UNIVERSITÀ DEL PIEMONTE ORIENTALE

UNIVERSITY OF EASTERN PIEDMONT

MEDICAL SCHOOL

MASTER DEGREE IN MEDICAL BIOTECHNOLOGIES

Director: Prof. Francesca Boccafoschi

Genetic Screening in familial melanoma: Results from a Multigene Panel Analysis and investigation of a large pedigree with multiple familial tumor syndromes

Supervisor

Prof. Mara GIORDANO

Firmato digitalmente da Mara Giordano
Data: 30.06.2026 08:53:19 CEST
Organizzazione: UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE/01943490027

Candidate

Simone CABRI

Matricula

20043267

Academic year: 2025-2026

INDEX

ABSTRACT

Background: Melanoma is one of the most aggressive cutaneous malignancies, characterized by marked genetic heterogeneity. Although most cases are sporadic, a significant proportion is driven by germline mutations in high- and moderate-penetrance predisposing genes. This study aimed to evaluate the frequency of pathogenic variants in a cohort of Italian patients.Additionally, germline mutations in melanoma patients were investigated across both well-established and other genes predisposing to other form of cancer to further elucidate the genetic landscape of the disease and its clinical implications.

Materials and Methods: A cohort of 245 index cases referred to the Genetics Laboratory of the A.O.U. "Maggiore della Carità" in Novara between January 2025 and May 2026 was analyzed. Patients were enrolled by the hospital's Dermatology Clinic and other healthcare facilities, Genomic sequencing was performed using Sanger sequencing and Next-Generation Sequencing (NGS) methodologies. High-penetrance genes (*CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*) and moderate-penetrance genes (*MITF*, *MC1R*) were evaluated using a multigene panel (BML, "Breast-Melanoma-Lynch"). This panel also included 16 genes associated with other hereditary cancer syndromes
(*APC*, *ATM*, *BRCA1/2*, *BRIP1*, *CHEK2*, *EPCAM*, *MLH1*, *MSH2*, *MSH6*, *PALB2*, *PMS2*, *RAD50*, *RAD51C*, *RAD51D*, and *TP53*), which are routinely evaluated using the same methodology.

Results: Among the 245 patients analyzed, 34 (13.88%) carried pathogenic or likely pathogenic variants, whereas 6 patients (2.45%) harbored variants of uncertain significance. Overall, these findings indicate a relevant diagnostic yield for the multigene panel approach in this cohort of patients referred for hereditary melanoma predisposition assessment.

The most frequently affected gene was *CDKN2A*, with pathogenic variants identified in 22 patients, corresponding to 8.98% of the overall cohort and 64.7% of all positive cases. Within the *CDKN2A*-positive subgroup, the c.301G>T variant was the most recurrent alteration, being detected in 14 of 22 patients, 63.6%. Other pathogenic variants were less frequent and accounted for the remaining *CDKN2A*-positive cases.

Among the remaining 12 positive patients, pathogenic or likely pathogenic variants were identified in additional cancer predisposition genes included in the panel. These included *CHEK2* in 4 patients, 1.63% of the total cohort; *MITF* in 3 patients, 1.22%; *BRCA1/2* and *ATM* in 2 patients each,

0.82% each; and *POT1* in 1 patient, 0.41%. These results show that, although *CDKN2A* remains the major contributor to hereditary melanoma susceptibility in this cohort, the use of an expanded panel allowed the identification of clinically relevant variants in other genes associated with melanoma risk or broader hereditary cancer predisposition.

In addition, we report a large pedigree in which segregation analysis demonstrated the familial distribution of pathogenic variants in *CDKN2A* and *MLH1 (*correlated to colorectal cancer), both identified through the multigene panel approach.

Conclusions: This study underscores how the implementation of comprehensive multigene panels enables the identification of actionable mutations that are not traditionally or directly linked to melanoma development. This large pedigree illustrated, shows the added value of extended genetic testing, as it allowed the detection of coexisting hereditary cancer susceptibility factors. The integration of molecular findings with segregation analysis and family history therefore provided a more comprehensive assessment of inherited cancer risk within this pedigree.

2. INTRODUCTION

2.1 DEFINITION

Cutaneous melanoma is a highly aggressive malignant neoplasm originating from the oncogenic transformation of melanocytes, which are primarily located in the basal layer of the epidermis and mucous membranes.

Although less frequently, melanocytes can also give rise to tumors at extracutaneous sites, including the uveal tract, meninges and inner ear.

Characterized by a remarkably high metastatic potential, melanoma accounts for the vast majority of skin cancer-related deaths.

The clinical and biological progression of the disease is a multistep process, with each stage displaying distinct histopathological features and specific molecular alterations [1] .

From a clinical standpoint, primary melanoma is traditionally classified into four major histopathological subtypes [2] :

1) Superficial spreading melanoma(SSM): This subtype represents the most common variant and is biologically characterized by an initial phase of radial and horizontal growth.

During this stage, atypical melanocytes proliferate in a disorganized manner within the intraepidermal layer, either as single units (in situ) or in nests among normal keratinocytes.

This horizontal phase is subsequently followed by a vertical growth phase, marked by dermal invasion and an increased metastatic potential [3] .

2) Lentigo maligna melanoma(LMM): Considered a significantly more aggressive clinical entity compared to SSM, this variant typically develops from pre existing lentigo maligna lesions on chronically sun-damaged skin. Its progression is characterized by rapid, invasive dermal infiltration and a high propensity for systemic dissemination.

3) Acral lentiginous melanoma(ALM): This distinct clinical-pathological variant typically presents as a lesion associated with chronic friction rather than direct solar radiation, frequently manifesting in elderly subjects.

Biologically, ALM represents the invasive progression into the dermis of a pre-existing melanoma in situ or acral lentigo [4] .

4) Nodular melanoma(NM): This aggressive variant occurs with a higher frequency on the palmar, plantar, particularly affecting individuals with dark phototypes.

Histopathologically, NM presents with a prominent, vertical-oriented proliferation of atypical melanocytes along the dermal-epidermal junction, bypassing a significant radial growth phase and rapidly invading the deeper layers of the epidermis and dermis [3] .

2.2 EPIDEMIOLOGY

Historically considered a rare malignancy until a few decades ago, the incidence of cutaneous melanoma has experienced a steady and significant worldwide increase, with numerous epidemiological studies suggesting that the number of cases has effectively doubled over the last 10 years.

Globally, it is estimated that over the past decade, cutaneous melanoma diagnoses have reached approximately 100,000 cases per year, representing an increase of roughly 15% compared to the previous decade [1].

Focusing on the national landscape, data from the last five years indicate that deaths attributed to cutaneous melanoma in Italy have reached approximately 4,000 among males and over 3,000 among females, corresponding to an average mortality rate of 5 and 6 per 100,000 inhabitants per year, respectively [1].

According to the latest available national registry data (2024), an estimated 12,941 new diagnoses of melanoma were recorded, showing a higher prevalence in the male population with 7,069 cases compared to 5,872 cases among women [5].

Furthermore, current epidemiological evidence suggests that melanoma prevalence is strongly age-dependent. While incidence rates remain higher among women until the age of 40, a significant gender shift occurs later in life; by age 75, the incidence in men becomes threefold that of women(145.6 vs. 47.3 per 100,000).

Nevertheless, although the overall risk of developing melanoma progressively increases with advancing age and the highest incidence is concentrated among older populations, this malignancy consistently ranks among the most frequently diagnosed cancers in young adults **[**6].

This epidemiological trend is clearly reflected in national cancer registries; as illustrated in **Table 1**, melanoma represents the second most frequent malignancy in males and the third in females within the younger age cohort (0-49 years), accounting for 10% and 9% of all cancer diagnoses in these group, respectively.

Table 1: Ranking of the five most frequently diagnosed cancers in the Italian population, stratified by gender and age group, highlighting the significant burden of melanoma in the younger cohort .

RANK	Male			Female		
	Age			Age		
	0 - 49	50 - 69	70	0 - 49	50 - 69	70
Total incidence cases	100% n=15.829	100% n=76.201	100% n=102.724	100% n=29.918	100% n=66.446	100% n=85.493
1°	Testicle 12%	prostate 22%	prostate 20%	breast 41%	breast 35%	breast 22%
2°	**melanoma** **10%**	lung 14%	lung 17%	Thyroid 15%	colon-rectal 11%	colon-rectal 16%
3°	NHL 8%	colon-rectal 12%	colon-rectal 14%	**melanoma** **8%**	uterus body 7%	lung 8%
4°	Thyroid 8%	bladder 9%	bladder 11%	colon-rectal 4%	lung 7%	pancreas 6%
5°	colon-rectal 7%	upper aerodigestive tract 5%	stomach 5%	uterus cervix 4%	Thyroid 5%	stomach 5%

2.3 RISK FACTORS

2.3.1 ENVIRONMENTAL FACTORS

The most critical and potentially modifiable environmental risk factor for the development of malignant melanoma(MM)is exposure to ultraviolet(UV) radiation, primarily due to its intrinsic genotoxic effects.

Solar ultraviolet radiation is traditionally categorized into three distinct wavelengths: UVC, UVB and UVA, each exhibiting different biophysical properties and degrees of pathogenicity regarding the skin and eyes.

Among these, UVC radiation represents the most energetic and dangerous waveband; fortunately, it is entirely absorbed by the stratospheric ozone layer before reaching the Earth's surface.

Conversely, UVB radiation is only partially filtered and represents the second most powerfull component.

It primarily penetrates the superficial layers of the epidermis and serves as the principal biological cause of sunburn, as well as a major driver in the pathogenesis of cutaneous malignancies, including basal cell carcinoma, squamous cell carcinoma, and melanoma.

Lastly, UVA radiation constitutes the least energetic but most abundant form of solar radiation, accounting for approximately 95% of the total UV light reaching the Earth.

Due to its longer wavelength, UVA penetrates deeply into the dermal layers, leading to chronic dermal remodelling, and premature skin aging.

Furthermore, it significantly exacerbates UVB-induced damage, collectively increasing the overall risk of developing melanoma and non melanoma skin cancer (IARC) [7].

From a mechanistic standpoint, the primary molecular damage induced by these radiations is indirect, driven by the intracellular generation of reactive oxygen species (ROS)through photolysis processes.

These unstable molecules behave as potent oxidants; when present in excess, they overwhelm cellular physiological antioxidant defenses, causing widespread oxidative damage to crucial biomolecules including nucleic acids, lipids, proteins, and carbohydrates. Regarding genomic stability, ROS-mediated stress manifest as single-and double-strand DNA breaks, the inactivation of key enzymatic pathways involved in DNA repair, and the direct oxidation of purine and pyrimidine bases. The main products of this oxidative stress are thymine glycol and 8-oxo-7,8-dihydroguanine.

These chemical alterations introduce critical structural disruptions that promote incorrect base pairing, specifically inducing mispairing between thymine glycol and cytosine, as well as between 8-oxo-7,8-dihydroguanine and adenine [8].

The physical penetration capabilities of these different ultraviolet wavelengths into cutaneous strata, along with the protective interference of chemical or physical sunscreens. are schematically illustrated in **Figure 1**.

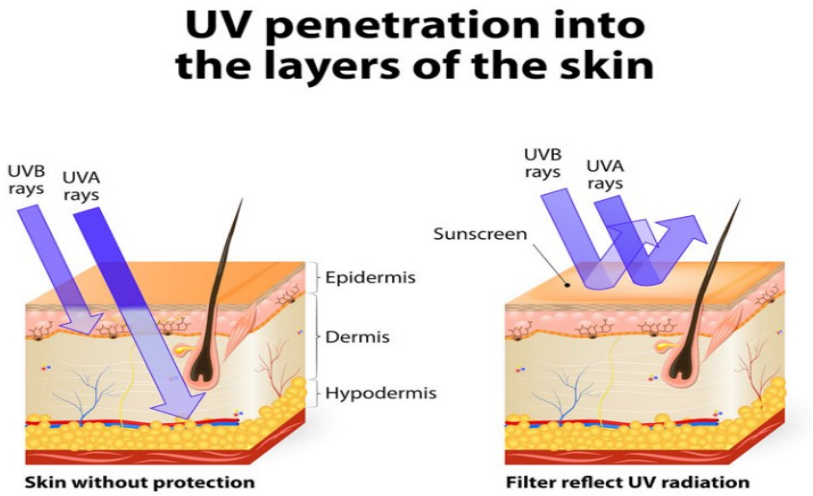

Figure 1: Schematic representation of ultraviolet(UV) radiation penetration depths into unprotected human skin layers(A) compared to reflective and protective mechanisms offered by topical sunscreen filters(B).

Another fundamental determinant of cutaneous melanoma susceptibility is an individual's constitutional phenotype, specifically defined by the concept of "skin phenotype". This term refers to an objective classification system based on baseline epidermal melanin production and the skin's specific constitutional reaction to solar radiation.

This clinical paradigm was originally developed by American dermatologist Thomas B. Fitzpatrick in 1975 to evaluate patient responsiveness to ultraviolet therapy, and it remains the global standard in clinical dermatology to assess individual risks regarding acute sun damge, photoaging, and skin oncogenesis.

The classification stratifies human skin into six distinct categories(Types I to VI), moving progressively from highly sensitive, pale phenotypes to deeply pigmented, radiation-resistant profiles, as detailed in **Table 2** [9].

Table 2: The Fitzpatrick Skin Phototype Classification System, illustrating the relationship between constitutional pigmentation features, acute solar radiation reactivity and relative risk of melanoma development.

Phototypes	**Constitutional Characteristics**	**Sun exposure reaction**	**Melanoma and Photo-damage Risk**
Skin type I	Very fair skin, frequent freckles, red or blond hair, ligh-colored eyes.	Always burns, never tans.	Very high risk
Skin type II	Fair skin, blond or light brown hair, light-colored eyes.	Usually burns, tans with difficulty.	High risk
Skin type 1II	Fair to light-olive skin, brown hair, brown or green eyes.	Sometimes burns, tnas with difficulty.	Moderate risk
Skin type IV	Moderately dark or olive skin, dark brown or black hair, brown eyes.	Rarely burns, tans easily.	Low risk
Skin type V	Dark skin, black hair, dark eyes.	Very rarely burns, tans rapidly and deeply.	Low risk(but not zero)
Skin type VI	Deeply pigmented dark skin, black hair, dark eyes.	Never burns, acclimated deeply to sun.	minimal risk

2.3.2 GENETIC FACTORS

While the vast majority of cutaneous melanoma cases manifest as sporadic events primarily driven by environmental insults, a significant subset-estimated to account for approximately 10% of al diagnoses- exhibits a hereditary pattern.

This familial clustering is strongly associated with inherited germline mutations in specific susceptibility genes [10 -11].

Biologically the loci involved in conferring an elevated risk of developing melanoma are predominantly associated with critical cellular pathways, including cell cycle regulation, cellular differentiation, DNA damage response mechanisms, and telomere stabilization.

These genetic determinants are traditionally stratified into three distinct categories based on their relative penetrance [12].

- **High-penetrance genes**: *CDKN2A, CDK4, BAP1, POT1, ACD, TERT, TERF2IP*. These genes are responsible for 50% of hereditary melanomas.
- **Medium-penetrance genes**: *MC1R* and *MITF*.
- **Low-penetrance genes**: *OCA2, TYR, TYRP1, SLC45A2.*

Table 3*: High-risk melanoma susceptibility genes and associated clinicopathological features, detailing their protein function, associated phenotypes, and most prevalent histopathological sybtypes in hereditary melanoma predisposition.*

	Protein function	**Phenotype**	**most common histopathological subtype of melanoma**
CDKN2A	Tumor suppressor	melanocytic nevi, melanoma, respiratory cancer	superficial spreading melanoma, spindle cell morphology in vertical growth phase
CDK4	Oncogene	melanocytic nevi, melanoma, pancreatic cancer	superficial spreading melanoma, pigmentation and patogetoid scatter.
POT1	costituent of shelterin complex	melanoma, breast cancer, lung cancer, glioma and other cancers.	superficial spreading melanoma

2.4 GERMLINE MUTATIONS

Germline mutations are inherited genetic alterations transmitted via parental gametes, meaning they are passed to offspring and become constitutively present in every somatic cell of the organism. Unlike somatic mutations which are non-heritable and acquired over time within specific tissues due to environmental carcinogens (such as ultraviolet radiation) germline variants establish a baseline genetic susceptibility that significantly lowers the threshold for oncogenesis. In cutaneous melanoma, these pre-existing alterations predominantly disrupt critical tumor suppressor genes and cell cycle regulators. According to Knudson's "two-hit" model of hereditary cancer, individuals carrying a germline mutation already possess a disruptive alteration in one allele across all cells (the first hit). Consequently, malignant transformation is accelerated when a stochastic or environmentally induced somatic mutation inactivates the remaining wild-type allele (the second hit) within a specific melanocytic lineage. Ultimately, the phenotypic manifestation and clinical penetrance of these germline variants are heavily modulated by complex interactions with low-penetrance modifier genes and external risk factors. It is this interplay that drives the distinct clinical-pathological features observed in hereditary melanoma families.

2.4.1 *CDKN2A*

Identified in 1994, *CDKN2A* remains the most significant high-risk susceptibility gene for malignant melanoma (MM). It is mutated in approximately 20% to 40% of high-risk families, though this prevalence varies considerably depending on specific selection criteria and the geographical cohort analyzed [13-15]. Located at the 9p21 locus, *CDKN2A* possesses a unique genomic architecture that allows it to encode two distinct tumor suppressor proteins **$p16^{INK4a}$** and **$p14^{ARF}$** through alternative reading frames and differential splicing:

- **The alpha-transcript** (comprising exons 1α, 2 and 3) encodes $p16^{INK4a}$, which promotes cell cycle arrest in the G1 phase by inhibiting cyclin-dependent kinase 4 and 6 (CDK4/6)-mediated phosphorylation of the retinoblastoma (RB) protein.

- **The beta-transcript** (comprising exons 1β, 2 and 3) encodes $p14^{ARF}$, which acts upstream of the p53 pathway to induce cell cycle arrest or trigger apoptosis [16].

Together, p16INK4a and p14ARF coordinate critical cellular defenses, playing a vital role in both the DNA damage response and cellular senescence [17].

To establish the precise clinical impact of these alterations, Bishop et al. evaluated 80 families with documented germline *CDKN2A* mutations and multiple cases of cutaneous melanoma.

Their findings demonstrated a cumulative mutation penetrance of 30% by age 50, which rises sharply to 67% by age 80 [18].

Consistent with these familial observations, the probability of detecting *CDKN2A* mutations in sporadic multiple primary melanoma (SMP) patients correlates directly with the total number of primary tumors. Conversely, the likelihood of identifying germline *CDKN2A* mutations in sporadic, single-melanoma patients with no personal or family history of the disease remains remarkably low, at approximately 1% [19].

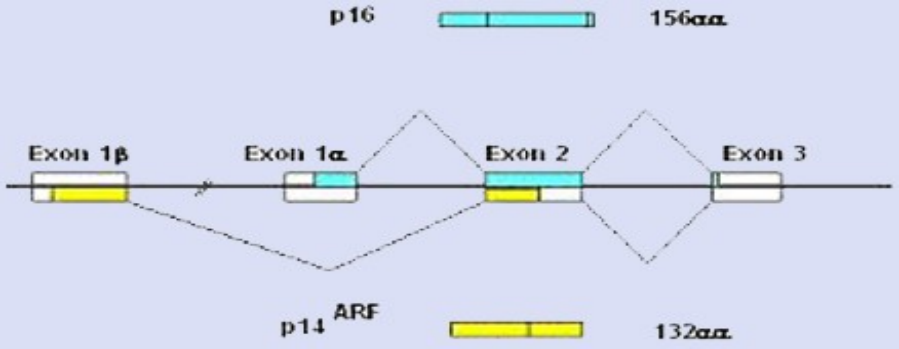

Figure 2: The *CDKN2A* locus and its transcripts, p16 and $p14^{ARF}$, generated by alternative splicing. Boxes indicate exons, and coloured sections the protein coding regions within them.

p16: 3 exons (1a, 2 and 3) encoding 156 amino acids
$p14^{ARF}$: 3 exons (1b, 2 and 3) encoding 132 amino acids

2.4.2 *CDK4*

Cyclin-dependent kinase 4 (*CDK4*), located within the 12q14 chromosomal region, was the second high-risk melanoma susceptibility gene to be identified. As an oncogene, it encodes a catalytic protein subunit crucial for driving cell cycle progression through the G1 phase.

Phenotypically, families harboring *CDK4* mutations are virtually indistinguishable from those carrying *CDKN2A* alterations; both cohorts present with early-onset cutaneous melanoma, multiple primary melanomas, a high burden of clinically atypical nevi, and an increased association with various extracutaneous malignancies [20].

At the molecular level, *CDK4* mutations predominantly affect the arginine residue at position 24 (specifically the CDK4-R24C variant), which is a critical point within the $p16^{INK4a}$ -binding domain. These specific variants—currently identified in melanoma patients with a strong familial predisposition—structurally prevent $p16^{INK4a}$ from binding to the catalytic subunit. As corroborated by extensive preclinical models, this lack of inhibition triggers the constitutive activation of the kinase, conferring a biological behavior that closely mimics $p16^{INK4a}$ loss [21-23].

Consequently, without the inhibitory control of $p16^{INK4a}$, the complexes formed by *CDK4* and D-type cyclins promote uncontrolled cellular proliferation, causing *CDK4* mutation carriers to

behave phenotypically like their $p16^{INK4a}$ deficient counterparts [24].

Mechanistically, the hyperactivation of this pathway disrupts the downstream pocket protein machinery:

- **Phosphorylation:** Harbour et al. demonstrated that phosphorylation of the C-terminal region of RB1 by the *CDK4/CDK6* complex initiates successive intramolecular interactions between its C-terminal domain and the central pocket.
- **Conformational Shift:** This conformational shift displaces histone deacetylase (HDAC) from the pocket structure.
- **E2F Activation:** This displacement effectively blocks the active transcriptional repression of E2F by RB, driving the cell past the G1/S restriction point and committing it to division [25].

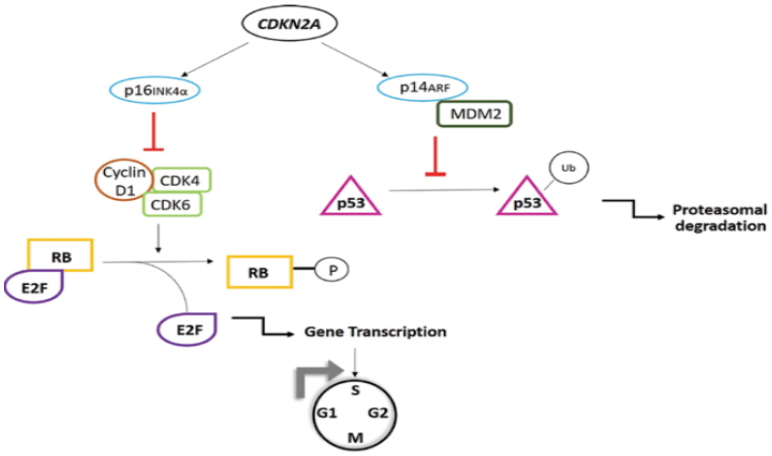

Figure 3: Schematic representation of the molecular pathways involved in high-risk melanoma susceptibility.
The diagram illustrates the integrated role of *CDKN2A(*$p16^{INK4\alpha}$ and $p14^{ARF}$) and *CDK4* in cell cycle regulation, highlighting the cascade leading to E2F-mediated gene transcription and the MDM2-dependent proteosomal degradation of p53.

2.4.3 *MC1R*

Located on chromosome 16q24, **MC1R** is classified as a moderate-risk susceptibility gene that encodes a G-protein coupled receptor (GPCR) specific for the melanocyte-stimulating hormone (α-MSH). Beyond its independent role in melanoma predisposition, *MC1R* acts as a potent genetic modifier in carriers of *CDKN2A* mutations; specifically, its co-inheritance significantly elevates the baseline risk, accelerating oncogenesis [26-27]. Furthermore, this highly polymorphic gene exhibits a strong association with *BRAF* mutations in melanomas arising on non-chronically sun-damaged skin, suggesting a distinct pathogenic pathway wherein *MC1R* variants inherently predispose melanocytes to acquiring somatic *BRAF* hits [28].

Within Caucasian populations, specific germline allelic variants most notably Arg151Cys, Arg160Trp, and Asp294His are fundamentally linked to the "red hair phenotype" (RHC) and collectively double the baseline risk for malignant melanoma [29-31].

At the biochemical level, these functional alterations disrupt the melanogenic cascade:

- **Signaling Impairment:** The variants compromise either α-MSH binding affinity or downstream cAMP signaling.
- **Pigment Shift:** This impairment shifts melanin synthesis, lowering the ratio of photoprotective eumelanin to pro-mutagenic pheomelanin.
- **Oxidative Stress:** The resulting overproduction of pheomelanin generates a pro-oxidative cutaneous microenvironment that damages melanocytic DNA even in the absence of UV radiation [32].

Interestingly, the clinical relevance of *MC1R* may extend beyond cutaneous malignancies; recent evidence has evaluated its expression in uveal melanoma, demonstrating a significantly higher expression profile compared to other traditional melanoma markers [33].

Structurally, the canonical *MC1R* gene encodes a highly conserved 317-amino-acid protein featuring seven hydrophobic transmembrane domains, an extracellular N-terminus with a glycosylation site, and an intracellular C-terminal extension stabilized by a palmitoylation site [34-38]. However, the locus can also undergo intergenic splicing, giving rise to complex chimeric

transcripts such as MC1R-TUBB3 (β-tubulin III), alongside at least two alternative intragenic splice variants [39-40]. Although these non-canonical mRNAs generate proteins with a longer C-terminal extension, they fully preserve the core seven-transmembrane architecture characteristic of the GPCR superfamily [41].

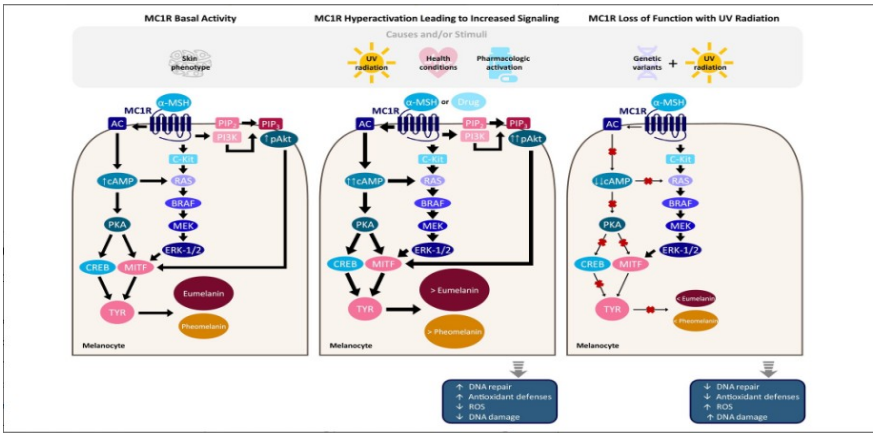

Figure 4: Schematic representation of the *MC1R* signaling pathway under distinct physiological and pathological conditions: basal physiologic signaling (left), hyperactivation (middle) and UV radiation-induced loss of function (right).

Variations in line thickness represent the relative intensity of downstream signaling cascades, while red indicators signify pathway suppression. The shifting dimensions of the eumelanin and pheomelanin schemas correspond to quantitative changes in melanin pigment synthesis.

2.4.4 *MITF*

Located within the 3p14 chromosomal region, Microphthalmia-associated transcription factor **(MITF)** is classified as a moderate-risk melanoma susceptibility gene. Functioning as the master regulator of melanogenesis, *MITF* was characterized over two decades ago as a critical lineage-survival oncogene. It plays a pivotal role in melanoma initiation and progression by stringently regulating cellular proliferation and apoptosis [42-44]. At the transcriptomic level, *MITF* is frequently overexpressed in both cutaneous and uveal melanomas, exhibiting genomic amplification in 10% to 20% of patients diagnosed with advanced-stage disease [45].

Structurally, the locus encodes multiple isoforms that exhibit either ubiquitous or strict tissue-specific expression patterns.

Among these, **MITF-M** serves as the primary and most functionally prominent isoform driving the melanocyte lineage.

The pathogenic relevance of germline alterations at this locus is strongly highlighted by specific rare variants:

- **The E318K Variant:** Bertolotto et al. (2011) identified a recurrent germline missense substitution resulting in the **E318K** amino acid change, which occurs with a significantly higher frequency in genetically enriched melanoma cohorts [46-47].
- **Molecular Mechanism:** Mechanistically, the E318K mutation severely impairs the sumoylation of the protein.
- **Downstream Effects:** This impairment enhances the binding affinity of MITF toward the *HIF1A* promoter, significantly upregulating its transcriptional activity compared to wild-type *MITF* and fostering a pro-tumorigenic microenvironment.

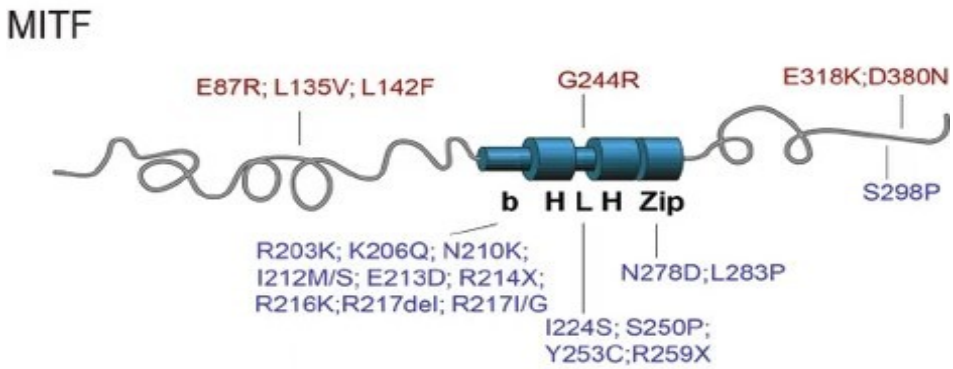

Figure 5: A graphic depiction of the human MITF protein with the location of the mutations indicated. The mutations found in melanoma patients are indicated in red (on top). These mutations are located at the amino- and carboxyl ends, and only one mutation is located in the helix– loop– helix domain (G244R).

2.4.5 *POT1*

Protection of Telomeres 1 **(*POT1*)** is a crucial, highly conserved component of the shelterin complex [11]. Together with **ACD** (also known as TPP1), *POT1* binds directly to the overhanging 3′ single-stranded telomeric DNA (ssDNA) at the ends of chromosomes via its two oligonucleotide - oligosaccharide-binding (OB) domains [48-50].

This interaction facilitates the stable assembly of the shelterin complex onto genomic DNA and promotes the configuration of ssDNA into a protective **"t-loop."** Mechanistically, this loops shields chromosome ends, preventing free telomere termini from being inappropriately recognized by the cell's DNA repair machinery or prematurely extended by telomerase [51].

In a clinical context, both missense and loss-of-function variants in *POT1* have been identified in a small proportion (approximately 4%) of *CDKN2A*-negative familial melanoma cohorts.

At the molecular level, mutated POT1 is structurally unable to complex with telomeric DNA. Strikingly, this disruption of telomeric cap integrity causes carriers of *POT1* mutations to exhibit abnormally elongated telomeres, a hallmark feature that drives genomic instability and oncogenesis [52-53].

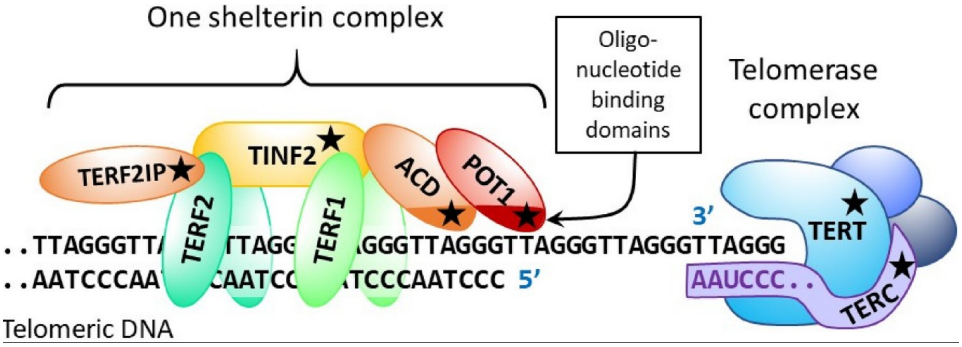

Figure 6:Diagram of the shelterin and telomerase complexes on a telomere. The DNA 3′ overhang is normally arranged within the T-loop (not shown).

Component with germline mutations or GWAS locus (*TERC*) linked to melanoma susceptibility.

The clinical and molecular characteristic of the main susceptibility genes involved in melanoma are summarized in **table 4** below.

Table 4: Summary of the primary high, medium and low penetrance susceptibility genes implicated in melanoma pathogenesis, including their physiological functions and the functional consequences of their mutations.

Gene penetrance	Gene	Normal biological function	effect of mutation
HIGH	*CDKN2A* p16	Senescence effector, tumor suppressor	Impaired cell senescence
	CDKN2A ARF	Tumor suppressor, activator of p53	Impaired p53-dependent cell apoptosis.
	CDK4	Cell-cycle regulator, senescence	Impaired cell senescence
	TERT	Telomere maintenance and repair	Overexpression. Delay or absence of cell senescence
	POT1	Telomere protection	Delayed cell senescence
MEDIUM	*MITF*	Melanocyte development and differentiation	Melanoma risk possibly through impaired cell senescence
LOW	*MC1R*	UV response and melanin synthesis; melanocyte differentiation	Reduced UV protection, higher risk of mutation.

2.5 SOMATIC MUTATIONS: THE *BRAF-NRAS* GENES.

2.5.1 *BRAF*

While germline alterations discussed in the preceding section delineate the inherited genetic background of predisposed individuals, the evolutionary progression toward cutaneous melanoma is fundamentally driven by somatic mutations. These genetic events events are not inherited through the germline but are stochastically acquired during an individual's lifetime, frequenty secondary to environmental insults such as ultraviolet (UV) radiation exposure.

In cutaneous melanomas, somatic mutations within the *BRAF* oncogene represent the most prevalent oncogenic drivers, occurring in approximately 50% of diagnosed patients.

Interestingly, *BRAF* alterations are significantly more frequent in younger patient populations who experienced intermittent, occasional sun exposure, contrasting with lower mutational rates observed in individuals with histories of chronic, lifelong occupational sun damage.

BRAF-mutated melanomas display unique clinical and pathological features.

Characteristically, these tumors exhibit a significantly more aggressive biological behavior compared to their BRAF-wild type (WT) counterparts.

Patients harboring these alterations demonstrate a higher propensity for systemic dissemination, particularly showing a pronounced tropism for brain metastases, which clinically correlates with a shorter overall survival and an increased likelihood of presenting with advanced stage IV disease [54].

Table 5:Pathogenic missense *BRAF* variants detected in melanoma and their frequency.

***BRAF* variants**	**Frequency in melanoma**	**Aminoacid Change**
p.V600E c.1799 T>A	70-88%	valine to glutamate
p.V600K c.1798_1799delGTinsAA	10-20%	valine to lysine
p.V600R c.1798_1799delGTinsAG	<5%	valine to arginine
p.V600D c.1799_1800delTGinsAC	<5%	valine to aspartate

The single nucleotide substitutions at codon 600 culminate in specific amino acid changes that induce a costitutive hyperactivation of the kinase, with the canonical **p.V600E** alteration provoking an approximate 480-fold increase in kinase activity.

Clinically, non-canonical variants such as p.V600K-positive melanomas present an even more aggressive phenotype relative to p.V600E cases, frequently correlating with a higher overall mutational burden.

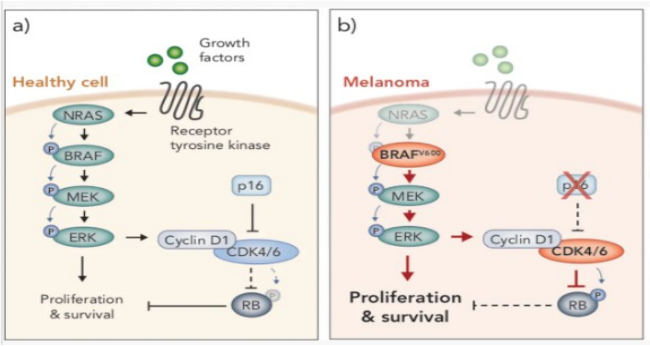

Figure 7: Schematic comparison of the MAPK/ERK and p16/Cyclin D-CDK4/6/RB signaling axes in physiological conditions and melanoma pathogenesis.

In healthy cells (Panel A), mitogenic stimuli trigger a controlled cascade of phosphorylation events along the MAPK/ERK pathway to regulate cell proliferation.

Concurrently, $p16^{INK4a}$ exerts tight inhibitory control over the CDK4/6 complex, preserving the hypophsphorylated, active tumor-suppressive state of RB.

Conversely, in melanoma cells (Panel B), oncogenic BRAF mutations induce constitutive kinase activation, driving continuous transcription independently of upstream growth factors. This aberrant signaling operates in tandem with the functional loss of p16, which unleashes CDK4/6 activity to hyperphosphorilate and inactive RB, thereby promoting unconstrained cellular survival and proliferation.

2.5.2 NRAS

Activating mutations within the *RAS* oncogene family are identified in approximately one-third of allhuman malignancies, with specific *NRAS* characterizing 15% to 20% of cutaneous melanomas.

Biochemically , *NRAS* encodes a small GTPase that acts as a critical upstream molecular switch in the MAPK/ERK cascade.

Structurally and functionally, it was the first oncogene formally identified in melanoma and remains a hallmark of a distinct mutational subtype.

Clinically, patients harboring mutant *NRAS* tumors represent a well-defined demographic, as they tend to be older and present with documented clinical history of chronic, cumulative ultraviole (UV) radiation exposure [55-57].

Histologically, the *NRAS*-mutant subset represents an exceptionally aggressive disease profile associated with unfavorable prognostic outcomes compared to non-*NRAS*-mutant melanomas.

At the bioptic level, these tumors are characteristically characterized by significantly thicker primary lesions, elevated mitotic activity, and substantially higher rates of regional lymph-node metastasis [58-59].

From a therapeutic and evolutionary perspective, oncogenic alterations in *BRAF* and *NRAS* sre typically onsidered mutually exclusive during early oncogenesis due to redundant signaling activation. However, subclonal co-mutations can emerge under selective pressure; notably, the

detection of double-mutated cellular clones within a single tumor mass is often indicative of acquired drug resistance.

Consequently the isolated finding of such co-mutations is insufficient to dictate subsequent monotherapies, highlighting why sophisticated, combinational therapeutic strategies designed to simultaneously target parallel and downstream pathways are strictly necessary to overcome resistance mechanism in these patients[60-63].

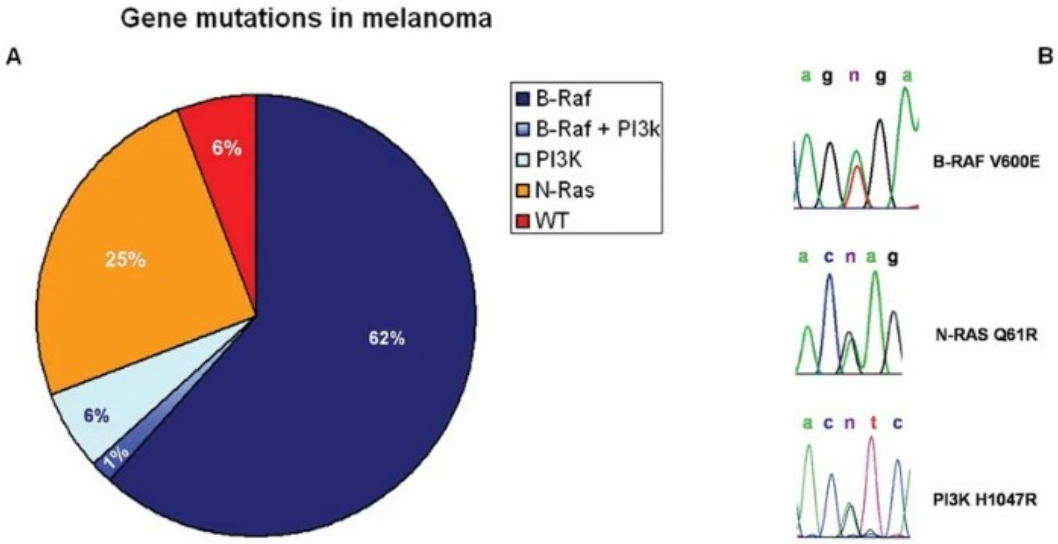

Figure 8:Mutational landscape and molecular hot-spots in cutaneous melanoma.

A) Pie chart representing the percentage distribution of major somatic drivers(*BRAF, NRAS*, AND *PI3K*) within a melanoma case series, highlighting the relative prevalence of each oncogenic subtype alongside wild-type (WT) cases.

B)Representative sequencing chromatograms displaying specific hot-spot missense mutations, including *BRAF* V600E, *NRAS* Q61R and *PI3K* and I1047R.

The vast majority of oncogenic *NRAS* alterations (accounting for >80%) cluster at Codon 61 (predominantly Q61R and Q61L), where they functionally impair intrinsic GTPase activity, effectively locking the protein in a costitutively active, GTP-bound conformation.

Recent functional characterizations demonstrate that these specific position-61 variants exhibit an enhanced capacity to hyperactivate the mitogen-activated protein kinase (MAPK) cascade compared to less frequent alternative *NRAS* mutations located at codons 12, 13, or parallel domains, such as G12D, G13D, G13R, Q61H and Q61P [64].

2.6 CRITERIA FOR GENETIC EVALUATION

Clinical criteria for referring patients to genetic counseling vary significantly according to regional melanoma incidence rates.

In geographical areas characterized by a medium-to-high risk profile such as the United States or Northern Europe, where the estimated incidence exceeds 10 cases per 100,000 individuals- referral thresholds are traditionally less restrictive.

Conversely, in regions with lower baseline incidence such as Italy, stricter screening protocols are required to optimize the diagnostic yield of multi-gene panels.

In these populations, the lower background rate of sporadic, environmentally-driven melanomas increases the probability that clustering or early onset cases are linked to high-penetrance germline mutations.

The latest **AIOM guidelines** (2024) recommend the use of a multigene panel for patients presenting with:

- **Melanoma and a positive family history** (at least two affected members in the same family) or a suggestive personal history (multiple primary melanomas);
- **Melanoma and a personal or family history** of associated malignancies, including pancreatic adenocarcinoma, uveal melanoma, pleural or peritoneal mesothelioma, and renal tumors;
- **A personal history** of excised atypical Spritz nevi;
- **Clinical features fulfilling the diagnostic criteria** for melanoma-related syndromes, particularly hamartoma syndromes associated with *PTEN* mutations and *BAP1* tumor predisposition syndrome [65-66].

3. MATERIALS AND METHODS

Between January 2025 and May 2026, a sample size comprising 245 individuals, including index cases and respective family members who are mutation-carrier were enrolled in this study.

Genetic screening was performed at the Genetics Laboratory within the S.C.D.U Clinical Biochemistry of the "Maggiore della Carità" University Hospital in Novara.

Patient eligibility for genetic evaluation was determined based on direct clinical referrals from the institutional Dermatology Clinic and collaborating regional oncology networks.

The primary operational objective of the laboratory pipeline was to identify pathogenic germline variants correlating with cutaneous melanoma susceptibility or broader cancer-predisposing syndromes.

Prior to peripheral blood collection, all participants received appropriate genetic counseling and provided written informed consent in accordance with the institutional ethical protocols.

3.1 GENOMIC DNA EXTRACTION

Total genomic DNA (gDNA) was extracted from peripheral blood leukocytes. The isolation protocol was performed utilizing an initial volume of 200 μL of whole blood, processed via the ReliaPrep^{TM} Blood gDNA Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Purified gDNA was eluted in nuclease-free water to achieve a final volume of 50 μL.

Quantitative and qualitative DNA assessments were performed via spectrophotometric analysis using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

High-quality genomic DNA criteria were defined by target absorbance ratios of 1.8-2.0 for A_{260}/A_{280} and 2.0-2.2 for A_{260}/A_{230} alongside a minimum concentration threshold of >30 ng/μL.

3.2 SANGER SEQUENCING

Sanger sequencing, based on the dideoxy chain-termination methodology, was employed to analyze targeted genomic regions.

This classic sequencing approach relies on the selective incorporation of fluorescence-labeled dideoxynycleotides (ddNTPs) during in vitro DNA replication, enabling high-resolution fragment identification.

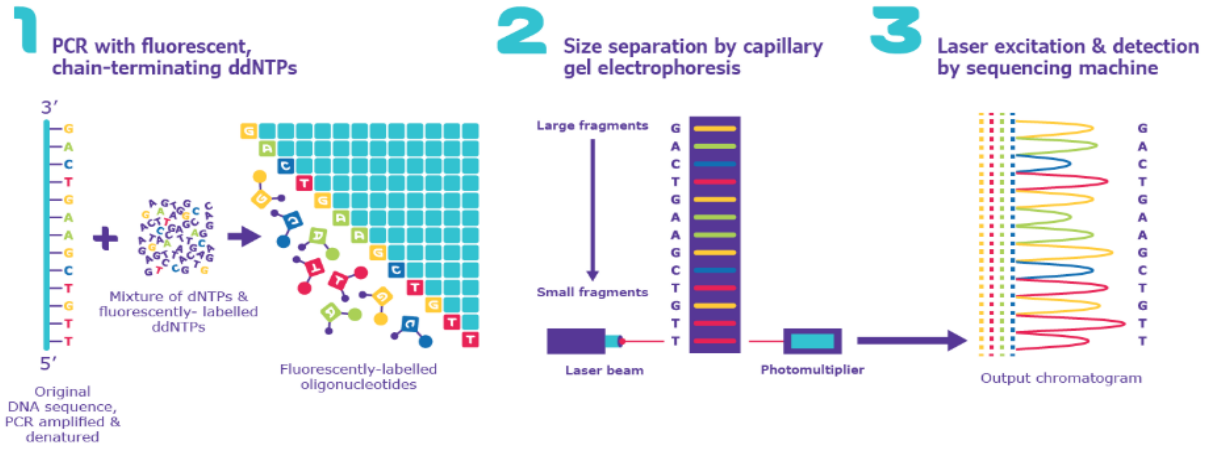

Figure 9: Methodological workflow of Sanger sequencing by chain termination. The process integrates three sequential technical steps: 1) PCR amplification utilizing fluorescently labeled chain-terminating dideoxynycleotides (ddNTPs).
2) High resolution size separation of the resulting single-stranded fragments via capillary gel electrophoresis.
3) Automated laser excitation and fluorescence detection to generate the corresponding nucleotide sequence chromatogram.

In this study, Sanger sequencing was utilized to evaluate the presence of germline point mutations within the coding regions of the *CDKN2A* gene, corresponding to the entire sequence of the $p16^{INK4a}$ and $p14^{ARF}$ proteins (exons 1α, 1β, 2, and 3), as well as the flanking exon-intron junctions at the canonical splice sites. Concurrently, exon 2 of the *CDK4* oncogene was analyzed through targeted amplification and direct sequencing following an independent secondary DNA extraction protocol.

3.2.1 SANGER SEQUENCING WORKFLOW

The analytical pipeline initiated with the site-specific amplification of target genomic DNA via Polymerase Chain Reaction (PCR), employing customized complementary forward and reverse primer pairs selected to guarantee high selectivity for the loci of interest.

To verify successful fragment amplification and evaluate amplicon size, a fraction of the PCR product was subjected to horizontal agarose gel electrophoresis in 1 X Tris-Acetate-EDTA (TAE) buffer.

Subsequently enzymatic purification of the remaining PCR volume was performed to eliminate excess unincorporated deoxynucleotide triphosphates (dNTPs) and residual single-stranded primers, utilizing the Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Weltham, MA, USA).

The purified amplicons then underwent a secondary asymmetric PCR profile, structurally defining the "chain termination" reaction. This phase integrated dideoxynycleotides (ddNTPs), which mimic conventional dNTP substrates but lack the critical 3'-hydroxyl (-OH) moiety essential for phosphodiester bond formation, thus halting DNA chain elongation upon incorporation.

Given that each of the four distinct ddNTP bases is covalently labeled with a specific, non-overlapping fluorophore, the reaction yielded an array of variable-length DNA fragments, each terminating with a base-specific fluorescent tag.

Following a final purification step designed to precipitate and remove residual salts, enzymes, and unincorporated dye terminators, the labeled fragments were loaded into the automated capillary electrophoresis sequencer. Electrokinetic injection guided the fragments through the capillary matrix, enabling high-resolution separation based on molecular weight and the subsequent reconstruction of the definitive digital nucleotide chromatogram.

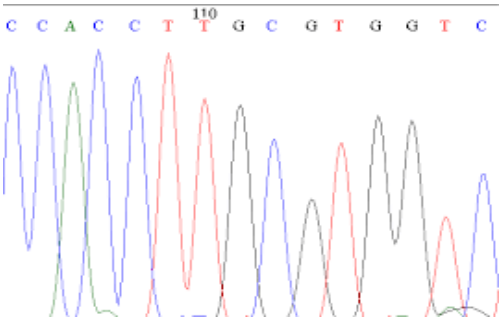

Figure 10: Representative capillary electrophoresis Sanger sequencing chromatogram.

The electropherogram displays a high-resolution, distinct single-color peak profile corresponding to each incorporated dye-terminator base.

3.3 NEXT GENERATION SEQUENCING (NGS)

For high-throughput parallel genetic analysis, Next-Generation Sequencing (NGS) was utilized as the preferred methodological approach, employing a targeted multi-gene panel specifically designed to screen 23 high and medium penetrance genes implicated in hereditary cancer syndromes. This customized diagnostic panel, designed as the Breast-Melanoma-Lynch (BML) panel, allows for the concurrent evaluation of constitutional susceptibility across multiple syndromic lineages. The specific genomic composition is detailed below in **Table 6**.

Table 6: Comprehensive locus composition of the custom Breast-Melanoma-Lynch (BML) multi-gene panel.

APC	*EPCAM*	*RAD50*
ATM	*MC1R*	*RAD51C*
BAP1	*MITF*	*RAD51D*
BRCA1	*MLH1*	*TERT*
BRCA2	*MSH2*	*TP53*
BRIP1	*MSH6*	
CDK4	*PALB2*	
CDKN2A	*PMS2*	
CHEK2	*POT1*	

Bold formatting highlights the core predisposing genes specifically evaluated for cutaneous melanoma susceptibility configurations in this cohort.

Target enrichment and library preparation were performed utilizing the SureSelect XT HS2 DNA kit (Agilent Technologies, Santa Clara, CA, USA), defining a cumulative target coverage of 131,335 bp. The bioinformatic alignment and variant calling analysis focused strictly on the coding exons and adjacent flanking exon-intron boundaries (20 base pairs) at the canonical splice sites of the primary melanoma-associated loci, including ***BAP1***, ***CDKN2A*** (exons 1α, 1β, 2, and 3, coding regions for $p16^{INK4a}$ and $p14^{ARF}$), ***CDK4, MC1R, MITF, POT1*** and ***TERT***, alongside secondary core genes included in the custom sequencing architecture.

Concurrently, Clinical Focused Exome Sequencing (CES) was implemented using high-capacity NGS methodologies. Clinical exome screening offers a highly strategic diagnostic architecture by selectively targeting clinically relevant, disease associated loci rather than processing the entire Whole Exome Sequencing (WES) landscape, thereby optimizing sequencing depth and variant interpretation efficiency. Within this pipeline, the specialized genomic downstream analysis was restriction-mapped to evaluate the complete coding sequences and respective exon-intron splice junction of 5,629 well-characterized disease associated genes.

3.3.1 NEXT GENERATION SEQUENCING WORKFLOW

Fluorometric DNA quantification was critically performed utilizing the Qubit™ 1X dsDNA HS (High Sensitivity) Assay Kit on a Qubit™4.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) to ensure accurate initial template concentrations. For library preparation of the custom BML 23-gene panel, target enrichment was executed according to the Agilent SureSelect XT HS2 DNA System protocol (Agilent Technologies, Santa Clara, CA, USA) optimized for Illumina platform.

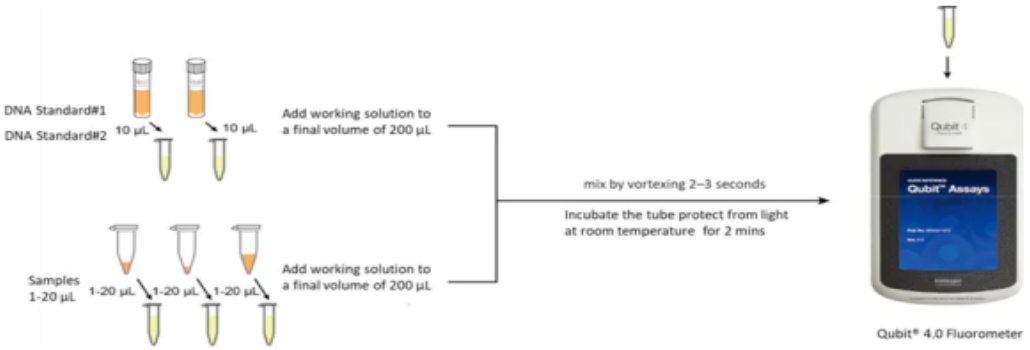

Figure 11: Analytical workflow for fluorometric DNA quantification utilizing the Qubit™ dsDNA HS Assay configuration.

The schematic illustrates the sequential preparation steps, initiating with the 1:200 operational diluition of the target-specific fluorescent dye within the dedicated buffering matrix to generate the working solution. Following the quantitative incorporation of pre-calibrated standard references and individual genomic DNA isolates (1-20 μL), the mixture undergoes brief vortexing and a 2-minute incubation period protected from light to stabilize the selective fluorophore-target complex. Final fluorescence emission profiles are captured via the automated optical system of the Qubit™ 4.0 Fluometer, ensuring highly selective and precise target concentration metrics prior to downstream next-generation sequencing library processing.

The comprehensive library preparation pipeline required an approximate 10-hour operational workflow, initiating with the enzymatic fragmentation of genomic DNA to generate fragments with an average size distribution of approximately 400 base pairs (bp).

Following fragmentation, end repair and A-tailing protocols were implemented to facilitate the subsequent ligation of platform-specific molecular adapters and unique dual indexing barcodes, which individually discriminate patient samples within multiplexed pool. The adapter ligated DNA structures were then hybridized with customized, biotinylated capture probes complementary to the target interest regions.

Streptavidin-coated magnetic beads were utilized to capture the probe template hybrids, followed by stringent washing cycles designed to eliminate non-specific genomic material prior to final PCR amplification of the target-enriched libraries.

The average size distribution and structural integrity of the final library fragments were assessed qualitatively and quantitatively via automated capillary electrophoresis using the 4150 TapeStation System coupled with the High Sensitivity D1000 ScreenTape Kit and associated reagents (Agilent Technologies). To ensure uniform data generation across all multiplexed channels, an equimolar library pool was constructed. The operational pooling strategy calculated the final required volume of the desired pool (V_f , typycally targeted between 100-150 μL for a 32 library multiplex)and the final concentration (C_f, optimized around 4-6 nM) against the initial library concentration of each individual sample (C_i), as determined by the TapeStation metrics.

High-throughput sequencing architectures were segregated based on panel requirements. For the targeted 23-gene BML panel, sequencing was conducted on the MiSeq Dx instrument utilizing MiSeq Reagent Kits v2 or v3, selected relative to sample cohort sizes. Conversely, downstream processing for the broader Clinical Exome Sequencing (CES) configuration was performed on a NextSeq 1000 platform using the NextSeq 1000/2000 P2 Standard SBS Reagent Kit
(Illumina, San Diego, CA, USA).

During the clusters generation phase on the flow cell, platform adapters mediated the bridge amplification process, yielding dense, clonal clusters of identical single-stranded DNA templates.

To allow bidirectional paired-end sequencing, the antisense strands were enzymatically cleaved and removed, and the 3' ends were blocked to ensure directional fidelity.

Sequencing by Synthesis (SBS) chemistry progressed via successive reaction cycles, where fluorescence-labeled reversible terminator nucleotides competed for incorporation into the growing nascent strand.

Laser excitation captured the unique emission spectrum of each incorporated base in real-time, enabling automated base calling.

Following Read 1 completion, a sodium hydroxide (NaOH) denaturation step removed the extended complementary strand, and the process was systematically repeated from the opposite direction to generate Read 2, producing millions of high-accuracy paired-end reads in parallel.

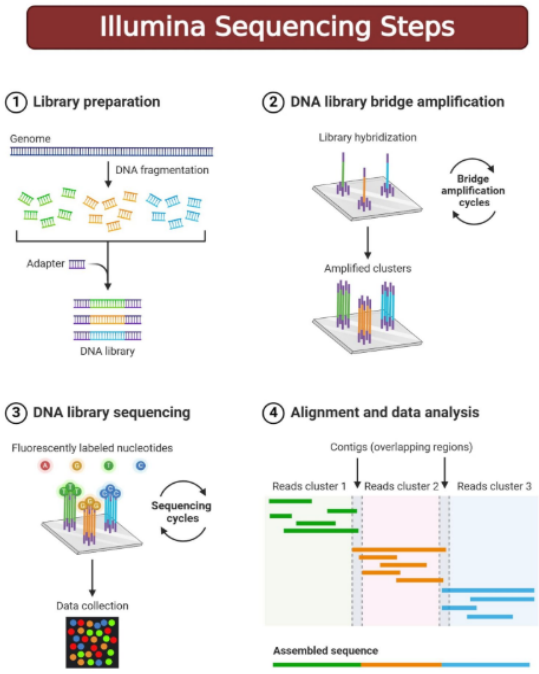

Figure 12: Core operational stages of high-throughput sequencing on Illumina architectures. The diagram provides a macro-level overview of the downstream analytical pipeline, consolidating four sequential phases: 1) generation of the adapter-ligated DNA library, 2) automated clonal amplification via bridge replication cycles on the flow cell matrix to optimize signal density, 3) real-time cyclic sequencing tracks driven by fluorescence-labeled reversible terminators, and 4) automated computational sequence alignment against reference genomes for definitive variant calling within target predisposing loci.

3.3.2 BIOINFORMATIC PIPELINE AND VARIANT ANNOTATION

Paired-end high throughput sequencing systematically generated two distinct FASTQ raw data files, representing Read 1 and Read 2 operations for each interrogated genomic fragment. Initial sequencing quality control and raw read filtering were conducted to eliminate low-score data channels. Surviving high-quality forward and reverse tracks were subsequently paired into contigous genomic structures and computationally aligned against the canonical human reference genome assembly GRCh37/hg19.

Downstream bioinformatics alignment and definitive variant calling operations were segregated based on the strategic assay design:

- For patient sample cohorts processed via the customized 23-gene BML panel on the MiSeq Dx platform, data architecture management was executed utilizing the Alissa Reporter software package (Agilent Technologies) .
- Conversely, secondary and tertiary sequencing analyses for Clinical Exome Sequencing (CES) raw datasets were driven by the enGenome-eVai platform and the wANNOVAR annotation

computational framework.

To characterize and prioritize the clinical significance of the detected nucleotide alterations, candidate variants were comprehensively cross-referenced against internationally recognized population and locus-specific genomic databases. Minor allele frequencies (MAF) and baseline genomic polymorphisms were evaluated using dbSNP, the Genome Aggregation Database (gnomAD), the Exome Variant Server (EVS/ESP), and the 1000 genomes project platform.

Definitive clinical interpretation, pathogenicity classification, and clinical correlation profiles of the targeted loci were established via systematic querying of the National Center for Biotechnology Information (NCBI) ClinVar registry.

Despite the rigorous analytical sensitivity of the deployed high-throughput methodologies, specific technical constraints must be acknowledged. The custom bait-capture enrichment chemistry is inherently restricted to coding exons and flanking canonica splice-site donor-acceptor regions, thus limiting the direct detection of deep intronic alterations or distant regulatory elements located outside the pre-defined target boundaries.

Furthermore, rare pathogenic variants located within sub-optimally enriched gene domains or regions characterized by inadeguate sequencing depth (coverage threshold strictly below 20 filtered reads) might escape robust detection criteria, potentially resulting in false negative calling profiles.

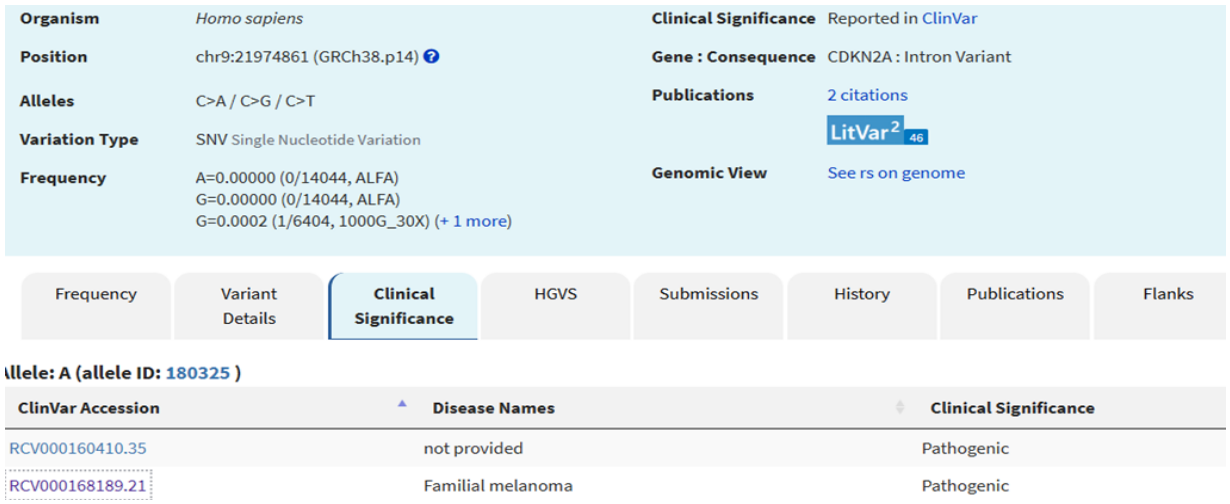

Figure 13: Representative interface of the National Center for Biotechnology Information ClinVar database utilized for the clinical classification of identified variants.

3.4 MULTIPLEX-LIGATION-DEPENDENT-PROBE-AMPLIFICATION (MLPA)

To complement nucleotide variant screening and detect large genomic copy number variations (CNVs), including exonic deletions or duplications that escape NGS capture architectures, Multiplex Ligation-dependent Probe Amplification (MLPA) was performed.

Constitutional copy number status of the high-penetrance *CDKN2A* and *CDK4* loci was systematically evaluated using commercial SALSA MLPA probemix configurations (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's protocols.

The standardized semi-quantitative workflow integrated four sequential stages: 1)thermal DNA denaturation followed by overnight target-specific probe hybridization, 2) covalent enzymatic probe ligation, 3) multiplex PCR amplification using a single fluorscence-labeled universal prime pair and 4) high-resolution fragment separation via capillary electrophoresis on an ABI 3500 Series Genetic Analyzer (Applied Biosystems). Relative peak area quantification and automated dosage ratio calculations were executed using Coffalyser.Net software (MRC Holland).

Genomic deletion profiles were defined by an analytical probe ratio threshold below 0.7 (heterozygous loss), whereas exonic duplications were characterized by a dosage ratio shifts exceeding 1.3 relative to wild type reference controls.

4. RESULTS

4.1)PATIENTS

From January 2025 to May 2026, a total of 245 patients were referred to the Genetics laboratory, operating within the S.C.D.U. Clinical Biochemistry of the A.O.U "Maggiore della Carità" in Novara.

Referral cohorts were selected strictly adhering to current national and international guidelines for hereditary melanoma predisposition.

Out of the 245 patients evaluated, 34 (13,88%) tested positive for at least one pathogenic germline alterations, 6 (2,45%) have VUS germline alteration, whereas the remaining 205 (83,67%) exhibited wild-type genetic profile (**figure 14**).

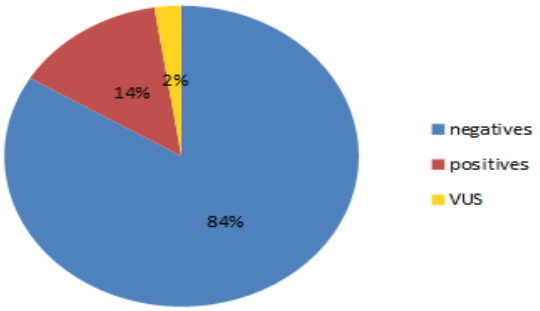

Figure 14: Overall diagnostic yield of germline genetic testing within the study cohort (January 2025-May 2026).
The pie chart illustrates the proportion of patients with a negative result (84%, n=205) those who harbored a pathogenic or likely pathogenic germline variant (14%, n=34), and those who harbored a VUS alteration (2,45%, n=6), among the 245 total individuals referred to the Novara center.

The resulting molecular data were thorougly collected and standardized in **Tables 6-7**. The screening primarily focused on *CDKN2A*, the main susceptibility gene associated with familial cutaneous melanoma, and was subsequently extended to a panel of secondary genes. These included factors involved in Mismatch Repair (MMR) system as well as tumor suppressors implicated in various hereditary cancer syndromes such as BRCA1 and BRCA“.

For each identified variant, the descriptive nomenclature was standardized according to the HGVS (Human Genome Variation Society) guidelines, reporting the alterations at the nucleotide level (cDNA) and the corresponding effect on the protein chain. Variant pathogenicity was interpreted in accordance with the standardized criteria of the ACMG (American College of Medical Genetics and Genomics).

Variants detected in the patients

Table 6 reports the genes associated with melanoma syndrome development and Table 7 details the genes linked to other hereditary cancer syndromes

Table 6: Gene associated with melanoma development

GENE	ID/number of patients	cDNA	Protein	ACMG classification
CDKN2A	**14 patients**	***c.301G>T*** NM_000077.5	**p.Gly101Trp**	**Pathogenic**
	#31-25	***C.79G>T*** NM_000077.5	**p.Glu27Ter**	**Pathogenic**
	#1514-25	***c.142C>A*** NM_000077.5	**p.Pro48Thr**	**Pathogenic**
	#176-26			
	#824-21			
	#1577-25	***c.71G>C*** NM_000077.5	**p.Arg24Pro**	**Likely pathogenic**
	#303-26			
	#1752-25	***c.377T>A*** NM_000077.5	**p.Val126Asp**	**Likely pathogenic**
	#350-26	***c.379G>C*** NM_000077.5	**p.Ala127Pro**	**Likely pathogenic**
BAP1	**#383-25**	***c.1139G>A*** NM_004656.4	**p.Gly380Asp**	**VUS**
CDK4	**#1576-25**	***c.458C>T*** NM_000075.4	**p.Thr153Ile**	**VUS**
POT1	**#987-25**	***c.259_260insT*** NM_015450.3	**p.Gln87LeufsTer4**	**Likely pathogenic**
Moderate risk genes				
ATM	**#1703-25**	***c.3511C>T*** NM_000051.4	**p.Gln1171***	**Pathogenic**
	#27-26	***c.6629del*** NM_000051.4	**p.Gln2210Argfs*25**	**Pathogenic**

GENE	ID/number of patients	cDNA	Protein	ACMG classification
MITF	**#28-25**	**c.*32C>T*** NM_001354607.2	**p.Pro11Leu**	**VUS**
	#611-25	***c.1273G>A*** NM_001354604.2	**p.Glu425Lys**	**Likely pathogenic**
	#20-26			
	#1013-25			
TERT	**#779-25**	***c.556C>T*** NM_198253.3	**p.Pro186Ser**	**VUS**

Table 7: Gene that are generally not associated with melanoma development.

GENE	ID/number of patients	cDNA	Protein	ACMG classification
BRCA1/2	**#1078-25**	***c.5509T>C*** NM_007294.4	**p.Trp1837Arg**	**Pathogenic**
	#1078-25	***c.8186A>G*** NM_000059.4	**p.Lys2729Arg**	**VUS**
BRCA1	**#74-26**	***c.3257T>G*** NM_007294.4	**p.Leu1086***	**Pathogenic**
MSH6	**#1799-22**	***c.3844_3845insTAT*** NM_000179.3	**p.Thr1282delinsIleSer**	**VUS**
CHEK2	**#634-25**	***c.1427C>T*** NM_007194.4	**p.Thr476Met**	**Pathogenic**
	#101-26			
	#697-25	***c.85C>T*** NM_007194.4	**p.Gln29Ter**	**Pathogenic**
	#1899-24	***c.1100del*** NM_007194.4	**p.Thr367MetfsTer15**	**Pathogenic**

The prevalence of specific high and moderate risk variants among the 245 analyzed patients, is detailed in **Table 8** below.

Table 8: Prevalence and percentage distribution of germline mutations identified via multigene panel testing in the study cohort (N=245).

Category	**Gene panel**		***CDKN2A***		***Non-CDKN2A***		***MITF***		***CHEK2***		**BRCA1/2**		**ATM**		**POT1**	
	Mut	%	Mut	%	mut	%	mut	%	mut	%	mut	%	mut	%	mut	%
Total(N=245)	34	**13.88**	22	**8.98**	12	**4,9**	3	**1.22**	4	**1.63**	2	**0.82**	2	**0,82**	1	**0.41**

As summarized in Table 8, the implementation of an expanded multigene panel provided a substantial clinical advantage over traditional diagnostic strategies, increasing the detection rate of variants in genes associated with hereditary cancer syndromes. However, the clinical relevance of variants identified in non-classical melanoma-associated genes requires careful interpretation, particularly in light of each patient's personal and family history.Specifically, the *CDKN2A* detection rate was **8.98%** (22 cases), whereas adding other risk genes to the multigene-panel increased the total detection rate up to **13.88%** (34 cases).

Among patients carrying *CDKN2A* pathogenic variants, the most frequent alteration was c.301G>T, accounting for 63.6% of *CDKN2A*-mutated cases (N=14/22). Other recurrent pathogenic variants included c.142C>A (N=3/22, 13.6%) and c.71G>C (N=2/22, 9.1%).

After *CDKN2A*, the most frequently altered genes in the panel were *CHEK2* and *MITF*. *MITF* is recognized as a moderate-risk melanoma susceptibility gene, whereas the role of *CHEK2* in melanoma predisposition remains less clearly defined and should be interpreted in the broader context of hereditary cancer risk and family history. Variants in *CHEK2* and *MITF* accounted for 4/34 (11.8%) and 3/34 (8.8%) of all identified pathogenic variants, respectively, corresponding to prevalences of 1.63% and 1.22% in the entire study cohort (N=245).

4.2 Identification of Dual Hereditary Cancer Predisposition in a Large Pedigree

A particularly interesting case concerns a large pedigree in which germline pathogenic variants in both *CDKN2A* and *MLH1*, the latter associated with Lynch syndrome, were identified and shown to segregate within the family.

Genetic investigation was initiated in the proband, III-7, who had a personal history of early-onset melanoma and was affected by colorectal cancer at the time of genetic testing. This clinical presentation raised the suspicion of an underlying hereditary cancer predisposition syndrome. In particular, the coexistence of melanoma and colorectal cancer suggested that a broader genetic approach, rather than testing limited to classical melanoma-predisposition genes, could be clinically informative.

For this reason, multigene panel testing was performed in the proband. This analysis led to the simultaneous identification of pathogenic variants in *CDKN2A*, a major melanoma susceptibility gene, and *MLH1*, a mismatch repair gene associated with Lynch syndrome. The detection of both variants in the same individual represented a key finding, as it provided a molecular explanation for the complex cancer phenotype observed in the family and highlighted the possible coexistence of two distinct hereditary cancer predisposition conditions.

Following this result, cascade genetic testing was extended to at-risk relatives across generations III, IV, and V. Segregation analysis allowed the familial distribution of the two pathogenic variants to be clarified, distinguishing relatives carrying the *CDKN2A* variant, the *MLH1* variant, both variants, or neither. This information was clinically relevant because it enabled a more precise assessment of individual cancer risk and supported tailored surveillance strategies according to the specific genetic status of each family member.

Overall, this pedigree illustrates the added value of multigene panel testing in families with complex or partially overlapping cancer histories. In this case, the use of an expanded approach allowed the identification of dual hereditary cancer predisposition involving both familial melanoma and Lynch syndrome, which may have been missed or only partially characterized by a single-gene testing strategy.

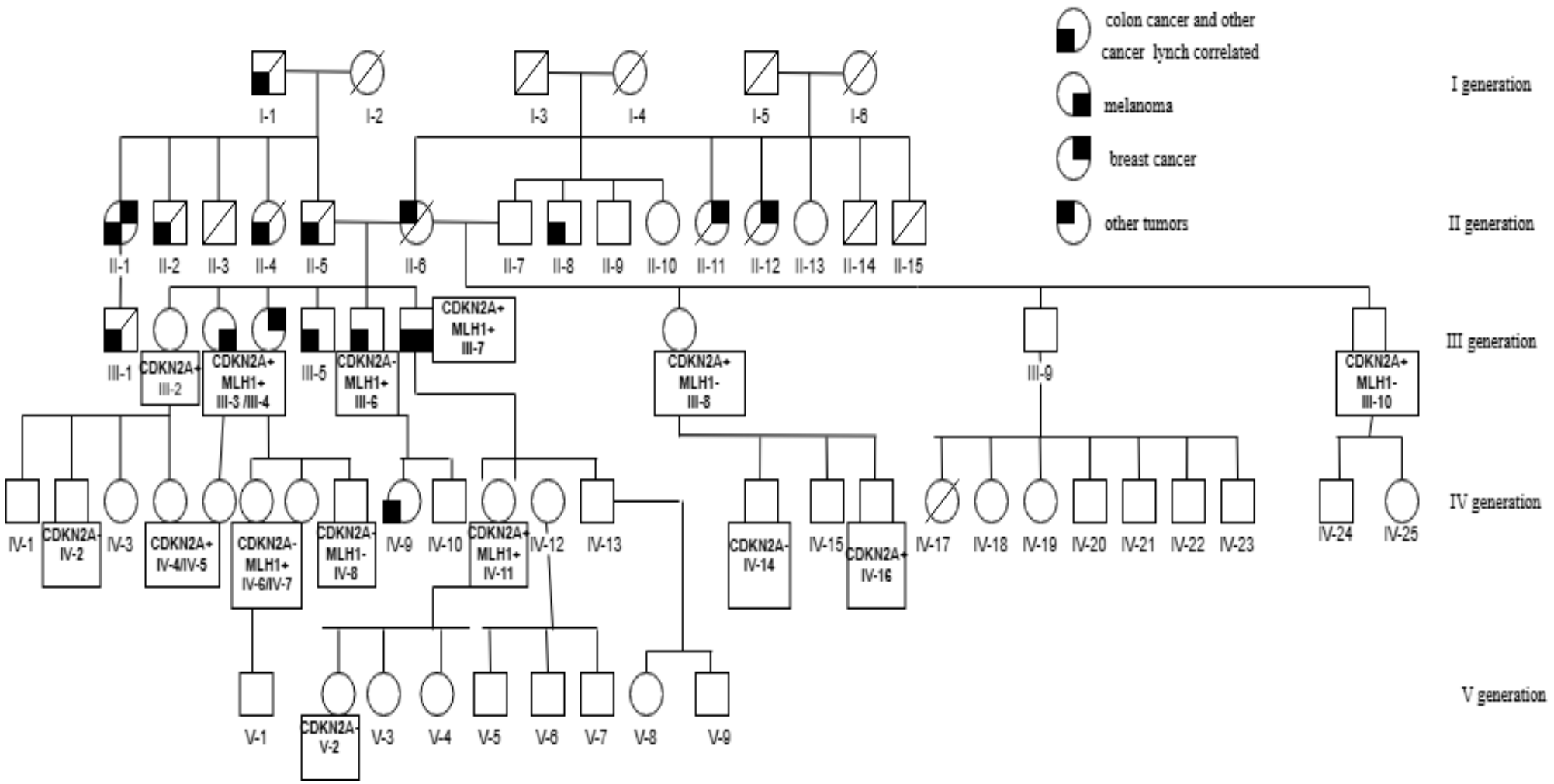

Figure 15: Pedigree chart of the analyzed multi-generational family showing the germline co-segregation of *CDKN2A* and *MLH1* mutations. Squares represent males, circles represent females, and diagonal lines indicate deceased individuals. Shaded quadrants within symbols define specific tumor phenotypes as indicated in the legend.

To complement the visual representation of the pedigree and provide a clearer integration of these clinical findings, the germline genotypes, detailed tumor manifestations, and respective age of onset for each evaluated family members are comprehensively summarized in **Table 9**. Molecular testing for the familial pathogenic variants in *MLH1* and *CDKN2A* allowed the genetic status of several at-risk relatives to be defined and clarified the segregation pattern within the pedigree.

Four individuals were found to carry both pathogenic variants. Among them, one had a personal history of both colorectal cancer and melanoma, one had breast cancer, one had melanoma, and one was unaffected at the time of testing. Six individuals carried only the *CDKN2A* pathogenic variant; notably, none of these relatives had a personal history of cancer at the time of evaluation. Four individuals carried only the *MLH1* pathogenic variant, including one affected by colorectal cancer, one by glioblastoma, one by Wilms tumor, and one individual with no personal history of cancer.

In contrast, five tested relatives were negative for both familial variants and had no reported personal history of cancer. Nine family members were not available for genetic testing, including one individual with cervical cancer and eight unaffected relatives.

Overall, this segregation analysis showed the coexistence of two independent hereditary cancer predisposition variants within the same family. The identification of relatives carrying both variants, only one variant, or neither variant provided clinically relevant information for risk stratification and personalized surveillance. In particular, carriers of the *CDKN2A* variant may benefit from melanoma- and pancreatic cancer-oriented surveillance, whereas carriers of the *MLH1* variant require management according to Lynch syndrome recommendations. Individuals carrying both variants represent a particularly relevant subgroup, as their clinical follow-up should take into account the combined cancer risks associated with both genetic conditions.

Table 9: Genotype-phenotype correlation, tumor manifestations, and age of onset of tested family members.

ID	*CDKN2A* status	*MLH1* status	Phenotype
III-2	+	not tested	Healthy
III-3	+	+	Melanoma at 34y and another one at 57y.
III-4	+	+	Breast cancer at 64y.
III-6	-	+	Colorectal cancer at 36y.
III-7	+	+	Colorectal cancer at 40y and melanoma at 56y
III-8	+	-	Healthy
III-10	+	-	Healthy
IV-2	-	not tested	Healthy
IV-4	+	not tested	Healthy
IV-5	+	not tested	Healthy
IV-6	-	+	Healthy
IV-7	-	+	Wilms tumor 5-6y
IV-8	-	-	Healthy
IV-9	not tested	not tested	Glioblastoma at 19y
IV-11	+	+	Healthy
IV-14	-	not tested	Healthy
IV-16	+	not tested	Healthy
V-2	-	not tested	Healthy

Abbreviations: +, mutated status; -, wild type status; not tested, analysis not performed for the specific locus; y, years of age at diagnosis.

5. DISCUSSION

The genetic risk factors for melanoma are complex, involving both familial and environmental components.

Of the thousands of cases diagnosed each year, only a fraction are due to inherited cases.

These familial melanoma typically present in younger individuals and are frequently associated with genetic factors that predispose patients to other malignancies.

To date, *CDKN2A* and CDK4 represent the most well-characterized mutations, identified in up to 40% of familial cases.

Historically, *CDKN2A* was one of the earliest susceptibility genes identified. Together with *CDK4*, it accounts for the majority of familial melanoma syndromes. In recent years, however, other genes such *BRCA2, BAP1,* and *POT1* also been shown to play a significant role [67-69].

These pathway and loci alterations include genetic changes (amplifications, deletions, mutations, translocations) as well as epigenetic modifications like promoter hypermethylation.

Oncogenesis begin with a 'driver' event, an initial genetic or epigenetic shift that triggers tumor development [70] .

This is followed by 'passenger' events, which accumulate further genomic insults and propagate the molecular cascade driving melanoma progression [67] .

In this context, analyzing the germline mutational spectrum of high and moderate risk genes is essential to understand the specific genetic architecture of familial melanoma in clinical practice.

The present study evaluated a selected cohort of 245 patients with familial melanoma using an expanded multigene panel.

Our findings revealed a total germline mutation detection rate of 13,88% (34 of 245 patients), underscoring the high prevalence of inherited susceptibility within this clinically selected population.

Consistent with published literature, *CDKN2A* represented the predominant susceptibility gene identified in our cohort, showing a mutation frequency of 8.98% (*n*=22) [71].

Looking closely at the mutational spectrum of *CDKN2A*, the c.301G>T pathogenic variant was highly predominant, accounting for 63.6% (*n*=14/22) of all *CDKN2A* mutated cases.

This specific nucleotide substitution is widely recognized in the literature as a classic founder mutation in the Italian population [72].

Beyond *CDKN2A*, the inclusion of moderate-risk susceptibility genes in the panel provided critical diagnostic insights.

An interesting result that emerged from our study is the presence of patients harboring mutations in the *CHEK2* gene (1.63%).

Although germline variants in *CHEK2* are traditionally recognized as susceptibility factors for

hereditary breast cancer, the increasing use of multigene panel testing has broadened the clinical spectrum associated with this gene, suggesting a possible involvement in additional malignancies, including cutaneous melanoma.

From a biological perspective, *CHEK2* encodes checkpoint kinase 2, a key protein involved in the DNA damage response. CHEK2 is activated following genomic stress, particularly in response to DNA double-strand breaks induced by ionizing radiation and other genotoxic insults. Acting in a pathway functionally connected to *ATM*, CHEK2 contributes to the recognition and signaling of DNA damage and phosphorylates downstream targets such as p53, BRCA1, and other proteins involved in cell cycle control. Through these mechanisms, CHEK2 promotes cell cycle arrest, DNA repair, or apoptosis, thereby helping to preserve genomic stability.

Among the deleterious variants described in this gene, *CHEK2* c.1100delC is one of the most widely studied. This frameshift variant is predicted to generate a truncated, non-functional protein, resulting in impaired DNA damage response activity [73]. Its possible association with melanoma risk has been previously investigated [74]. In particular, Danish and German case-control studies, together with meta-analyses, reported an approximately twofold increased risk of malignant melanoma in individuals heterozygous for *CHEK2* c.1100delC compared with non-carriers. However, the strength and clinical relevance of this association remain less clearly established than for classical melanoma susceptibility genes.

Consistent with these observations, in our diagnostic cohort, *CHEK2* pathogenic variants represented one of the most frequent non-*CDKN2A* findings. Notably, the c.1100delC variant was identified in one of the four patients carrying pathogenic alterations in *CHEK2*. Although this finding alone does not allow definitive conclusions regarding causality, it supports the relevance of considering *CHEK2* within a broader hereditary cancer predisposition framework, especially in patients with melanoma and a personal or family history suggestive of additional tumor susceptibility.

Mechanistically, a contribution of *CHEK2* pathogenic variants to melanoma susceptibility appears biologically plausible. Melanocytes are chronically exposed to ultraviolet radiation, which induces several forms of DNA damage and increases genomic instability. In individuals carrying loss-of-function *CHEK2* variants, a reduced ability to activate appropriate DNA damage checkpoints and repair pathways may contribute to the accumulation of mutations and, consequently, to melanoma development [75–76]. Therefore, while *CHEK2* should not currently be considered a classical high-penetrance melanoma gene, its identification in melanoma-prone individuals may provide clinically useful information, particularly when interpreted alongside personal history, family history, and the presence of other hereditary cancer features.

Family with the co-segregation of *CDKN2A* and *MLH1* mutations

The proband (**III-7**), who was referred to genetic screening following a clinical history of cutaneous melanoma diagnosed at age 40 and colorectal cancer (CRC) at age 56, was found to carry pathogenic germline variants in both *CDKN2A* and *MLH1* genes. This dual molecular finding perfectly mirrors the striking multi-organ oncological aggregation observed across the pedigree.

The proband's family history reveals a heavy burden of malignancies on both parental sides: the father (II-5) was diagnosed with colorectal cancer at age 50, a classic hallmark of Lynch syndrome, while the mother (II-6) developed a cutaneous squamous cell carcinoma in her late seventies. Familial segregation analysis performed on the proband's siblings demonstrated an extraordinarily high mutation transmission rate. Among the tested siblings, 3 out of 5 carried the *CDKN2A* pathogenic variant (**III-2, III-3, III-4**), while all 3 evaluated individuals (3/3) tested positive for the *MLH1* mutation (**III-3,III-4, III-6**), confirming a high penetrance and an independent segregation of the two loci within this branch.

Interestingly, the right side of the pedigree originating from the mother's second partner (II-7) shows an isolated segregation of the *CDKN2A* mutation alone, with a complete absence of the *MLH1* alteration.

This distinct distribution clearly identifies the maternal lineage as the vehicle for *CDKN2A* susceptibility, whereas the *MLH1* defect tracks back to the paternal branch.

Given the high risk of multiple primary malignancies in these mutation carriers, extensive clinical surveillance and further cascade genetic testing on currently untested relatives are absolutely mandatory to implement timely, life-saving screening protocols for both melanoma and colorectal carcinoma.

The *MLH1* gene, located at **chromosome 3p22.2**, encodes a 756-amino-acid protein that plays a pivotal role as a central coordinator within the DNA mismatch repair (MMR) system.

This cellular quality-control mechanism functions as a heterodimeric complex primarily with PMS2 (forming the MutLα complex) to detect and correct base substitution mismatches and insertion-deletion loops that arise during DNA replication. Constitutional disruption of this pathway leads to Lynch syndrome, characteristically predisposing individuals to early-onset colorectal and endometrial malignancies.

While germline mutations in *CDKN2A* are classically associated with **familial atypical multiple mole melanoma** (FAMMM) syndrome, multi-gene panel testing has increasingly identified its involvement in wider oncogenic spectrums, including familial pancreatic cancer (FPC) [77].

Cases of germline co-inheritance involving *CDKN2A* and mismatch repair (MMR) genes are exceptionally rare in the literature; intriguingly, the pathogenic co-segregation of *MLH1* and

CDKN2A has been documented in connection with highly aggressive phenotypes.

For instance, a 2020 study investigated a cohort of 43 pancreatic ductal adenocarcinomas (PDAC) and identified constitutional pathogenic variants in both *MLH1* and *CDKN2A* in 3 out of 43 cases (7%) [78].

Although germline mutations in *CDKN2A* remain rare across broader familial pancreatic cancer (FPC) cohorts, individuals belonging to FAMMM syndrome families carry a significantly elevated risk of developing pancreatic malignancies [79-81].

Determining the individual genetic status is essential to establish appropriate surveillance strategies and optimize genetic counselling. In some family members carrying pathogenic variants in *MLH1* and/or *CDKN2A*, tumors belonging to the clinical spectra associated with the two hereditary cancer syndromes were observed. The coexistence of both genetic conditions required the development of personalized surveillance programs. All tumors diagnosed in the studied individuals occurred before the identification of the double genetic risk within the family. Following genetic diagnosis, each HEALTHY carrier was enrolled in a dedicated surveillance protocol, with the expectation that this will reduce cancer diagnoses among at-risk relatives in subsequent generations.

6. BIBLIOGRAPHY

1) https://www.epicentro.iss.it/melanoma/

2)Saginala K, Barsouk A, Aluru JS, Rawla P, Barsouk A. Epidemiology of Melanoma. Med Sci (Basel) 2021;9:63.

3)Guo W, Wang H, Li C. Signal pathways of melanoma and targeted therapy. Signal TransductmTarget Ther. 2021 Dec 20;6(1):424.

4)Scatena C, Murtas D, Tomei S. Cutaneous Melanoma Classification: The Importance of High-Throughput Genomic Technologies. Front Oncol. 2021 May 28;11:635488.

5)https://www.salute.gov.it/new/it/pubblicazione/i-numeri-del-cancro-italia-2025/

6)Fidler MM, Gupta S, Soerjomataram I, Ferlay J, Steliarova-Foucher E, Bray F. Cancer incidence and mortality among young adults aged 20-39 years worldwide in 2012: a population-based study. Lancet Oncol. 2017;18(12):1579-1589. doi: 10.1016/S1470-2045(17)30677-0

7)Https://www.who.int/data/gho/data/themes/topics/topic-details/GHO/ultraviolet-(uv)-radiation#

8)Cadet J, Wagner JR. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harb Perspect Biol. 2013 Feb 1;5(2):a012559.

9)https://www.ide.it/patologie/fototipo/

10) Read, J.;Wadt, K.A.W.; Hayward, N.K. Melanoma Genetics. J. Med. Genet. 2016, 53, 1–14.

11)Šerman, N.; Vrani´c, S.; Glibo, M.; Šerman, L.; Mokos, Z.B. Genetic Risk Factors in Melanoma Etiopathogenesis and the Role of Genetic Counseling: A Concise Review. Bosn. J. Basic Med. Sci. 2022, 22, 673–682.

12)https://www.ioveneto.it/tumori-trattati/melanoma-familiare/

13) Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA and Dracopoli NC. Germline p16 mutations in familial melanoma. Nat Genet 1994; 8: 15-21.

14) Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, Hussey C, Tran T, Miki Y, Weaver-Feldhaus J, et al. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat Genet 1994; 8: 23-26.

15)Danishevich A, Bilyalov A, Nikolaev S, Khalikov N, Isaeva D, Levina Y, Makarova M, Nemtsova M, Chernevskiy D, Sagaydak O, Baranova E, Vorontsova M, Byakhova M, Semenova A, Galkin V, Khatkov I, Gadzhieva S, Bodunova N. CDKN2A Gene Mutations: Implications for Hereditary Cancer Syndromes. Biomedicines. 2023 Dec 18;11(12):3343.

16) Chudnovsky Y, Khavari PA, Adams AE. Melanoma genetics and the development of rational therapeutics. J Clin Invest 2005;115:813-24.

17) Lou Z, Chen J. Cellular senescence and DNA repair. Exp Cell Res 2006

18) Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, Chompret A, Ghiorzo P, Gruis N, Hansson J, Harland M, Hayward N, Holland EA, Mann GJ, Mantelli M, Nancarrow D, Platz A, Tucker MA and Melanoma Genetics Consortium. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst 2002; 94: 894-903.

19) Harland M, Cust AE, Badenas C, et al. Prevalence and predictors of germline CDKN2A mutations for melanoma cases from Australia, Spain and the United Kingdom. Hered Cancer Clin Pract 2014

20) Ribeiro Moura Brasil Arnaut J, Dos Santos Guimarães I, Evangelista Dos Santos AC, de Moraes Lino da Silva F, Machado JR, de Melo AC. Molecular landscape of Hereditary Melanoma. Crit Rev Oncol Hematol. 2021 Aug;164:103425.

21) Tsao, H.; Chin, L.; Garraway, L.A.; Fisher, D.E. Melanoma: From Mutations to Medicine. *Genes Dev.* 2012, *26*, 1131–1155.

22) Rane, S.G.; Cosenza, S.C.; Mettus, R.V.; Reddy, E.P. Germ Line Transmission of the Cdk4R24C Mutation Facilitates Tumorigenesis and Escape from Cellular Senescence. *Mol. Cell. Biol.* 2002, *22*, 644–656

23) Sotillo, R.; Garcia, J.F.; Ortega, S.; Martin, J.; Dubus, P.; Barbacid, M.; Malumbres, M. Invasive Melanoma in Cdk4-Targeted Mice. *Proc. Natl. Acad. Sci. USA* 2001, *98*, 13312–13317.

24) Puntervoll HE, Yang XR, Vetti HH, et al. Melanoma prone families with CDK4 germline mutation: phenotypic profile and associations with MC1R variants. J Med Genet 2013

25) Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., Dean, D. C. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98: 859-869, 1999.

26) Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA and Hayward NK. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet 2001; 69: 765-773

27)van der Velden PA, Sandkuijl LA, Bergman W, Pavel S, van Mourik L, Frants RR and Gruis NA. Melanocortin-1 receptor variant R151C modifies melanoma risk in Dutch families with melanoma. Am J Hum Genet 2001; 69: 774-779.

28) Landi, M. T., Bauer, J., Pfeiffer, R. M., Elder, D. E., Hulley, B., Minghetti, P., Calista, D., Kanetsky, P. A., Pinkel, D., Bastian, B. C. MC1R germline variants confer risk for BRAF-mutant melanoma. Science 313: 521-522, 2006.

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

30) Palmer JS, Duffy DL, Box NF, Aitken JF, O'Gorman LE, Green AC, Hayward NK, Martin NG and Sturm RA. Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? Am J Hum Genet 2000; 66: 176-186.

31) Box NF, Wyeth JR, O'Gorman LE, Martin NG and Sturm RA. Characterization of melanocyte stimulating hormone receptor variant alleles in twins with red hair. Hum Mol Genet 1997; 6: 1891-1897.

32) Dessinioti C, Antoniou C, Katsambas A, et al. Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol 2011.

33) Lopez, M. N., Pereda, C., Ramirez, M., Mendoza-Naranjo, A., Serrano, A., Ferreira, A., Poblete, R., Kalergis, A. M., Kiessling, R., Salazar-Onfray, F. Melanocortin 1 receptor is expressed by uveal malignant melanoma and can be considered a new target for diagnosis and immunotherapy. Invest. Ophthal. Vis. Sci. 48: 1219-1227, 2007.

34) Geschwind, I.I. Change in hair color in mice induced by injection of α MSH. *Endocrinology* 1966, *79*, 1165–1167

35) Sawyer, T.K.; Hruby, V.J.; Wilkes, B.C.; Draelos, M.T.; Hadley, M.E.; Bergsneider, M. Comparative Biological Activities of Highly Potent Active-Site Analogues of α-Melanotropin. *J. Med. Chem.* 1982, *25*, 1022–1027

36) García-Borrón, J.C.; Sánchez-Laorden, B.L.; Jiménez-Cervantes, C. Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res.* **2005**, *18*, 393–410

37) Wolf Horrell, E.M.; Boulanger, M.C.; D'Orazio, J.A. Melanocortin 1 receptor: Structure, function, and regulation. *Front. Genet.* **2016**, *7*, 95.

38) Chen, S.; Zhu, B.; Yin, C.; Liu, W.; Han, C.; Chen, B.; Liu, T.; Li, X.; Chen, X.; Li, C.; et al. Palmitoylation-dependent activation of MC1R prevents melanomagenesis. *Nature* **2017**, *549*, 399–403.

39) Dalziel, M.; Kolesnichenko, M.; Das Neves, R.P.; Iborra, F.; Goding, C.; Furger, A. α-MSH regulates intergenic splicing of MC1R and TUBB3 in human melanocytes. *Nucleic Acids Res.* 2011, *39*, 2378–2392.

40) Martínez-Vicente, I.; Abrisqueta, M.; Herraiz, C.; Jiménez-Cervantes, C.; García-Borrón, J.C.; Olivares, C. Functional characterization of a C-terminal splice variant of the human melanocortin 1 receptor. *Exp.*

Dermatol. 2020, *29*, 610–615.

41) Herraiz, C.; Olivares, C.; Castejón-Griñán, M.; Abrisqueta, M.; Jiménez-Cervantes, C.; García-Borrón, J.C. Functional characterization of MC1R-TUBB3 intergenic splice variants of the human melanocortin 1 receptor. *PLoS ONE* 2015, *10*, e0144757.

42) Goding, C. R. et al.MITF–the first 25 years. Genes Dev. 33, 983–1007 (2019).

43) Garraway, L. A. et al. Integrative genomic analyses identifyMITF as a lineage survival oncogene amplified in malignant melanoma. Nature 436, 117–122 (2005).

44) Garraway, L. A. et al. Lineage dependency and lineagesurvival oncogenes in human cancer. Nat. Rev. Cancer 6, 593–602 (2006).

45) Ugurel, S. et al. Microphthalmia-associated transcription factor gene amplification in metastatic melanoma is a prognostic marker for patient survival, but not a predictivemarker for chemosensitivity and chemotherapy response. Clin. Cancer Res. 13, 6344–6350 (2007).

46) Bertolotto, C., Lesueur, F., Giuliano, S., Strub, T., de Lichy, M., Bille, K., Dessen, P., d'Hayer, B., Mohamdi, H., Remenieras, A., Maubec, E., de la Fouchardiere, A., and 45 others. A SUMOylation-defective MITF germline mutation predisposes to melanoma and renal carcinoma**.** Nature 480: 94-98, 2011. Note: Erratum: Nature 531: 126 only, 2016

47) Yokoyama, S., Woods, S. L., Boyle, G. M., Aoude, L. G., MacGregor, S., Zismann, V., Gartside, M., Cust, A. E., Haq, R., Harland, M., Taylor, J. C., Duffy, D. L., and 35 others. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma**.** Nature 480: 99-103, 2011

48)L. G. Aoude, K. A. Wadt, A. L. Pritchard, and N. K. Hayward, "Genetics of Familial Melanoma: 20 Years After CDKN2A," *Pigment Cell & Melanoma Research* 28 (2015): 148–160.

49)L. Zocchi, A. Lontano, M. Merli, et al., "Familial Melanoma and Susceptibility Genes: A Review of the Most Common Clinical and Dermoscopic Phenotypic Aspect, Associated Malignancies and Practical Tips for Management," *Journal of Clinical Medicine* 10, no. 16 (2021).

50)S. E. Artandi and R. A. DePinho, "Telomeres and Telomerase in Cancer," *Carcinogenesis* 31, no. 1 (2010)

51)K. K. Takai, T. Kibe, J. R. Donigian, D. Frescas, and T. de Lange, "Telomere Protection by TPP1/POT1 Requires Tethering to TIN2," *Molecular Cell* 44, no. 4 (2011)

52)J. Shi, X. R. Yang, B. Ballew, et al., "Rare Missense Variants in POT1 Predispose to Familial Cutaneous Malignant Melanoma," *Nature Genetics* 46, no. 5 (2014)

53)C. D. Robles-Espinoza, M. Harland, A. J. Ramsay, et al., "POT1 Loss-Of- Function Variants Predispose to Familial Melanoma," *Nature Genetics* 46, no. 5 (2014)

54) Castellani G, Buccarelli M, Arasi MB, Rossi S, Pisanu ME, Bellenghi M, Lintas C, Tabolacci C. BRAF Mutations in Melanoma: Biological Aspects, Therapeutic Implications, and Circulating Biomarkers. Cancers (Basel). 2023 Aug 8;15(16):4026. doi: 10.3390/cancers15164026. PMID: 37627054; PMCID: PMC10452867.

55)Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353(20):2135–2147.

56)Jakob JA, Bassett RL, Jr, Ng CS, et al. NRAS mutation status is an independent prognostic factor in metastatic melanoma. Cancer. 2012;118(16):4014–4023.

57)Lee JH, Choi JW, Kim YS. Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: a meta-analysis. Br J Dermatol. 2011;164(4):776–784.

58)Thumar J, Shahbazian D, Aziz SA, Jilaveanu LB, Kluger HM. MEK targeting in N-RAS mutated metastatic melanoma. Mol Cancer. 2014;13:45.

59)Devitt B, Liu W, Salemi R, et al. Clinical outcome and pathological features associated with NRAS mutation in

cutaneous melanoma. Pigment Cell Melanoma Res. 2011;24(4):666–672.
60)Robert C, Schachter J, Long GV, et al. KEYNOTE-006 investigators Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med. 2015;372(26):2521–2532.
61)Ascierto PA, McArthur GA, Dréno B, et al. Cobimetinib combined with vemurafenib in advanced BRAF(V600)-mutant melanoma (coBRIM): updated efficacy results from a randomised, double-blind, phase 3 trial. Lancet Oncol. 2016;17(9):1248–1260.
62)Dhillon S. Dabrafenib plus trametinib: a review in advanced melanoma with a BRAF (V600) mutation. Target Oncol. 2016;11(3):417–428.
63)Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med. 2015;373(1):23–34.
64) Murphy BM, Terrell EM, Chirasani VR, et al. : Enhanced BRAF engagement by NRAS mutants capable of promoting melanoma initiation. Nat Commun 13:3153, 2022
65) https://www.iss.it/documents/20126/8403839/LG+127_Melanoma_agg-ago2023_rev-nov.pdf/d1e6e188-6e08-8cd6-5ac7-3e1f83dce7c6?t=1702303171666
66)"Raccomandazioni per consulenza e test genetico nel melanoma ereditario", SIGU 2005
67) Meyle KD, Guldberg P. Genetic risk factors for melanoma. Hum. Genet. 2009;126(4):499–510. doi: 10.1007/s00439-009-0715-9.
68) Newton Bishop JA, Bishop DT. The genetics of susceptibility to cutaneous melanoma. Drugs Today (Barcelona). 2005;41(3):193–203. doi: 10.1358/dot.2005.41.3.892524.
69) Fargnoli MC, Argenziano G, Zalaudek I, Peris K. High- and low-penetrance cutaneous melanoma susceptibility genes. Expert Rev. Anticancer Ther. 2006;6(5):657–670. doi: 10.1586/14737140.6.5.657.
70) Mehnert JM, Kluger HM. Driver mutations in melanoma: lessons learned from bench-to-bedside studies. Curr. Oncol. Rep. 2012;14(5):449–457. doi: 10.1007/s11912-012-0249-5.
71) Palmieri G, Colombino M, Casula M, Budroni M, Manca A, Sini MC, Lissia A, Stanganelli I, Ascierto PA, Cossu A. Epidemiological and genetic factors underlying melanoma development in Italy. Melanoma Manag. 2015 May;2(2)
72) Pastorino L, Bonelli L, Ghiorzo P, Queirolo P, Battistuzzi L, Balleari E, Nasti S, Gargiulo S, Gliori S, Savoia P, Abate Osella S, Bernengo MG, Bianchi Scarrà G. CDKN2A mutations and MC1R variants in Italian patients with single or multiple primary melanoma. Pigment Cell Melanoma Res. 2008.
. **73)**https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/chek2
74)https://www.jidonline.org/article/S0022-202X(15)35596-2/fulltext
75) Rastogi RP, Richa, Kumar A, Tyagi MB, Sinha RP. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. J Nucleic Acids. 2010 Dec 16;2010
76)Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell. 2003 May;
77) Lehman B, Matthäi E, Gercke N, Denzer UW, Figiel J, Hess T, Slater EP, Bartsch DK. Characteristics of familial pancreatic cancer families with additional colorectal carcinoma. Fam Cancer. 2023 Jul;22(3):323-330.
78) Earl J, Galindo-Pumariño C, Encinas J, Barreto E, Castillo ME, Pachón V, Ferreiro R, Rodríguez-Garrote M, González-Martínez S, Ramon Y Cajal T, Diaz LR, Chirivella-Gonzalez I, Rodriguez M, de Castro EM, García-Seisdedos D, Muñoz G, Rosa JMR, Marquez M, Malats N, Carrato A. A comprehensive analysis of candidate genes in familial pancreatic cancer families reveals a high frequency of potentially pathogenic germline variants. EBioMedicine. 2020
79) Hu C., Hart S.N., Polley E.C., Gnanaolivu R., Shimelis H., Lee K.Y. Association between inherited germline mutations in cancer predisposition genes and risk of pancreatic cancer. JAMA. 2018;319(23):2401–2409.

80) Cremin C., Howard S., Le L., Karsan A., Schaeffer D.F., Renouf D. CDKN2A founder mutation in pancreatic ductal adenocarcinoma patients without cutaneous features of familial atypical multiple mole melanoma (FAMMM) syndrome. Hered Cancer Clin Pract. 2018.

81) McWilliams R.R., Wieben E.D., Chaffee K.G., Antwi S.O., Raskin L., Olopade O.I. CDKN2A germline rare coding variants and risk of pancreatic cancer in minority populations. Cancer Epidemiol Biomark Prev. 2018.

Table 1: (AIOM-AIRTUM Working Group, 2020-numeri del cancro in Italia)

Figure 1: https://www.aimatmelanoma.org/it/melanoma-101/prevenzione/cosa-sono-i-raggi-ultravioletti-UV/

Figure2: https://atlasgeneticsoncology.org/cancer-prone-disease/10088/familial-melanoma

Figure3:https://www.researchgate.net/figure/Pathways-of-high-risk-genes-involved-in-melanoma-susceptibility-A_fig1_340846505

Figure 4: https://onlinelibrary.wiley.com/doi/full/10.1111/jdv.20269

Figure5:https://www.researchgate.net/figure/MITF-mutations-found-in-melanoma-WS2A-and-TS-patients-A-A-graphic-depiction-of-the_fig1_240308577

Figure 6:Constantinou SM, Bennett DC. Cell Senescence and the Genetics of Melanoma Development. Genes Chromosomes Cancer. 2024 Oct;63(10):e23273. doi: 10.1002/gcc.23273. PMID: 39422311.

Figure7: https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2021.661737/full#f1

Figure8:https://www.researchgate.net/figure/A-NRAS-BRAF-PI3K-mutations-in-a-melanoma-case-series-B-Hot-spot-mutations-of_fig3_24410466

Figure9:https://www.sigmaaldrich.com/IT/it/technical documents/protocol/genomics/sequencing/sanger-sequencing

Figure 10: https://www.researchgate.net/figure/Figura-20-Elettroferogramma_fig11_285725055

Figure11: https://www.yeasenbio.com/it/blogs/ngs/qubit-what-a-powerful-tool-for-ngs-library-quantification

Figure 12: https://microbenotes.com/Illumina-sequencing/

Figure 13: https://www.ncbi.nlm.nih.gov/snp/rs1800586