UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Health Sciences

Master's Degree in Medical Biotechnologies

Graduation thesis

Germline variants detected by multigene panel testing in oncology patients with unexpected gene-associated phenotypes

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SUMMARY

Introduction and Background: Hereditary predisposition to cancer represents a crucial area of modern oncology and personalized medicine. Among the most widely studied autosomal dominant conditions are Hereditary Breast and Ovarian Cancer (HBOC) syndrome, linked to high-penetrance germline variants in the *BRCA1* and *BRCA2* genes involved in DNA double-strand break repair, familial cutaneous melanoma (associated with genes such as *CDKN2A* and *MITF*). The introduction of Next-Generation Sequencing (NGS) technologies has enabled the expansion of diagnostic testing beyond classic single genes through the use of extended multigene panels.

Objective: This thesis aims to evaluate the occurrence of genotype-phenotype discrepancies within a large cohort of oncology patients. Specifically, the study seeks to quantify the diagnostic and clinical utility of a massive parallel sequencing approach extended to all genes included in a custom panel named "BML" (Breast, Melanoma, Lynch), regardless of the patient's initial clinical suspicion or specific referral criteria.

Materials and Methods: The study was conducted on a total cohort of 1064 patients, divided into two main subgroups: breast cancer (HBOC, N=662) and cutaneous melanoma (N=402). The molecular and bioinformatic workflow involved automated (QIAsymphony platform) or manual genomic DNA extraction from peripheral blood leukocytes, followed by quality control and spectrophotometric (NanoDrop) and fluorometric (Qubit) quantification. Library preparation was performed using the Agilent SureSelect XT HS2 protocol, and sequencing was carried out on Illumina platforms. Pathogenic variants identified were subsequently validated via Sanger sequencing.

Results: Global genetic analysis revealed that 12.22% of the analyzed subjects (representing a large portion of the cohort) carry at least one pathogenic or likely pathogenic (P/LP) variant. In the HBOC cohort, the majority of variants were concentrated in the *BRCA1*, *BRCA2*, *PALB2*, *ATM*, and *CHEK2* genes, whereas in the melanoma cohort, the primary gene was *CDKN2A*, followed by *MITF*, consistent with international literature. The most significant finding was the detection of "unexpected" P/LP variants: mutations in typical melanoma genes (*CDKN2A*, *MITF*) and Lynch syndrome genes (*MSH2*, *MSH6*, *PMS2*) were identified within the HBOC cohort; conversely, variants in genes traditionally linked to the HBOC spectrum (*BRCA1*, *CHEK2*, *ATM*, *BRIP1*) were identified in the melanoma cohort. Finally, the distribution of variants was found to be homogeneous between sexes within both cohorts (HBOC: 16.35% in females vs. 18.46% in males; Melanoma: 15.31% in females vs. 16.02% in males).

Discussion and Conclusions: The obtained results confirm that genetic predisposition to cancer can no longer be interpreted in a rigidly compartmentalized manner or restricted to specific organ-gene associations. Instead, the extensive phenotypic overlap observed reflects shared, transversal biological mechanisms of cell cycle control and genomic stability maintenance. The adoption of expanded multigene panels proves to be a superior diagnostic tool compared to traditional targeted testing, playing a fundamental role in optimizing family risk stratification, enabling access to molecularly targeted therapies and implementing personalized and effective cancer surveillance pathways.

However, it remains to be clarified whether the identification of pathogenic or likely pathogenic variants in genes that are not classically investigated for that specific tumor type reflects a true biological and clinical association, or whether these findings represent incidental detections resulting from the broader analytical scope of multigene panel testing. Further studies, including larger cohorts, family segregation analyses, and functional investigations, will be necessary to determine the actual pathogenic relevance and clinical impact of these non-classical variants in the patient’s disease.

INTRODUCTION

2.1: TUMOR ASSOCIATED TO HBOC SPECTRUM

Hereditary Breast and Ovarian Cancer (HBOC) is a genetically predisposed condition characterized by an increased risk of developing tumors, mainly of the breast and ovary, but also of other organs such as the pancreas, prostate, and to a lesser extent can also predispose to melanoma. Overall, approximately 5-10% of breast cancers and 10-15% of ovarian cancers are attributable to an inherited predisposition of genes associated with the HBOC spectrum.

HBOC is caused by the presence of germline pathogenic variants in genes involved in DNA repair mechanisms, particularly homologous recombination repair (HRR). BRCA1 and BRCA2 are the genes most often associated with these types of tumors, in fact, they are considered the genes with high penetrance.

These genes encode proteins that play an important role in maintaining genomic stability through the repair of DNA double-strand breaks. If these genes are mutated or silenced, there will be an accumulation of genetic alterations.

Using next-generation sequencing (NGS) technologies, in addition to BRCA1 and BRCA2, other genes associated with increased tumor risk have been identified, including PALB2, ATM, CHEK2, TP53, PTEN, CDH1, STK11, RAD51C, RAD51D, BRIP1, BARD1, and other genes involved in the DNA damage response.

Some of these confer high penetrance, while others result in a moderate increase in risk. Therefore, it is necessary to study each patient's variant individually, in order to create a personalized therapy.

HBOC transmission follows an autosomal dominant inheritance pattern. So every child of a carrier of a pathogenic variant has a 50% chance of inheriting the mutation, regardless of gender. However, clinical expressiveness is variable, so not all carriers will necessarily develop cancer, and the risk depends on the interaction between individual genetic makeup, environmental factors, and lifestyle.

HBOC is considered a "spectrum" of hereditary tumors rather than a syndrome limited exclusively to the breast and ovary. In particular, patients carrying variants on the BRCA1 AND BRCA2 genes are at increased risk of developing breast and ovarian cancer if they are women, and pancreatic and prostate cancer if they are men. In some cases mutations on these genes may increase the risk of cutaneous melanoma [1].

The presence of defects in homologous recombination makes tumor cells particularly sensitive to poly(ADP-ribose) polymerase inhibitors (PARP inhibitors), such as Olaparib.

PARP proteins, particularly PARP1 and PARP2, play an essential role in the repair of single-strand DNA breaks through the base excision repair pathway. PARP inhibitors block the enzymatic activity of these proteins and promote their "trapping" on DNA, blocking the proper completion of DNA repair processes. As a result, during cellular replication, single-strand breaks are converted into double-strand breaks.

In normal cells, these lesions can be repaired through homologous recombination. However, in tumor cells harboring mutations in these genes (BRCA1 and/or BRCA2), homologous recombination is impaired, and DNA damage progressively accumulates, ultimately leading to genomic instability and cell death.

This phenomenon, known as "synthetic lethality," constitutes the main therapeutic mechanism of PARP inhibitors and represents the biological basis for their efficacy in tumors with homologous recombination deficiency [2].

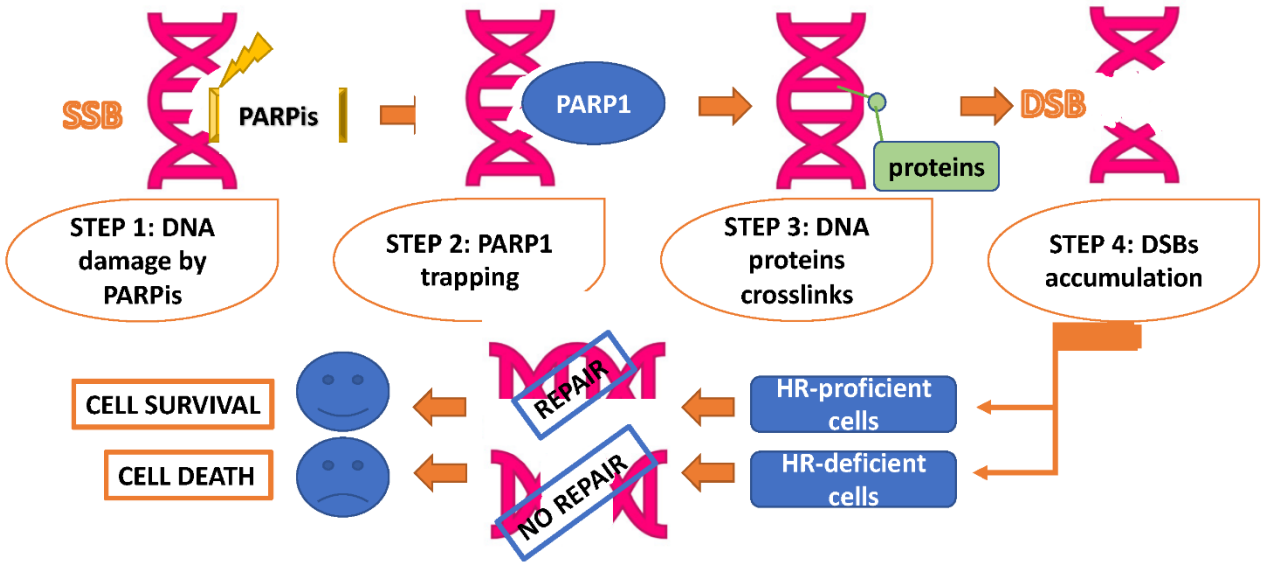

***Image 1**: PARP-inhibitors block the PARP1 enzyme, which normally repairs single-strand breaks in DNA. This leads to damage accumulation and "trapping" of PARP1 on DNA, creating obstacles to replication.*
During DNA duplication, these blocks turn into double-strand breaks, which are much more serious. Cells with a functioning HR repair system are able to repair them and survive, while those with HR defects (e.g. BRCA mutations) are unable to and die.
This mechanism exploits "synthetic lethality", selectively targeting tumor cells[3].

2.1.2: BREAST CANCER

Breast cancer is the most common global malignancy, with an annual incidence of over 1.1 million women worldwide [4] and it defines a range of malignancies occurring in the mammary glands.

The majority of breast cancers are adenocarcinomas and the pathology ranges from ductal carcinoma in situ, to invasive carcinoma which has spread beyond the basement membrane into adjacent breast parenchyma.

Other forms of breast cancer include Paget's disease of the breast, inflammatory breast cancers and papillary carcinomas. Sarcomas, such as malignant phyllodes and angiosarcomas, appear to be rare.

Breast cancer commonly manifests as a mass in the breast and it is usually painless in the first stages of development. However, usually 90% of breast masses are benign. Among the different benign manifestations fibroadenoma, cysts are included (World Health Organization, 2021) [5] .

2.1.1.1: CLASSIFICATION

From a molecular perspective, breast carcinomas can be classified into four main subtypes: Luminal A, Luminal B, HER2-positive, and triple negative. This classification enables prognostic stratification and supports the implementation of novel strategies in personalized medicine, thereby avoiding unnecessary and ineffective treatments that could potentially worsen the patient's clinical condition.

Luminal A and Luminal B

Luminal A breast carcinoma is characterized by the expression of estrogen receptors (ER) and/or progesterone receptors (PR), the absence of HER2 overexpression, and a low proliferative index Ki-67, typically below 20%.

This subtype typically exhibits a low histological grade, slow growth, a lower risk of recurrence, and it is the most favorable prognosis among invasive breast cancers.

The **Luminal B** subtype is also ER-positive, but it may exhibit reduced PR expression, higher proliferative activity, and, in some cases, HER2 positivity.

Luminal B tumors are also associated with a poorer prognosis than Luminal A tumors [6].

Ki-67 is a nuclear protein expressed in actively proliferating cells (i.e., during the G1, S, G2, and mitotic phases) while it is absent in quiescent cells (in the G0 phase). For this reason, it is used as a specific immunohistochemical marker of cellular proliferation recognition.

In breast carcinoma, the Ki-67 index represents an important prognostic factor. High Ki-67 values are generally associated with greater tumor aggressiveness, a less favorable prognosis, and a higher risk of recurrence.

For this reason, Ki-67 is tightly used in the distinction between the Luminal A and Luminal B subtypes, as it is typically higher in the latter [7].

HER2-positive

HER2-positive breast carcinoma is characterized by amplification of the ERBB2 gene and the resulting overexpression of the HER2 protein, a tyrosine kinase receptor involved in the regulation of cell growth and proliferation. This subtype rapresents approximately 20% of invasive breast cancers and it is associated with high aggressiveness, rapid growth, and an unfavorable prognosis in the absence of specific treatments [8].

Over the past decades, the introduction of anti-HER2 targeted therapies, expecially monoclonal antibody such as **Trastuzumab**, has significantly altered the natural course of the disease, leading to a substantial improvement in the survival of patients affected by this tumor subtype[9].

Triple negative

This subtype is characterized by the absence of both estrogen and progesterone receptors plus the ulterior absence or very low HER2 expression. It accounts for approximately 20% of cases and represents the most aggressive form of breast carcinoma, with a high rate of bone metastasis.

This marked aggressiveness is primarily attributed to a high mutational burden and genomic instability, which contributes to a reduced efficacy of conventional chemotherapeutic treatments. For this reason, recently, novel immunotherapeutic approaches have been investigated [10].

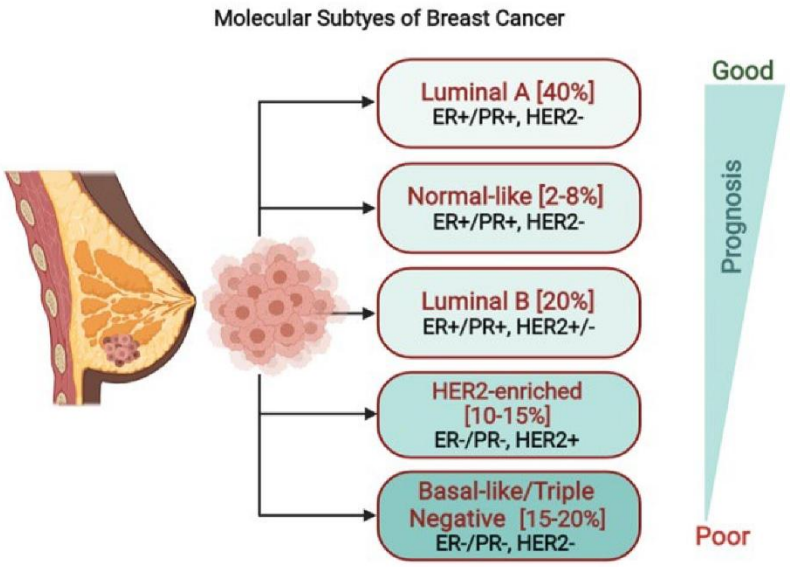

***Image 2**: classification of different types of breast cancer with relative prognosis [11].*

2.1.3: OVARIAN CARCINOMA

Ovarian carcinoma represents a heterogeneous group of malignant neoplasms, among which high-grade serous ovarian carcinoma (HGSOC) is the most frequent and clinically relevant subtype for aggressiveness and prognosis. Pathogenetically, epithelial ovarian cancer should not be considered a single entity, but rather a collection of tumors distinguished by cellular origin, molecular profile, and biological behavior.

In particular, HGSOC is characterized by high genomic instability and the almost constant presence of inactivating TP53 mutations, accompanied in a significant proportion of cases by alterations in the BRCA1 and BRCA2 genes.

The disease is frequently diagnosed at an advanced stage due to nonspecific symptoms and the absence of effective screening programs in the general population.

However, the recognition of mutations on BRCA genes has enabled important therapeutic developments, such as PARP inhibitors, which have significantly improved the outcome in selected patients [12].

2.1.4: PANCREATIC CARCINOMA

Approximately 90–95% of pancreatic tumors are pancreatic ductal adenocarcinoma (PDAC), a neoplasm that arises from the epithelium of the pancreatic ducts.

The high mortality of pancreatic cancer is mainly attributable to the absence of specific symptoms in the early stages of the disease. Clinical signs, such as abdominal pain, weight loss, obstructive jaundice, and new-onset diabetes, tend to appear when the tumor is already locally advanced or metastatic. As a result, over 80% of patients are diagnosed at a stage where surgery is no longer possible, which is currently the only curative option.

Treatment depends on the stage of the disease at the time of diagnosis. Surgery is the treatment of choice and is generally combined with adjuvant chemotherapy to reduce the risk of recurrence. In locally advanced or metastatic cases, treatment is predominantly based on systemic chemotherapy. Precision medicine and immunotherapy for specific molecular subgroups are currently still under study.

The incidence of pancreatic cancer is constantly increasing worldwide, especially in industrialized countries. Among the main recognized risk factors are cigarette smoking, advanced age, obesity, diabetes mellitus, and chronic pancreatitis.

Pancreatic cancer is characterized by mutations in the KRAS, TP53, CDKN2A, and SMAD4 genes [13].

2.1.5: PROSTATE CANCER

Globally, prostate cancer is the most diagnosed cancer in men, it is particularly common in developed countries.

The incidence of prostate cancer varies greatly worldwide, is very high in men living in the United States and very low in Asian men living in their native country. Incidence is influenced by both differences in PSA screening and environmental and lifestyle factors.

Prostate cancer is the fifth leading cause of cancer death worldwide. Mortality is highest in LDCs, especially in the Caribbean and Africa, and lowest in Asia. Mortality has decreased in Western countries, likely due to early diagnosis through PSA screening and timely treatment.

The risk and prognosis of prostate cancer depend on biological factors and lifestyle. Because the disease is clinically heterogeneous, it is essential to distinguish risk factors associated with indolent forms from those related to aggressive or lethal forms [14].

2.1.6: GENES ASSOCIATED WITH THE HBOC SPECTRUM

Several genes are associated with HBOC spectrum tumors. Most notably include *BRCA1* and *BRCA2*, but there are other common gene mutations which confer cancer risk, including: *PTEN*, *TP53*, *CHEK2*, *STK11*, *ATM*, and *CDH1* [15].

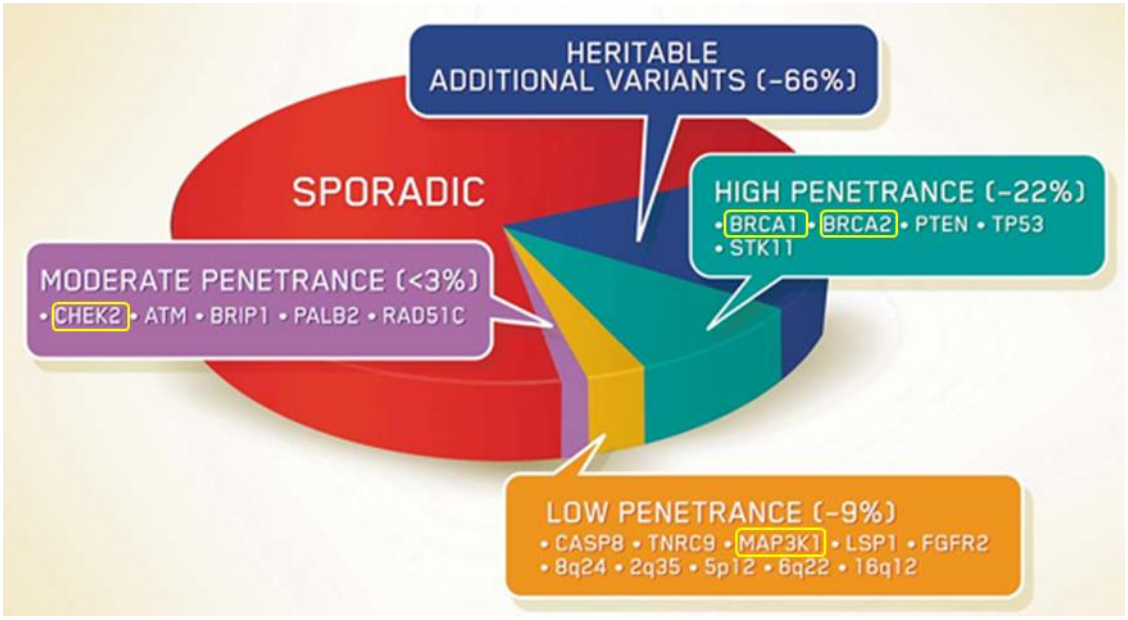

***Image 3**: Familial BC (~30% of patients), often observed in families with a high incidence of BC, has been associated with a number of high-, moderate-, and low-penetrance susceptibility genes [15].*

2.1.6.1: *BRCA1* and *BRCA2 (BReast CAncer gene 1/2)*

Tumor predisposition due to mutations in BRAC1-2 is an autosomal dominant disorder, and the mutations confer high risks for additional tumors, including ovarian, male breast, prostate, and pancreatic cancer.

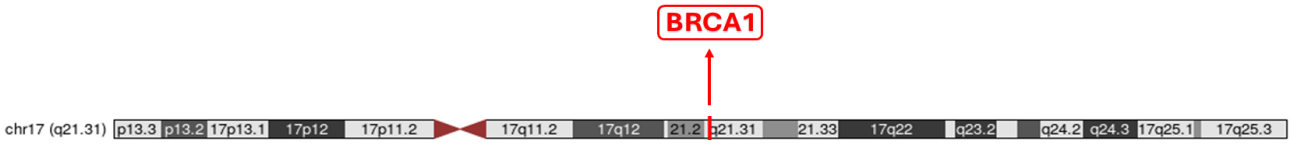

***Image 4**: Localization of the BRCA1 gene on chromosome 17 (region 17q21.31) visualized using the UCSC Genome Browser [16].*

BRCA1 is a gene located on chromosome 17q21.31 and functions as a tumor suppressor. It contributes to the maintenance of genomic stability by facilitating the repair of DNA double-strand breaks through homologous recombination. Moreover, this gene interacts with multiple

proteins involved in cell cycle regulation and chromosome stability, contributing to tumor suppression.

***Image 5**: Localization of the BRCA2 gene on chromosome 13 (region 13q13.1) visualized using the UCSC Genome Browser[16].*

The ***BRCA2*** gene, located on chromosome 13q12.3, is also a tumor suppressor gene that plays an essential role in DNA repair through the homologous recombination pathway. The BRCA2 protein contributes to the maintenance of genomic stability through RAD51 protein recruitment, that will localize onto the DNA damage spot and will look for the sister chromatide in order to copy the right sequence[17].

Hereditary pathogenic mutations in *BRCA1* and *BRCA2* impair the efficiency of DNA repair processes, leading to the accumulation of genetic alterations [15].

BRCA1 and BRCA2 act in the same genomic stability maintenance pathway, but with different roles. BRCA1 intervenes in the early stages of the DNA damage response, coordinating the recognition of breaks and the preparation of DNA ends for repair. In particular, it promotes processes that allow access to the damage site and the removal of associated protein structures, thus facilitating entry into the homologous recombination pathway.

BRCA2, on the other hand, acts at a more advanced stage of the process, playing a central role in the direct mediation of homologous recombination. Its main function is to regulate and target the RAD51 protein to single-stranded DNA generated by damage, promoting the formation of the nucleoprotein strand necessary for homology search and strand exchange. In this way, BRCA2 ensures that the repair takes place perfectly using the sister chromatid as a mold [18].

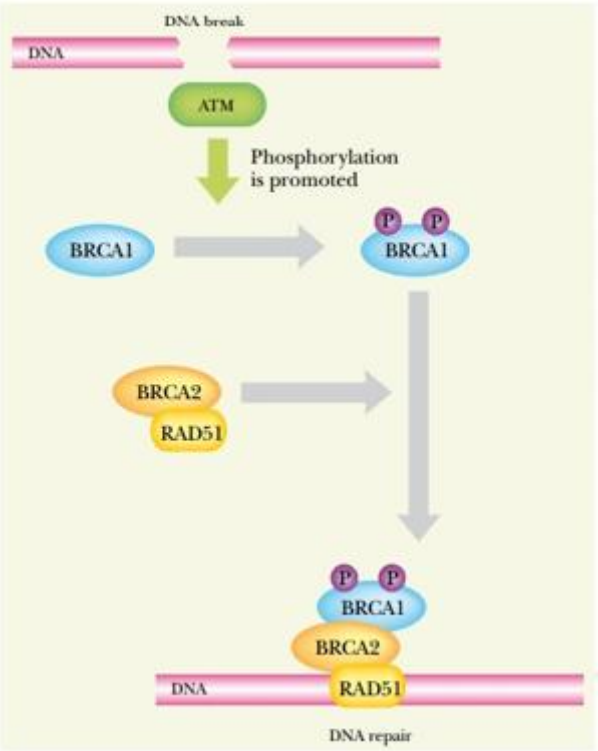

***Image 6**: BRCA1 and BRCA2 bind to RAD51. The presence of a double-stranded DNA break triggers the phosphorylation of BRCA1 via the ATM protein. BRCA2 plus RAD51 then bind to phosphor-BRCA1 and take part in the DNA repair process [19].*

Approximately 52% of familial cases are associated with BRCA1, while approximately 32% are associated with BRCA2. Families with BRCA1 mutations more frequently have associations with ovarian cancer, while those linked to BRCA2 more often include cases of breast cancer even in male subjects.

Finally, penetrance analysis indicates that both genes confer a high lifetime risk of developing breast cancer, although with possible differences in risk distribution between the two genetic groups [17].

2.1.6.2: *CHEK2 (Checkpoint Kinase 2)*

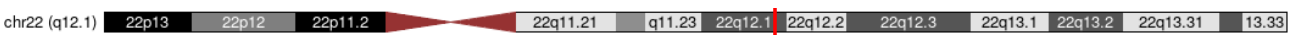

***Image 7**: Localization of the CHEK2 gene on chromosome 22 (region 22q12.1) visualized using the UCSC Genome Browser[16].*

The CHEK2 gene, located on chromosome 22q12.1, encodes a serine/threonine kinase that plays a key role in the DNA damage response and in the maintenance of genomic stability.

In the presence of DNA damage, CHEK2 is activated by the ATM protein and participates in cell cycle regulation by promoting cell cycle arrest, thereby allowing DNA repair or the activation of apoptosis. This effect is mediated through interactions with some tumor suppressor proteins, including p53, BRCA1, and others.

Pathogenic variants of the CHEK2 gene impair these control mechanisms, favoring the accumulation of genetic alterations and increasing the probability of the development of various neoplasms [20].

Mutations in this gene are associated with a moderate increase in the risk of hereditary breast carcinoma. Unlike *BRCA1* and *BRCA2*, which are considered high-penetrance genes, *CHEK2* is classified as a moderate-penetrance gene, associated with a lower but still clinically relevant risk [21].

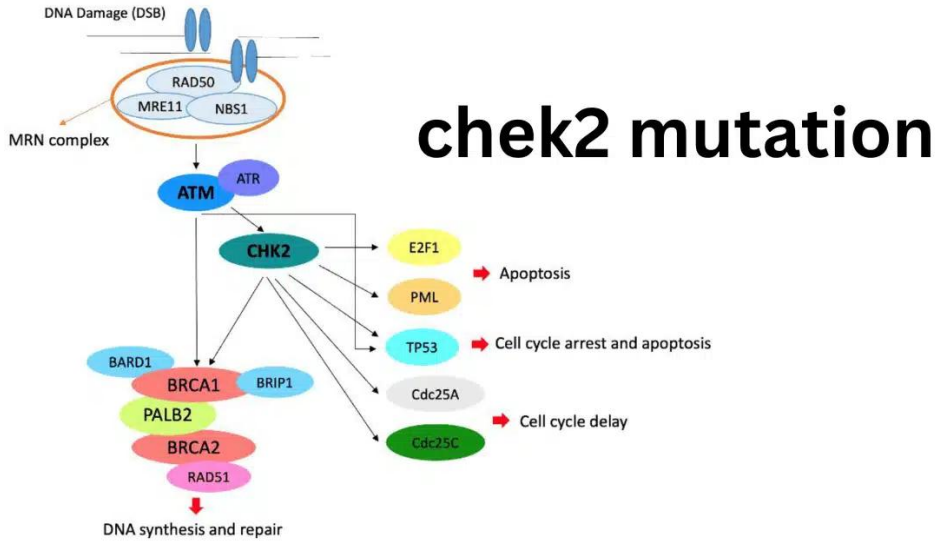

***Image 8**: CHEK2 pathway[22].*

2.1.6.3: *PALB2 (partner and localizer of BRCA2)*

***Image 9**: Localization of the PALB2 gene on chromosome 16 (region 16p12.2) visualized using the UCSC Genome Browser[16].*

The PALB2 gene, located on chromosome 16p12.2, encodes a protein that contributes to the maintenance of genomic stability. PALB2 acts as a binding protein between BRCA1 and BRCA2, participating in the repair of DNA double-strand breaks through the homologous recombination pathway. Pathogenic alterations in *PALB2* impair these mechanisms [23].

Germline mutations in *PALB2* are associated with a significant increase in the risk of hereditary breast carcinoma, with lower risk levels than those observed for *BRCA1* and *BRCA2,* but higher than those associated with many other moderate-susceptibility genes.

For this reason, *PALB2* is considered one of the major breast cancer predisposition genes after *BRCA1* and *BRCA2* [24].

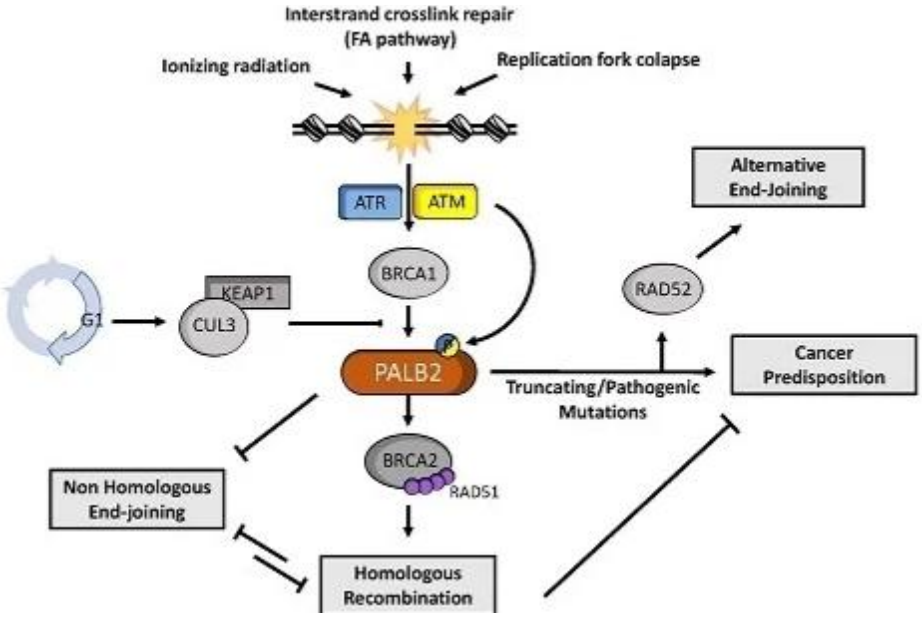

Image 10*: The role of PALB2 in the DNA Damage Response and cancer predisposition* [25].

2.1.6.4: *MAP3K1 (Mitogen-Activated Protein Kinase Kinase Kinase 1)*

Image 11*: Localization of the MAP3K1 gene on chromosome 5 (region 5q11.2) visualized using the UCSC Genome Browser*[16].

The *MAP3K1* gene, located on chromosome 5q11.2, encodes a serine/threonine kinase that contributes to NF-κB pathway activation, placing it at a central control point for cellular signaling responses.

Somatic mutations in *MAP3K1* have been identified in approximately 6–10% of breast carcinomas. These alterations affect MAP3K1 signal transduction, with consequent impacts on the regulation of cell proliferation and survival [26].

Furthermore, *MAP3K1* has been shown to contribute to the growth of cancer cells and to be involved in the development of resistance to certain endocrine therapies [27].

Preclinical studies have demonstrated that inhibition of *MAP3K1* through artificial microRNAs reduces proliferation, invasiveness, and metastatic potential in murine models of breast carcinoma, suggesting the potential of this kinase as a therapeutic target [28].

Recently, multi-omics analyses have shown that MAP3K1 mutations contribute to the modulation of the immune microenvironment in HR+/HER2− breast carcinoma. In particular, they promote immune evasion by reducing MHC class I–mediated antigen presentation and consequently attenuating CD8+ T cell responses [29].

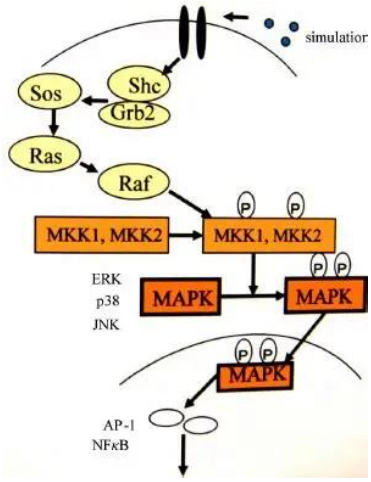

***Image 12**: Mitogen-activated protein kinases (MAPKs) play a crucial role in the transduction of signals through protein kinases and protein phosphatases. The MAPK pathways are a ubiquitous group of protein serine and threonine kinases that regulate gene expression through transcription factor activity. The stimulus may transduce to the nucleus to regulate gene expression through a distinct set of MAPK signal transduction cascades, including extracellular signal- regulated kinases 1 and 2 (ERK1, ERK2), p38 mitogen- activated protein (p38), and the c-Jun NH2-terminal kinase (JNK). These pathways are important mediators of the signal transduction responsible for cell growth and proliferation. The nuclear targets of these MAPK signaling pathways are transcriptional factors, such as transcriptional factor activator protein-1 (AP-1) and nuclear factor-kappa B (NF κ B), which regulate the expression of various genes [30].*

2.1.7: Eligibility Criteria for Clinical BRCA1/BRCA2 Pathogenic Variant Testing

The Italian guidelines of the Italian Society of Human Genetics (SIGU, 2021) define the clinical criteria for access to BRCA1/BRCA2 genetic testing in the context of hereditary breast and ovarian cancer predisposition, distinguishing between diagnostic indications (affected individuals) and predictive testing (unaffected at-risk individuals).

Testing is recommended both in the oncological (diagnostic) and predispositional (predictive) settings.

INDICATIONS IN AFFECTED INDIVIDUALS (PROBAND)

BRCA1/BRCA2 testing is indicated in the presence of one or more of the following conditions:

1. Breast cancer

- Diagnosis at age ≤ 45 years
- Triple-negative breast cancer diagnosed at age ≤ 60 years
- Male breast cancer
- Bilateral breast cancer (synchronous or metachronous)
- Second primary breast cancer at any age

2. Ovarian / fallopian tube / primary peritoneal cancer

- All non-mucinous epithelial ovarian cancers, regardless of age

3. Other BRCA-associated tumors

- Pancreatic cancer (particularly pancreatic ductal adenocarcinoma)
- Metastatic or high-risk prostate cancer

INDICATIONS IN UNAFFECTED INDIVIDUALS (PREDICTIVE TESTING)

Genetic testing is indicated in unaffected individuals when at least one of the following family history criteria is present:

1. A known pathogenic BRCA1/BRCA2 variant identified in a family member
2. Two or more cases of breast cancer in first- or second-degree relatives
3. Ovarian cancer in a first- or second-degree relative
4. Combination of breast and ovarian cancer within the same family lineage
5. Male breast cancer in the family
6. Familial clustering of pancreatic cancer associated with breast/ovarian cancer
7. Early-onset breast cancer in the family (typically <45–50 years, depending on clinical context) [31].

2.2: CUTANEOUS MELANOMA

Cutaneous melanoma is a malignant neoplasm arising from melanocytes, specialized cells located in the basal layer of the epidermis responsible for the production of melanin pigment. This type of melanoma represents the most aggressive form of skin cancer.

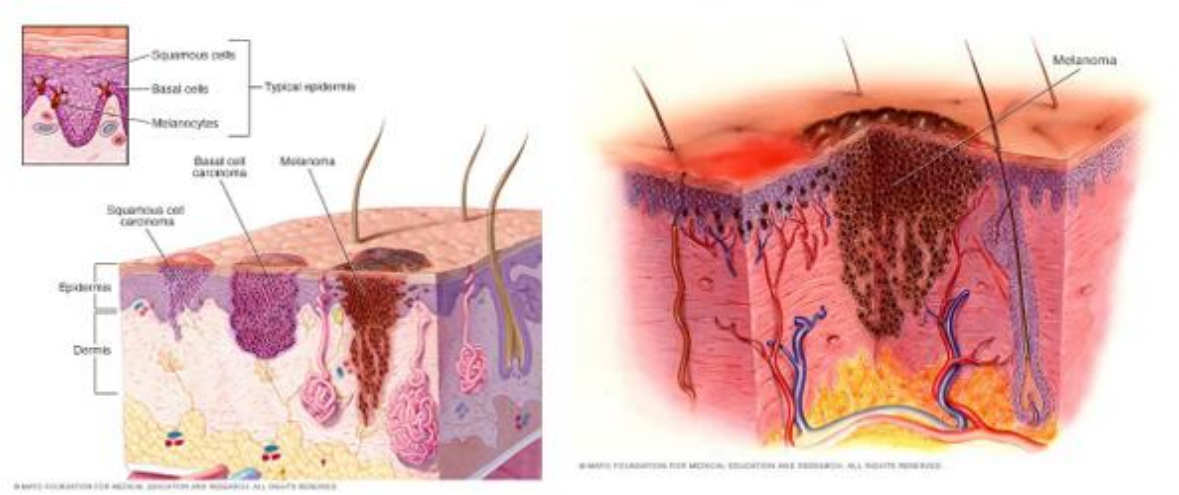

Image 13: *Diagrams illustrating the main skin cancers, with detail (right) of melanoma cells extending from the skin surface to the deeper skin layers (©Mayo Foundation for Medical Education and Research).*

Melanoma is classified into four major histopathological subtypes based on its clinical and microscopic characteristics: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM). SSM is the most common subtype and is characterized by an initial radial growth phase within the epidermis, followed by vertical invasion. In contrast, NM exhibits rapid vertical growth from the early stages and is associated with a more aggressive clinical course and a higher metastatic potential. LMM develops from lentigo maligna, a melanoma *in situ* typically associated with chronic sun exposure in elderly individuals, and progresses through dermal invasion. ALM primarily occurs on acral sites, such as the palms, soles, and nail beds, is more frequent in individuals with darker skin types, and is characterized by the proliferation of atypical melanocytes along the dermoepidermal junction, with possible invasion of the epidermis [32].

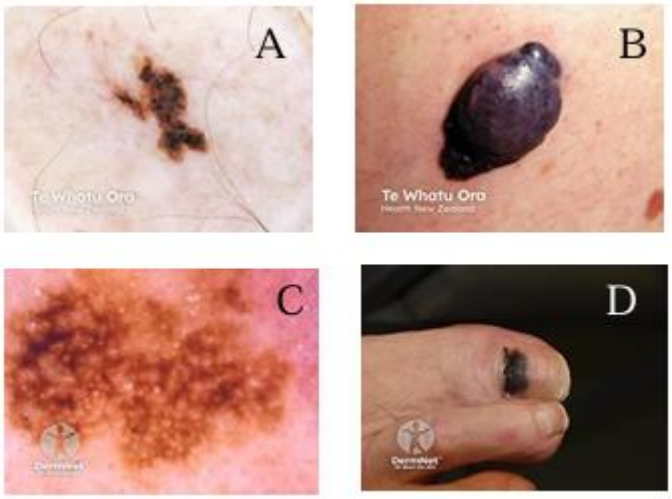

Image 14: *Clinical manifestations of the four main subtypes of melanoma: A) superficial spreading melanoma with characteristics of asymmetry, irregular borders, multiple colours and darker raised areas; B) nodular melanoma with characteristic protuberance; C) lentigo maligna melanoma with flat, irregularly shaped lesions (macules or plaques) and brown or black spots (some darker) that are scattered irregularly; D) acral lentiginous melanoma, with subungual manifestation. Images selected from: https://dermnetnz.org/*

The incidence of cutaneous melanoma has increased steadily over the past decade, making it one of the most common cancers worldwide. Incidence varies according to geographic location, ethnicity, age, and sex, with the highest rates observed in fair-skinned populations living in regions with high UV exposure. In Italy, melanoma incidence continues to rise in both men and women, with the trunk being the most frequently affected anatomical site[33].

The prognosis of melanoma is strongly dependent on the stage at diagnosis. There is a high probability of survival when the tumor is treated surgically at an early stage; however, survival rates decrease dramatically in the presence of metastatic disease [34].

2.2.1: FAMILIAL MELANOMA

Familial melanoma accounts for approximately 5–10% of all cases of cutaneous malignant melanoma, highlighting the contribution of genetic susceptibility to disease development. However, inherited predisposition alone is not sufficient to cause melanoma, as environmental factors, particularly ultraviolet (UV) radiation exposure, also play a crucial role. Consequently, melanoma susceptibility is considered multifactorial and only partially predictable. Familial melanoma is defined by the occurrence of melanoma in two or more relatives across two or three generations within the same family branch. In a subset of these families, specific germline pathogenic variants are responsible for hereditary melanoma, a rarer condition typically

inherited in an autosomal dominant pattern with incomplete penetrance and variable expressivity. Compared with sporadic melanoma, familial cases generally present at a younger age (30–40 years versus 50–60 years), are frequently associated with atypical nevi, and show an increased risk of developing multiple primary melanomas. Melanoma susceptibility genes are currently classified according to their relative risk into high-, intermediate-, and low-penetrance categories, reflecting their different contributions to disease predisposition [35].

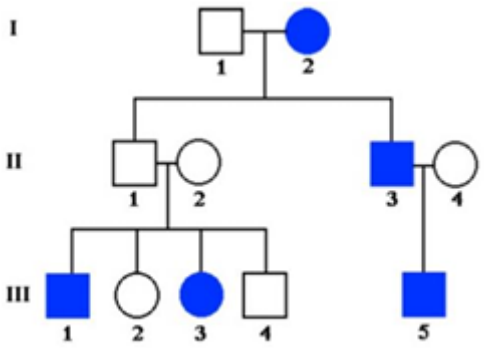

***Image 15**: Example family tree representing an Autosomal Dominant inheritance pattern with incomplete penetrance.*

2.2.2: RISK FACTORS

The development of cutaneous melanoma is a multifactorial process resulting from the interaction between genetic, predisposing, and environmental factors.

Among predisposing and environmental factors, the following play a critical role in determining melanoma susceptibility:

Skin phototype: The Fitzpatrick scale classifies skin into six phototypes according to its response to ultraviolet (UV) radiation, reflecting the skin's ability to produce melanin, tan, and resist sunburn. Individuals with phototypes I and II have the highest susceptibility to UV-induced skin damage and skin cancer due to their limited tanning ability and tendency to burn easily. Phototypes III and IV show progressively greater tanning capacity and a lower risk of sunburn, while phototypes V and VI, characterized by higher melanin content, are naturally more protected against UV damage and have a reduced risk of melanoma development [36].

Exposure to ultraviolet radiation (UV): Ultraviolet (UV) radiation is the primary environmental risk factor for skin cancer and is classified into three types according to wavelength: UVA, UVB, and UVC. While UVC is absorbed by the atmosphere and does not reach the Earth's surface, UVA (95%) and UVB (5%) contribute to skin damage through distinct

mechanisms. UVA penetrates deeply into the dermis, inducing indirect DNA damage via the generation of reactive oxygen species (ROS), which promote oxidative stress, photoaging, and mutations if not efficiently repaired by the base excision repair (BER) pathway. In melanoma, ROS play a dual role by promoting tumour progression while, at high levels, also inducing tumour cell death. In contrast, UVB radiation mainly affects the epidermis and directly damages DNA by inducing cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts, lesions that are primarily repaired through the nucleotide excision repair (NER) pathway. Failure of these repair mechanisms results in mutation accumulation, increasing the risk of skin carcinogenesis. Overall, due to its ability to induce DNA damage, oxidative stress, and chronic inflammation, UV radiation is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [37].

Atypical nevi: A high number of melanocytic nevi and the presence of atypical (dysplastic) nevi are well-established risk factors for melanoma. Individuals with numerous or atypical nevi have a significantly increased risk of developing melanoma, although only about one third of melanomas arise from pre-existing nevi, while most develop *de novo*. Therefore, regular clinical surveillance is recommended for individuals with atypical nevi [38].

2.2.2: GENETIC PREDISPOSITIONS

The major genetic risks are represented by germline mutations in the genes CDKN2A, CDK4, BAP1, and MITF. Germline mutations are genetic alterations present in germline cells (oocytes and spermatozoa) and are therefore transmissible to all the cells of the offspring.

Furthermore, at the somatic level, approximately 50% of melanomas are caused by activating mutations in the BRAF gene or by alterations that dysregulate the MAPK pathway [39]. These genetic alterations cause constitutive and uncontrolled activation of proliferative signaling, contributing to the neoplastic transformation of melanocytes [40].

2.2.3.1: *CDKN2A (Cyclin Dependent Kinase Inhibitor 2A)*

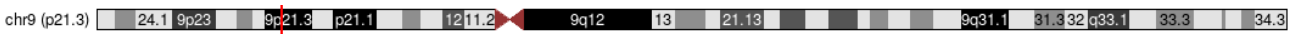

***Image 16**: Localization of the CDKN2A gene on chromosome 9 (region 9p21.3) visualized using the UCSC Genome Browser[16].*

The CDKN2A gene is one of the main tumor suppressor genes associated with genetic predisposition to familial cutaneous melanoma. It is located on chromosome 9p21 and encodes two distinct proteins, p16INK4a and p14ARF, generated through alternative splicing mechanisms. The p16INK4a protein negatively regulates the cell cycle by inhibiting the cyclin-dependent kinases CDK4 and CDK6, thereby controlling the transition from the G1 phase to the S phase, whereas p14ARF contributes to the stabilization of p53 through the inhibition of MDM2, promoting cell cycle arrest or apoptosis in response to DNA damage [41].

Germline mutations in *CDKN2A* lead to the loss of its regulatory functions, resulting in uncontrolled melanocyte proliferation and a significantly increased risk of developing cutaneous melanoma.

This genetic predisposition is often associated with an early onset of the disease and with a higher incidence of multiple tumors in the same individual or within the same family.

CDKN2A is considered a key gene in hereditary susceptibility to melanoma and an important marker for clinical surveillance programs in high-risk individuals.

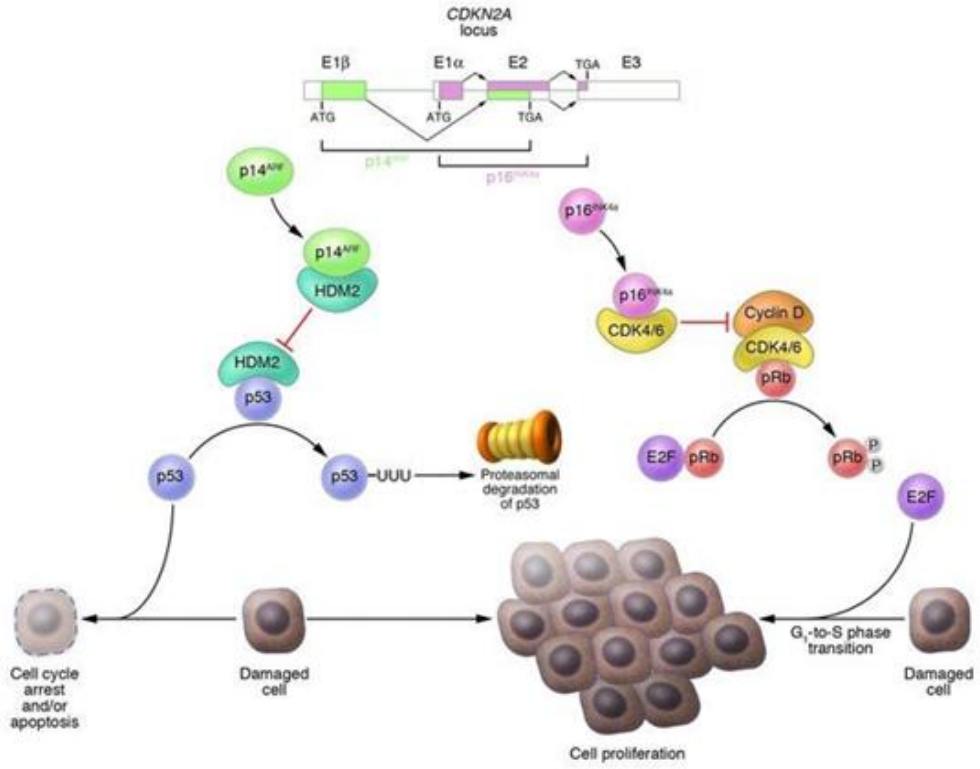

Image 17: *Organization and function of the CDKN2A locus on chromosome 9p21.3: alternative splicing generates two distinct tumor suppressors— p16INK4Ais translated from the splice product of exons E1α, E2 and E3, while p14ARF is translated from the splice product of exons E1β and E2 (Chudnovsky Y et al., 2005)*[42]

2.2.3.2: *CDK4 (Cyclin-dependent kinase 4)*

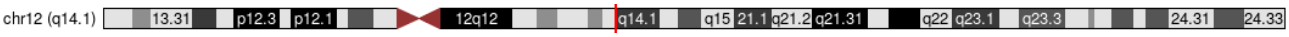

***Image 18**: Localization of the CDK4 gene on chromosome 12 (region 12q14.1) visualized using the UCSC Genome Browser*[16].

The *CDK4* gene, located on chromosome 12q14.1, encodes a serine/threonine kinase that plays a fundamental role in cell cycle regulation, particularly in the transition from the G1 phase to the S phase.

CDK4 forms a complex with cyclin D proteins, and its activation leads to phosphorylation of the retinoblastoma protein (pRb), resulting in the release of E2F transcription factors and the entry of the cell into the DNA synthesis phase [43].

In cutaneous melanoma, genetic alterations that increase CDK4 activity or reduce its regulation may promote uncontrolled cell proliferation. Activating germline mutations in *CDK4* have been associated with rare forms of familial melanoma, highlighting its role in tumor predisposition.

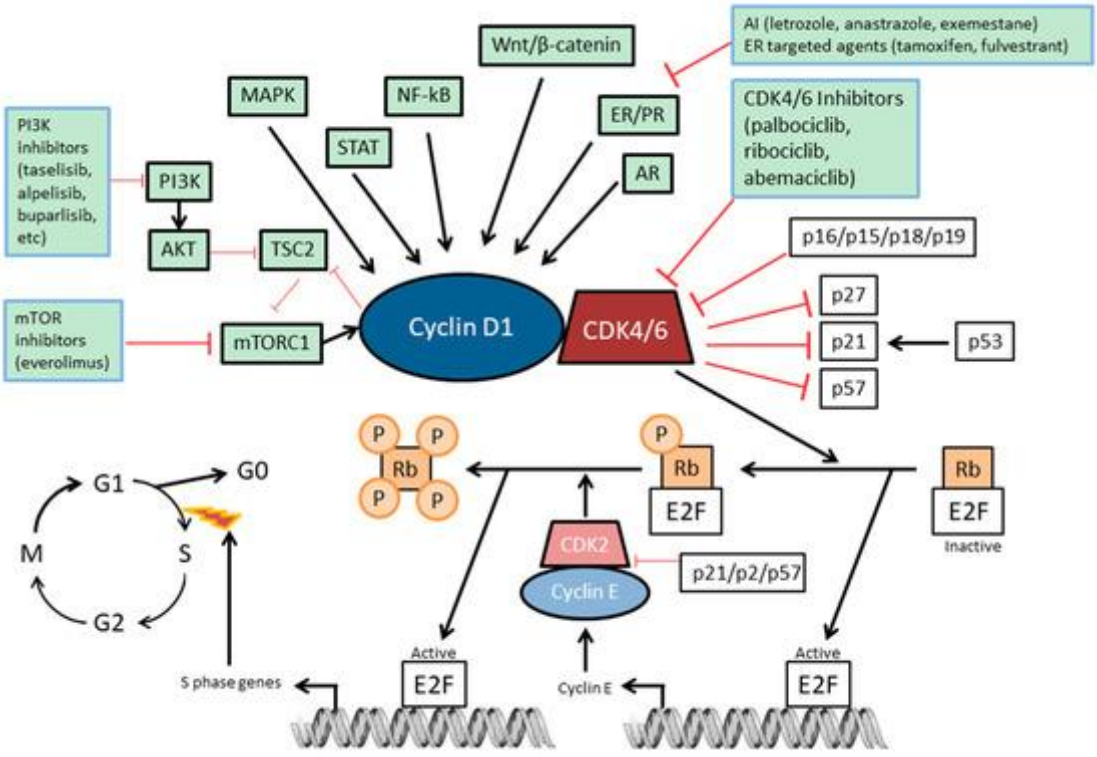

***Image 19:** CDK4/6 signaling pathway and therapeutic targets in melanoma.The diagram illustrates the central role of the CDK4/cyclin D complex in driving cell cycle progression via phosphorylation of Rb and activation of E2F, promoting G1/S transition. In melanoma, aberrant activation of upstream pathways (e.g., PI3K/AKT/mTOR, HER2 signaling) can enhance CDK4 activity. CDK4/6 inhibitors block Rb phosphorylation, halting proliferation. Additional therapeutic targets include PI3K, AKT, mTOR, and immune checkpoints (e.g., PD-L1), as well as HDAC and PARP inhibitors that modulate cell survival*

and apoptosis. Resistance and pathway cross-talk highlight the importance of combination strategies in melanoma treatment [44].

2.2.3.3: *BAP1 (BRCA1 Associated Protein-3)*

***Image 20**: Localization of the BAP1 gene on chromosome 3 (region 3p21.1) visualized using the UCSC Genome Browser*[16].

The BAP1 gene is a tumor suppressor gene located on chromosome 3p21.1, that encodes a nuclear deubiquitinase involved in the regulation of numerous cellular processes, including DNA repair, cell cycle control, and the modulation of gene transcription.

BAP1 interacts with protein complexes involved in the genomic damage response and contributes to the maintenance of chromosomal stability.

Germline mutations in *BAP1* cause a cancer predisposition syndrome known as **BAP1 tumor predisposition syndrome**.

Loss of BAP1 function leads to alterations in epigenetic control mechanisms and in the DNA damage response, promoting neoplastic transformation and tumor progression [45].

Furthermore, germline and somatic mutations in BAP1 represent an important diagnostic and prognostic biomarker, especially for uveal and cutaneous melanoma.

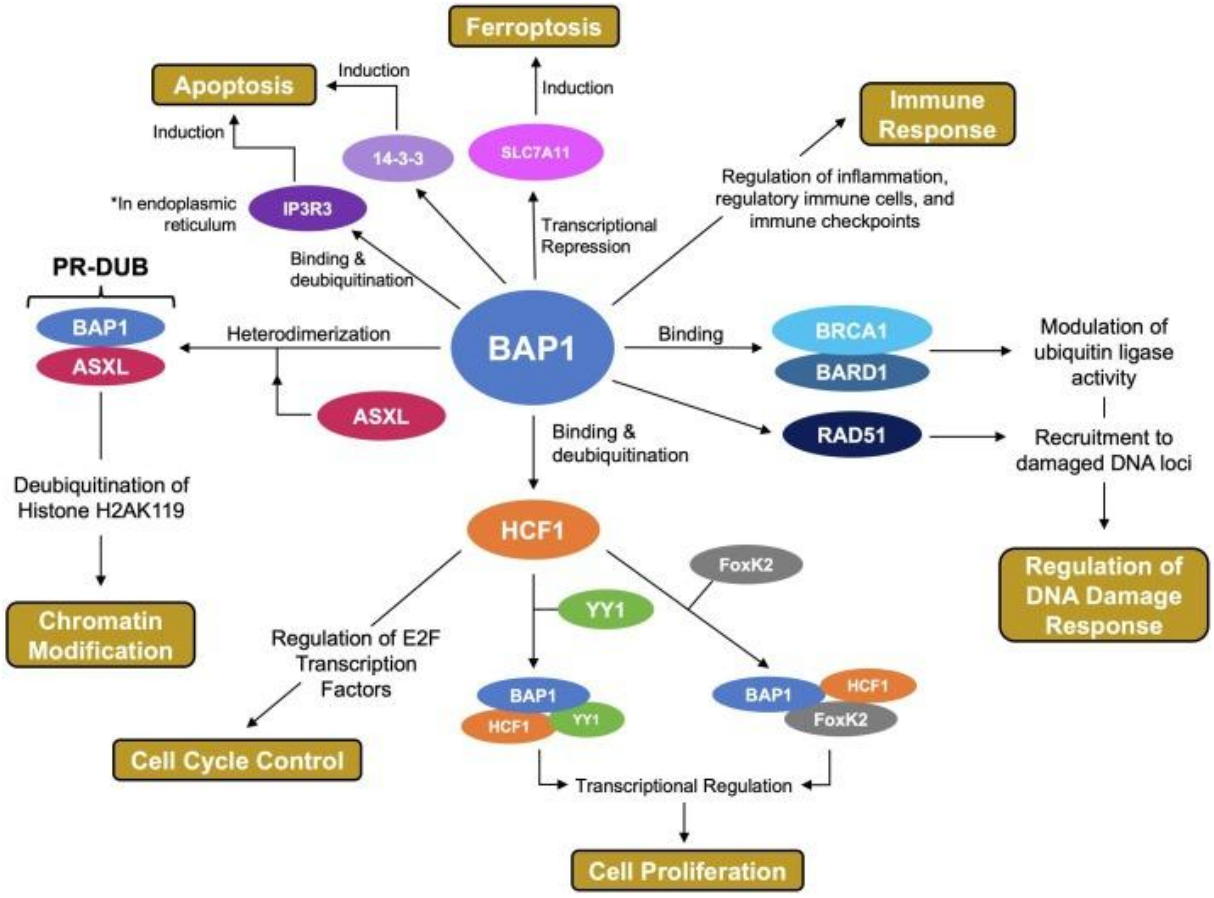

***Image 21**: BAP1 is a tumor suppressor gene that plays a central role in melanoma by regulating chromatin remodeling, cell cycle progression, DNA repair, apoptosis, ferroptosis, and immune responses. Through interactions with proteins such as ASXL, HCF1, BRCA1, and IP3R3, BAP1 controls gene transcription and maintains cellular homeostasis. Loss of BAP1 function disrupts these regulatory pathways, promoting melanoma development, tumor progression, immune evasion, and resistance to therapy [46].*

2.2.3.4: *MITF (Microphthalmia-associated transcription factor)*

***Image 22**: Localization of the MITF gene on chromosome 3 (region 3p14.1/3p13) visualized using the UCSC Genome Browser[16].*

The MITF gene, located on chromosome 3 between regions 3p14.1 and 3p13, encodes a transcription factor that plays a role in the development, differentiation, and survival of melanocytes by regulating the expression of several genes involved in melanogenesis and cell proliferation.

Under physiological conditions, MITF integrates signals from the MAPK and cAMP pathways, regulating the balance between cellular differentiation and growth.

Genetic alterations or amplifications of *MITF* may contribute to melanocytic tumorigenesis.

In particular, a specific germline variant, ***E318K***, has been identified and it is associated with a moderate increase in the risk of familial cutaneous melanoma [47].

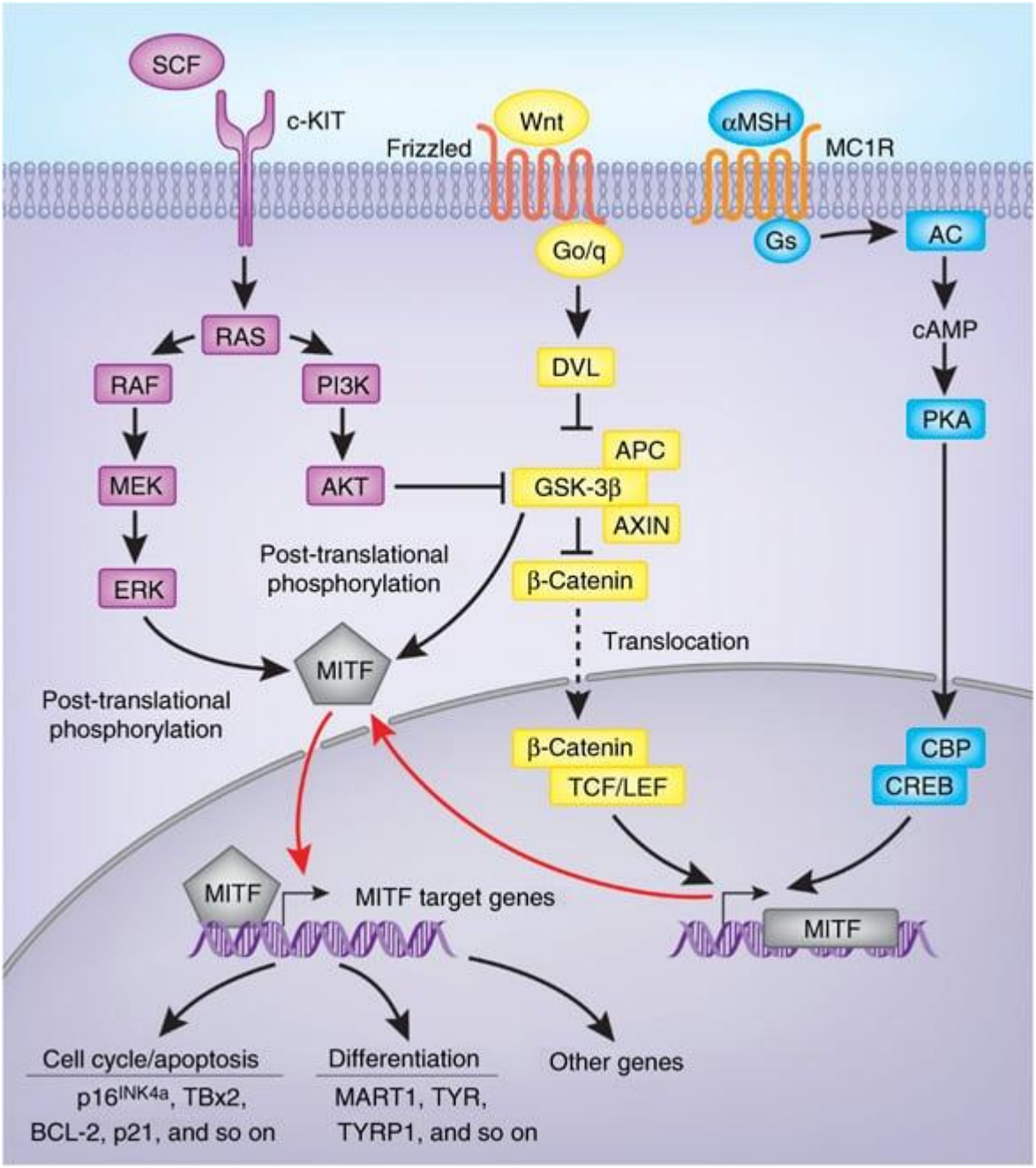

Image 23*: MITF is the master transcription factor regulating melanocyte differentiation and melanoma biology. Its activity is controlled by major signaling pathways, including MAPK, PI3K/AKT, Wnt/β-catenin, and cAMP/PKA, which modulate its expression and post-translational modifications. In turn, MITF regulates genes involved in cell proliferation, apoptosis, and differentiation. Dysregulation of these signaling pathways alters MITF activity, promoting melanoma progression and phenotypic plasticity* [48].

2.2.4: CRITERIA FOR MELANOMA PATIENTS' GENOMIC EVALUATION

International guidelines establishing clinical criteria for selecting patients to undergo genetic testing were first published in 2009, primarily addressing CDKN2Amutations (Leachman SA et al., 2009 e 2017) [49-50].

The guidelines, refined in 2017, vary by geographic region. In countries with a low incidence of melanoma (< 10 cases per 100,000 inhabitants), such as Italy, France and Brazil, a stringent "rule of two" is recommended.

Based on this approach, eligibility for genetic testing is based on:

1. ≥ 2 histologically confirmed invasive primary melanomas in the proband (index case);
2. invasive melanomas in first- and/or second-degree relatives;
3. pancreatic tumours in the proband or blood relatives.

In Italy, the Italian Society of Human Genetics (Gruppo SIGU-ONC, 2005) [51] recommends genetic testing for familial melanoma to assess genetic risk in patients with a personal or family history including one or more of the following items:

1) A known pathogenic mutation in a predisposing gene (CDKN2A, CDK4);
2) Two or more first/second degree relatives with melanoma;
3) Presence of multiple primary melanomas in the same patient;
4) Presence of atypical/dysplastic nevi associated with melanoma;
5) Personal or family history of both melanoma and pancreatic cancer;
6) Diagnosis of melanoma at a young age (< 40 years).

Conversely, in geographical areas with a medium-high risk of melanoma (estimated as an incidence rate greater than 10 per 100,000 individuals), such as the United States of America or Northern Europe, the criteria are less restrictive. In fact, the "rule of three" is followed, meaning that individuals or families with a total of 3 or more invasive primary melanomas (or a combination of melanoma and pancreatic cancer) are referred directly for genetic counselling (Leachman SA et al., 2017) [50].

2.2.5: THERAPIES

The treatment of cutaneous melanoma largely depends on the stage of the disease. The most effective option is certainly localized surgery; however, in recent years, immunotherapy and targeted therapies have also shown significant improvement.

Among target therapies, inhibitors of the MAPK pathway are of great importance, particularly *BRAF* and *MEK* inhibitors.

In parallel, immune checkpoint inhibitors such as Ipilimumab for *CTLA-4* and Nivolumab for *PD-1* or Atezolizumab for *PD-L1* have also led to a significant improvement in long-term survival.

Despite the evident progress and highly positive outcomes, research aimed at identifying predictive biomarkers of response remains ongoing [52].

2.3: MOLECULAR DIAGNOSIS

Next-generation sequencing (NGS) technologies have changed the approach to the molecular diagnosis of hereditary cancer syndromes by enabling the analysis, at the same time, of numerous genes associated with cancer predisposition through the use of multigene panels, as in this case, in which the BML panel was employed.

These panels now represent a fundamental diagnostic tool, as they allow the identification of pathogenic variants in high-, moderate-, or low-penetrance genes involved in the development of specific neoplasms [53].

With regard to breast cancer, the Genetic Laboratory of the Maggiore Hospital in Novara analyses, in addition to high-risk genes (*BRCA1* and *BRCA2*), other genes involved in DNA repair mechanisms and cell cycle control, including *PALB2, ATM, CHEK2, TP53, PTEN, CDH1, STK11, BARD1,* and *BRIP1*.

The analysis of all genes related to breast cancer increases the diagnostic yield and provides a genetic overview of the patient, in order to support the development of target therapy.

Similarly, for cutaneous melanoma, the multigene BML panel allows the analysis of genes involved in cell cycle regulation and tumor suppression. Among these, *CDKN2A* represents the main susceptibility gene, followed by *CDK4, BAP1,* and *MITF*.

Regarding Lynch syndrome, the genes analyzed include *MLH1, MSH2, MSH6, PMS2,* and *EPCAM*.

In addition, the panel used in this laboratory also investigates genes directly involved in DNA repair, such as *RAD51C, RAD51D,* and *NBN*.

The use of multigene panels also involves some critical aspects, including the identification of variants and their classification into different categories, such as benign, Likely Benign (LB), Variants of Uncertain Significance (VUS), Likely Pathogenic (LP) and pathogenic.

These different variants require careful interpretation within the patient's clinical and family context, carried out by medical geneticists and supervising physicians.

The NGS panel used in this study also included genes associated with **Lynch syndrome**. However, these genes were not investigated in the present work, as the aim of this study was to evaluate genetic variants specifically associated with cutaneous melanoma and breast cancer susceptibility. Lynch syndrome is an inherited cancer predisposition syndrome rather than a disease associated with a single tumor type. It is caused by germline pathogenic variants in DNA mismatch repair (MMR) genes and confers an increased lifetime risk of developing a broad spectrum of malignancies, most notably colorectal and endometrial cancers, but also ovarian, gastric, small bowel, pancreatic, urinary tract, brain, and several other cancers. Therefore, the analyses presented in this thesis were restricted exclusively to cohorts of patients affected by cutaneous melanoma and breast cancer.

OBJECTIVE OF THE THESIS

This study investigates gene-phenotype discordance in patients undergoing germline multigene panel testing for suspected hereditary cancer predisposition.

Specifically, we analyzed a cohort of individuals referred for suspected hereditary breast cancer or cutaneous melanoma who were tested using a custom Next-Generation Sequencing (NGS) panel, designated BML (Breast-Melanoma-Lynch).

The primary objective is to characterize the spectrum and frequency of pathogenic (P) and likely pathogenic (LP) germline variants within this cohort.

Crucially, the analysis extends beyond genes classically linked to the patients' initial clinical indications to encompass all cancer predisposition genes included in the BML panel.

By evaluating variants in these non-canonical genes, this work aims to assess the occurrence of unexpected genotype-phenotype associations, thereby elucidating the clinical utility and contribution of broader multigene testing in hereditary cancer risk assessment.

MATERIALS AND METHODS

4.1: NEXT-GENERATION SEQUENCING (NGS)

4.1.1: NGS MULTIGENE PANEL

Following the development of sequencing technologies, the mutational status of patients affected by breast cancer, Lynch syndrome, and familial melanoma was investigated using Next-Generation Sequencing (NGS).

A custom multigene panel comprising 23 genes associated with the most common hereditary cancer syndromes was designed based on evidence reported in the literature. Target enrichment was performed using the SureSelect XT HS2 DNA kit (Design ID: 3426591; Agilent Technologies, USA).

The genes included in the custom panel, named Breast–Melanoma–Lynch (BML), are listed in Table X. In addition, copy number variation (CNV) analysis, including the detection of deletions and duplications affecting these genes, was performed using EnGenome software (Evai GUI version 2.9).

Genes associated with breast cancer / HBOC	*BRCA1*
	BRCA2
	PALB2
	ATM
	CHEK2
	BARD1
	BRIP1
	CDH1
	PTEN
	STK11
	TP53
Genes associated with Lynch syndrome	*MLH1*
	MSH2
	MSH6
	PMS2
	EPCAM
Genes associated with familial melanoma	*CDKN2A*
	CDK4
	BAP1
	MITF
DNA repair genes	*RAD51C*
	RAD51D
	NBN

***Table 1**: list of the 23 genes included in the custom panel, named BML (Breast, Melanoma, Lynch).*

4.1.2: GENOMIC DNA EXTRACTION

Genomic DNA was extracted from buffy coat samples using the QIAsymphony DSP DNA Mini Kit (QIAGEN, Hilden, Germany) on the automated QIAsymphony SP platform, following the manufacturer's *DNA_Buffy_Coat_200_V7_DSP* protocol. This automated system enables the purification of both genomic and mitochondrial DNA from human blood samples with high reproducibility and minimal manual handling.

Buffy coat samples were obtained from peripheral blood collected in tubes containing anticoagulants (EDTA, citrate, or heparin). Whole blood was centrifuged at 900–1100 × *g* for 10 minutes at room temperature (15–25 °C), resulting in the separation of three distinct layers: plasma (upper layer), leukocyte-rich buffy coat (intermediate layer), and erythrocytes (lower layer). Approximately 1 mL of buffy coat was recovered from 10 mL of whole blood, corresponding to a 5- to 6-fold enrichment of leukocytes. For DNA extraction, 200 µL of buffy coat were used as the input material.

DNA purification was performed using magnetic particle–based technology, in which nucleic acids selectively bind to magnetic beads and are subsequently purified through a series of automated washing steps to remove proteins, cellular debris, and other contaminants. Purified DNA was eluted in a dedicated buffer, with a final elution volume ranging from 200 to 400 µL according to downstream molecular applications.

The extraction workflow was fully automated, requiring the loading of reagent cartridges, disposable filter tips, and sample preparation cartridges into the appropriate instrument compartments. All extraction steps were controlled by the instrument software, thereby reducing hands-on time and minimizing the risk of cross-contamination. DNA eluates were stored at 2–8 °C, −20 °C, or −80 °C until further analyses, avoiding repeated freeze–thaw cycles to preserve DNA integrity.

Overall, the automated extraction procedure provided a standardized, reproducible, and reliable method for isolating high-quality genomic DNA from leukocyte-enriched blood fractions, yielding nucleic acids suitable for both diagnostic and research molecular applications.

4.1.3: MANUAL EXTRACTION OF GENOMIC DNA

Genomic DNA was extracted from peripheral blood leukocytes using 200 µL of whole blood collected in EDTA anticoagulant tubes and processed with the **ReliaPrep Blood gDNA Miniprep System** (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. Purified DNA was eluted in nuclease-free water to a final volume of 50 µL.

4.1.4: NANODROP 2000 QUANTIFICATION

DNA quality and concentration were assessed using a **NanoDrop One spectrophotometer** (Thermo Fisher Scientific, USA). DNA purity was evaluated by measuring the absorbance ratios at 260/280 nm and 260/230 nm, with values between 1.8 and 2.0 considered indicative of high-quality DNA. Samples with a DNA concentration ≥ 30 ng/µL were considered suitable for subsequent molecular analyses.

4.1.5: DNA quantification by Qubit fluorimetric assay

Many molecular biology applications, including PCR, library preparation, and Next-Generation Sequencing (NGS), require accurate DNA quantification. Although spectrophotometric analysis provides a rapid assessment of DNA concentration and purity, it may overestimate DNA content due to the presence of contaminants. Therefore, DNA concentration was further determined using the Qubit dsDNA High Sensitivity (HS) Assay Kit on a Qubit 4 Fluorometer (Invitrogen™, Thermo Fisher Scientific, USA), which provides a more accurate and selective quantification of double-stranded DNA (dsDNA).

The Qubit dsDNA HS assay is based on a fluorescent dye that selectively binds dsDNA, generating a fluorescence signal proportional to the amount of DNA present while exhibiting minimal interference from RNA, proteins, or other contaminants. The assay is optimized for DNA concentrations within the range recommended by the manufacturer, making it particularly suitable for downstream molecular applications requiring precise DNA input.

The working solution was prepared by diluting the Qubit dsDNA HS reagent in the supplied buffer at a 1:200 ratio. Instrument calibration was performed using the two standards provided with the kit, according to the manufacturer's instructions. For sample quantification, 2 µL of

DNA was mixed with 198 µL of working solution, briefly vortexed, and incubated at room temperature for approximately 2 minutes to allow complete dye–DNA binding. Fluorescence was then measured using the Qubit 4 Fluorometer, which automatically calculated DNA concentration based on the calibration curve generated from the standards.

Samples with DNA concentrations exceeding 60 ng/µL were diluted 1:2 (v/v) with nuclease-free water before quantification to ensure that measurements fell within the assay's dynamic range.

4.1.6: AUTOMATED LIBRARY PREPARATION

After loading the strips containing the diluted DNA samples, together with the pre-aliquoted kit reagents that had been appropriately resuspended according to the manufacturer's instructions, the Agilent SureSelect XT HS2 DNA workflow was performed using the SSELDNA-XT-HS2-ILM protocol, optimized for sequencing on Illumina platforms (Illumina, Inc., San Diego, CA).

The automated library preparation procedure, which requires approximately 10 hours, included enzymatic DNA fragmentation to generate fragments of approximately 400 bp, followed by end repair to produce ligation-compatible DNA ends. Sequencing adapters containing sample-specific index sequences were then ligated to the DNA fragments, enabling both cluster generation on the Illumina flow cell and multiplex sequencing of multiple samples within the same run.

Subsequently, target enrichment was achieved through hybridization with biotinylated capture probes specific for the genomic regions of interest. After a series of washing steps to remove non-specifically bound DNA fragments, the enriched libraries were PCR-amplified to generate the final target-enriched sequencing libraries for downstream NGS analysis.

Image 24:

(A) Workflow for SureSelect XT HS2 DNA target enrichment using the Magnis NGS Prep System (Agilent Technologies, Inc., USA).

(B) Images of the Magnis NGS Prep System, shown externally and with the internal work deck loaded with all the components required for protocol execution, as provided in the kit (top right).

Image A adapted from: https://www.agilent.com/cs/library/usermanuals/public/G9751-90000.pdf;
Images B sourced from: https://www.agilent.com/en/product/next-generation-sequencing/ngs-automation-platforms/magnis-ngs-prep-system-430157429

4.1.7: PREPARATION OF THE POOL FOR SEQUENCING

Following library quality assessment, individual indexed libraries were pooled in equimolar amounts to ensure equal representation of each sample during sequencing. The volume of each library to be included in the pool was calculated according to the manufacturer's instructions using the formula reported below, based on the desired final pool volume and concentration, the number of indexed libraries, and the concentration of each individual library.

$$\frac{V(f)\times C(f)}{\#\times C(i)}$$

- V(f) indicates the desired final volume of the pool (typically around 100–150 µL for 32 libraries);
- C(f) indicates the desired final concentration of total DNA in the pool (generally 4–6 nM);
- # indicates the number of each index, as provided by the method manufacturer;

- C(i) indicates the initial concentration of each sample library, determined using the TapeStation 4150 System.

4.1.8: SEQUENCING USING ILLUMINA INSTRUMENTATION

(Illumina, Inc., San Diego, CA), illustrated in *Figure 18*.

For the 23-gene panel, the MiSeq Dx platform was used with MiSeq Reagent Kits v2 or v3 (depending on the number of samples).

Image 25: *Illumina (Illumina, Inc., San Diego, CA) NGS sequencers: MiSeq Dx (left) and NextSeq 1000 (right). Images sourced from: https://www.illumina.com/systems/sequencing-platforms.html*

The use of Agilent SureSelect XT HS2 target-enriched libraries is fully compatible with the Illumina sequencing workflow, which is based on the sequencing-by-synthesis (SBS) technology (Figure X). During library preparation, sequencing adapters are ligated to both ends of each DNA fragment. These adapters enable fragment immobilization on the flow cell through hybridization with complementary oligonucleotides and provide the binding sites required for sequencing primer annealing. In addition, they allow paired-end sequencing, in which each DNA fragment is sequenced from both ends, thereby improving mapping accuracy, particularly across repetitive genomic regions, and increasing variant detection reliability.

Following library loading, adapter-ligated DNA fragments hybridize to complementary oligonucleotides immobilized on the flow cell surface. Through repeated cycles of extension and denaturation, fragments undergo bridge amplification, generating clusters of clonally amplified DNA molecules that provide sufficient fluorescence intensity for sequence detection.

After cluster generation, one DNA strand is selectively removed, leaving single-stranded templates for sequencing. The 3′ ends are then blocked, and the sequencing primer for Read 1 is annealed. DNA synthesis proceeds through iterative incorporation of fluorescently labeled reversible terminator nucleotides. During each sequencing cycle, only the nucleotide

complementary to the template strand is incorporated, and its fluorescent signal is detected by the instrument, allowing base identification. After image acquisition, the fluorescent label and terminator group are chemically removed, enabling the next sequencing cycle. This cyclic process constitutes the sequencing-by-synthesis (SBS) technology.

Upon completion of Read 1, the newly synthesized strand is removed by denaturation, and the complementary template is regenerated. The sequencing primer for Read 2 is then annealed, and a second round of sequencing-by-synthesis is performed in the opposite direction, producing paired-end reads. The number of sequencing cycles determines the final read length.

By simultaneously sequencing hundreds of millions of DNA clusters in parallel, the Illumina platform provides high-throughput, highly accurate sequencing, making it particularly well suited for large-scale genomic analyses, including targeted sequencing applications.

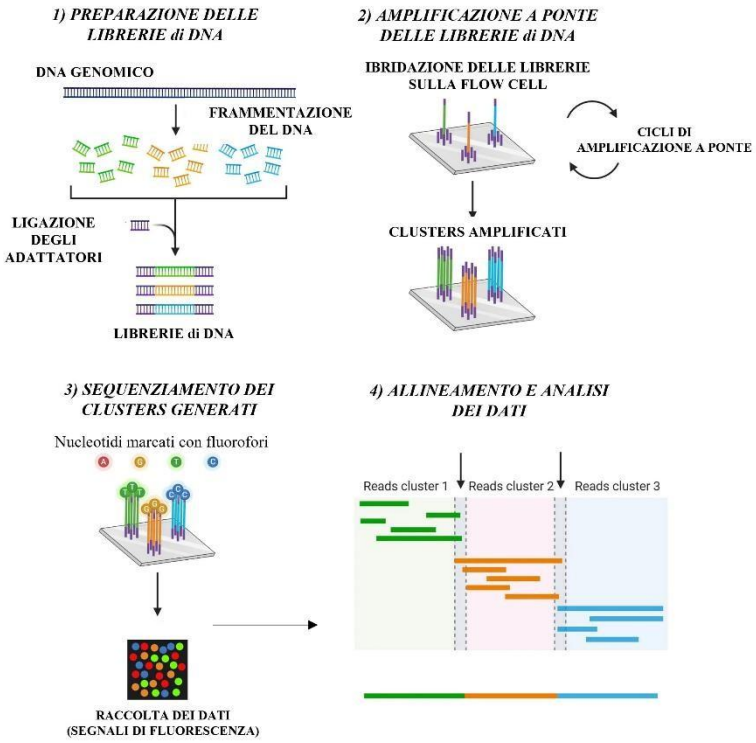

Image 26: *Schematic representation of the different stages of Illumina sequencing.*

Image adapted from: https://microbenotes.com/illumina-sequencing/

4.1.9: DATA ANALYSIS

Paired-end sequencing generates two separate FASTQ files (Read 1 and Read 2), containing sequences derived from both ends of each DNA fragment. At the end of the sequencing run,

millions of reads are produced per sample; however, the presence of unique index sequences enables the assignment of reads to their corresponding samples through a demultiplexing step.

Following initial quality assessment of the raw reads, high-quality paired reads are retained and the corresponding forward and reverse sequences are processed for downstream analysis. Where appropriate, overlapping read pairs are merged to generate contiguous sequences. The resulting reads are then aligned to the human reference genome (GRCh37/hg19) for subsequent variant analysis.

4.1.10: INTERPRETATION OF THE IDENTIFIED VARIANTS

The final phase of the workflow consists of the evaluation and validation of the identified genetic variants through Sanger sequencing, performed on a newly extracted DNA sample. As expected, the number of variants requiring review increases proportionally with the size of the multigene panel analyzed.

Detected variants are systematically compared with those reported in publicly available population databases, including dbSNP, the Exome Sequencing Project (ESP), ExAC, the 1000 Genomes Project, and the Genome Aggregation Database (gnomAD). Variants with a population frequency greater than 1% are considered common polymorphisms and are therefore excluded from further analysis. Likewise, variants classified as benign or likely benign according to the recommendations of the American College of Medical Genetics and Genomics (ACMG) Laboratory Practice Working Group (Richards et al., 2015) are not reported. Variant pathogenicity is assessed in accordance with the ACMG guidelines, integrating evidence from major clinical interpretation databases, including Franklin by Genoox, VarSome, and ClinVar (NCBI). Furthermore, genotype–phenotype correlations are investigated through consultation of the OMIM® and Orphanet databases.

Despite its high analytical performance, this approach presents some limitations. Specifically, intronic variants located outside the canonical splice sites, as well as variants affecting regulatory regions not included in the targeted panel, cannot be detected. In addition, pathogenic variants located in genomic regions that are either not covered or insufficiently covered by the sequencing assay may remain undetected.

4.2: SANGER SEQUENCING

Next-generation sequencing (NGS) enables the simultaneous analysis of multiple genomic regions, allowing the identification of a wide range of genetic variants in a single assay. However, despite its high throughput and sensitivity, variants identified by NGS are commonly validated using Sanger sequencing.

Sanger sequencing is widely regarded as the gold standard for the confirmation of genetic variants, owing to its high accuracy and reliability. This technique is routinely employed to verify NGS findings, thereby excluding false-positive results arising from sequencing artifacts, amplification biases, or bioinformatic errors. Furthermore, its relatively rapid turnaround time and lower cost for targeted analyses make Sanger sequencing the preferred confirmatory method before the clinical reporting of pathogenic or likely pathogenic variants.

4.2.1: PCR: Polymerase Chain Reaction

PCR is a molecular biology technique developed by Kary Mullis (Nobel Prize in Chemistry in 1993) in 1983 that enables rapid and specific amplification of defined DNA sequences. PCR produces billions of copies of a specific DNA fragment, facilitating its analysis.

The reaction takes place in an instrument called a thermal cycler, which precisely controls the temperature changes required for the process. PCR is based on repeated cycles of heating and cooling, each consisting of three main steps.

The first step is denaturation (96 °C). At this high temperature, the DNA double strand is separated into two single strands by breaking the hydrogen bonds between complementary nucleotide bases, making the DNA accessible to the other reaction components.

This is followed by the annealing step (50–65 °C). During this phase, short synthetic DNA sequences called primers bind to the DNA strands. The primers are designed to recognize and hybridize to regions flanking the target DNA segment to be amplified. The temperature of this step is critical and is chosen according to the composition and length of the primers to ensure stable and specific binding.

The third step is extension (72 °C). This phase relies on the activity of Taq polymerase, a thermostable enzyme capable of withstanding high temperatures. This DNA polymerase adds nucleotides to the primers, synthesizing a new DNA strand complementary to the template

strand and thereby copying the target sequence. Taq polymerase is named after the thermophilic bacterium *Thermus aquaticus*, from which it was originally isolated.

These three steps constitute a single PCR cycle. At the end of each cycle, the number of target DNA molecules doubles, as each strand can serve as a template for the synthesis of a new one. By repeating the process for 20–40 cycles, exponential amplification is achieved, leading to the production of billions of copies of the desired sequence.

4.2.2: GEL ELECTROPHORESIS

Gel electrophoresis is a molecular biology technique used to separate nucleic acids (DNA or RNA) or proteins according to their size and, in the case of proteins, their charge. Although primarily considered a qualitative rather than a quantitative method, it is routinely employed to assess the quality of PCR amplification products and to verify the absence of contamination before downstream applications.

For DNA analysis, the agarose gel is prepared by dissolving 0.75 g of agarose powder in 50 mL of 1× Tris–Acetate–EDTA (TAE) buffer. The mixture is heated until the agarose is completely dissolved and then poured into a casting tray, where it is allowed to cool and solidify, forming a gel with wells for sample loading.

Prior to electrophoresis, PCR products are mixed with the nucleic acid stain SYBR Green, an intercalating dye that enables DNA visualization following electrophoretic separation. Once the gel has solidified, the prepared samples are carefully loaded into the wells together with an appropriate DNA molecular weight marker.

Electrophoretic separation is achieved by applying an electric field across the gel using a power supply. Because DNA molecules possess a negatively charged phosphate backbone, they migrate toward the positive electrode (anode). The agarose matrix acts as a molecular sieve, allowing smaller DNA fragments to migrate more rapidly through its pores, while larger fragments experience greater resistance and therefore migrate more slowly. Electrophoresis is typically performed at approximately 140 V for 15 minutes.

Following electrophoresis, the gel is visualized under ultraviolet (UV) illumination. The intercalating dye fluoresces upon exposure to UV light, allowing the DNA bands to be observed and their size to be estimated by comparison with the molecular weight marker.

4.2.3: EXO/SAP PURIFICATION

Exo/SAP purification is an enzymatic cleanup method commonly used to remove residual primers and unincorporated deoxynucleotide triphosphates (dNTPs) from PCR products prior to Sanger sequencing. This purification step improves the quality of sequencing reactions by eliminating components that could interfere with DNA polymerase activity and compromise sequence accuracy.

The Exo/SAP reagent consists of two enzymes with complementary functions: Exonuclease I (Exo I), which selectively degrades residual single-stranded primers, and Shrimp Alkaline Phosphatase (SAP), which dephosphorylates unincorporated dNTPs, thereby rendering them unavailable for subsequent enzymatic reactions.

Before use, the Exo/SAP reagent is diluted 1:20 in nuclease-free water and added directly to the PCR products. The enzymatic reaction is then performed in a thermal cycler using a two-step incubation protocol consisting of 15 minutes at 37 °C, during which primer degradation and dNTP dephosphorylation occur, followed by 15 minutes at 80 °C to inactivate both enzymes. Enzyme inactivation is essential to prevent interference with the subsequent sequencing reaction.

4.2.4: SEQUENCING AND RESULTS READING

Sanger sequencing, developed in 1977 by Frederick Sanger, is the most widely used method for accurately determining the order of bases in a DNA fragment.

This technique is based on the use of dideoxynucleotides (ddNTPs), chemically modified nucleotides that terminate DNA strand elongation. They lack a 3′-OH group, and therefore DNA polymerase is unable to form the subsequent phosphodiester bond, resulting in termination of DNA synthesis.

The process starts from the DNA fragment obtained through PCR, to which several components are added, including:

- Primers: required to initiate synthesis
- DNA polymerase
- Deoxynucleotides (dNTPs)
- Fluorescently labeled ddNTPs (each nucleotide is labeled with a different fluorescent dye for identification)

The result of this process is an electropherogram, i.e., a graph composed of colored peaks representing the bases:

• A (adenine): green

• T (thymine): red

• C (cytosine): blue

• G (guanine): black

This allows the nucleotide sequence of the region of interest to be obtained (*Figure below*).

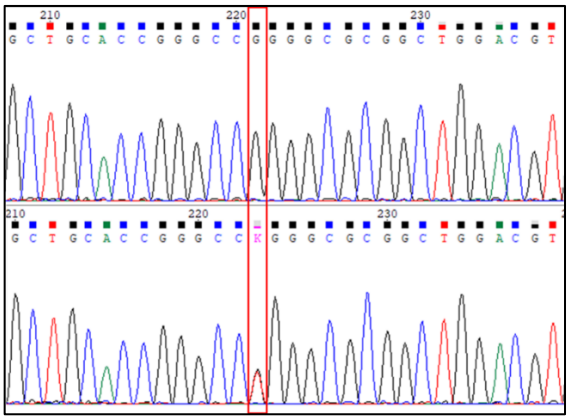

Image 27: *Two examples of Sanger sequencing electropherograms, showing a wild-type sequence (G) at the top and a heterozygous mutation (G>T) at the bottom.*

RESULTS

5.1: STUDY COHORT

A total of 1064 patients were analyzed, including 728 (68.42%) females and 336 (31.58%) males, who underwent genetic testing at the Genetics Laboratory of the Clinical Biochemistry Unit at the Maggiore Hospital in Novara (Italy), between January 2024 and December 2025.

The tests were requested by specialists from the same hospital and other hospitals within the quadrant, with the aim of confirming the presence or absence of one or more mutations causing cutaneous melanoma or HBOC spectrum tumors and investigating the degree of pathogenicity of related mutations.

Patients underwent peripheral venous blood sampling for genetic analysis after completing and signing an informed consent form. This documentation was submitted to the laboratory together with the relevant personal and family clinical history, which was archived in compliance with data privacy regulations.

Of the 1064 patients included in the study, 662 (62.22%) are patients with HBOC spectrum tumors (Hereditary Breast and Ovarian Cancer syndrome), while 402 (37.78%) have cutaneous melanoma.

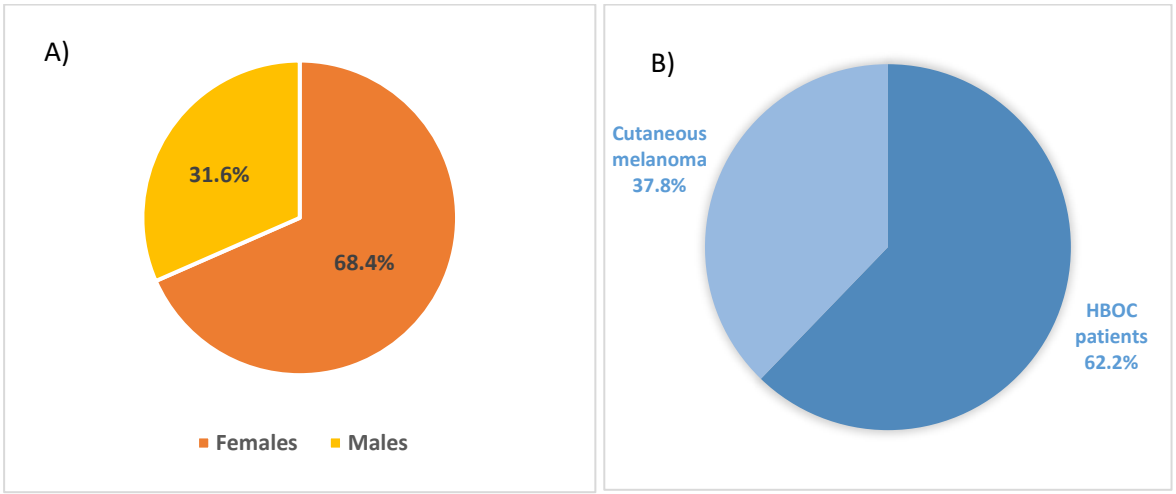

Image 28: *Distribution of the study cohort by sex and tumor type:*
(a) Distribution of the 1064 patients included in the study by sex, with women (68.4%) compared to men (31.6%). (b) Distribution of patients according to the type of cancer analyzed: 662 patients (62.2%) belonging to the HBOC spectrum tumors (Hereditary Breast and Ovarian Cancer) and 402 patients (37.8%) with cutaneous melanoma.

5.2: GENETIC RESULTS IN THE GENERAL COHORT

Within the general cohort, 174 (16.35%) patients have mutations in the genes included in the BML panel used.

Within the group of patients carrying variants, there were 117 (67.24%) females and 57 (32.76%) males.

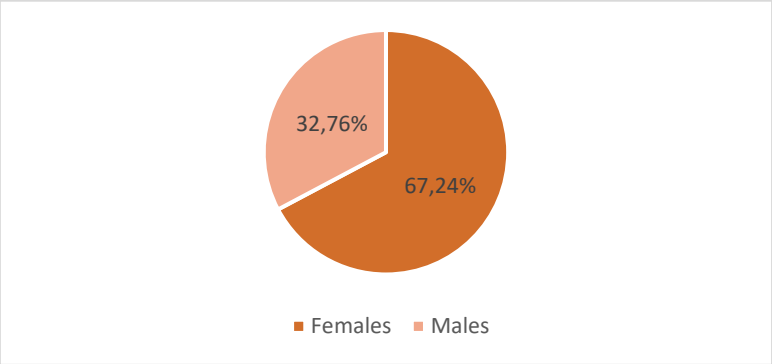

***Image 29**: Distribution by sex of patients carrying genetic variants in the general cohort*

The identified variants have been subdivided into the following classes: Variants of Uncertain Significance (VUS), Likely Pathogenic (LP) and Pathogenic (P).

The results are presented in the table below, which shows the number of patients belonging to each class based on the mutational variant found, their frequency within the cohort of mutated subjects (174/1064 patients) and the ratio between males and females.

Percentages exceed 100% because some patients carried more than one variant, therefore, the same individual may be counted more than once.

Variant class	N. patients	Frequency within mutated cohort	Sex	N. patients
VUS	44	25.29%	females	31 (70.45%)
			males	13 (29.55%)
LP	38	21.84%	females	26 (68.42%)
			males	12 (31.58%)
P	92	78.63%	females	60 (65.22%)
			males	32 (34.78%)

***Table 2.** Stratification by sex and pathogenicity class of variants identified in the analyzed patients. For each category the absolute number of carriers and their percentage frequency on the mutated cohort.*

5.3: COHORT OF PATIENTS INVESTIGATED FOR TUMORS OF THE HBOC SPECTRUM

The analyzed cohort includes 662 patients; among these, 16.77% (111/662 patients) have mutations in the genes analyzed by the BML panel.

Among patients with mutations associated with breast, ovarian and prostate cancer, 87 (78.38%) are females and 24 (21.62%) are males.

Another relevant finding is the frequency of women carrying mutations compared to the total of women tested for breast and ovarian cancer. Overall, 532 women underwent genetic testing and 87 (16.35%) of these were found to have mutations in the genes of interest.

For the male population, 130 individuals underwent genetic testing; of these, 24 (18,46%) carryed variants in the analyzed genes.

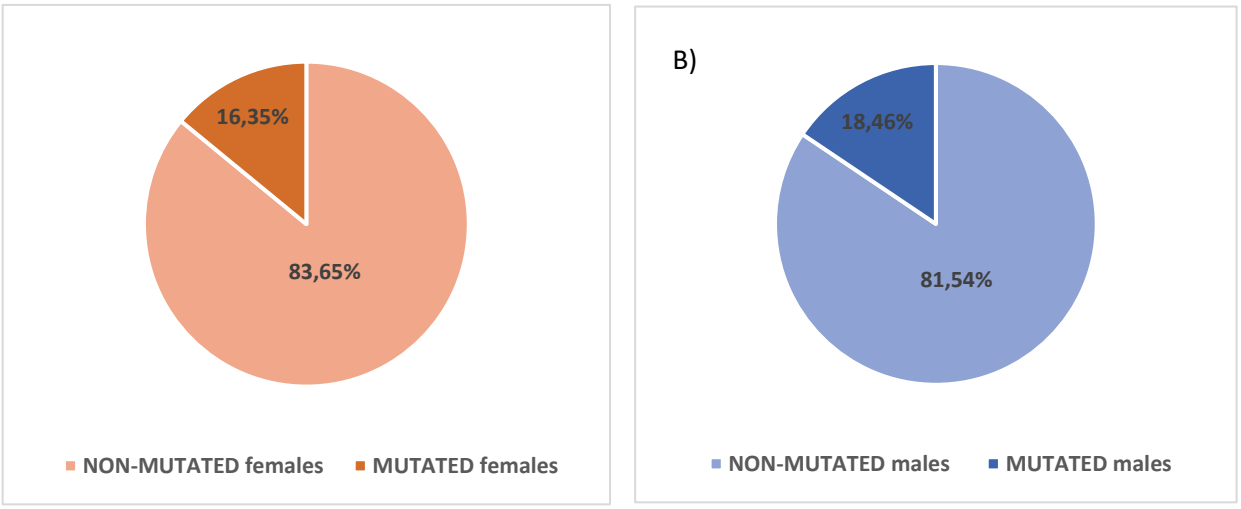

Image 30. *Prevalence of mutated genetic variants in the population under study. (a) Percentage distribution of mutations in genes of interest within the female sample (N = 532). (b) Percentage distribution of mutations within the male sample (N = 130).*

Variants identified by the genetic panel do not exclusively concern canonical genes associated with the HBOC spectrum, but also other genes involved in DNA repair mechanisms.

These variants have been classified based on their pathogenicity into the following categories: Variants of Uncertain Significance (VUS), Likely Pathogenic (LP) and Pathogenic (P).

The following table shows, for each variant class, the number of carrier patients and their frequency, stratified by sex and calculated on the total of analyzed HBOC patients of the respective sex.

Variant class	Sex	N. patients carrying the variant	Frequency
VUS	Females (N=532)	25	4.70%
	Males (N=130)	6	4.62%
Likely Pathogenic	Females (N=532)	22	4.14%
	Males (N=130)	3	2.31%
Pathogenic	Females (N=532)	40	7.52%
	Males (N=130)	15	11.54%

Table 3. *Stratification by sex and pathogenicity class of variants identified in the analyzed patients. For each category the absolute number of carriers and their percentage frequency, calculated on the total of the respective samples (Females = 532; Males = 130).*

The total number of mutations found exceeds that of the analyzed patients, as some individuals are carriers of variants located in several genes.

Patients with P/LP mutations are 80, of which 62 (77.5%) women and 18 (22.5%) men.

Gene	N. patients	Prevalence (variant/662)	Correlation with the patient's tumor phenotype (yes/no)
BRCA1	21	3.17%	Yes
BRCA2	28	4.23%	Yes
PALB2	6	0.91%	Yes
CHEK2	4	0.60%	Yes
ATM	6	0.91%	Yes
BRIP1	1	0.15%	Yes
RAD50	1	0.15%	Yes
RAD51C	1	0.15%	Yes
CDKN2A	3	0.45%	No
MITF	4	0.60%	No
MSH2	3	0.45%	No
MSH6	3	0.45%	No
PMS2	1	0.15%	No

Table 4. *Distribution of pathogenic or likely pathogenic mutations (P/LP) found in the individual genes analyzed. For each gene, the absolute number of carrier patients, the percentage prevalence calculated over the entire cohort (N=662) and the presence or absence of a specific correlation with the patient's tumor phenotype are indicated.*

Each of the genes listed above has different pathogenic or likely pathogenic variants. Some of these occur more frequently within the cohort of mutated patients, while others have been found in a single case.

GENE	VARIANTS	N. patients	P/LP	Frequency (N/662)	gnomAD*
BRCA1	c.4889_4892del (p.T1630Ifs*2)	1	P	/	/
	c.5030_5033del (p.Thr1677Ilefs*2)	2	P	/	/
	c.5251C>T (p.Arg1751*)	1	P	0.0015	0.00001414
	c.798_799del (p.Ser267LysfsTer19)	3	P	/	/
	c.5509T>C (p.Trp1837Arg)	3	P	/	/
	c.5193+2T>G	1	P	/	/
	c.303T>A (p.Y101*)	1	P	/	/
	c.3823A>G (p.I1275V)	1	P	0.0015	0.0001522
	c.1297delG (p.A433Pfs*8)	1	P	/	/
	c.3257T>G (p.Leu1086Ter)	1	P	/	/
	c.514del (p.Gln172AsnfsTer62)	1	P	0.0015	0.000003977
	c.3928dupA (p.Thr1310fs)	1	P	/	/
	c.4484G>T (p.Arg1495Met)	1	P	0.0015	0.000003981
	c.3767_3768del (p.Thr1256ArgfsTerm 10)	1	P	/	/
	c.ex1_2del	1	P	/	/
BRCA2	c.5909C>A (p.Ser1970Ter)	2	P	/	/
	c.7506dupC (p.V2503Rfs*36)	1	LP	/	/
	c.3881T>G (p.Leu1294Ter)	1	P	/	/
	c.1446_1447insCAGT (p.Ala483GlnfsTer32)	1	LP	/	/
	c.8754+4A>G	3	LP	/	/
	c.4695_4698dup (p.Leu1567Aspfs*9)	1	P	/	/
	c.7877G>A (p.Trp2626*)	1	P	/	/
	c.5721dup (p.Leu1908Serfs*3)	1	LP	/	/
	c.8878C>T (p.Gln2960*)	2	P	/	/
	c.5645C>A (p.Ser1882Ter)	1	P	0.0015	0.00001604
	c.5130_5133del (p.Tyr1710_Val1711delinsTer)	1	P	0.0015	0.000004408
	c.4693_4694insGACC (p.Lys1565ArgfsTer11)	1	LP	/	/
	c.7007G>A (p.Arg2336His)	1	P	/	/
	c.7007+5G>A	1	LP	/	/
	c.5410_5411del (p.Val1804LysfsTer2)	1	P	/	/
	c.4478delAAAAG (p.Glu1493fs)	2	P	0.0030	0.00001598
	c.7504dup (p.Arg2502ProfsTer37)	1	LP	/	/
	c.9770_9773del (p.Lys3257fs)	1	LP	0.0015	0.000003978
	c.8486A>G p.(Gln2829Arg)	1	P	/	/
	c.5851_5854del (p.Ser1951TrpfsTerm 11)	1	P	/	/
	c.2808_2811del (p.Ala938ProfsTer21)	1	P	0.0015	0.000007973
	c.7871A>G (p.Tyr2624Cys)	1	P	0.0015	0.000003979
PALB2	c.2285A>G (p.His762ArgfsTer8)	1	P	/	/

	c.ex11dup	1	LP	/	/
	c.509_510delGA (p.Arg170fs)	1	P	0.0015	0.00003579
	c.1451T>G (p.Leu484Ter)	1	P	/	/
	c.ex6del	1	LP	/	/
	c.740_741del (p.Thr247Serfs*9)	1	P	/	/
CHEK2	c.1160C>T (p.Thr387Ile)	1	LP	0.0015	0.000003984
	c.1420C>T (p.Arg474Cys)	1	P	0.0015	0.00001711
	c.190G>A (p.Glu64Lys)	2	LP	0.0030	0.0001591
ATM	c.2501dupA (p.V835Sfs*7)	1	P	0.0015	0.000003978
	c.3746+2T>G	1	LP	/	/
	c.7327C>T (p.Arg2443*)	1	P	0.0015	0.000003983
	c.2413C>T (p.Arg805Ter)	1	P	0.0015	0.00003981
	c.7753A>T (p.Lys2585Ter)	1	P	/	/
	c.1920_1923del (p.Glu641AsnfsTer7)	1	P	/	/
BRIP1	c.1A>G (p.Met1Val)	1	LP	0.0015	0.00001593
RAD50	c.3164+2T>C	1	P	/	/
RAD51C	c.904+5G>T	1	LP	0.0015	0.00001597
CDKN2A	c.301G>T (p.Gly101Trp)	3	P	/	/
MITF	c.1273G>A (p.Glu425Lys)	4	LP	0.0060	0.001365
MSH2	c.2581C>T (p.Gln861*)	2	P	/	/
	c.484G>A (p.Gly162Arg)	1	P	0.0015	0.000003976
MSH6	c.2315G>A (p.Arg772Gln)	1	LP	0.0015	0.00001770
	c.4079del (p.Leu1360TyrfsTer11)	1	LP	/	/
	c.741dupA (p.Arg248Thrfs*8)	1	P	/	/
PMS2	c.2T>C	1	P	0.0015	0.000003996

Table 5: *This table describes all the P/LP variants found in the HBOC spectrum genes and DNA repair mechanisms. The variants of each gene, the number of carriers in the sample and the comparison between the internal frequency of the study (N=662) and the global frequency extracted from the gnomAD database are indicated.*

5.4: COHORT OF PATIENTS INVESTIGATED FOR CUTANEOUS MELANOMA

The analyzed cohort includes 402 patients; among these, 15.67% (63 patients) have mutations in the genes analyzed by the BML panel.

Among patients with mutations related to this type of cancer, 30 (47.61%) are females and 33 (52.38%) males.

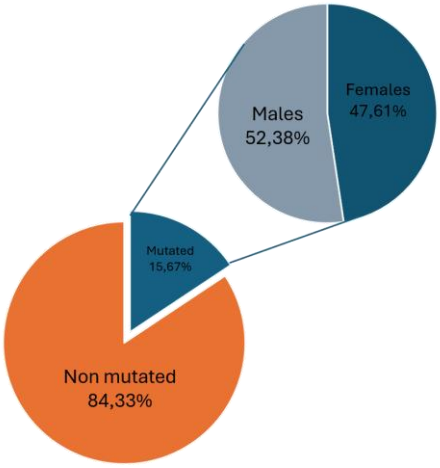

Image 31: *Percentage of mutated and non-mutated patients in the BML panel and relative detail of the distribution by sex within the quota of positive subjects.*

Another relevant data concerns the frequency of women carrying mutations compared to the total of women analyzed for cutaneous melanoma. Overall, 196 women underwent genetic testing and 30 (15.31%) were found to have mutations in the genes of interest.

As regards the male population, 206 men underwent genetic testing; of these, 33 (16.02%) showed mutations in the analyzed genes.

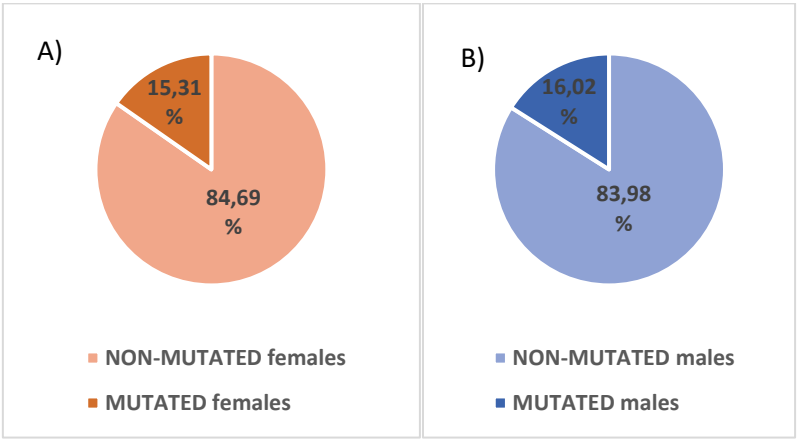

Image 32: *Prevalence of mutated genetic variants in the population under study. (a) Percentage distribution of mutations in genes of interest within the female sample (N = 196). (b) Percentage distribution of mutations within the male sample (N = 206).*

Variants identified through the genetic panel are not exclusively located in canonical genes associated with cutaneous melanoma, but also affect other genes defined as 'non-canonical'.

These variants have been classified by level of pathogenicity, which include: Variants of Uncertain Significance (VUS), Likely Pathogenic (LP), and Pathogenic (P).

The table below shows, for each class, the distribution of patients by sex, distinguishing the number of males and females.

Variant class	Sex	N. patients carrying the variant	Frequency
VUS	Females (N=196)	6	3.06%
	Males(N=206)	7	3.40%
Likely Pathogenic	Females (N=196)	4	2.04%
	Males(N=206)	9	4.37%
Pathogenic	Females (N=196)	20	10.20%
	Males(N=206)	17	8.25%

Table 6. *Stratification by sex and pathogenicity class of variants identified in the analyzed patients. For each category the absolute number of carriers and their percentage frequency, calculated on the total of the respective samples (females = 196; Males = 206).*

Patients with P/LP mutations are 50, of which 24 (48%) women and 26 (52%) males.

Gene	N. patients	Prevalence (variant/402)	Correlation with the patient's tumor phenotype (yes/no)
CDKN2A	31	7.71%	Yes
CDK4	0	0%	Yes
BAP1	0	0%	Yes
MITF	4	0.99%	Yes
ATM	1	0.25%	No
BRCA1	2	0.50%	No
BRCA2	0	0%	No
BRIP1	2	0.50%	No
CHEK2	7	1.74%	No
MC1R	0	0%	No
MLH1	1	0.25%	No
PMS2	1	0.25%	No
POT1	1	0.25%	No
TERT	0	0%	No

Table 7. *Distribution of pathogenic or likely pathogenic mutations (P/LP) found in the individual genes analyzed. For each gene, the absolute number of carrier patients, the percentage prevalence calculated over the entire cohort (N=402) and the presence or absence of a specific correlation with the patient's tumor phenotype are indicated.*

Each gene listed above has different pathogenic variants or likely pathogenic variants. Some variants were recurrent within the cohort, whereas others were identified in a single patient.

GENE	VARIANTS	N. patients	P/LP	Frequency (N/402)	gnomAD*
CDKN2A	c.301G>T (p.G101W)	26	P	/	/
	c.79G>T (p.E27*)	1	P	/	/
	c.199G>T (p.G67C)	2	LP	/	/
	c.71G>C (p.R24P)	1	LP	0.0024	0.00001690
	c.377T>A (p.V126D)	1	LP	/	/
MITF	c.1273G>A (p.E425K)	4	LP	0.00995	0.001365
ATM	c.3511C>T (p.Q1171*)	1	P	/	/
BRCA1	c.5030_5033del (p.T1677Ifs*2)	1	P	/	/
	c.5509T>C (p.W1837R)	1	P	/	/
BRIP1	c.2392C>T (p.R798X)	1	P	0.0024	0.0001581
	c.751C>T (p.R251C)	1	LP	0.0024	0,00001194
CHEK2	c.1427C>T (p.T476M)	2	P	0.0049	0.0003130
	c.1175C>T (p.A392V)	1	LP	0.0024	0.00003585
	c.1141A>G p.(M381V)	1	LP	0.0024	0.00003542
	c.190G>A (p.E64K)	1	LP	0.0024	0.0001591
	c.1100del p.(Thr367MetfsTer15)	1	P	0.0024	0.002108
	c.85C>T (p.Q29T*)	1	P	0.0024	0.000008018
MLH1	c.676C>T (p.Arg226*)	1	P	0.0024	0.000003984
PMS2	c.137G>T (p.S46I)	1	P	0.0024	0.0001732
POT1	c.259_260insT (p.Q87LfsTerm 4)	1	LP	/	/

Table 8: *The table describes all the P/LP variants found in cutaneous melanoma genes and DNA repair mechanisms. The variants of each gene, the number of carriers in the sample and the comparison between the internal frequency of the study (N=402) and the global frequency extracted from the gnomAD database are indicated.*

Among the non-canonical genes included in the study, *CHEK2* showed the highest number of detected variants. To estimate the overall contribution of these alterations, the cumulative frequency of pathogenic and likely pathogenic *CHEK2* variants was calculated separately in the study cohort and in the reference population.

In the study group, 7 patients carried a pathogenic or likely pathogenic variant in *CHEK2*, corresponding to an internal frequency of 1.7% (0.017). In contrast, the cumulative frequency of the same variants in the gnomAD population database was 0.27% (0.00266). This difference suggests an enrichment of pathogenic *CHEK2* variants in the analyzed cohort compared with the general population, supporting the potential relevance of *CHEK2* in the broader genetic landscape of melanoma susceptibility and hereditary cancer predisposition.

In the study cohort, 7 out of 402 patients carried a pathogenic or likely pathogenic variant in *CHEK2*, corresponding to a frequency of 1.74%. This frequency was markedly higher than the cumulative frequency of the same variants in gnomAD, estimated at 0.27% among approximately 120,000 individuals. Overall, this corresponds to an approximately 6.45-fold enrichment in the study cohort. Fisher's exact test confirmed that this difference was statistically significant, $p \approx 0.00014$. Although this enrichment was statistically significant, the comparison with gnomAD should be interpreted cautiously, as differences in cohort selection, ancestry background, sequencing methods, and variant classification criteria may influence frequency estimates

Gene	N. patients	Cumulative frequency inside the cohort	Cumulative frequency gnomAD
CHEK2	7	0.017 (1.7%)	0,00266 (0,27%)

Table 9: *Comparison of the cumulative frequency of CHEK2 variants in the cohort under study (n=7) and gnomAD population database.*

5.5: VARIANTS OF UNCERTAIN SIGNIFICANCE (VUS) IN CUTANEOUS MELANOMA AND BREAST CANCER

Genomic analysis detected uncertain significance variants (VUS) in both tumor phenotypes, localized in both canonical and non-canonical genes.

Overall, 44 patients carrying VUS were identified, of which 31 related to the HBOC spectrum (70.45%) and 13 to cutaneous melanoma (29.55%).

In HBOC tumors, 33 VUS variants have been described (28 in canonical genes and 5 in non-canonical genes), while in cutaneous melanoma 14 variants were found, of which 6 in canonical genes and 8 in non-canonical genes.

Tumor phenotype	Gene	Variant	Frequency	gnomAD
Breast cancer (N=662)	*BRCA1*	c.5129G>A (p.G1710E)	/	/
		c.1333G>C (p.E445Q)	0.0015	0.00002790
	BRCA2	c.6665A>G (p.Y2222C)	0.0015	0.00001595
		c.1064T>C (p.Val355Ala)	/	/
		c.6332A>C (p.K2111T)	/	/
		c.6431A>G (p.E2144G)	0.0015	0.000004330
		c.6323G>C (p.R2108P)	/	/
		c.7505G>A (p.R2502H)	0.0015	0.00006364
		c.5345A>C (p.Q1782P)	0.0015	0.000003991
		c.7559G>C (p.R2520P)	/	/
		c.5663A>G (p.K1888R)	0.0015	0.00001202

		c.3509C>T (p.A1170V)	0.0015	0.00001595
		c.9118-17del	/	/
		c.7628A>G (p.Y2543C)	0.0015	0.00003189
		c.6770C>G (p.P2257R)	/	/
	PALB2	c.2379C>T (p.G793G)	0.0015	0.00008132
		c.3089C>A (p.T1030N)	/	/
		c.2767G>T (p.V923F)	0.0015	0.000003993
		c.3350G>A (p.R1117K)	/	/
		c.3296C>T (p.T1099M)	0.0015	0.00001193
		c.3451C>T (p.L1151F)	0.0015	0.000007953
		c.769G>A (p.G257S)	0.0015	0.00001193
	CHEK2	c.727T>C (p.F243L)	0.0015	0.00002122
		c.428T>C (p.I143T)	0.0015	0.00002388
		c.444+3A>G	0.0015	0.000007959
	ATM	c.4952T>C (p.L1651S)	/	/
		c.1235+4_1235+5del	0.0015	0.000003984
	BRIP1	c.861G>C (p.E287D)	/	/
	MLH1	c.2084C>G (p.S695W)	0.0015	0.00001594
		c.1154G>A (p.R385H)	0.0015	0.00005308
	MSH2	c.366+3A>T	/	/
	MSH6	c.3518T>C (p.V1173A)	/	/
	RAD51C	c.428A>G (p.Q143R)	0.0015	0.00003579
Cutaneous melanoma (N=402)	*CDKN2A*	c.127A>G (p.S43G)	0.0025	0.000008113
		c.142C>A (p.P48T)	/	/
	CDK4	c.458C>T (p.T153I)	/	/
	BAP1	c.1139G>A (p.G380D)	/	/
	MITF	c.1252G>A (p.E418K)	/	/
		c.354+4C>A	0.0025	0.00002015
	ATM	c.8147T>C (p.V2716A)	0.0025	0.00003183
	BRCA2	c.572A>T (p.D191V)	0.0025	0.000003978
		c.8186A>G (p.K2729R)	0.0025	0.000007977
	MC1R	c.916C>T (p.R306C)	0.0025	0.00002496
		c.83_84insA (p.N29QfsTer14)	/	/
		c.487C>T p.R163*	0.0025	0.00002961
	MSH6	c.3844_3845insTAT (p.T1282delinsIS)	/	/
	TERT	c.556C>T (p.P186S)	/	/

Table 10. *Mutational spectrum and frequencies of the uncertain significance variants (VUS) identified in the cohorts of breast carcinoma (HBOC, N=662) and cutaneous melanoma (N=402). For each variant, the molecular nomenclature (cDNA and protein), the internal allelic frequency observed in the study and the population reference value extracted from the gnomAD database are reported. The symbol (/) indicates the absence of the variant in public databases or data not available.*

DISCUSSION AND CONCLUSIONS

The present study aimed to evaluate the presence of genotype-phenotype discrepancies in patients undergoing genetic testing for suspected hereditary predisposition to breast/ovarian cancer (HBOC) or cutaneous melanoma. Unlike the traditional approach, the analysis was extended to all genes included in the BML panel, regardless of the initial clinical indication, to evaluate the diagnostic contribution of using an extended multigenic panel.

Analysis of 1064 patients showed that 12.22% of subjects carried at least one pathogenic or probably pathogenic variant. As expected, in the HBOC cohort the variants were predominantly localized in the BRCA1, BRCA2, PALB2, ATM and CHEK2 genes, while in the melanoma patient cohort the gene most involved was CDKN2A, followed by MITF. These results are fully consistent with current knowledge on the main susceptibility genes associated with the two tumor phenotypes.

The most relevant aspect that emerged from the present work, however, concerns the identification of pathogenic or probably pathogenic variants in genes not traditionally associated with the tumor phenotype for which the patient had been referred for genetic testing. In particular, mutations in the CDKN2A and MITF genes, typically related to melanoma predisposition, as well as in the mismatch repair system genes (MSH2, MSH6, and PMS2) were identified in the HBOC cohort. In parallel, variants in the BRCA1, CHEK2, ATM, and BRIP1 genes were found in the melanoma cohort, generally associated with hereditary predisposition to breast cancer. These findings demonstrate that genetic predisposition to cancer cannot be interpreted solely through an association between gene and phenotype, but reflects the sharing of numerous biological mechanisms involved in DNA repair, maintaining genomic stability, and cell cycle control.

In agreement with what has been reported in the literature, the CDKN2A gene was found to be the main susceptibility gene in the cohort of patients with cutaneous melanoma, representing over 60% of pathogenic and probably pathogenic variants. This data confirms the central role of this gene in the hereditary predisposition to melanoma. In parallel, the identification of variants in genes not traditionally associated with melanoma, such as BRCA1, CHEK2, ATM, and BRIP1, highlights how the use of extensive multigenic panels allows us to identify clinically relevant alterations that would not have emerged through an approach limited to canonical genes alone.

The analysis of *CHEK2* variants revealed a significant enrichment of pathogenic and likely pathogenic alterations in the study cohort compared with the reference population. Specifically,

7 out of 402 patients carried a P/LP variant in *CHEK2*, corresponding to a frequency of 1.74%, whereas the cumulative frequency of the same variants in gnomAD was 0.27%. This represents an approximately 6.45-fold enrichment, with Fisher's exact test confirming a statistically significant difference between the two groups.

This finding is noteworthy because *CHEK2* is not traditionally considered a classical high-penetrance melanoma susceptibility gene, unlike *CDKN2A*. However, *CHEK2* is a well-established cancer predisposition gene involved in the DNA damage response, and its association with a broader tumor spectrum has increasingly emerged through multigene panel testing. The observed enrichment in our cohort may therefore support the hypothesis that *CHEK2* contributes to melanoma susceptibility, at least in a subset of patients, particularly in the context of broader hereditary cancer predisposition.

From a biological perspective, this association is plausible. *CHEK2* encodes checkpoint kinase 2, which plays a central role in detecting DNA damage and activating cell cycle arrest, DNA repair, or apoptosis. Since ultraviolet radiation is a major mutagenic factor in melanoma and induces DNA damage in melanocytes, impaired checkpoint activation due to pathogenic *CHEK2* variants may facilitate the accumulation of genomic alterations and contribute to melanoma development. In this context, *CHEK2* variants may not act as strong monogenic drivers, but rather as moderate-risk alleles or genetic modifiers that increase susceptibility in combination with environmental exposure, family history, or additional inherited variants.

The statistically significant enrichment observed in our cohort also supports the clinical utility of including *CHEK2* in expanded multigene panels for patients referred for melanoma predisposition assessment. Although the identification of a *CHEK2* variant should not 2be interpreted in the same way as a pathogenic variant in *CDKN2A*, it may still provide clinically relevant information, particularly when associated with a personal or family history of breast, colorectal, prostate, thyroid, or other *CHEK2*-related malignancies. Therefore, these findings highlight the importance of interpreting *CHEK2* results within a broader oncological and familial context.

Nevertheless, this result should be interpreted with caution. The comparison with gnomAD is informative but not equivalent to a matched case-control analysis. Differences in ancestry composition, cohort selection, sequencing technologies, variant calling pipelines, coverage of specific loci, and variant classification criteria may influence frequency estimates. In addition, gnomAD represents a population reference database and is not specifically designed as a

cancer-free control cohort. Therefore, while the observed enrichment is statistically significant, it should be considered as evidence of overrepresentation rather than a direct estimate of melanoma risk.

Overall, the increased frequency of P/LP *CHEK2* variants in the study cohort suggests that this gene may contribute to the genetic background of melanoma susceptibility, particularly within the context of multigene hereditary cancer predisposition. Further studies in larger, ancestry-matched melanoma cohorts, ideally including segregation analysis, detailed family history, and comparison with matched controls, are needed to clarify the magnitude of melanoma risk associated with *CHEK2* variants and to define their clinical management implications.ù

From a clinical point of view, these observations take on important relevance. Limiting genetic analysis to genes classically associated with the diagnostic suspicion could in fact lead to the failure to identify clinically significant variants, with important repercussions on the management of the patient and his or her family members. Identifying an unexpected pathogenic variant can modify clinical surveillance pathways, guide personalized prevention strategies, influence potential treatment choices, and allow genetic testing to be extended to at-risk family members through cascade testing.

The use of multigenic panels, however, also entails some limitations. Simultaneous analysis of numerous genes inevitably increases the number of variants of uncertain significance (VUS), the interpretation of which still represents a limitation. These variants in fact require a continuous reevaluation of the patient's clinical picture.

Overall, the results obtained confirm the clinical value of extended multigenic panels compared to a strictly phenotype-driven approach. The identification of unexpected genotype-phenotype associations demonstrates how tumor syndromes share common molecular bases and suggests that a broader diagnostic approach may improve the identification of at-risk individuals, allowing for a more comprehensive assessment of genetic predisposition to cancer.

Therefore, the integration of multigenic panels into clinical practice represents a fundamental tool for promoting increasingly personalized precision medicine, improving not only the diagnosis of hereditary syndromes, but also prevention and clinical surveillance.

APPENDIX

GENE ACRONYM	*FULL NAME*	*OMIM ID*	*BIOLOGICAL FUNCTION*
HER-2	Human Epidermal Growth Factor Receptor 2	164870	Receptor tyrosine kinase involved in cell growth and proliferation signaling
BRCA1	Breast Cancer 1	113705	DNA repair tumor suppressor involved in homologous recombination and maintenance of genomic stability
BRCA2	Breast Cancer 2	600185	DNA repair protein involved in homologous recombination and maintenance of genomic stability
PTEN	Phosphatase and Tensin Homolog	601728	Tumor suppressor phosphatase that negatively regulates the PI3K/AKT signaling pathway
TP53	Tumor Protein p53	191170	Tumor suppressor protein involved in DNA damage response, cell cycle arrest, and apoptosis
CHEK2	Checkpoint Kinase 2	604373	Serine/threonine kinase involved in DNA damage checkpoint signaling and cell cycle arrest
STK11	Serine/Threonine Kinase 11	607707	Serine/threonine kinase involved in regulating cell polarity, energy metabolism, and tumor suppression
ATM	Ataxia Telangiectasia Mutated	607585	Serine/threonine protein kinase involved in DNA double-strand break repair and activation of the DNA damage response
CDH1	Cadherin 1	192090	Cell-cell adhesion glycoprotein important for

			epithelial cell integrity and suppression of invasion/metastasis
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1	600982	Serine/threonine kinase involved in MAPK signaling pathways regulating cell proliferation, differentiation, and apoptosis
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	600160	Tumor suppressor that regulates the cell cycle by inhibiting CDK4/6 and stabilizing p53 pathway activity
CDK4	Cyclin Dependent Kinase 4	603629	Cyclin-dependent kinase that regulates cell cycle progression from G1 to S phase by phosphorylating RB protein
BAP1	BRCA1 Associated Protein 1	603089	Deubiquitinating enzyme that regulates chromatin dynamics, DNA repair, and tumor suppression pathways
MITF	Microphthalmia-Associated Transcription Factor	156845	Transcription factor that regulates melanocyte development, pigment production, and cell differentiation
BRAF	B-Raf Proto-Oncogene	164757	Serine/threonine kinase that mediates MAPK/ERK signaling to regulate cell growth, differentiation, and survival
MEK	Mitogen-Activated Protein Kinase Kinase (MAP2K)	176872	Dual-specificity kinase (MAP2K) that activates ERK in the MAPK signaling pathway regulating

			cell proliferation and survival
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4	123880	Immune checkpoint receptor that inhibits T-cell activation and downregulates immune responses
PD-1	Programmed Cell Death Protein 1	600244	Immune checkpoint receptor that inhibits T-cell activation and regulates immune tolerance
PD-L1	Programmed Death-Ligand 1	605402	Immune checkpoint ligand that binds PD-1 to suppress T-cell activation and modulate immune responses
MLH1	MutL Homolog 1	120436	DNA mismatch repair protein that maintains genomic stability by correcting replication errors
MSH2	MutS Homolog 2	609309	DNA mismatch repair protein that forms a complex with MSH6 to recognize and repair base-base mismatches and insertion-deletion loops
MSH6	MutS Homolog 6	600678	DNA mismatch repair protein that partners with MSH2 to recognize and initiate repair of replication errors
PMS2	Postmeiotic Segregation Increased 2	600259	DNA mismatch repair endonuclease involved in correcting replication errors and maintaining genomic stability
EPCAM	Epithelial cell adhesion molecule	185535	Transmembrane epithelial glycoprotein that mediates calcium-

			independent cell–cell adhesion and also participates in epithelial signaling, proliferation, differentiation, and barrier maintenance.
BARD1	BRCA1 associated RING domain 1	601593	BARD1 forms a heterodimer with BRCA1 and functions as an E3 ubiquitin ligase involved in DNA damage repair, cell-cycle checkpoint control, and maintenance of genomic stability
BRIP1	BRCA1 interacting helicase 1	605882	BRIP1 is a DNA-dependent ATPase/helicase that interacts with BRCA1 and participates in homologous recombination and the Fanconi anemia DNA interstrand crosslink repair pathway, helping maintain genomic stability.
RAD51C/D	RAD51 paralog C/D	602774/ 602954	homologous recombination DNA repair proteinS that help repair double-strand DNA breaks, promote RAD51 filament function, and contribute to genome stability
NBN	Nibrin	602667	NBN encodes a core component of the MRN complex that senses DNA double-strand breaks, promotes DNA damage signaling and checkpoint activation, and

	supports homologous recombination and genome stability

TABLE 11*: Summary list of the genes mentioned in the paper, specifying the abbreviation, full name, gene OMIM ID, and their respective biological function.*

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