated melanoma. *Concordant positive BRAF^{V600E} in melanoma and nevus: (A) Hematoxylin and Eosin staining. (B) BRAF^{V600E} VE1 staining. Magnification ×20.*

Correlation between molecular analysis and immunohistochemistry

BRAF^{V600E} VE1 immunostaining was consistent with *BRAF* molecular findings in 56 of 59 (94.9%) tissues, with 23 (23/56, 41.1%) *BRAF*^{V600E} mutated and 33 (34/56, 60.7%) wild type (Table 5). The 10 (10/69, 14.5%) samples harboring a *BRAF*^{V600K} mutation were indeed negative on IHC VE1 staining and were not included in this analysis. Discrepant findings were found in 3 of 59 (5.1%) samples: all cases were positive for immunostaining but wild type on molecular testing (both Sanger sequencing and competitive allele TaqMan^{TM} PCR). Two discordant (2/3, 66.7%) cases (1 primary melanoma and 1 metastasis) had a moderate and heterogeneous BRAF^{V600E} VE1 staining pattern, while the remaining sample (1 primary melanoma) presented a strong positive and homogeneous staining. *BRAF*^{V600E} VE1 antibody sensitivity was 100.0%, specificity 91.7%, accuracy 94.9%. Overall, the agreement between molecular testing and IHC was *"almost perfect*" (Cohen's kappa = 0.90; p<0.001).

SP174 *NRAS*^{Q61R} immunostaining and *NRAS* molecular analysis showed a high rate of consistency (60 of 61 cases, 98.4%), with 8 (8/60, 13.3%) tissues carrying the *NRAS*^{Q61R} mutation and 52 (52/60, 86.7%) the wild-type genotype. Cases carrying other

NRAS^{Q61} mutations showed no IHC SP174 staining and were not included in the analysis. Only one primary melanoma resulted positive for IHC *NRAS*^{Q61R} with weak and heterogeneous staining pattern but wild type at the molecular analysis. The sensitivity of the SP174 NRAS^{Q61R} antibody was 100.0%, specificity 96.2%, accuracy 96.7%. Overall, the agreement between molecular testing and IHC was *"almost perfect*" (Cohen's kappa = 0.87; $p<0.001$).

Regarding *c-KIT* gene, no mutation was found in all 16 samples with a high level of CD117/c-KIT by IHC while 2 of the 3 (66.7%) cases with *c-KIT* gene amplification showed an increased expression of CD117/c-KIT.

2.5 Discussion

In our study, we identified *BRAF* mutations in 46.7% of primary melanomas and in 48.7% of metastases and *NRAS* mutations in 20% and in 25.6%, respectively. The intra-patient molecular heterogeneity between primary melanoma and related metastases was detected in 13.3% of patients for both *BRAF* and *NRAS* genes and was not associated with clinicopathological characteristics of melanoma or metastases. We demonstrated consistency of *BRAF*^{V600E} and *NRAS*^{Q61R} mutational findings obtained by molecular analysis and IHC immunostaining for both overall mutation frequencies and intra-patient heterogeneity with an "almost perfect" agreement.

Recently, four different molecular melanoma subtypes were proposed based on the type of driver MAPK activating gene mutation, i.e. *BRAF* (35%-50% of cases), *NRAS* (10%-25%), *NF1* (~ 15%) mutated and triple-wild-type melanomas (~ 10%) including *c-KIT* mutated lesions [53]. These oncogenic alterations have been associated with different clinicopathological aspects of patients or tumors, such as age, anatomical site and degree of cumulative sun exposure.

Intra-patient molecular heterogeneity between primary melanoma and related metastases has important implications in clinical practice when metastatic patients with discordant lesions need to be treated with targeted therapy. In addition, comparison between primary lesions and related metastases can provide insights into the processes involved in metastatic progression. We observed an intra-patient discrepancy of *BRAF* mutations between primary and metastatic lesions in 13.3% of patients as evaluated by

molecular methods, in line with the discordance rate of 13.4% and 15.5% reported in two recent meta-analyses [53] [58]. A true biological manifestation of tumor heterogeneity but also technical issues (molecular-based methods compared to IHC-based) have been hypothesized to explain these discrepancies [53] [58] [59]. A higher mutational discordance rate has been reported with the increasing number of metastases, i.e. 8% in patients with one metastasis, 18% in patients with two metastases, and 20% in patients with three metastases [53]. All our patients with multiple metastases (23.3%) showed a consistent *BRAF* status between primary melanoma and all metastatic tissues.

Regarding *NRAS*, the intra-patient discordance rate was reported to range from 3% to 14.3% of the cases in few small studies [60] [61] [62] while no discrepancy was found in one [63]. A 13.3% discordant rate was observed in our patients, with the majority of them acquiring the *NRAS* mutation over time as part of disease progression.

Controversial results were reported on intra-patient *BRAF* or *NRAS* discrepancies rate according to the site of metastasis with a suggested, but not confirmed, higher rate of discordance between primary melanoma and visceral metastases as compared to lymph node metastases. The discrepancy rate for locoregional lymphatic metastases was indeed reported to range from 9.2 to 38% across four previous studies [60] [63] [64] [65] [66], while for visceral metastases, including brain, from 13 to 50% [60] [64] [67]. In our study, we did not observe any differences in the heterogeneity rate between primary melanoma and lymph node or visceral metastatic sites.

The intra-patient heterogeneity between primary melanoma and related metastases has been hypothesized to be due to the specific detection method with a higher rate of heterogeneity for molecular methods than for IHC approaches [58] [59] [65] [68] [69] [70]. Therefore, we compared $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutations using molecular methods (Sanger sequencing and allele specific Taqman® assays) and IHC. We observed very consistent findings between immunostaining and molecular methods for $BRAF^{V600E}$ mutation as detected in 95% of our samples, and for $NRAS^{Q61R}$ found in 98% of cases. Interpretation issues have been reported for weak and moderately stained lesions, as in 3 of our 4 discordant cases, since they have been considered either positive or negative in the literature, thus suggesting that caution is necessary in case of unclear staining [71] [72]. In addition, a rare VE1 antibody cross-reactivity with an unknown epitope may also be a possible explanation for false positive staining [73] [74]. Overall, in our cases the intra-patient heterogeneity between primary melanoma and metastatic tissues does not

seem to be attributable to methodological aspects since it was similar for IHC and molecular methods thus possibly reflecting the true biological heterogeneity during melanoma progression.

Few small studies investigated the concordance rate of *BRAF*^{V600E} and *NRAS*^{Q61R} mutations between nevus and melanoma in nevus-associated melanomas [55] [75] [76] [77]. For *BRAF* gene, a concordance rate varying from 75% to 100% was reported in 4 studies [55] [75] [76] [77] and for *NRAS*, 91% of melanomas and associated nevi were concordant in one study [76]. All 11 nevus-associated melanomas of our series showed a concordant of BRAF^{V600E} and NRAS^{Q61R} status between melanoma and nevus cells by immunostaining.

This study has a few limitations. *NF1* mutational analysis was not performed since the clinical significance of *NF1* mutations in melanoma is unknown and the interest as a potential therapeutic target is currently scarce. In addition, this study did not have adequate statistical power to evaluate intra-patient heterogeneity of *c-KIT* mutations, probably due to the low prevalence of acral lentiginous and mucosal melanoma subtypes. Finally, our results on intra-patient heterogeneity are mainly referred to lymph node metastasis due to the low number of visceral metastasis in our samples that might have underestimated the overall discordance rate. However, the evaluation of the discordance rate between primary melanoma and nodal metastases in disease-free patients is nowadays important for administration of targeted therapies in the adjuvant setting.

2.6 Conclusions

Our findings confirm that a relevant intra-patient heterogeneity between primary melanoma and related metastases exists independently of the technical approach, thus supporting the polyclonal model of melanoma progression. In addition, IHC and molecular methods provided highly consistent results in the detection of BRAF^{V600E} and NRAS^{Q61R} mutations supporting IHC as a rapid and cost-effective screening method in melanoma although a combined approach is necessary in cases with negative or doubtful immunostaining.

Chapter 3

Project 2: "Genetic susceptibility to familial melanoma in Mediterranean populations"

3.1 Introduction

Familial melanoma represents about 5-12% of melanoma cases [78]. "Familial melanoma" is defined if at least two individuals affected by melanoma are first-degree relatives or when three affected members have a second-degree relativeness in the same branch of the family tree. Since, in large population-based samples of families, the majority of melanoma kindreds failed to demonstrate a single major gene responsible for melanoma transmission, a more complex polygenic mechanism of melanoma susceptibility has also been hypothesized [79].

About 30-40% of familial melanoma cases are due to mutations in high-penetrance genes. The most frequently mutated gene is *CDKN2A* (20-40%), while *CDK4, TERT, POT1* and BRCA1 associated protein 1 (*BAP1)* are involved in about 10% of the cases [80]. The remaining 55% of familial melanoma seems due to polymorphisms in low-penetrance genes in combination with exposure to environmental risk factors [81].

Among intermediate-penetrance genes, the melanocortin 1 receptor (*MC1R)* gene is a highly polymorphic gene in white populations and one of the key regulators of human skin and hair pigmentation [20]. Carriers of *MC1R* variants show characteristic phenotypic traits (i.e. red hair, pale skin, and freckles) and an increased sensitivity to sunlight [82], and are at greater cutaneous melanoma risk compared to wild-type individuals [83] [84]. In addition, inheritance of *MC1R* variants with *CDKN2A* mutations has been shown to increase penetrance of melanoma in families carrying *CDKN2A* mutations [84].

3.1.1 High-penetrance genes

CDKN2A and *CDK4* are well-known high-risk melanoma susceptibility genes, identified about 20 years ago [85] [86]. Germline mutations of the *CDKN2A* gene allow cells to escape cell cycle arrest in G1, leading to uncontrolled cell proliferation and growth

[87]. and are described in 20% of familial melanoma patients. The frequency increases to 40% in families with three or more affected members. Early age at diagnosis, presence of multiple primary melanoma (MPM) and pancreatic cancer within the family have shown a significant association with *CDKN2A* mutations, although the effects vary widely across continents [88].

The *CDK4* oncogene plays an important role at the G1/S phase cell cycle checkpoint [89]. Mutations in *CDK4* are rare, with only 18 families carrying the mutation worldwide [90].

The introduction of Next-Generation Sequencing (NGS) techniques led to the identification of new melanoma susceptibility genes implicated in melanoma development.

BAP1, was identified as a tumor suppressor gene playing a role in DNA damage repair [91]. Later observations tested *BAP1* as a candidate susceptibility locus for familial melanoma, because of the identification of *BAP1* mutations in families, with individuals who developed cutaneous melanoma. Additionally, other studies showed *BAP1* increases predisposition to other cancer types. To date, only 15% of *BAP1* mutation carriers are reported with cutaneous melanoma [92] [93].

POT1 gene is a crucial member of the shelterin complex proteins, important for telomere maintenance [94] and was identified as a new high-penetrance susceptibility gene for familial melanoma. Mutations in *POT1* gene have been identified in few melanoma families. The p.S270N germline variant has been reported as a founder mutation in five families in Emilia-Romagna where the frequency of this variant is comparable with that of *CDKN2A* mutations [95] [96].

Additional shelterin complex genes, such as adrenocortical dysplasia protein homolog (*ACD)* and TERF2 interacting protein *(TERF2IP)* were later found to be mutated in familial melanoma patients. Whole-genome, exome and targeted sequencing methods were used to screen 510 melanoma families and authors found novel *ACD* and *TERF2IP* mutations [97]. Overall, mutations in three of the shelterin genes, *ACD*, *TERF2IP* and *POT1*, account for approximately 9% of high-density cutaneous melanoma families lacking mutations in the other high-penetrance genes [95] [96].

TERT is the ribonucleoprotein complex that maintains telomere length [98]. *TERT* mutations induce increased expression of telomerase, promoting telomere stabilization

and acting on cell aging, turnover and senescence [80]. Huang and colleagues showed the role of *TERT* in melanoma using whole-genome sequencing to discover recurrent somatic *TERT* promoter mutations. Results reported the presence of -124 bp and -146 bp in 17 of 19 melanoma cell lines, suggesting that mutations in this region may play an important role in melanoma [99]. A novel germline variant occurring in the promoter region of the *TERT* gene was identified as a high-risk predisposition allele in two melanoma-dense families from Germany and UK [98] [100].

3.1.2 Intermediate-penetrance genes

In almost half of melanoma families, a polygenic susceptibility as result of coinheritance of multiple intermediate- and/or low-risk alleles has been suggested. To date, two intermediate-penetrance genes, *MC1R* and melanocyte inducing transcription factor (*MITF)*, predisposing to melanoma have been identified [80].

The *MC1R* gene has a key role in cutaneous pigmentation [101], [102]. Specific *MC1R* variants (R142H, R151C, R160W, and D294H) are classified as red hair color (RHC) or "R" variants and have been strongly associated with fair skin, freckling, UV radiation sensitivity, and inability to tan [103], [104]. In addition, inheritance of *MC1R* variants with *CDKN2A* mutations has been shown to increase penetrance of melanoma in families carrying *CDKN2A* mutations [84].

The *MITF* gene is a master regulator of melanocyte homeostasis encoding a lineage-specific transcription factor, involved in cell survival, differentiation, and proliferation [105]. A rare functional variant p.E318K in the *MITF* gene has been implicated in familial melanoma because it alters MITF stimulation, increasing the MITF transcriptional activity with upregulation of downstream genes [106].

3.1.3 Low-penetrance genes

In addition to intermediate-penetrance genes, other low-penetrance genes have been associated with melanoma. Agouti signaling protein (*ASIP*), tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*) and oculocutaneous albinism type II (*OCA2*) are involved in pigmentation [107] [108] [109] [110]. *ASIP* encodes for an antagonist of α-MSH, which competitively binds to MC1R, thereby preventing MC1R-mediated stimulation of eumelanin synthesis. Similarly to *MC1R* RHC variants, melanoma-

associated *ASIP* single-nucleotide polymorphisms (SNPs) have been linked to red hair and skin freckling. TYR impacts eye color and tanning response, where activity of the enzyme tyrosinase influences the ratio of eumelanin to pheomelanin, and thus TYR alterations can contribute to a fair skin phenotype. *TYR* SNPs associated with blue eye color and sun sensitivity have been significantly associated with melanoma. TYRP1 stabilises the protein encoded by TYR, and thus mutations in this gene can also affect tanning response [111]. Further to known phenotypic associations with melanoma, increased risk has been reported for pigment related SNPs in the HERC2/OCA2 region on chromosome 15q13.1. The two SNPs most significantly associated with melanoma risk are rs1129038 and rs12913832, the latter being a key determinant of human blue-brown eye color [78] [112] [113].

Familial melanoma may also occur in the clinical context of hereditary syndromes, such as Li-Fraumeni syndrome, Breast and Ovaric Cancer hereditary syndrome (*BRCA1* e *BRCA2*) or xeroderma pigmentosum (*XPA-XPG*) [91].

Concerning molecular testing for genetic screening of familial melanoma, discrepant indications have been suggested. According to the AIOM 2019 guidelines, *CDKN2A* genetic testing can be recommended in patients with familial and multiple primary melanoma, even if it is more important for prevention than for clinical diagnosis.

Variability of melanoma incidence and penetrance of molecular alterations of the *CDKN2A* gene among different countries makes difficult to define unique guidelines. The recommended rule for the recruitment of eligible patients for genetic testing is specified as follows:

- In countries with low incidence of melanoma, the presence of two melanomas or a melanoma and a pancreatic cancer in the family is a sufficient condition.
- In countries with medium- or high incidence of melanoma, the appropriate candidates are those with three or more primary melanomas and/or families with three cases of invasive melanomas or two cases of melanoma and a pancreatic cancer (Figure 12) [114].

Geographic area/population with low melanoma incidence	Geographic area/population with moderate-high melanoma incidence
• Two (synchronous or metachronous) primary melanomas in an individual and/or • Families with following clinical features in first- or second-degree relatives on the same side of the family: o Two cases of melanoma (one invasive) or o One case of melanoma and one case of pancreatic cancer	• Three primary melanomas in an individual and/or • Families with the following clinical features in first- or second-degree relatives on the same side of the family: o Three cases of melanoma (one invasive) or o Two cases of melanoma and one case of pancreatic cancer or o One case of melanoma and two cases of pancreatic cancer

Figure 12. *Recommendations for CDKN2A molecular testing* [80].

3.2 Aim

We investigated the mutational profile of high and intermediate-penetrance genes in familial melanoma cases collected from centres of Mediterranean countries and included in the international database of the MelaNostrum Consortium. The aim of the project was the evaluation of the genetic predisposition in this population, which has peculiar characteristics of sun exposure and has not been well characterized in large global genetic studies until now.

3.3 Materials and Methods

A. Consortium Melanostrum

The MelaNostrum consortium is an association of researchers and clinicians from Mediterranean countries. Its primary goals are as follows:

(i) to identify genetic, environmental and phenotypic features associated with melanoma risk;

(ii) to investigate disease development and clinical outcomes using molecular classification of melanoma;

(iii) to study the role of genetics and immunity in melanoma progression and response to treatment.

MelaNostrum collects and analyzes data and biological samples from 16 centres across the Mediterranean area. MelaNostrum centres are illustrated in Figure 13.

Figure 13. *Geographic distribution of participating centres of Melanostrum Consortium.*

B. Families and sample selection

Melanoma families were enrolled from 2019 to 2020 at MelaNostrum centres in Spain (Barcelona and Valencia), Greece (Athens) and Italy (L'Aquila, Rome, Cesena, Genoa and Padua). Inclusion criteria were as follows: presence of at least two 1st or 2nd degree relatives suffering from melanoma in the family and availability of information on the genetic status of the high-penetrance genes *CDKN2A* and *CDK4*. In addition, the pedigree was drawn for each family.

For each familial member, demographic and phenotypic characteristics were collected through the administration of a specific questionnaire developed by each centre.

The questionnaire included also information on personal and family history of melanoma and/or other cancers, solar exposure habits, skin reaction after 30 minutes of sun exposure and tan intensity after a prolonged exposure to UV. Moreover, the dermatological physical examination allowed to collect data on hair and eye color, on the presence of solar freckles, lentigos and actinic keratoses, on the number of common melanocytic nevi and on the presence of clinically atypical nevi. The following histopathological features of melanoma were also recorded: clinical-pathological variant, Breslow thickness, regression, ulceration and presence of mitosis.

Considering the differences in terms of variables and categories used across centres to collect data, we harmonized the information and created a homogeneous database to limit missing data or misclassification issues.

We merged data from all participating Centres in a comprehensive single database, divided into two parts. The first part included family information, such as country, number of affected members in the family, degree of relatedness, presence of MPM or other cancers and available genetic data for high and intermediate risk genes. In the second part, epidemiological, clinical and genetic characteristics of each affected or unaffected member of those families were recorded. In detail, age, sex, skin type, eye and hair color, number of melanocytic nevi, personal history of cancers, available individual genetic data for high and intermediate risk genes and, for patients, histological characteristics of the melanoma were collected.

C. ***Molecular analysis***

For each patient, germline genetic analysis of melanoma predisposition genes *CDKN2A*, *CDK4*, and, for some patients, of the promoter region of *TERT*, *TERF1P*, *BAP1*, *ACD* and *POT1* was carried out at the reference laboratory of each centre or at the National Institutes of Health (NIH), USA.

Regarding samples collected in L'Aquila, DNA was extracted from whole blood using QIAmp DNA Blood Midi Kit (QIAgen, Hilden, Germany).

Mutational screening of exons 1α, 1β, 2, and 3, including the exon–intron boundaries of *CDKN2A*, exon 2 of *CDK4*, promoter region of the *TERT* gene (from −497 bp to the ATG start site) and the entire open reading frame of *MC1R*, was performed by PCR and direct sequencing on a 3500 Genetic-Analyzer (Thermo-Fisher, Foster City, California, USA).

PCR amplification of the regions of interest was performed in a Simply-Amp PCR-System (Thermo Fisher, Foster City, CA), using primers reported in Table 2. PCR experiments were performed with 1.25 U of AmpliTaq Gold-360 (Thermo Fisher) in a 50-µL volume, containing the 1X reaction buffer provided by the manufacturer, 1.6 mmol/L of $MgCl_2$, 200 µmol/L of each deoxynucleoside triphosphate, 0.2 µmol/L of each primer, and 50 ng of genomic DNA template. 10% glycerol (for *TERT* promoter amplification) was added to the reaction solution.

PCR amplification of *CDKN2A* and *CDK4* genes was performed as follows: 7 min at 95°C, followed by 35 cycles of 94°C for 1 min, Tm for 1 min and 72°C for 1 min, and in the end 7 min at 72°C. For the promoter region of *TERT* PCR amplification was performed as previously described for somatic analysis [3]. Water controls and positive controls were run in parallel with patient's DNA samples.

Table 6. *List of primers used for PCR amplification.*

Target gene	Primer ID	Sequence (5' > 3')	Tm [°C]	Reference
CDKN2A exon 1α	forward	CTCCAGAGGATTTGAGGGAC	54.0	[115]
	reverse	GCGCTACCTGATTCCAATTC		
CDKN2A exon 1β	forward	GGTCCCAGTCTGCAGTTAAG	51.5	
	reverse	GTCTAAGTCGTTGTAACCCG		
CDKN2A exon 2	forward	GGAAATTGGAAACTGGAAGC	53.0	
	reverse	GATTGGCGCGTGAGCTGA		
CDKN2A exon 3	forward	TGGACCTGGAGCGCTTGA	55.0	
	reverse	GAAAACTACGAAAGCGGGG		
CDK4 exon 2	forward	CTGCAGGCTCATACCATCCT	59.0	
	reverse	ATCATCACACCCCACCTATAGG		
TERT	forward	CACCTTCCAGCTCCGCCTCCT	60.0	
	reverse	CCCACGTGCGCAGGAC		

Tm: melting temperature; *CDKN2A*: cyclin dependent kinase inhibitor 2A; *CDK4*: cyclin dependent kinase 4; *TERT*: telomerase reverse transcriptase

Genotyping assay of the g.124493086C>T (p.S270N) mutation of the *POT1* gene was carried out using TaqMan SNP Genotyping Assays (Thermo-Fisher). PCRs containing 40 ng of DNA, ×1 TaqMan Genotyping Master Mix; ×1 TaqMan genotyping assay mix (Thermo-Fisher), and water to reach the final volume of 10 µl were performed in 96-well plates using the standard TaqMan protocol on a 7500 Fast Real Time-PCR System (Thermo-Fisher). Water controls and positive controls were run in parallel with patients' DNA samples.

Analysis of the *TERF1P*, *BAP1, ACD* and *POT1* genes was performed by whole exome sequencing at the NIH.

For each mutation of all specimens included in the study, we assigned a reference SNP ID number, or "rs" ID according to the National Center for Biotechnology

Information (NCBI) database. Then, we annotated each SNPs on the Genome Reference Consortium Human Build 38 patch release 13 (GRCh38.p13), on the base of the Human Genome Variation Society (HGVS)-nomenclature to define the genomic position of each variant.

Variants were then classified for functional significance, using ClinVar archive and Varsome database [116] as "Benign", "Likely benign", "Variant of Uncertain Significance (VUS), "Likely pathogenic" and "Pathogenic". In case of disagreement between ClinVar and Varsome, functional published studies were reviewed to assess the significance of the variant. Finally, the clinical meaning of variants was classified in five categories: 1=Benign; 2= Likely benign; 3= VUS, 4= Likely pathogenic and 5=Pathogenic according to the American College of Medical Genomics (ACMG) and the Association of Molecular Pathology (AMP) guideline [117].

D. Statistical Analysis

Variables regarding melanoma families were categorized as follows: geographic origin (Italy, Spain or Greece), number of affected members in the family (2 or > 2), number of MPM cases (none, 1, 2 and >2), grade of relatedness (first or second). Family history of other malignancies was grouped as present or absent.

We evaluated and grouped the following clinical characteristics for each individual family member:

- ✓ age at diagnosis: ≤40; >40
- ✓ phototype: I/II, III/IV
- ✓ Hair color: red/blond, light brown, dark brown/black
- ✓ Eye color: blue/green, light brown, dark brown
- ✓ Total number of melanocytic nevi: ≤50; >50
- ✓ Breslow thickness: in situ, invasive (≤1 mm; >1mm)
- ✓ Histological subtype: superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma and others (such as spitzoid melanoma, unknown primary melanoma)

The mutational status of the *CDKN2A* gene was classified into 3 groups: wild type and classes 1-2 (benign/ likely benign); class 3 (VUS); classes 4-5 (likely pathogenic/ pathogenic).

For the other high-penetrance genes, the mutational status was categorized as wild-type or mutated.

Patients were categorized as wild type or mutated for the *MC1R* gene if they presented at least one *MC1R* variant; in addition, individuals with *MC1R* variants were divided into two groups: "RHC" patients, who presented at least one RHC variant (D84E, R142H, R151C, R160W, D294H, I155T) and "NRHC" patients, who carried any other variants.

Chi square test or Fisher's exact test were used, as appropriate, to test for the significance of the mutation frequency according to the characteristics of families. Results were considered statistically significant with a p-value of <0.05. Logistic regression and decision tree classification were used to perform the multivariate analysis.

Statistical analysis was realized by the SPSS statistical package (IBM) version 25.0 (SPSS Inc., Chicago, IL, USA) and using the software GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).

3.4 Results

3.4.1 Families

A. Families' characteristics

We analyzed 852 Melanostrum melanoma families: 520 families (61.0%) from Spain, 318 (37.3%) from Italy and 14 (1.6%) from Greece.

Families with 2 affected members were 641 (75.2%), those with 3 affected members were 155 (18.2%) and those with 4 or more were 56 (6.6%). Families with 1st degree affected relatives were 725 (85.0%) and those with 2nd degree relatedness were 128 (15.0%). MPM occurred in 28.5% (243/852) of the families. Of those, families with one MPM patient were 213 (87.6%), those with 2 or more MPM members were 30 (3.5%).

Overall, family history of other malignancies (excluding keratinocytes cancers, KC) was present in 608 (71.4%) families. In detail, breast cancer was detected in 162 families (162/852, 19.0%), colon cancer in 92 families (92/852, 10.8%), kidney cancer in 26 families (26/852, 3.0%), lung cancer in 124 families (124/852, 14.5%), pancreatic cancer in 51 families (51/852, 6.0%), prostatic cancer in 76 families (76/852, 8.9%), and sarcoma in 12 families (12/852, 1.4%). Families with other types of cancers were 200 (200/852, 23.5%) (Table 7).

Table 7. *Clinicopathological characteristics of families.*

Characteristics		**Families N=852 (%)**
Country	Greece	14 (1.6)
	Italy	318 (37.3)
	Spain	520 (61.0)
Grade of relatedness	1st degree	725 (85.0)
	2nd degree	128 (15.0)
Affected members	2 members	641 (75.2)
	3 members	155 (18.2)
	≥4 members	56 (6.6)
Number of MPM in families	Single	609 (71.5)
	Multiple	243 (28.5)
	1 MPM patient	213 (87.6)
	2 MPM patients	29 (12.0)
	3 MPM patients	1 (0.4)
Family history of cancer	No	244 (28.6)
	Yes	608 (71.4)
	Missing	488 (35.7)
Type of other cancers	KC	118 (8.6)
	Breast	34 (2.5)
	Colon	9 (0.6)
	Kidney	6 (0.4)
	Lung	14 (1.0)
	Pancreas	5 (0.3)
	Prostate	10 (0.7)
	Sarcoma	2 (0.1)
	Other	32 (2.3)

MPM: multiple primary melanoma; KC: keratinocytic cancer

B. Mutational findings

We analyzed the germline mutational status of all families. Regarding high-risk susceptibility genes, we identified 124 *CDKN2A* variants; of those, 74.2% were class 4-5 (Likely pathogenic/Pathogenic), variants, 10.5% were class 3 (VUS) and 15.3% were class 1-2 (Benign/Likely benign).

Overall, 12.3% (105/852) of families carried the class 3 to 5 variants and 2.2% (19/852) the class 1-2, that were considered as wild-type families for the following analysis (Figure 14). Stratifying by country, in Greece there were 7 families with *CDKN2A* variants (7/14, 50%), in Italy 29 (29/318, 9.1%), in Spain 88 (88/520, 17.0%). Details of *CDKN2A* mutations across countries are reported in Table 8.

Table 8. *CDKN2A variants across countries.*

	***CDKN2A* nucleotide change**	**p16INK4a amino acid change**	**Greece N= 7 (%)**	**Italy N= 29 (%)**	**Spain N= 88 (%)**
5' UTR	c.-25C>T	-	0	0	3 (3.4)
	c.-33G>C	-	0	2 (6.9)	0
	c.-34 G>T	-	0	0	3 (3.4)
	c.60ins16	-	0	0	1 (1.1)
Exon 1α	c.13G>A	p.A5T	0	0	1 (1.1)
	-	p.S7Efs*8	0	0	1 (1.1)
	c.31C>A	p.P11T	0	0	1 (1.1)
	c.71G>C	p.R24P	6 (85.7)	3 (10.3)	0
	c.92C>T	p.T31M	0	1 (3.4)	0
	c.104G>A	p.G35E	0	0	2 (2.3)
	c.106delG	p.A36Rfs*17	0	0	6 (6.8)
	c.116A>G	p.N39S	0	0	1 (1.1)
	c.131insA	-	0	0	1 (1.1)
	c.142C>A	p.P48T	0	6 (20.7)	0
	c.149A>G	p.Q50R	0	0	2 (2.3)
	-	p.P75fs	0	1 (3.4)	0
Exon 2	c.151-2A>G	-	0	0	0
	c.151-13T>C/c.151-18T>C	-	0	1 (3.4)	0
	c.164G>T	p.G55V	0	0	1 (1.1)
	c.167G>T	p.S56I	0	1 (3.4)	0
	c.176T>G	p.V59G	0	1 (3.4)	9 (10.2)
	c.194T>C	p.L65P	0	1 (3.4)	1 (1.1)
	c.212A>G	p.N71S	0	1 (3.4)	5 (5.7)
	c.238C>T	p.R80*	0	0	1 (1.1)
	c.241C>T	p.P81S	0	0	1 (1.1)
	c.249C>A	p.H83Q	0	1 (3.4)	0
	c.250G>T	p.D84Y	0	0	1 (1.1)
	c.259C>T	p.R87W	0	1 (3.4)	2 (2.3)
	c.262G>T	p.E88*	0	0	1 (1.1)
	c.280_282insAG	-	0	1 (3.4)	0
	c.295C>T	p.R99W	0	0	1 (1.1)
	c.301G>T	p.G101W	0	6 (20.7)	28 (31.8)
	c.305C>T	p.A120V	0	0	1 (1.1)
	c.318G>A	p.V106V	0	0	1 (1.1)
	c.335G>C	R112P	0	0	1 (1.1)
	c.341C>T	p.P114L	0	1 (3.4)	0
	c.352G>A	p.A118T	0	0	0
	c.358delG	p.E120fs*145	0	0	4 (4.5)
	c.361G>A	p.A121T	0	0	0
	c.370C>T	p.R124C	0	0	1 (1.1)
	c.379G>T	p.A127S	0	0	2 (2.3)
	c.444C>A	p.A148T	2 (28.5)	0	0
	c.457G>A	p.D153N	0	0	2 (2.3)
Intron 2	IVS1+37G>C	-	0	0	2 (2.3)
	IVS2-2A>G	-	0	0	1 (1.1)
	IVS2-105A>G	-	0	0	1 (1.1)

CDKN2A: cyclin dependent kinase inhibitor 2A; UTR: untraslated region

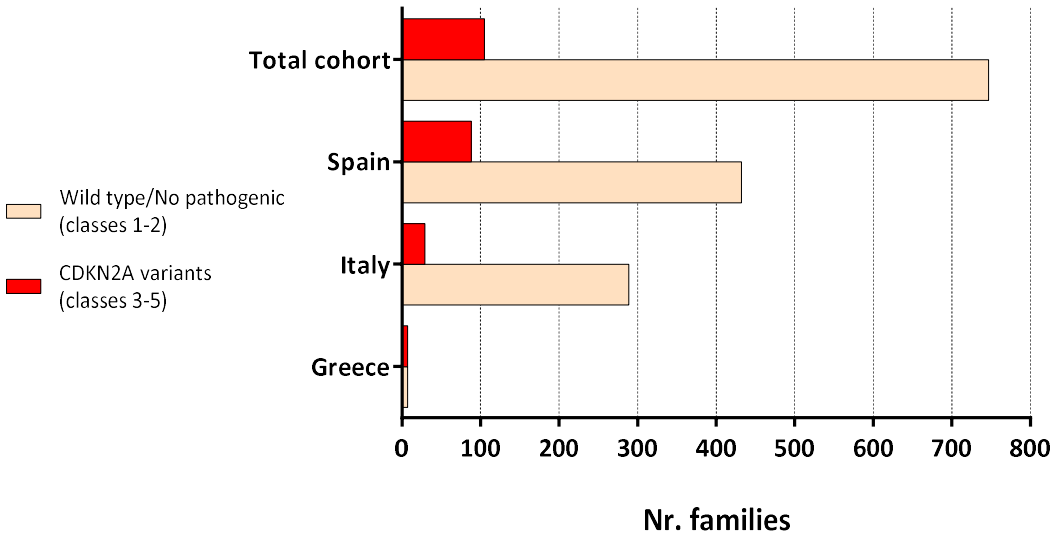
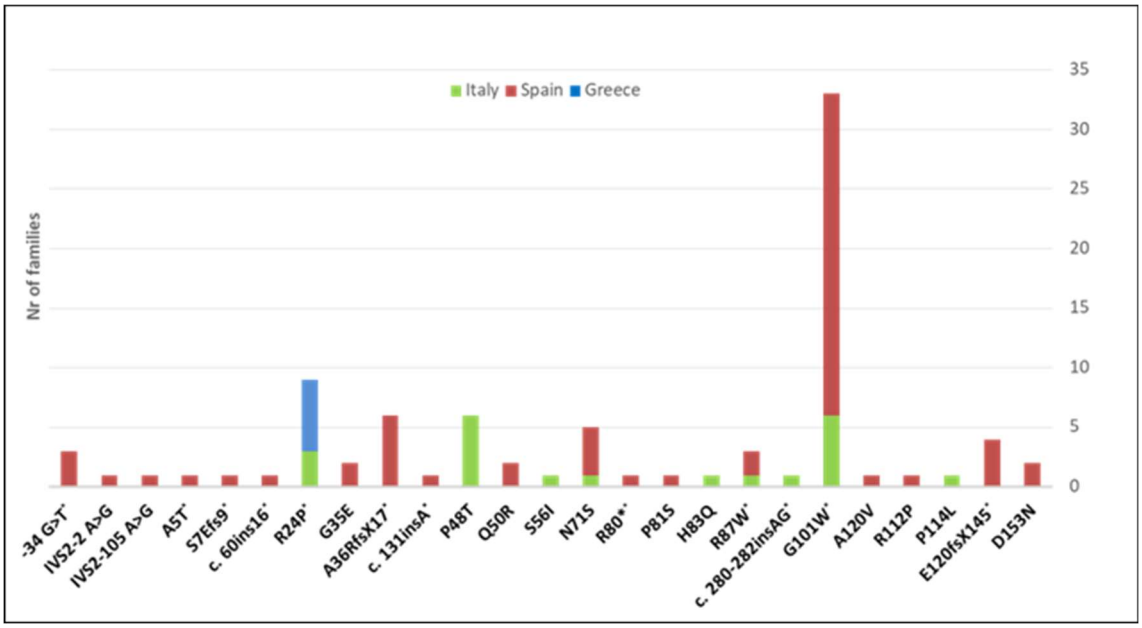


Figure 14. *Frequency of CDKN2A variants stratified by country.*

The most frequent *CDKN2A* variant was G101W, identified in 34 families (27.4%); of these, 17.6% (6/34) and 82.4% (28/34) were Italian and Spanish families, respectively. Greek families did not carry this variant. The second most frequent variant was R24P, detected in 0.9% of the families; of this, 62.5% were Greek families and 37.5% were Italian families. This variant was not found in Spanish families (Figure 15).



X: class 5 variants

Figure 15. *Classes 4 and 5 CDKN2A variants identified in the study.*

Considering *CDKN2A* wild-type families, *POT1* gene mutations were reported in 15 of the 382 tested families (15/382, 3.9%), with the most frequent mutation, the S270N, detected in 5 families (5/15, 33.3%). Mutations of *TERF2IP* gene were identified in 14 of 374 screened families (14/374, 3.7%); in particular, class 4-5 variants (Q354*, T346A, T346I, A348S, L350V) were found in 5 families each (5/15, 33.3%). *ACD* gene mutations were identified in 4 families (4 of 376 tested, 1.1%), with the D392fs pathogenetic variant found in 1 family (1/4, 25%); finally, only one family presented a *BAP1* mutation (1 of 407 screened, 0.2%), the S455P (Figure 16). Details of identified gene mutations are reported in Table 9.

No families showed mutations in the core promoter region of *TERT* and in the *CDK4* gene.

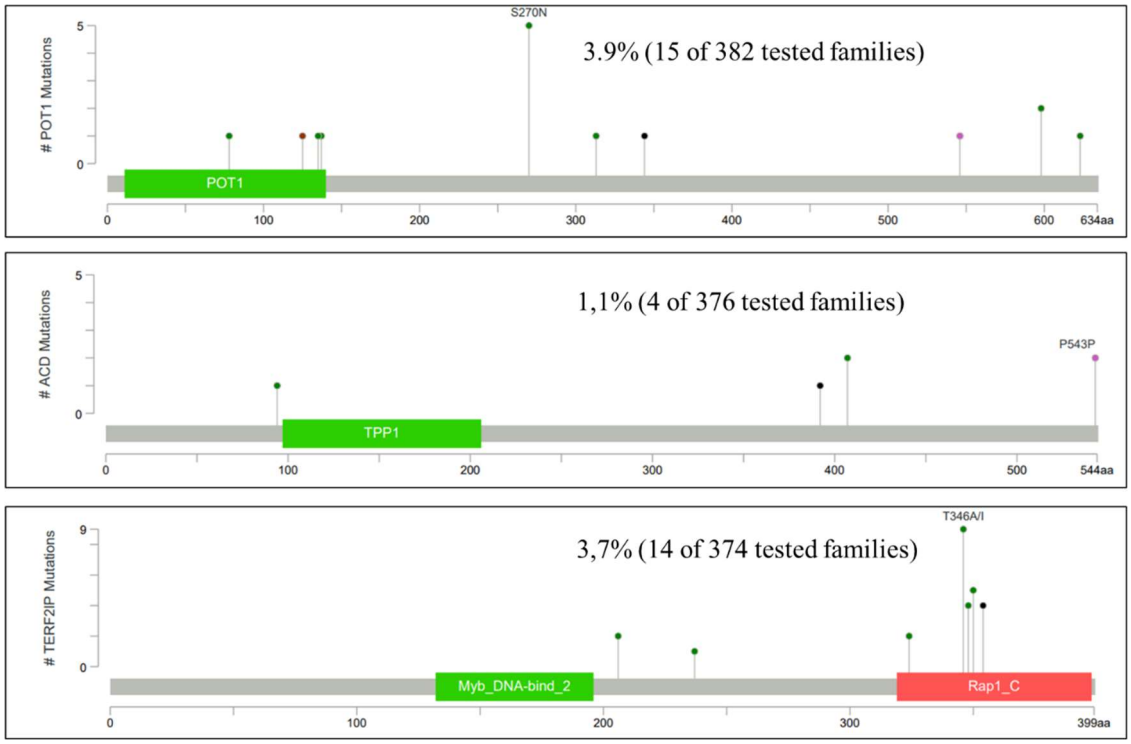


***Figure 16**. Distribution of POT1, ACD and TERF2IP variants across gene sequence.*

Table 9. *Details of mutations in ACD, BAP1, POT1, and TERF2IP genes.*

Genes	**Amino-acid change**	**Greece N=7 (%)**	**Italy N=237 (%)**	**Spain N=132 (%)**
ACD	V94I	1 (14.3)	0	0
	D392fs	0	0	1 (0.7)
	S407L	1 (14.3)	1 (0.4)	0
	Amino-acid change	**Greece N=7 (%)**	**Italy N=237 (%)**	**Spain N=163 (%)**
BAP1	S455P	0	0	1 (0.6)
	Amino-acid change	**Greece N=7 (%)**	**Italy N=241 (%)**	**Spain N=134 (%)**
POT1	r.125_255del	0	0	1 (0.7)
	I78T	0	0	1 (0.7)
	A135T	0	1 (0.4)	0
	R137H	0	1 (0.4)	0
	S270N	0	5 (2.1)	0
	D313N	0	0	1 (0.7)
	E344*	0	0	1 (0.7)
	D598N	0	0	2 (1.5)
	Q623H	0	2 (0.8)	0
	Amino-acid change	**Greece N=7 (%)**	**Italy N=237 (%)**	**Spain N=130 (%)**
TERF2IP	S206Y	0	2 (0.8)	0
	E237Q	0	1 (0.4)	0
	K324E	1 (14.3)	0	0
	T346A	0	4 (1.7)	2 (1.5)
	T346I	0	4 (1.7)	2 (1.5)
	A348S	0	5 (2.1)	2 (1.5)
	L350V	0	4 (1.7)	2 (1.5)
	Q354*	0	4 (1.7)	1 (0.8)

ACD: adrenocortical dysplasia protein homolog, *BAP1*: BRCA1 associated protein 1, *POT1*: protection of telomeres 1, *TERF2IP*: TERF2 interacting protein

C. Association of mutational status with family characteristics

We analyzed the association of epidemiological and clinical characteristics of the families with the presence of a specific germline mutational status.

Regarding *CDKN2A*, we found a significant association of *CDKN2A* mutations with the number of affected members (OR 2.75, CI 95% 1.80-4.20, p <0.0001) and with the number of MPM in the family (OR 5.19, CI 95% 3.38-9.97, p <0.0001). In detail, families with a higher number of affected relatives (more than 2) or a higher number of patients with MPM in the family had higher frequency of *CDKN2A* mutations. Instead,

we did not observe significant association of *CDKNA* mutations with the degree of relatedness (first or second) (p=0.672).

Considering the occurrence of additional cancers in the family, we observed a higher frequency of *CDKN2A* mutations in families with pancreatic (OR 5.43, CI 95% 2.97-9.96, p<0.0001) and breast cancers (p=0.045), while no additional association was observed with other cancers. In addition, when we analyzed KC no difference was observed in the rate of *CDKN2A* variants between families with KC (10.4%) and without (13.6%) (p=0.458). Multivariate analysis confirmed the occurrence of pancreatic carcinoma (OR 4.55, IC 95% 2.32-8.91), the presence of MPM cases (OR 4.34, IC 95% 2.77-6.80) and the number of affected members (OR 2.12, IC 95% 1.34-3.37) as independent predictors of *CDKN2A* mutations in the family (Table 10).

When we stratified the analysis by country for the univariate analysis, we confirmed the significant association of *CDKN2A* mutations with pancreatic cancer in Italy (p=0.009) and in Spain (p<0.0001), but not in Greece. Moreover, we observed a borderline significant association of *CDKN2A* mutations and the presence of KC in Spain (p=0.051). We did not find significant association of *CDKN2A* mutations with the grade of relatedness across countries.

Table 10. *Significant associations of CDKN2A with clinical features of families.*

	Tot Nr (%)	*CDKN2A* wild type/no pathogenic Nr (%)	*CDKN2A* variants Nr (%)	OR	95% CI		p*	OR	95% CI		p#
				Univariate analysis				*Multivariate analysis*			
Families	852 (100)	747(87.7)	105 (12.3)								
Nr. of affected members				**2.75**	1.80	4.20	<0.0001	**2.12**	1.34	3.37	0.001
2	641 (100)	582 (90.8)	59 (9.2)								
≥3	211 (100)	165 (78.2)	46 (21.8)								
Presence of MPM				**5.19**	3.38	7.97	<0.0001	**4.34**	2.77	6.80	<0.0001
No	609 (100)	569 (93.4)	40 (6.6)								
Yes	243 (100)	178 (73,3)	65 (26,7)								
Pancreatic cancer				**5.43**	2.97	9.96	<0.0001	**4.55**	2.32	8,91	<0.0001
No	801(100)	716 (89.4)	85 (10.6)								
Yes	51 (100)	31 (60.8)	20 (39.2)								
Breast cancer				**1.67**	1.07	2.60	0,045				ns
No	670 (100)	595 (88.8)	75 (11.2)								
Yes	162 (100)	139 (82.7)	28 (17.3)								

CDKN2A: cyclin dependent kinase 2A; OR: odd ratio; CI: confidence interval; MPM: multiple primary melanoma

The decision tree regression analysis stratified by country identified different algorithms of classification across Mediterranean populations (Figure 17). In Italy, evaluation of the number of affected members followed by family history of pancreatic cancer can be used to predict a higher risk of carrying *CDKN2A* mutations, while in Spain the algorithm including first the presence of MPM patients and then the family history of pancreatic cancer can predict the presence of *CDKN2A* mutations.

The limited number of families from Greece did not allow to perform the decision tree regression analysis in those families.

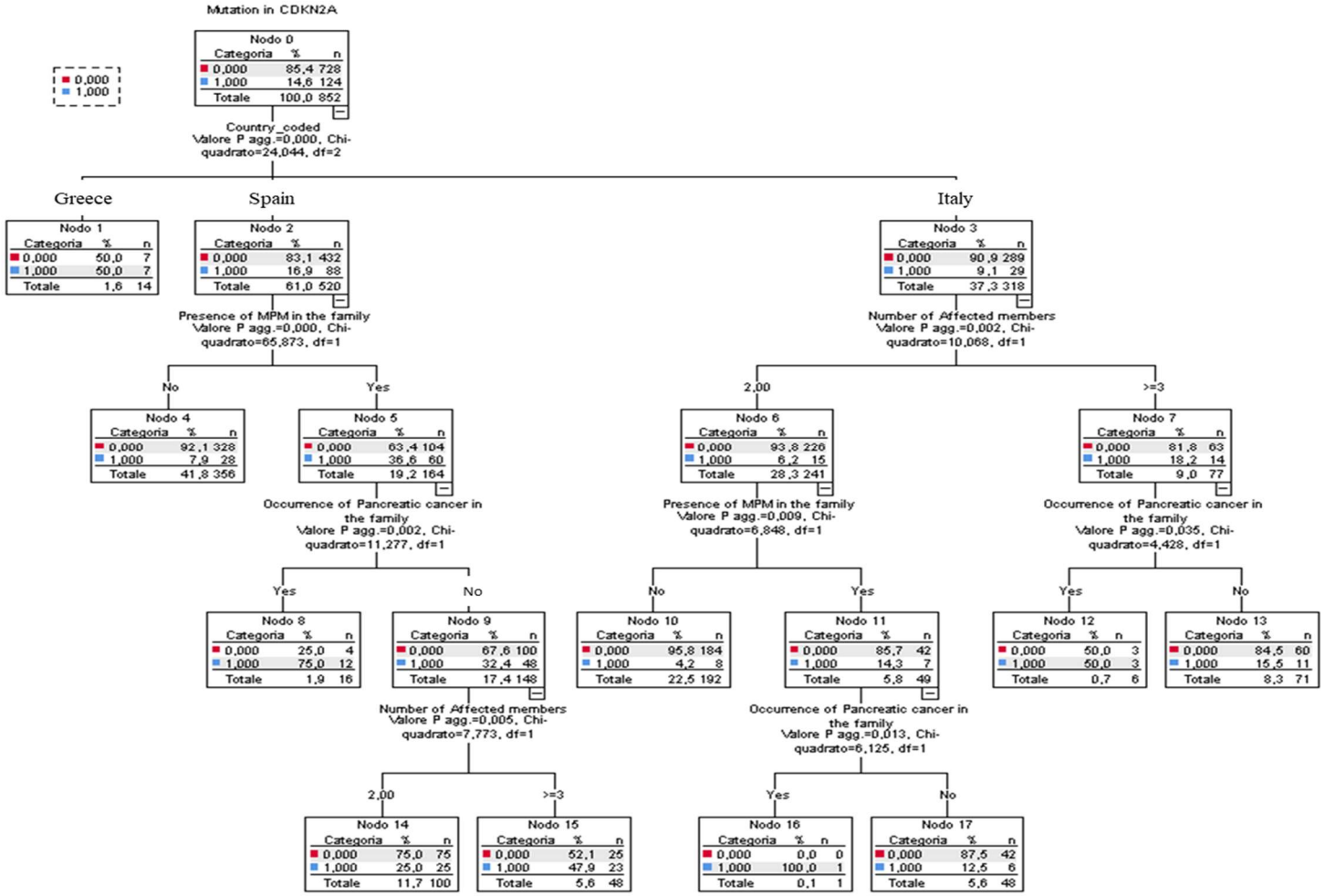


Figure 17. *Decision tree algorithm for CDKN2A variants stratified by country.*

TERF2IP variants were more frequent in families with a history of kidney cancer (p=0.015), while no other association was found between the mutational germline status and other family characteristics (Figure 18).

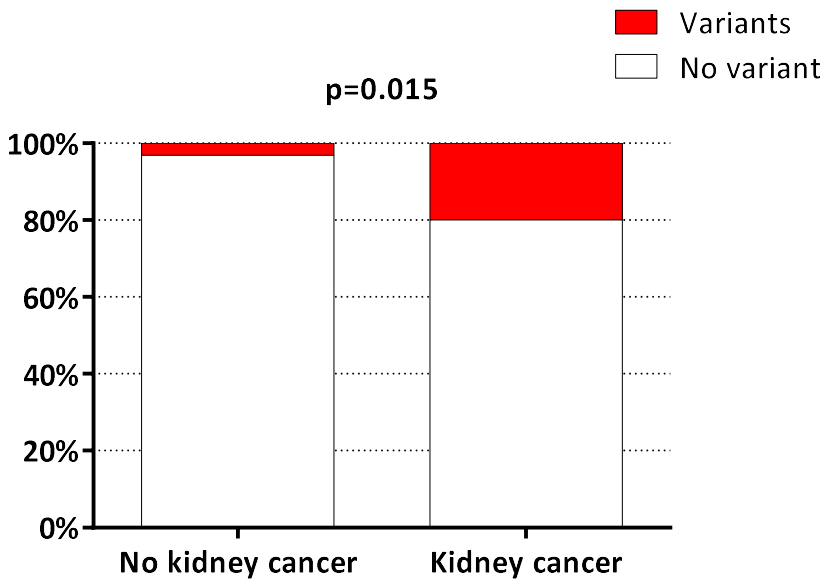


Figure 18. *Occurrence of kidney cancer in families with TERF2IP variants.*

3.4.2 Individuals

A. Individuals' characteristics

A total of 3488 individuals from 852 families, including 1365 melanoma patients and 2123 unaffected members, were enrolled in the study. All the following analyses were performed for affected individuals.

Melanoma patients included 803 (803/1365, 58.8%) females and 562 (562/1365, 41.2%) males, with a median age at first diagnosis of 48 years (range 7-99 years). Concerning phenotypical characteristics, 48.1% (657/1365) showed a blue/green eye color, whereas 35.5% (485/1365) had a medium/dark color. Red hair patients were 81 (81/1365, 6.0%), blond 684 (684/1365, 50.1%), brown 240 (240/1365, 17.6%) and dark brown/black 76 (76/1365, 5.5%). Regarding total number of nevi, patients with <10 nevi were 9.0%, those with 10-50 nevi were 24.3% and those with >50 nevi were 31.0%. Most affected individuals had phototype II (41.6%).

A personal history of MPM was present in 19.3% (263/1365) of the patients and 16.2% (221/1365) were diagnosed with other cancers in addition to melanoma. The most

frequent cancer type was KC (118/221, 53.4%), followed by breast cancer (34/221, 15.4%) and kidney cancer (32/221, 14.5%). Pancreatic cancer was diagnosed in 2.3% (5/221) of melanoma patients (Table 11).

Table 11. *Clinicopathological characteristics of affected individuals.*

Characteristics		**Affected individuals N= 1365 (%)**
Sex	Females	803 (58.8)
	Males	562 (41.2)
Age	Median Age (range)	48.3 (7-99)
Hair color	Red	81 (6.0)
	Blond	684 (50.1)
	Brown	240 (17.6)
	Black	76 (5.5)
	Missing	284 (20.8)
Eye color	Light	657 (48.1)
	Medium/Dark	485 (35.5)
	Missing	223 (16.3)
Phototype	I	73 (5.3)
	II	568 (41.6)
	III	413 (30.3)
	IV	97 (7.1)
	V	7 (0.5)
	Missing	207 (15.2)
Number of nevi	<10	122 (9.0)
	10-50	332 (24.3)
	>50	423 (31.0)
	Missing	488 (35.7)
Type of other cancers	KC	118 (8.6)
	Breast	34 (2.5)
	Colon	9 (0.6)
	Kidney	6 (0.4)
	Lung	14 (1.0)
	Pancreas	5 (0.3)
	Prostate	10 (0.7)
	Sarcoma	2 (0.1)
	Other	32 (2.3)
Characteristics of primary melanoma		**Affected individuals N= 1365 (%)**
Breslow thickness	Median value (range)	5.0 (0.05-99)
Histopathological subtype	In situ	291 (21.3)
	Invasive	834 (61.1)
	SSM	53
	NM	644
	ALM	101
	LMM	19
	Uveal	6
	Other	1
	Missing	240 (17.6)

SSM: superficial spreading melanoma, NM: nodular melanoma, ALM: acral lentiginous melanoma, LMM: lentigo maligna melanoma

The majority of melanomas were invasive (834/1365, 61.1%), with a mean Breslow thickness of 1.52 mm (±3.15). The most frequent subtype was nodular melanoma (644/1365, 47.2%) followed by acral lentiginous melanoma (101/1365, 7.4%) and superficial spreading melanoma (4.0%). The remaining 2% showed a histologic subtype of lentigo maligna, uveal or other types of melanoma.

B. Mutational findings

Concerning high-risk melanoma susceptibility genes, *CDKN2A* class 3 to 5 variants were present in 176 of 1264 (13.9%) patients with available *CDKN2A* information. The remaining 86.1% (1088/1264) were *CDKN2A* wild-type individuals.

Stratifying by country, 137 (137/860, 16.0%) individuals with mutations were from Spain, 32 (32/490, 6.5%) from Italy, 7 (7/15, 46.6%) from Greece.

The most frequent variant was G101W found in 61 (61/1264, 4.8%); individuals, of this, 85.2% (52/61) were Spanish and 9.8% (9/61) Italians. Greek patients did not carry the G101W variant.

In *CDKN2A* wild-type individuals (n=1058), *POT1* gene mutations were detected in 23 individuals of the 652 tested (3.5%) with the most frequent mutation, the S270N, identified in 10 (10/21, 47.6%) individuals. Mutations of the *TERF2IP* gene were observed in 17 (17/643, 2.6%) cases; in particular, the "Likely pathogenic/Pathogenic" variants (Q354*, T346A, T346I, A348S, L350V) were found in 7 (7/17, 41.2%) individuals each. *ACD* gene mutations were found in 4 (4/643, 0.6%) individuals, with the D392fs pathogenetic variant found in 1 (1/4, 25%) case; finally, two (2/665, 0.3%) individuals presented a *BAP1* mutation, the S455P.

No mutations were detected in the core promoter region of *TERT* nor in the *CDK4* gene.

Individuals with available *MC1R* data were 932; of these, 67.3% presented at least 1 *MC1R* variant, and 35.7% ≥ 2 *MC1R* variants. RHC variants were observed in 52.1% of the cases, with the most frequent variant being R151C (17.8%). Among NRHC variants, the most frequently observed variant was V60L (37.8%) (Table 12).

Table 12. *Frequency of MC1R variants among affected individuals.*

***MC1R* variants**	**Number of individuals N=932 (%)**
C35Y	1 (0.1)
c.86 insA	1 (0.1)
c.86dupA	1 (0.1)
L60L	1 (0.1)
V60L	237 (25.4)
S83L	1 (0.1)
S83P	2 (0.2)
D84E[×]	9 (1.0)
M92M	2 (0.2)
V92M	98 (10.5)
T95M	2 (0.2)
G104S	2 (0.2)
V119V	1 (0.1)
V122M	7 (0.7)
R123W	1 (0.1)
M128T	1 (0.1)
A139T	1 (0.1)
H142H	1 (0.1)
R142H[×]	44 (4.7)
A149V	1 (0.1)
R151C[×]	112 (12.0)
Y152*	6 (0.6)
I155T[×]	15 (1.6)
R155C[×]	1 (0.1)
R160W[×]	51 (5.5)
R163Q	45 (4.8)
V174del	2 (0.2)
R213W	3 (0.3)
c.699 G>A	1 (0.1)
Q233Q	1 (0.1)
T242S	1 (0.1)
I264I	1 (0.1)
P268R	2 (0.2)
K278E	3 (0.3)
N279D	2 (0.2)
N290S	1 (0.1)
A291A	1 (0.1)
A291V	1 (0.1)
D294H[×]	93 (10.0)
Y298H	1 (0.1)
T308M	1 (0.1)
c.942A>G	1 (0.1)
T314T	60 (6.4)

MC1R: melanocortin 1 receptor, RHC: Red Hair Color. [×]RHC variants

C. Association of mutational status with individual characteristics

We analyzed the association of epidemiological and clinical characteristics of individuals with the presence of a specific germline mutational status.

We found a significant association of *CDKN2A* mutations (classes 3 to 5) with a lower age at melanoma diagnosis ($p<0.0001$) (Figure 19), a higher number of MPM ($p>0.0001$) and a total number of nevi >50 ($p=0.013$).

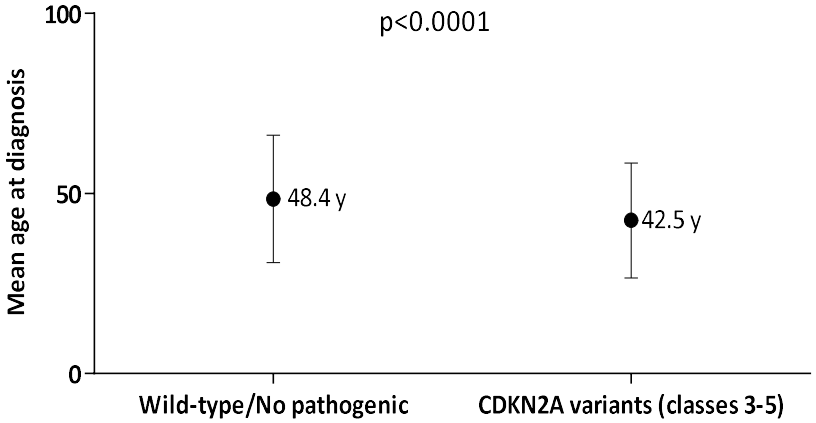


Figure 19. *Association of CDKN2A variants with age at diagnosis of melanoma.*

Regarding occurrence of other cancers in affecteds, we observed a higher frequency of *CDKN2A* mutations in individuals with pancreatic cancer ($p=0.001$).

For the other high-risk susceptibility genes, carriers of *POT1* variants were significantly associated with a higher Breslow thickness ($p=0.003$), while no other significant association was detected (Figure 20).

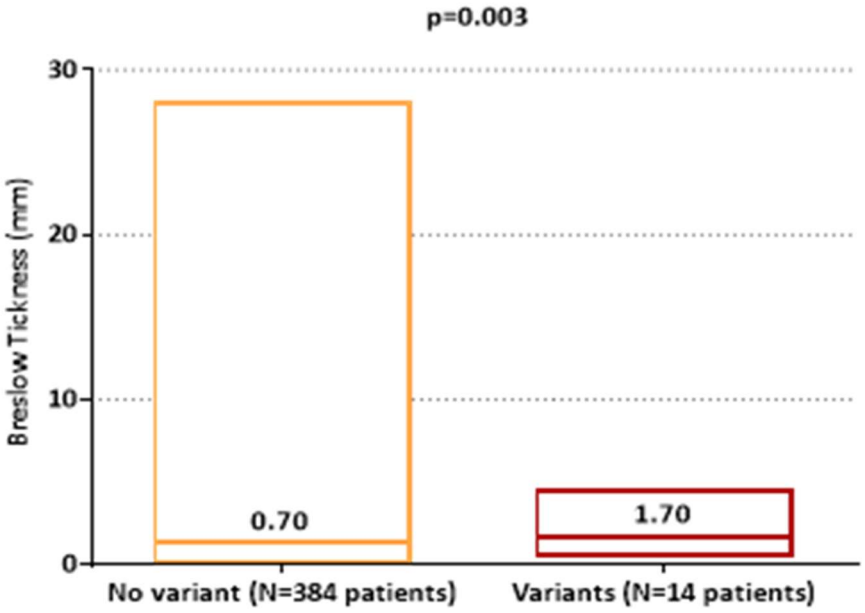


Figure 20. *POT1 variants were associated with a higher Breslow thickness.*

Carriers and non-carriers of *MC1R* variants showed a significant association with skin type I/II (p=0.02) and with red/blond hair color (p <0.0001) (Table 13).

Table 13. *Significant associations of MC1R variants with individuals' characteristics.*

		Affected patients					
			MC1R **Wild-type**	**Any *MC1R* variant**	**p[a]**	**RHC**	**p[b]**
Characteristics of patients		**TOT=932**	**N=305**	**N=627**		**N=327**	
Country	Greece	12 (100)	4 (33.3)	8 (66,7)	0.93	4 (50.0)	0.20
	Italy	280 (100)	94 (33,6)	186 (66,4)		97 (43.9)	
	Spain	640 (100)	207 (32.3)	433 (67,1		266 (51.2)	
Sex	M	551 (100)	185 (33,6)	366 (66,4)	0.52	195 (50.3)	0.39
	F	381 (100)	120 (31,5)	261 (68,5)		132 (46.8)	
Age at diagnosis	Mean value ± SD	46.9 ± 16.1	47,6± 15.0	45,7±15.7	0.09	45,6± 15.3	0.52
Skin type	I/II	578 (100)	198 (34,2)	380 (65,8)	**0.02**	194 (33.6)	**0.024**
	III/IV	247 (100)	64 (25,9)	183 (74,1)		97 (39.3)	
Hair color	Red-Blond	578 (100)	198 (34,3)	380 (65,7)	**<0.0001**	175 (30.2)	**<0.0001**
	Brown	185 (100)	55 (29,7)	130 (70,3)		72 (38.9)	
	Black	62 (100)	53 (85,5)	9 (14,5)		7 (11,3)	
Eye color	Light	479 (100)	158 (33,0)	321 (67,0)	0.59	161 (46.8)	0.28
	Medium/Dark	337 (100)	105 (31,1)	232 (68,9)		126 (51.4)	
Nevus count	< 50	114 (100)	40 (35,1)	74 (64,9)	0.16	40 (52.6)	0.64
	10-50	242 (100)	79 (32,6)	163 (67,4)		84 (46.9)	
	> 50	313 (100)	84 (26,8)	229 (73,2)		121 (50.6)	
Presence of MPM	No	718 (100)	245 (34,1)	473 (65,9)	0.10	243 (47,3)	0.17
	Yes	214 (100)	60 (28,0)	154 (72,0)		84 (53.8)	
Personal history of other cancers	No	758 (100)	257 (33,9)	501 (66,1)	0.13	253 (46.7)	0.02
	Yes	174 (100)	48 (27,6)	126 (72,4)		74 (57.8)	

MC1R: melanocortin 1 receptor, RHC: Red Hair Color; MPM: multiple primary melanoma. Numbers do not always add up to the total due to missing data. [a]p value calculated comparing *MC1R*-mutated vs MC1R wild-type patients; [b] p value calculated comparing RHC carriers vs *MC1R* wild type patients.

3.5 Discussion

We analyzed the clinical and genetic characteristics of 852 melanoma families with 1st or 2nd degree relatedness, collected from 2019 to 2020 in the database of the MelaNostrum Consortium. The *CDKN2A* gene was mutated in 12.3% of the families, while a very low frequency of mutations was observed in the other high-lowpenetrance genes*, POT1*, *ACD*, *TERF2IP*, and *BAP1*. No family carried mutations in the *CDK4* gene and in the core promoter region of *TERT*.

Our *CDKN2A* mutational frequency is in line with the literature where this gene is responsible for about 8-12% of familial melanoma [115] [118]. G101W was the most frequently identified *CDKN2A* mutation, being present in 27.4% of the families. Bishop et al. (2002) reported G101W as founder mutation in 16 families from Southern Europe [119].

We found a significant association between the presence of *CDKN2A* mutations and a higher number of affected members and MPM cases in the family. This evidence remained significant in Italy and in Spain but not in Greece, after stratification by country. Holland et al. (1999), more than 20 years ago, reported that the incidence of *CDKN2A* mutations was significantly higher in families with three or more melanoma cases (15.1%) than in those with only two affected relatives (1.5%) and that the presence of *CDKN2A* variants was also higher in families with at least one member with MPM (31.6%) compared to those without MPM cases (8.6%) [120].

Our findings also confirmed the results of the GenoMEL collaborative study by Goldstein et al. (2007), that evaluated 385 melanoma families from 17 GenoMEL centres. A frequency of *CDKN2A* mutations of 20% was observed in Australia, 45% in North America and 57% in Europe. An increase of *CDKN2A* mutation frequency was associated with a higher number of affected members in North American and Australian families, while in Europe (Sweden, France and in Mediterranean countries) the most important predictor was the presence of family members with MPM [88].

In a later Spanish study, Huerta et al. (2018) revealed a significant association between *CDKN2A* variants and families with more than two affected members, but no association was observed for degree of relatedness, as in our study [121].

Our results revealed a statistically significant association of *CDKN2A* variants with a lower age at diagnosis ($p<0.0001$) as previously reported by Karagianni et al. (2018) describing that age at diagnosis was significantly lower in patients harboring *CDKN2A* mutations compared with wild type patients ($p=0.001$) [122].

Regarding the association between *CDKN2A* mutations and other cancers in the families, we found a significant association with breast and pancreatic cancers, but not with prostatic, lung, colon, kidney cancers and sarcoma. Pancreatic cancer showed a significant association with *CDKN2A* mutations both in the totality of the families, and in Italy and Spain separately. No correlation was found in Greece, mainly due to the low number of enrolled families.

Similarly, in the GenoMEL study, a significant association between pancreatic cancer and *CDKN2A* mutations was described in North America ($p=0.02$), and in Europe ($p<0.001$) but no association was found in Australia ($p=0.38$) probably due to the divergent spectrum of mutations in Australian families compared to those from North America and Europe [88]. A borderline association between *CDKN2A* mutations and KC was observed only in Spanish families, but not in Greek nor in Italian. Recently, Pellegrini et al. (2017) reported a significant association between *CDKN2A* mutations and KC, in particular basal cell carcinoma, in a series of familial and MPM patients from Central Italy [115].

Multivariate analysis confirmed the independent significant association of *CDKN2A* mutations with the occurrence of pancreatic carcinoma, the presence of MPM cases and the number of affected members in the families. The decision tree allowed us to identify an algorithm, which is different for each country, useful to predict the presence of *CDKN2A* variants based on the characteristics of the families, that might help in selecting patients to be addressed for genetic testing.

Despite many screenings for *CDK4* alterations [123] [124] [125], fewer than 20 families were mutated worldwide [126]to, indeed, in our study we did not find any mutations in Mediterranean melanoma families.

Overall, the frequency of mutations in the other rare high-penetrance genes in our families was 9.3%.

No germline mutations were detected in the promoter region of *TERT* in our families. *TERT* promoter variants have been reported in only two families [98] [100]so far in the literature.

In the present study, p.S270N was the most frequent identified mutation in the *POT1* gene. Shi et al. (2014) reported 5 Italian families with the p.S270N variant. Carriers of this mutation had increased telomere length and elevated fragile telomeres suggesting its role in telomere maintenance [96].

We found mutations in the *ACD* and *TERF2IP* genes in 1.1% and 3.7% of tested families, respectively. As reported by Aoude et al. (2014), *ACD* mutations were observed in 6 of 510 (1.1%) melanoma families, similarly to our study. *TERF2IP* variants were detected in only 4 of 510 (0.8%) screened families, showing a lower frequency compared to our findings [97].

Concerning *MC1R*, the RHC R151C was found in 17.8% of patients, while V60L was the most frequent NRHC variants (37.8%). Demenais et al. (2010) analysed 473 individuals from 185 European, North American and Australian families, reporting that both RHC (R151C and R160W) and NRHC (V60L and V92M) variants are associated with increased melanoma risk, but with varying strengths in different continents. In Europe, this association was statistically significant for each variant, and the strongest association was noted for RHC variants followed by NRHC variants ($0.0002 \leq p \leq 0.03$). In North America, the association of *MC1R* variants with increased melanoma risk reached statistical significance for R151C, R160W, and V92M variants ($0.05 \leq p \leq 0.02$), whereas in Australia, the association reached statistical significance for V60L (p=0.009) and R151C (p=0.03) variants [127].

Furthermore, we found a significant association of *MC1R* variants with a fair phototype with a higher frequency of individuals with red/blond hair among RHC carriers compared to those carrying NRHC variants, confirming previous studies [104] [128].

Genetic screening is offered to melanoma-prone families to better understand their genetic susceptibility. It should be informative about the genetic risk for melanoma and other cancers, and the possible implications for other family members. Genetic testing to detect melanoma predisposition mutations can be used in clinical practice under adequate selection criteria. In countries with a low melanoma incidence, such as Southern European countries, selection criteria for genetic counseling and testing should follow the

rule of two: individuals with two (synchronous or metachronous) primary melanomas and/or families with at least one invasive melanoma and one or more other diagnoses of melanoma and/or pancreatic cancers among 1st- or 2nd-degree relatives on the same side of the family [20] [80].

To date, genetic testing for familial melanoma is mainly focused on *CDKN2A* and *CDK4* screening, although larger panel of genes are now available. Genetic panel offers a comprehensive, tailored gene assessment tool for families with tumor hereditary pattern, including melanoma. The implement of panel testing for melanoma could promote further understanding of the disease as well as it could provide in the future the state of art care for high-risk patients [81].

3.6 Conclusions

The frequency of *CDKN2A* mutations in melanoma families has been shown to vary considerably among different populations depending on baseline melanoma incidence, founder effects, and selection criteria of the study population. Our findings confirm the role of *CDKN2A* mutations in familial melanoma and that a high number of affected members within the family, the presence of multiple melanoma cases and pancreatic cancer cases are strong predictive factors for *CDKN2A* genetic screening. The presence of other high-risk variants besides *CDKN2A* are rare in this Mediterranean population.

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