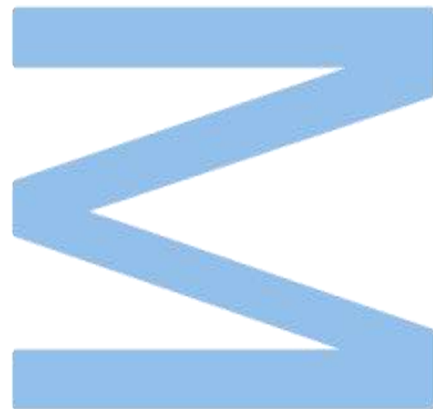
Study of genetic alterations in cutaneous melanoma



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“One day, in retrospect, the years of struggle will strike you as the most beautiful.”

— Sigmund Freud

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Resumo

O melanoma cutâneo (CM) é a forma mais letal de cancro da pele, devido a apresentar grande propensão para metastizar e à resistência às terapias em estadios avançados. O CM tem uma das maiores taxas de mutações de todos os tipos de cancro, maioritariamente adquiridas ao acaso, causadas pela exposição aos raios UV. O BRAF é o gene mais frequentemente mutado no CM (~50%), causando a sobre-activação da via de sinalização MAPK, sendo um alvo e o único biomarcador de resposta ao tratamento utilizado na prática clínica. Mutações no gene NRAS, que também levam à sobre-activação da via MAPK, representam 15-20% das mutações do CM, mas são menos recetivas às opções de tratamento. Além disso, mutações no promotor do gene da transcriptase reversa da telomerase (*TERTp*), que sintetiza o DNA telomérico, responsável pela manutenção do comprimento dos telómeros, já foram descritas como associadas a pior prognóstico dos pacientes com CM. O objetivo principal deste projeto é estudar as alterações genéticas encontradas em pacientes com melanoma e correlacioná-las com os dados clínico-patológicos. A análise molecular foi realizada em tumores primários, recidivas, gânglios sentinela e metástases recolhidos no Hospital Distrital de Santarém e Hospital dos Capuchos. Os casos foram reavaliados e organizados de acordo com as diretrizes da AJCC. O DNA foi extraído, amplificado por PCR e sequenciado pelo método de Sanger. A mutação *BRAF* foi observada em 36% dos 120 melanomas, sendo a mais prevalente a alteração V600E T>A, com 36% dos casos mutados. Como esperado, a alteração encontrada em menor frequência foi a mutação *NRAS*, encontrada em 18% dos 125 melanomas cutâneos, e destes, 17% eram Q61R A>G. A relação entre o estado mutacional dos genes e os parâmetros clínico-patológicos foi avaliada. As mutações do promotor *TERT* foram detetadas em 30% dos 99 melanomas cutâneos, sendo a alteração mais prevalente -124 G>A, representando 11% dos casos mutados. Os nossos resultados sugerem que alterações nos genes estudados desempenham um papel importante na biopatologia do melanoma. No nosso trabalho, a presença de mutações no gene *NRAS* parecem ter uma associação a caraterísticas de melhor prognostico. Em contrapartida, a presença de mutações no promotor da *TERT* parecem associar-se a menor sobrevivência de pacientes com melanoma cutâneo. Conhecer as associações entre o estado molecular dos genes e as características clínico-patológicas é fundamental, pois podem auxiliar na estratificação de risco dos pacientes e, consequentemente, no manejo da doença.

Palavras-chave: Melanoma, promotor da *TERT, BRAF, NRAS,* via MAPK.

Abstract

Cutaneous melanoma (CM) is the most lethal form of skin cancer, due to the high propensity to metastasize and the resistance to therapies in advanced stages. CM has one of the highest mutation rates from all types of cancers, mostly acquired by chance, caused by UV exposure. BRAF is the most frequently mutated gene found in CM (~50%), causing the upregulation of the mitogen-activated protein kinase (MAPK) pathway, being a target and the only biomarker of response to treatment used in the clinical practice. Mutations in the NRAS gene, which also leads to the upregulation of the MAPK pathway, represents 15-20% of CM mutations, but are less receptive to treatment options. Additionally, mutations in the promoter of the telomerase reverse transcriptase (*TERTp)* gene, that synthesizes telomeric DNA, responsible for maintaining telomere length, were already described as being associate with worse CM patients' prognosis. The main objective of this project is to study frequent genetic alterations found in melanoma patients and to correlate them with the clinic-pathological data. The molecular analysis was performed in primary tumors, recurrences, sentinel nodes and metastases collected at Hospital Distrital de Santarém and Hospital dos Capuchos. The cases were re-evaluated and staged according to AJCC guidelines. The DNA was extracted, submitted to PCR and sequenced using the Sanger method. The hotspots of the three genes were analyzed. The relationship between the mutational status of the genes and the clinicopathological parameters was evaluated. The *BRAF* mutation was observed in 36% of the 120 melanomas, the most prevalent being the V600E T>A alteration, in 36% of the mutated cases. As expected, the alteration with the lowest frequency was NRAS mutation, found in 18% of the 125 cutaneous melanomas, and of those, 17% harbor the Q61R A>G alteration. *TERT* promoter mutations were detected in 30% of the 99 cutaneous melanomas, and the most prevalent alteration was -124 G>A, representing 11 % of the mutated cases. Our results suggest that alterations in the genes studied play an important role in the biopathology of melanoma. In our work, the presence of *NRAS* mutations seem to associate with features of better prognosis. In contrast the presence of mutations in the *TERT* promoter seem to be associated with lower survival of cutaneous melanoma patients. The association of molecular status of the genes and clinical-pathological characteristics are fundamental, since they may help in the risk stratification of patients and, consequently, in the management of the disease.

Keywords: Melanoma, *TERT* promoter, *BRAF, NRAS,* via MAPK

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List of Abbreviations

A	Adenine
ACD	Shelterin Complex Subunit and Telomerase Recruitment Factor
AJCC	American Joint Committee on Cancer
ALM	Acral Lentiginous Melanoma
BAP1	Breast Cancer 1 Associated Protein 1
BCC	Basal Cell Carcinomas
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B1
C	Cytosine
CAD	Computer-Aided Diagnosis
CCND1	Cyclin D1
CDK4	Cyclin Dependent Kinase 4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2
CFC	Cardiofaciocutaneous Syndrome
CM	Cutaneous Melanoma
c-MET	Mesenchymal-Epithelial Transition Factor
CsA	Cyclosporine
CT	Chemotherapy
CTLA-4	Cytotoxic T Lymphocyte-Associated Antigen 4
ddNTPS	Dideoxynucleotides Triphosphates
DNA	Deoxyribonucleic Acid
dNTPS	Deoxyribonucleotide Triphosphate
ERCC	Excision Repair Cross Complementation
ERK	Extracellular Regulated MAP Kinase
FAMM	Familial Atypical Multiple Mole Melanoma
FGFR1	Fibroblast Growth Factor Receptor 1
G	Guanine
GDP	Guanosine 5′-Diphosphate
HC	Hospital Capuchos
HDS	Hospital Distrital de Santarém
HE	Haematoxylin – Eosin Staining
HIV	Human Immunodeficiency Virus
HR	Hazard Ratio

HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
hTERT	Human Telomerase Reverse Transcriptase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LB	Loading Buffer
LMM	Lentigo Maligna Melanoma
M1	Replicative Senescence Phase
M2	Crisis or Death Phase
MAPK	Mitogen-Activated Protein Kinase
MC1R	Melanocortin 1 Receptor
MEK	Mitogen-activated protein kinase kinase
MIC-1	Macrophage Inhibitory Cytokine-1
MITF	Microphthalmia-Associated Transcription Factor
NM	Nodular Melanoma
NMSC	Non-Melanoma Skin Cancer
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
P14ARF	ARF Tumor Suppressor
p16	Multiple Tumor Suppressor 1
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
POT1	Protection of Telomeres Protein 1
PTEN	Phosphatase and Tensing Homologue
RAF	Proto-Oncogene Serine/Threonine-Protein Kinase
RAS	Rat Sarcoma Viral Oncogenes Homolog
SCC	Squamous Cell Carcinoma
SF3B1	Splicing Factor 3B Subunit 1
SSM	Superficial Spreading Melanoma
T	Thymine
TERTp	Telomerase Reverse Transcriptase Promotor
TGF-BETA	Transforming Growth Factor Beta
TRF1	Telomere Repeat Factor 1
TRF2	Telomere Repeat Factor 2
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
VEGF	Vascular Endothelial Growth Factor

WT	Wild -Type
XP	Xeroderma Pigmentosum

1- Introduction

1.1- Skin cancer

The skin is constituted by the dermis and the epidermis. The most superficial layer of the skin, the epidermis, guarantees an impermeable barrier function [1]. It contains structural cells that represent about 90% of the layer, called keratinocytes, and also melanocytes, cells responsible for melanogenesis and melanin pigment synthesis [1]. Underlying the epidermis is the dermis, which provides structure and nutrients to the epidermis. It includes hair follicles and sweat glands. Human skin pigmentation is directly determined by the production of melanin, a pigment with a high molecular weight and with the main function of photoprotection [1, 2]. Melanin is divided into two forms, eumelanin, the black/brown pigment, and pheomelanin, the yellow/red pigment [3]. Eumelanin is a pigment that absorbs and disperses ultraviolet (UV) light, attenuates penetration and reduces harmful effects. Thus, individuals with high pigmentation tend to be more tanned and in turn have fewer sun burners than individuals with lighter skin color [4, 5]. On the other hand, pheomelanin produce high amounts of free radicals in response to UV exposure, capable of causing damage to the DNA, which may contribute to the phototoxic effects of UV radiation. Therefore, people with fair skin, with a lower amount of pheomelanin, have a higher risk of neoplasms, caused by excessive sun exposure [4].

Skin cancer is among the most common types of cancer worldwide and its incidence has increased over the last few years. It results from a combination of genetic and environmental factors [6]. Skin cancer can be divided in cancers derived from melanocytes (melanoma) and cancers derived from keratinocytes (non-melanoma skin cancer), being common in Caucasians. Regarding non-melanoma skin cancer (NMSC), basal cell carcinomas (BCC) are the most common form, usually have a slow growth and are locally invasive. Squamous cell carcinomas (SCC) are the second most common NMSC form, representing approximately 20%-30% of the cases [6, 7]. They are less aggressive than melanoma, especially if detected at an early stage [8, 9]. NMSCs account for about 95% of all skin cancers.

Over the last years, skin cancer has been considered an important public health problem, due to its high frequency and prevalence. Generally, incidence rates are higher in women than in men before the age of 50, although it is increasing in men, largely

reflecting differences in occupational habits and recreational exposure to ultraviolet (UV) radiation, as well as the excessive use of artificial tanners by young women [6].

1.2- Cutaneous melanoma

The most aggressive skin cancer form, cutaneous melanoma (CM), arises from epidermal melanocytes. Although it represents only 5% of all skin cancers, melanoma is the deadliest type [10, 11]. It is a highly heterogeneous tumor, containing several genetic and molecular alterations. It has a low overall survival and, therefore, new forms of treatment are necessary [12]. Several factors are associated with the risk of CM progression, such as family history of melanoma, prototypical fair skin, excessive sun exposure and/or increase incidence of sunburn, large number of common acquired nevi and occurrence of atypical nevi [11]. Most of the alterations that are implicit in the genetic basis of melanoma occur by mutations acquired by chance. However, the existence of hereditary germline variants can be considered a crucial factor in the sustainability of melanoma [13].

Benign nevi are neoplasms formed by melanocytes that appear on the skin, including nails, palms, and soles of the feet. They have a size of less than 6 mm, are symmetrical, with edges and homogeneous color. Its color may vary between individuals, depending on the nature of skin pigmentation [14]. On the other hand, dysplastic nevi are less frequent, and present an intermediate state between a benign nevus and a malignant melanoma [15], it is considered a pre-malignant lesion, which may arise from a pre-existing nevus or at a new location in the body, it is usually larger than 5 mm, with irregular borders and heterogeneous coloration [14]. Although melanocytes have a low proliferation potential [16, 17], these types of cells can develop the ability to proliferate horizontally, leading to radial growth in the epidermis, and due to the biochemical changes, that occur in malignant melanocytes, loss of E-cadherin expression may have an effect on cells, causing them to grow vertically, starting with an invasion of the dermis [18]. Over time, due to the continuous propagation, the cells can reach the lymph nodes and other organs, such as the brain, lung and breast [19].

Tumor progression is a fundamental clinical concept, as it provides an information base for early diagnosis. According to Foulds, at an early stage, a tumor is not usually malignant, but rather a benign tumor with a papilloma [20]. Over time, benign lesions can progress and acquire morphological and behavioral changes, such as increased size,

atypical macroscopic and microscopic morphology, and ability to metastasize. This can potentiate new proliferation patterns, turning an initial benign lesion into a malignant lesion [21]. In 1984's, Wallace H.Clark defined a 5-phase model of melanocytic nevi progression [22] (Figure 1). Starting with the benign melanocytic nevus, passing sequentially through dysplastic melanocytic nevus, melanoma in radial growth phase, and melanoma in vertical growth phase, until reaching the metastatic melanoma. Clark's model is based on the concept that melanocytic nevi and dysplastic melanocytic nevi are precursors of melanoma [23].

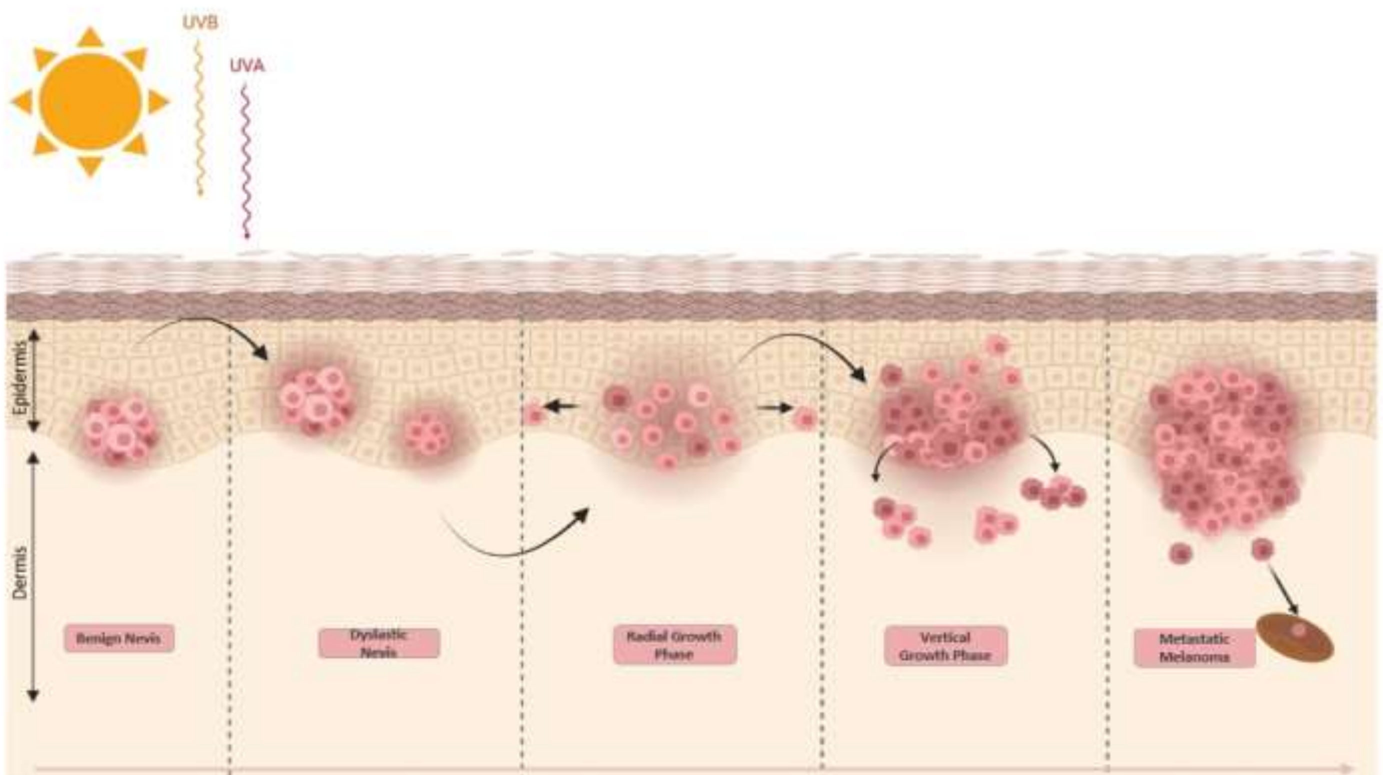


Figure 1 - Melanoma progression based on the Clark model. Melanocytes go through several changes for the transformation into melanoma. Melanocytes can initiate the formation of benign nevi, which transform into dysplastic nevi. In the growth phase, melanocytes progress and proliferate. In the last stage, metastatic melanoma spreads the malignant melanocytes in the vascular and lymphatic system to the lymph nodes, skin, subcutaneous soft tissues, lungs, and brain. Created in: BioRender.com

There are four main histological subtypes of melanoma: superficial spreading melanoma; nodular melanoma; lentigo maligna melanoma and acral lentiginous melanoma.

The most common subtype of cutaneous melanoma is superficial spreading melanoma, comprising about 70% of all primary melanomas in Caucasians [24]. It appears mostly between 40 and 50 years of age, and can be located anywhere in the body, but more frequently on the trunk in men and on the lower extremities in women, where the skin is more exposed to solar radiation [24]. It is a slow-growing and radial melanoma, with varied pigmentation and irregular contours, appearing mostly de novo, despite sometimes originating from pre-existing nevi [25]. The number and dimensions of pre-existing nevi are proportional to the risk of tumor development [25].

Nodular melanoma is the second most common type and encompasses about 10-15% of all cases. Has a rapid vertical growth, with a higher incidence at 50 years of age and predominant in men, it is preferentially located on the trunk, head and neck [26]. It develops mainly on skin with no history of pre-existing nevi. Nodular melanomas are frequently ulcerated and diagnosed as vascular neoplasms or basal cell carcinomas when amelanotic [26].

Lentigo maligna melanoma represent only 5-10% of all melanomas [24]. These melanomas develop from very slow growing colored patches of skin called lentigo maligna or Hutchinson's melanotic freckle [27]. The vast majority appears in patients with more advanced age, over 80 years old, and is located in areas of high sun exposure, such as the face and neck, thus being associated with chronic sun exposure [24]. It is considered a slow-growing lesion, taking decades to invade the papillary dermis. Chronic sun damage and, consequently, skin marked with solar elastosis constitute indicators of the higher risk for the development of this subtype of melanoma [27].

Representing only 2-8% of cases, acral lentiginous melanoma is characterized by a low percentage when compared to the other melanoma subtypes. Only 5 to 10 out of 100 people are diagnosed with this lesion, however, it is the most common melanoma in dark-skinned individuals. It is located on the glabrous skin and adjacent skin of the fingers, palmar and plantar surfaces [8]. It is a slow-growing tumor with varied pigmentation [27].

1.2.1- Cutaneous melanoma- epidemiology

According to GLOBOCAN, cutaneous melanoma accounts for approximately 1.7% of global cancer diagnoses, with an estimated 325,000 new cases in 2020 (Figure 2). The age-specific incidence is 3.0/100,000 for women and 3.8/100,000 for men, with a cumulative risk over the years of 0.33% and 0.42%, respectively. Non-melanoma skin cancer, with 106,000 new cases in 2020, represents about 5.6% of all cancer diagnoses. Melanoma has a high prevalence among light-skinned men, with an incidence of 34.7/100,000 compared to light-skinned women with 22.1/100,000. In darker-skinned individuals, the rate for men and women was 1.0 and 0.9, respectively, and 5.0 for both genders among Hispanics [28].

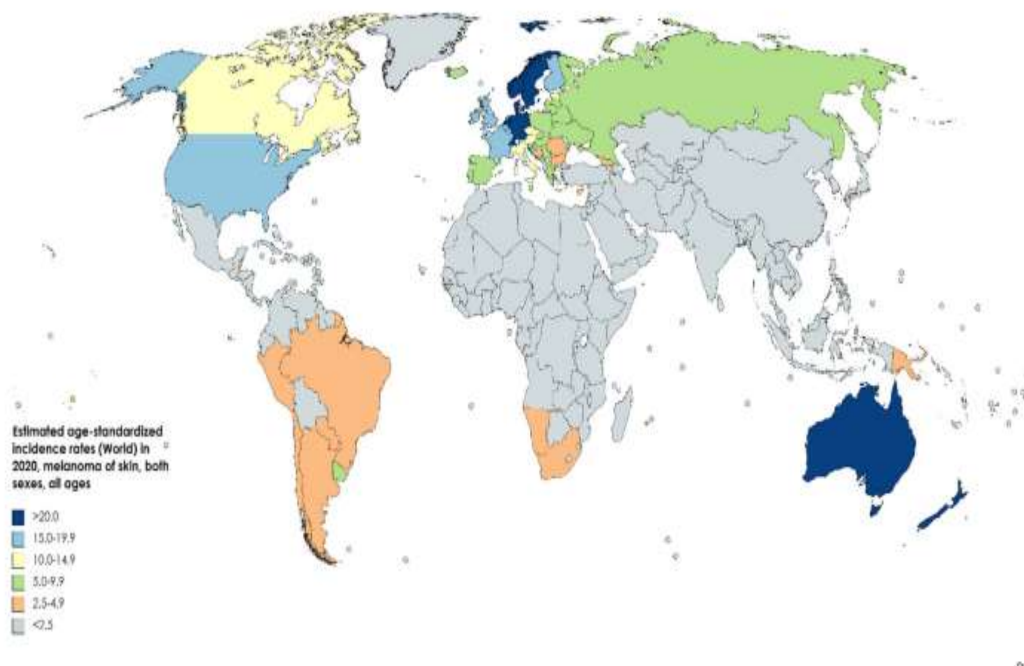


Figure 2 - Worldwide incidence of cutaneous melanoma. The map shows age and gender standardized incidence rates distributed across the world in 2020. Created with mapchart.net. Data obtained from Globocan 2020.

Melanoma has higher incidence in more developed countries. In the United States of America, the incidence increased by more than 320% over the last years, from 7.9/100,000 in 1975 to 25.3/100,000 in 2018 [28]. The incidence of cutaneous melanoma has been increasing in the last decades, and studies carried out point to that the US should peak around 2022-2026, and Sweden and northern Europe before 2030 [28].According to GLOBOCAM, an estimated 57,000 people died in 2020 from melanoma, resulting in an age-standardized mortality of 0.4/100,000 for women and 0.7/100,000 for men worldwide [28].

1.2.2- Cutaneous melanoma – risk factors

Currently, melanoma is considered a multifactorial disease, resulting from an interaction between genetic susceptibility and exposure to the environment. Risk factors can be divided into environmental, constitutional, genetic and others [14].

1.2.2.1- Sun Radiation

According to Elwood *et al*, sun exposure is a determining risk factor for the emergence and development of CM, due to its genotoxic effect [29].The sun emits UVA (320-400nm), UVB (280-315nm) and UVC (200-280nm) radiation. The UVC radiation is retained in the ozone layer. Of the UV radiation that reaches the earth's surface, 98.7% is UVA [30]. However, even at a low percentage, UVB radiation causes the formation of cyclobutene pyrimidine dimers and 4 to 6 photoproducts, causing DNA damage and cellular apoptosis [31]. During sun exposure, UVB radiation causes a covalent bond between thymine and cystine bases in DNA-forming dimers, which will cause a mutation during the DNA replication process [32]. Although previous published studies pointed that the main cause of melanoma development was UVB radiation, it is currently known that most cutaneous melanomas occur due to indirect damage caused by UVA radiation [30].

Throughout life, especially at an earlier age, high-intensity sun exposure is one of the greatest risk factors for the development of melanoma, especially associated with childhood sunburn [33]. However, it is important to emphasize that the site of the burn is not necessarily related to the melanoma area [6].

1.2.2.2- Skin Type

Individuals who have skin phototypes I and II in the Fitzpatrick classification have a higher risk of developing cutaneous melanoma than individuals who have never had a sunburn or tan easily (Table 1) [34].

Phenotypic characteristics of low melanin production are considered good markers for identifying a greater susceptibility to melanoma, such as, individuals with fair skin, blue or green eyes, blonde or red hair and freckles. Individuals with blue and green eyes have a relative risk of 1.6 to 3.0 and 1.1 to 2.5 respectively, compared with brown eyes, for developing melanoma [35]. Blonde hair is associated with a relative risk of 1.6 to 9.7. And individuals with red hair have a relative risk of 2.3 to 5.6 compared with darker hair [35].

Table 1 - Skin Phototypes (Adapted from Fitzpatrick, 1988) [34].

Skin Type	Skin color	Hair color	Eye color	History of sunburn	Ultraviolet radiation sensitivity
I	Pale, Fair	Blond	Blue	Always burns on minimal sun exposure	Extremely sensitive
II	Fair, Freackles	Blond, Red	Green	Burns very readily	Very sensitive
III	Light brown	Dark blond, Light brown	Hazel, Brown	May burn on regular sun exposure with no protection	Moderately sensitive
IV	Olive brown	Light brown, Brown, Black	Dark brown	Burns rarely	Relatively tolerant
V	Brown -Moderate constitutional pigmentation	Dark brown, black	Dark brown	Despite pigmentation, may burn surprisingly easily on sun exposure	Very variable
VI	Black- Marked constitutional pigmentation	Black	Black	Rarely burns, although sunburn is difficult to detect on very pigmented skin	Relatively insensitive

1.2.2.3- Tanning beds and UV lights

In the United States, artificial tanning regarding melanoma development, is included in the same classification as cigarettes to lung cancer. Studies have shown that tanning beds produce more than twelve times the UVA radiation of the sun [31]. Epidemiological studies as well as laboratory studies show that individuals who use indoor tanning beds before age 25 have a 6-fold increased risk of getting melanoma. Performing 1 to 14 sessions of tanning beds in a lifetime increase the risk of melanoma by 19%, between 15 and 30 sessions increase the risk by 31%, and individuals who have already exceeded 40 hours of artificial tanning, the risk may increase by 55% [30]. Surprisingly, in a large multinational study of individuals with melanoma, they reported that more than 70% of respondents had used tanning beds at least once in the past.

The age groups that are associated with the highest risk of melanoma, resulting from artificial tanning, are at a younger age. It is postulated that a more frequent use of tanning beds is found in young women than in men, and that is the reason for the increased incidence of melanoma in young women [36].

1.2.2.4- Age and Gender

A prognostic factor in cutaneous melanoma is age, which commonly appears in old people [37-39]. The average age of patients diagnosed with melanoma is 63 years and the highest percentage of deaths related to this pathology is in the 75-84 age group [40].

Differences in predisposition between women and men to develop cutaneous melanoma depend not only on gender differences but also on other factors. It is related to intact biological characteristics, such as cellular and hormonal features, or behavioral characteristics, such as patterns of UV exposure, clothing and sun protection [41-44]. Studies show that women under 50 show a higher incidence rate when compared to men of the same age group [45]. Of the differences that exist between genders, women have more tumors in the lower extremities, while men have more tumors in the trunk [46].

Males aged 75 years have an incidence of cutaneous melanoma almost 3 times higher when compared to women of the same age group [47]. Older men have a worse prognosis than women [48]. However, it is still unclear why men are at greater risk than

women, but it is believed that it may be correlated with different gender behaviors and sun protection strategies and less frequent use of sun protection, such as the skin type [32]. However, there is a high incidence in women up to the age of 40 and it is believed that the increase is due to the fact that there are more young women using artificial tanning [32].

1.2.2.5- Melanocytic Nevi

Studies carried out demonstrate a strong association between the number of melanocytic nevi and the development of melanoma. Melanocytic nevi are small clusters of melanocytes or nevus cells that can be congenital or acquired [48]. Several factors are determinant for the proliferation of nevi, such as genetic factors, immunological factors and sun exposure [14]. Studies show that approximately 25% of melanomas are derived from pre-existing nevi [14]. In addition, there is a strong correlation between the number of nevi and the risk of melanoma, varying based on nevi number, size, and type [49]. Therefore, patients with more than 100 nevi or with one or more atypical nevi are seven times more likely to be at risk compared to individuals with fewer nevi [33]. Regarding size, larger (>5mm) and giant (>20mm) nevi are also correlated with a higher risk of melanoma. Regarding the type of nevi, the presence of five atypical nevi increases the risk by six times. However, it is not only atypical nevi that are associated with an increased risk of melanoma, the unique presence of a nevus with atypical features such as variable pigmentation, irregular asymmetrical contour and blurred edges also increase that risk [50-52].

1.2.2.6- Family and Genetic Factors

Family history is considered an extremely important factor, as approximately 5% to 10% of melanomas occur in families with hereditary predisposition. Melanoma has a very high mutational load, and germ line mutations in *CDKN2A (p16 and p14ARF), and CDK4*, with higher frequency [31], and also *BAP1, POT1, MC1R, MITF, ACD,* and *TERF2IP*, with lower frequency, present a risk for the development of melanoma [52]. A good identification marker of individuals prone to develop melanoma are freckles, that do not appear in individuals who are not exposed to solar radiation.

Individuals who are carriers of xeroderma pigmentosum (XP) with association to different biallelic genes, *ERCC, POLH*, *XPA* and *XPC* [53-55] have a higher sensitivity to solar radiation and have a high number of freckles, and approximately 20% will develop melanoma during their lifetime [6].

Patients with B-K mole syndrome and familial atypical mole/melanoma (FAMM) have a very high risk for the development of cutaneous melanoma (including multiple primary cutaneous melanomas) and multiple primary malignancies. FAMM syndromes are both autosomal dominant traits with incomplete penetrance and may be related to an abnormality on chromosome 1P [14]. The incidence of melanoma in patients with FAMM is very high, reaching 85% in the middle age [49].

1.2.2.7- Personal History of Melanoma

Whatever the etiology of primary melanoma, the existence of a previous history is considered one of the most important risk factors for the development of melanoma. Individuals with a past history of melanoma have about a 3% to 8% probability of melanoma recurrence. And individuals with a family history of non-melanoma skin cancer have a relative risk between two and seven times [6].

1.2.2.8- Immunosuppression

Individuals with the HIV virus and patients who received immunosuppression throughout their lives have a higher incidence of cutaneous melanoma. The cumulative risk for the development of tumors, including skin cancer among others [56] is about 14% in the first 10 years after transplantation, and significantly increases to 40% in 20 years compared to the general population [57]. Tumor growth and development may be associated with the molecular effects of immunosuppressive drugs, such as cyclosporine (CsA) [58, 59] and azathioprine [60]. CsA is associated with inhibition of UVB-induced lesion turnover [61] and UVB-induced apoptosis in normal human epidermal keratinocytes [62]. It may also stimulate TGFbeta synthesis in tumor cells, thereby promoting cancer invasion and metastasis [58]. Recent studies demonstrate that azathioprine is shown to sensitize DNA to UVA radiation, thus resulting in increased mutagenesis [63].

1.2.3- Cutaneous melanoma - prevention

Primary and secondary prevention is extremely important for controlling skin cancer in the population [44, 64]. Primary prevention encompasses all behavioral strategies used daily to prevent the onset of melanoma, such as avoiding sun exposure at times of higher UV radiation intensity or the correct use of sunscreen [36, 44]. On the other hand, secondary prevention focuses on strategies for the early detection of melanoma, to provide a faster diagnosis and, in turn, a more effective treatment, contributing to the patient's survival [48]. According to the Portuguese Association of Cutaneous Cancer, skin self-examination is the best way to contribute to an earlier detection of melanomas. This method is supported by the Directorate-General for Health and is called the ABCDE method [31]. By observing the body's signs, such as asymmetry, border, color, dimension and evolution, the ABCDE method distinguishes normal nevi from melanomas [52]. The identification of two or more parameters is enough to be considered an atypical sign and indicates that the evaluation must be done by a specialist doctor [65].

1.2.4- Cutaneous melanoma – frequent molecular alterations

With the development of technology, in the last years, new information about the changes present in cutaneous melanoma appeared. New advances allowed a deeper diagnosis of the disease and, in turn, a better understanding of prognostic and molecular markers that led to the possibility of a more personalized and effective treatment of patients. Several investigators believe that the clustering of mutations affects genes fundamental to cell proliferation, differentiation and apoptosis, modifying normal cells into cancer cells, also leading to angiogenesis, invasion and metastasis. The most predominant molecular alteration in cutaneous melanomas is *BRAF V600E* [66]. The *TERT* promoter and *CDKN2A* mutation are also found in most cases. *PTEN* and *PT53* mutations can also be found in more advanced tumors. In acral and mucosal melanomas, *KIT* mutations are more frequently found, in addition to gene amplifications and structural rearrangements, most frequently of the *CCND1* and *SF3B1* gene [67]. Alterations in critical genes of the RAS-RAF-MEK-ERK and PI3K-AKT pathways, which

regulate signal transduction, such as genes involved in cell cycle regulation as *CDKN2A*, are important molecular features in the large part of the cases [68].

1.2.4.1- Activation of the MAPK signaling pathway

In melanoma, the mitogen-activated protein kinase (MAPK) signaling cascade is activated by a variety of mechanisms, thus it is a captivating target for pathway-targeting therapies. The MAPK signaling pathway is involved in cell maintenance, proliferation, senescence and survival [69]. The signaling pathway is composed of four well-studied cascades, sequentially transferring proliferative signals from cell surface receptors to the nucleus, through a series of phosphorylation events. The canonical cascade is the ERK pathway, also identified as the RAS-RAF-MEK-ERK pathway [70] (Figure 3).

This pathway acts through signal transducers between the extracellular and intracellular environment of the cell. Growth factors, hormones and cytokines are involved and interfere with cell receptors, causing conformational changes that lead to RAS activation. RAS proteins recruit RAF proteins that migrate to the cell membrane, thereby being activated. In turn, RAF proteins activate MEK proteins, which migrate and interfere with ERK, which will migrate to the cell nucleus and regulate various cellular processes [71, 72]. The role of this signaling pathway has been well studied, and it was revealed that about 30% of all cancers harbor mutations in proteins from this signaling pathway. About 90% of cutaneous melanoma present activated ERK, due to multiple mechanisms, including mutations in the *NRAS* and *BRAF*, stimulation of growth factor receptors such as c-MET and fibroblast growth factor receptors 1 (FGFR1), activation by αvβ 3 integrin or increased notch signals [73, 74]. Activation of this pathway reduces the expression the cell cycle inhibitor p12KIP1 and increases the expression of cyclin D1, which drives the growth and proliferation of melanoma cells. By regulating the function and expression of a set of proteins, this pathway controls the survival and apoptosis of melanoma tumor cells [75]. Numerous studies showed that the activation of the MAPK pathway is necessary for melanoma development, since the signal cascade facilitate the passage of tumor cells to the vascular system, surviving in the blood flow, and penetrating the endothelial lining of the vessels in the tissue, allowing the proliferation in the new tissue environment (Figure 3) [76].

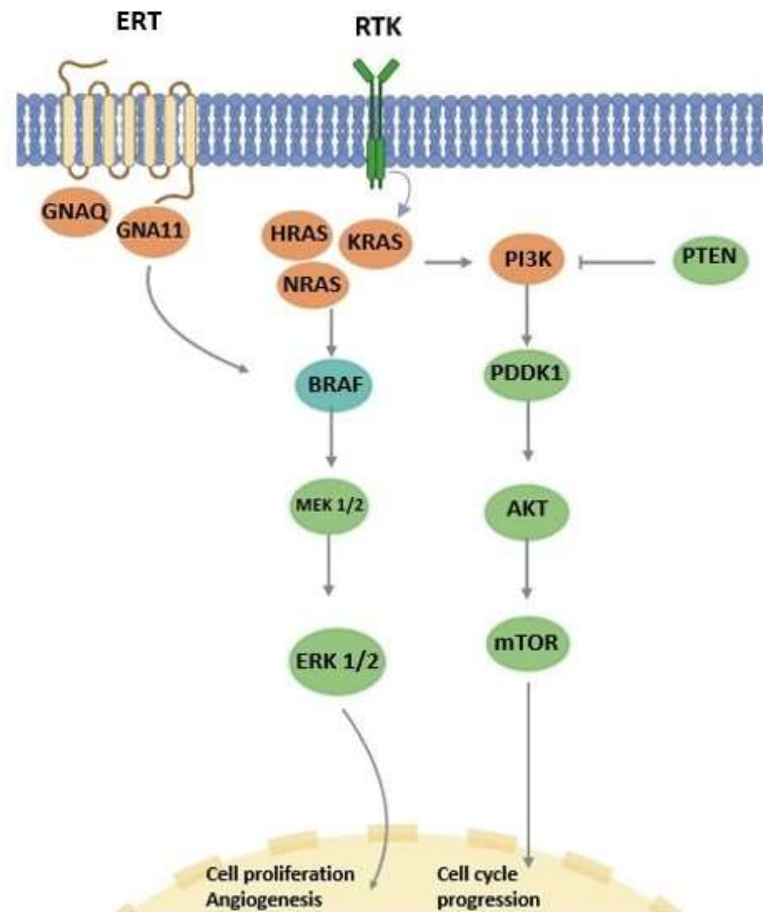


Figure 3 - Diagram of MAPK and PI3K/mTOR signaling pathways. The scheme shows the relationship between the MAPK and PI3K/mTOR pathway in the response to intracellular stimuli to control translation, cell survival, proliferation, motility, and metabolism. Genetic mutations in the MAPK pathway are related to melanoma cell proliferation, cell cycle progression, and the malignant phenotype. Created in: BioRender.com

1.2.4.1.1- *BRAF* mutations

Serine-threonine kinase (RAF) proteins emerge in 3 differentially regulated isoforms: A-RAF, B-RAF and C-RAF (RAF-1), and play an important role in signal transduction [77]. The B-RAF isoform (V-raf murine sarcoma viral oncogenes homolog B1) is the one with the highest basal activity and requires less phosphorylation to become active, making it the strongest activator of the MAPK pathway [69].

BRAF gene, located at 7q34, is actively mutated in several cancers, either by point mutations or chromosomal rearrangements. Thus, it leads to the loss of control of cellular metabolism, potentiates the emergence of malignancy, deregulates the cell cycle resulting in proliferation and disordered tissue growth and cell death [78].

Surprisingly, 80% of the mutations that occur in melanocytic nevi are in the *BRAF* gene, which is more prevalent in nevi than in melanoma, raising the question whether these genetic alterations are early events in the development of melanoma. However, two independent groups found that individuals with *BRAF* germline mutations develop cardiofaciocutaneous syndrome (CFC) but not cancer. Numerous lines of evidence have shown that *BRAF* mutations are insufficient, but in combination with other gene/protein changes, can lead to melanocytes transformation (Figure 3) [77].

BRAF mutations appear in approximately 30-60% of cutaneous melanomas, causing cell proliferation, tumor growth and metastases [68, 79-81]. The vast majority of *BRAF* mutations, approximately 90%, comprise the substitution of glutamic acid for valine in exon 15 (Val600Glu), representing a point mutation [82]. More than 50 distinct mutations in the BRAF gene have already been described [82]. Val600Lys and Val600Asp represented 16% and 3%, respectively, of mutations recorded in melanoma[83]. *BRAF V600E* promotes cell proliferation causing tumor growth and increased angiogenesis, consequently increases the expression of vascular endothelial growth factor (VEGF) and macrophage inhibitory cytokine-1 (MIC-1) [84]. This mutation also promotes the adhesion of melanocytes to the vasculature, boosting the appearance of metastasis [84]. Patients carrying these mutations are generally older, approximately 55 years old, than patients with *BRAF* mutations, and present chronic patterns of exposure to UV radiation. These lesions are localized and present higher levels of mitosis than melanomas with *BRAF* mutations [94, 95]. The mutational profile of the gene establishes a clinical parameter about the status and prognosis of diseases and is a marker for targeted therapy [85]. However, some authors describe that there is no relationship between the presence of BRAF mutations and patient prognosis [86, 87].

1.2.4.1.2- *RAS* mutations

NRAS, KRAS and *HRAS* belong to the group of *RAS* genes and represent a fundamental part in the signal transduction of MAPK and PI3K-AKT signaling [88]. The *RAS* (rat sarcoma virus oncogene homolog) genes encode small GTPase proteins that

alternate their state between active and inactive and are regulated, respectively, by GTP and GDP bonds. The *RAS* gene domain consists of two lobes, a first allosteric lobe that interacts with the cell membrane and an effector lobe that interacts with effector molecules of the signaling cascades. There are many homologies between the three different isoforms, each has a specific codon mutation signature, given that the C-terminal region of these proteins is hypervariable [89].

The most frequent *RAS* alterations found in human tumors are oncogenic missense mutations at codons 12, 13 or 61[90]. The first melanoma oncogene to be identified was *NRAS*, in 1984, in a screening of melanoma cell lines for genes that had transforming properties. *NRAS* activating mutations were found in 4/30 samples *NRAS, KRAS*, and *HRAS* mutations account for about 15-25 % [91], 2%, and 1% of all melanomas, respectively [92]. The most common *NRAS* mutation present in about 80% of the cases is the substitution of a glutamine for a leucine at position 61, the remaining mutations at positions 12 and 13 have a lower frequency. The *NRASQ61* induces constitutive RASGTPase activity and conformational changes towards the active GTP-bound state, while oncogenic changes at codons 12 or 13 impair hydrolysis mechanisms of the GTP [92, 93].

Previous studies show that the presence of *NRAS* gene mutations in cutaneous melanomas is associated with prognosis [94, 95]. Some studies demonstrate an association between the presence of the *NRAS* mutation and a high Breslow index which is considered an indicator of poor prognosis [96, 97]. However, there are authors who found no association between the presence of NRAS mutation in melanoma and prognostic characteristics [86].

1.2.4.2- *TERT* promotor mutations

Humans have telomere-protected ends on their chromosomes, which are nucleoproteins composed of 5 to 15 kb of 5'-TTAGGG-3' hexanucleotide repeats and shelter protein compounds (TRF1, TRF2, POT1). These complexes have the function of preventing the ends of chromosomes from being recognized as damaged DNA and preventing chromosomal fusion [98-100]. *hTERT* (human telomerase reverse transcriptase) is a gene located at 5p15.33 that encodes a ribonucleoprotein enzyme that catalyzes the extension of telomeric DNA in eukaryotic cells, by adding telomeric repeats to the G-rich strand, the last bases of the chromosomes. This process avoids

the successive shortening of telomeres and the loss of genomic information (Figure 4). As a natural consequence of cell division, during successive mitoses, telomeres shorten until they reach a critical point of length and instability. At that moment, the cell enters a crisis that triggers senescence. There are two checkpoints that are involved in cell control and prevention of cell immortalization, and transformation into malignant phenotypes, which are M1 and M2. The replicative senescence phase (M1) is where cell proliferation is inhibited, as telomeres have reached a critical length. This phase can also be overcome by transformed cells that have inactivated protective mechanisms, such as the p53 pathway, which leads to a continuous proliferation that keeps telomeres shortened, until a new crisis is reached (M2). This second crisis usually causes chromosomal instability, final fusion and apoptosis [101].

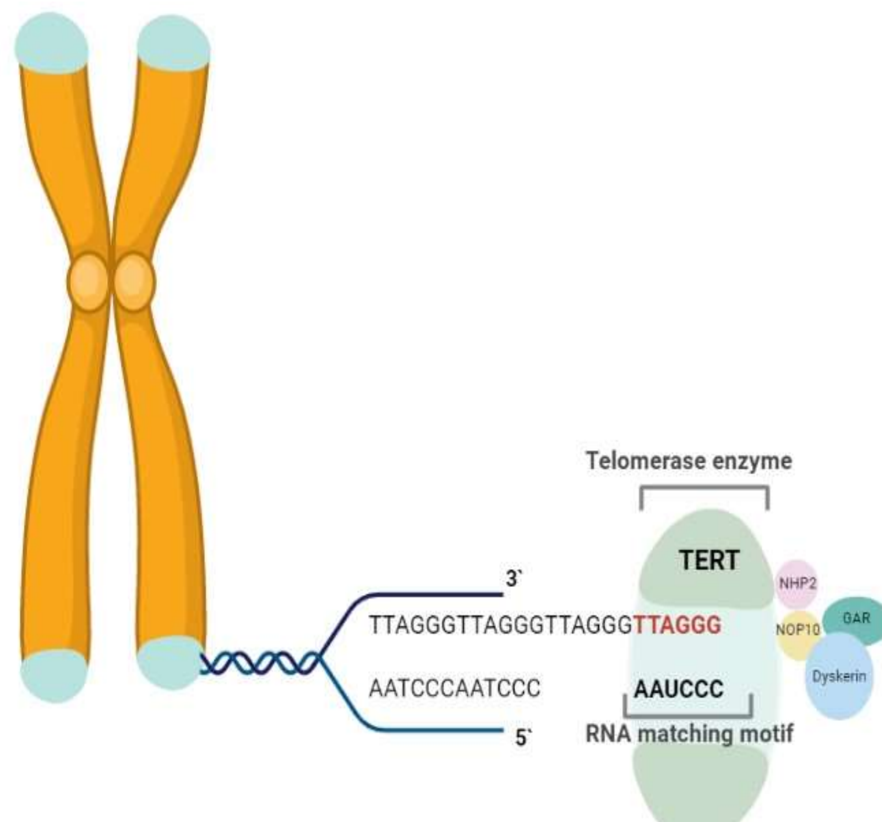


Figure 4 - Schematic representation of telomeric DNA and the shelter complex. The single G-rich tape allows the formation of a loop, meeting at the 3' end. At the 5' end, the overhang invades the terminal DNA double helix. Created in: BioRender.com

Approximately 90% of human cancers show telomerase activation. Several mechanisms have already been described as triggering telomerase activation, such as *TERT* promoter mutations, *TERT* amplification, epigenetic alterations and splicing [102]. Over the past few years, mutations in the *TERT promoter* region have been found in numerous types of cancer, such as bladder (59%), central nervous system (43%), melanoma (29-71%) [103-107] and thyroid (10%). Huang *et al.* and Horn *et al.* found a high frequency of two hotspots mutations in the *TERT* promoter gene in primary melanoma tumors and cell lines derived from metastatic melanomas [103]. The mutations, C228 T (124 C > T) and C250 T (146 C > T), respectively, corresponding to positions 124 and 146 base pairs, upstream of the ATG start site, come from a transcription of cytidine to thymidine, being an indication of damage induced by UV light. Tandem CC>TT mutations at positions 124/125 and 138/139, which create a CCGGAA/T motif for ETS transcription factors, were detected with a low frequency of about 5-10% in skin cancers. While some published studies do not associate the *TERTp* mutation with prognosis [108], others find an independent and significant association between the presence of *TERT* promoter mutations and poor prognosis in cutaneous melanoma [21]. According to Pópulo *et al*, the presence of *TERT* promoter mutations are associated with poor prognosis in primary cutaneous melanoma. The authors also showed that *TERTp* mutations are associated with subtypes of nodular melanoma and of superficial spreading melanomas, with greater thickness, ulceration, high mitotic rate and coexistence with the *BRAF* mutation. In addition, they also reported that mutations in the *TERT* promoter are more prevalent in cutaneous melanomas that occur in skin with intermittent exposure to the sun [109].

1.2.5 - Cutaneous melanoma - therapy

Metastatic cutaneous melanoma continues to kill thousands of people worldwide every year, with a median survival of 8 to 10 months [80]. Although the number of treatments has expanded over the years, until 2011 the only treatments available for treating stage V melanoma were chemotherapy (CT) and IL-2 immunotherapy. In recent years, the identification of new prognostic factors and therapeutic targets have been a priority for researchers and as of 2011, immunotherapy with immunological checkpoint inhibitors and target therapies with BRAF and MEK inhibitors are being used as treatments in BRAF mutated metastatic melanoma [110].

1.2.5.1- Immunotherapy

The immune system controls the duration and relevance of responses, thus controlling autoimmunity. This mechanism only happens through the interaction of specific T cell receptors with their ligands, leading to the activation of immunological control points with consequent blockage of the immune response [111].The hyperactivation of checkpoints of the immune system by melanoma cells, allow them to escape the natural immune control system [20].The approval of checkpoint inhibitors has transformed the treatment of patients with metastatic melanoma and has been one of the great advances in the field of immunotherapy. These inhibitors are monoclonal antibodies directed at PD-1 and CTLA-4 receptors, which block the binding to ligands and are essential for the activation of the immune system and consequent destruction of tumor cells. Currently, three monoclonal antibodies for the treatment of metastatic melanoma are available for treatment: ipilimumab (anti-CTLA-4 antibody), nivolumab and pembrolizumab (anti-PD-1 antibodies) [111].

1.2.5.2- Target therapy

New discoveries of somatic mutations and the understanding of signaling pathways have expanded the development of new therapeutic approaches. The inhibitors were developed to target the MAPK pathway in patients with metastatic melanoma harboring the molecular mutation V600 in the *BRAF* gene. Although the response rate is low, the main challenge focuses on the development of resistance up to 6 to 8 months after starting therapy, as the MAPK pathway is reactivated as a consequence of disease progression. Studies reveal that the reactivation of the MAPK pathway is one of the main mechanisms of resistance to B-RAF inhibition, that can occur by the reactivation of ERK signaling, through bypass mechanisms that are Ras/Raf dependent, as RAS activation [112, 113], or can also be by C-Raf upregulation [114]. Reactivation can also happen due to splicing or overexpression of the *BRAF* protein. The combination of *BRAF* inhibitors (vemurafenib, dabrafenib and encorafenib) with MEK inhibitors (cobimetinib, trametinib and binimetinib), respectively, has made it a standard therapy, as it delays the development of resistance by allowing greater inhibition of the MAPK pathway, and the decrease of cytotoxic effects that are associated with monotherapy with *BRAF* inhibitors [115]

2 – Aims

Cutaneous melanoma continues to kill thousands of people around the world every year, and although the range of treatments has expanded over the last years to include target and immunotherapies, the prognosis of patients with metastatic melanoma remains unsatisfactory, being the identification of new prognostic factors and therapeutic targets a priority for researchers.

Therefore, the overall aim of this project is to study of genetic alterations found in patients with melanomas and correlate them with the clinical and pathological data of the patients. With this project, we intended to obtain possible prognostic biomarkers for melanoma.

3 – Materials and Methods

3.1- Series selection and clinicopathological data

The data used in this work are included in 2 independent projects. The first project uses data collected from the Hospital Distrital de Santarém (HDS), from patients diagnosed with melanoma related events between 2012-2018. All metastatic cases were selected, to test the value of molecular markers in the prediction of metastatic disease, and non-metastatic random cases were also included in the series for comparison purposes. Tissue samples from patients encompassed primary, recurrent, locoregional, and distant tumors were collected. Clinicopathological and follow-up data were also provided by HDS. The majority of the series was previously collected and studied. Within this work, the series was completed and the missing cases were evaluated. All procedures were in accordance with institutional ethical standards and the study was approved by HDS Ethics Committee (Ref. HDS134A.05). The second project uses a new data series collected from Hospital dos Capuchos (HC) and aims to develop strategies to automatically analyze melanoma tumors using dermoscopy, trying to identify new predictive biomarkers for prognosis and/or therapy efficiency. In this project, primary tumors and clinicopathological data from melanoma patients were made available. The study procedures were in accordance with institutional ethical standards and the study was approved by HC Ethics Committee (Ref. INV195).

In this study, 128 patients with melanoma-related events were included, 55 patients from Hospital Distrital de Santarém and 72 from Hospital dos Capuchos. Tissue samples of cutaneous melanoma-related events were analyzed, corresponding to 158 formalin fixed paraffin-embedded samples, comprising primary tumors, recurrences, metastases and sentinel nodes (Table 2).

Table 2 - Description of the melanoma related events samples analyzed in this study for each gene.

Samples	Number of cases (%)	*BRAF* Number of cases (%)	*NRAS* Number of cases (%)	*TERTp* Number of cases (%)
Primary tumors	122 (77)	115 (76)	118 (76)	95 (76)
Recurrences	6 (4)	6 (4)	6 (4)	6 (5)
Metastases	18 (11)	18 (12)	18 (12)	13 (10)
Sentinel Lymph Nodes	12 (8)	12 (8)	12 (8)	11 (9)
Total	**158**	**151**	**154**	**125**

3.2- Haematoxylin- eosin staining

Formalin-fixed paraffin-embedded tissues were sectioned into 3 µm thick segments for hematoxylin-eosin staining (HE). The slides were initially deparaffinized with two successive incubations in xylene (Enzymatic, Santo Antão do Tojal, Portugal) for 10 min each, and then hydrated by applying decreasing concentrations of ethanol (Enzymatic, Santo Antão do Tojal, Portugal): two consecutive incubations in 100% ethanol for 5 min each, one incubation in 96% ethanol for 5 min and one incubation in 70% ethanol for 5 min. Then, the slides were washed in running water for 5 minutes, before staining with hermatoxylin (DIAPATH, Martinengo, Italy) for 4.30 min. The slides were then washed again in running water to remove excess dye before incubating for 5 min with 70% ethanol. Slides were stained with alcoholic eosin-Y (Richard Allan Scientific, ThermoScientific, Waltham, MA, USA) for 4 min. This was followed by one incubation in 96% ethanol and two incubations in absolute ethanol for 5 min each. To complete this process, two incubations of 10 min each in xylene were performed and then the slides were mount with mounting medium (Richard Allan Scientific, ThermoScientific, Waltham, MA, USA).

3.3- Histological revision

HE sections of all the cases (from HDS and HC) underwent by a final re-evaluation and staging according to AJCC guidelines, by a pathologist from Hospital São João. The HE staining also allowed for precise identification of the tumor's margins, which was useful for manual dissection of the samples sections.

3.4- Deparaffinization and DNA extraction

After being manually dissected from 10 µm thick sections, tissues were collected in 1.5 mL tubes. Then, the tissues were deparaffinized in 500 µL xylene (Valente and Ribeiro, Alcanena, Portugal) in 3 incubations of 10 min each, centrifuging between each at 1400 rpm, 5 min. Then, the tissues were washed with 500 µL 100% ethanol (Valente and Ribeiro, Alcanena, Portugal) in 3 incubations of 10 min each, centrifuging between each wash at 1400 rpm for 5 min.

Finally, two kits were used to perform the extraction: Grisp Genomic DNA Kit BroadRange (Waltham, USA) for cases larger than 5 nm and QIAmp DNA Investigator Kit (Hilden, Germany) for cases smaller than 5 mm, both according to the manufacturer's instructions.

3.4.1- DNA extraction by Grisp Genomic DNA kit Broad Range

DNA extraction was performed according to the manufacturer's instructions. All reagents used in the extraction were included in the Kit, except proteinase K (GRISP research solutions, Waltham, USA).

Integrity of the cells was interrupted by the addition of 200 µL of Buffer BR1 and 10 µL of proteinase K (20 mg/mL). Samples were incubated overnight at 60°C with 600 rpm. The next day, another 5 µL of proteinase K (20 mg/mL) were added, the eppendorfs were vigorously shaken and incubated for 1 h at 60°C. 200 µl of Buffer BR2 was added and all samples were shaken vigorously. 200 µL of Absolute Ethanol was added and immediately shaken vigorously for 10 sec. All samples were transferred to columns (provided by the kit) and centrifuged at 14,000G for 1 min. The contents of the tube were

discarded and a new one (provided by the kit) placed on the column. The samples were washed in 2 steps, initially with 400 µL of Wash Buffer 1 by centrifugation at 14,000 G for 30 sec, followed by a second wash with 600 µL of Wash Buffer 2 by centrifugation at 14,000 G for 30 sec. The contents of the collection tube were discarded and centrifuged again at 16,000 G for 3 min to dry the entire membrane. The columns were transferred to previously labeled 1.5 ml eppendorfs and 25-50 µL of Elution Buffer (previously heated to 60°C) added to the center of the membrane to elute the DNA. Samples were incubated for 5 min at room temperature and centrifuged at 14,000 G for 30 sec. The samples were stored at -20ºC.

3.4.2- DNA extraction by QIAmp DNA Investigator Kit

DNA extraction was performed according to the manufacturer's instructions. All reagents used in the extraction were included in the Kit, except proteinase K (GRISP research solutions, Waltham, USA).

Fifteen µl of Buffer ATL and 10 µl of proteinase K (20mg/ml) were added to each eppendorf. The tubes were vigorously shaken and incubated for approximately 3 hours at 60°C with 600rpm. 5 µl of Proteinase K were added and again incubated for 1 hour at 60°C with 600rpm. 25 µl of Buffer ATL and 50 µl of Buffer AL were added, all samples were shaken vigorously for 15 sec in order to keep the sample homogeneous. 50 µL of 100% Ethanol was added and the tubes were incubated for 5 min at room temperature. All samples were transferred to the 2 mL columns (provided by the kit) and centrifuged at 6000 G for 1 min. The contents of the tube were discarded and a new one (provided by the kit) placed on the column. The samples were washed in 3 steps, initially with 500 µL of Buffer AW1 by centrifugation at 6000G for 1 min, followed by a second wash with 700 µL of Buffer AW2 by centrifugation at 6000G for 1 min, and finally 700 µL of 100% Ethanol by centrifugation at 6000G for 1 min. The contents of the collection tube were discarded and centrifuged again at 20,000G for 3 min to dry the entire membrane. The columns were transferred to 1.5ml eppendorfs, previously identified and placed in the thermo-block at 56ºC for 3min. 20 µL of Elution Buffer ATE (previously heated to 60°C) was added to the center of the membrane to elute the DNA. The samples were incubated for 5 min at room temperature and centrifuged at 20,000G for 1 min. Samples were stored at -20°C.

3.5- DNA quantification

The DNA was quantified by spectrophotometry, in a NanoDrop® N-1000 Spectrophotometer 156 (Thermo Scientific, Lithuania, EU).

3.6- Multiplex polymerase chain reaction (Multiplex PCR)

For the PCR multiplex reaction (for the 3 genes simultaneously), genomic DNA was amplified using the following conditions: 15 min at 95ºC; 30 s at 95 ºC, 1:30 min at 63°C, 1 min at 72 °C for 40 cycles; and 10 min at 72ºC (Figure 5 and Table 3).

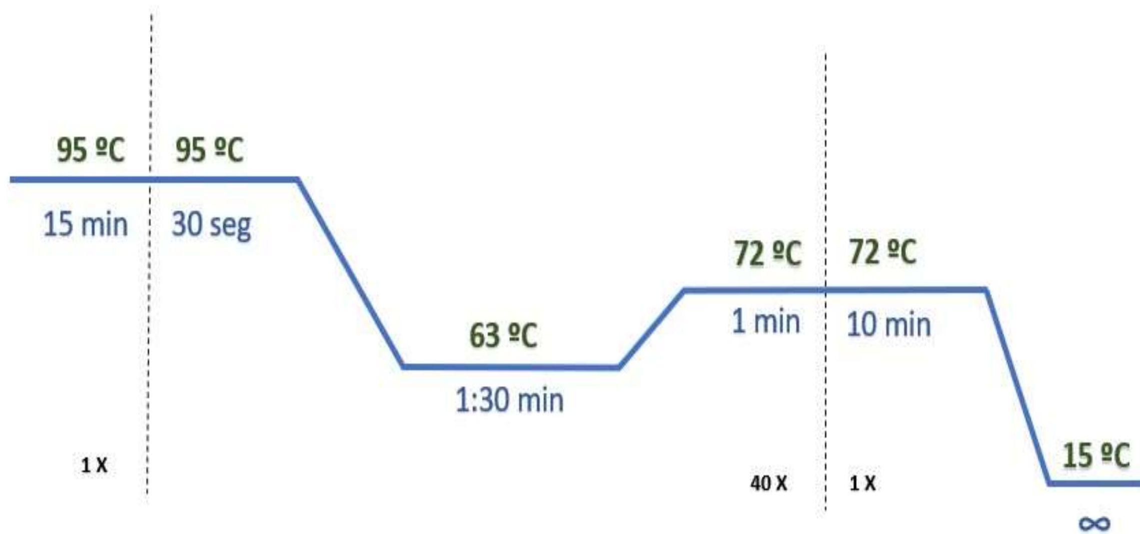


Figure 5 - Thermocycler conditions applied in the multiplex PCR.

3.7- Polymerase chain reaction (PCR)

Genomic DNA was amplified by PCR using the following cycling conditions for each gene: 2 min at 95ºC; 30 s at 95°C, 30 s at 58°C for BRAF and NRAS and 62°C for *TERT*, and 20 s at 72 °C for 40 cycles; and 10 min at 72ºC (Figure 6 and Table 3).

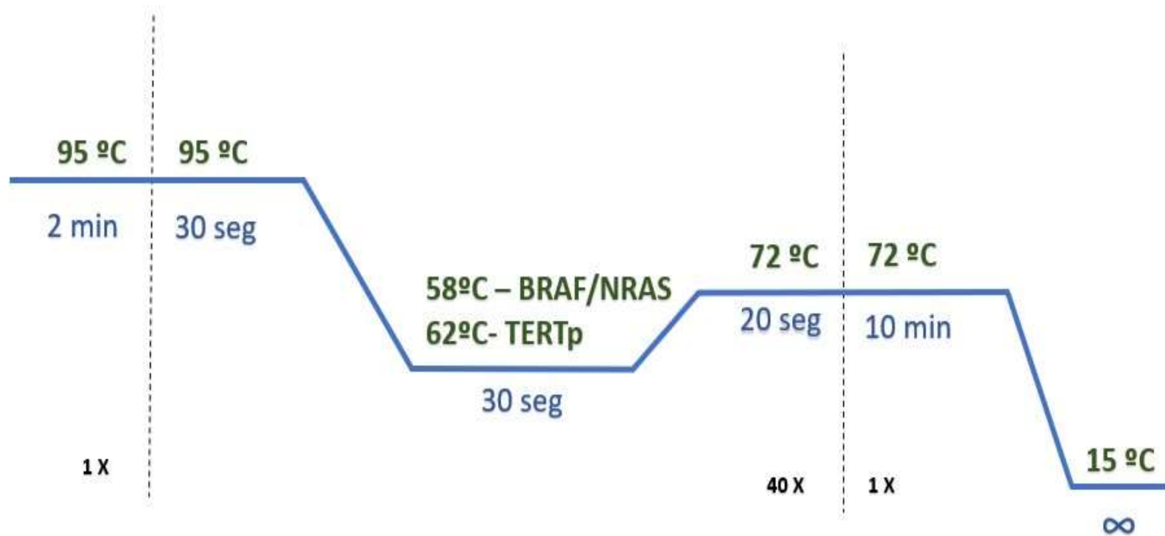


Figure 6 - Thermocycler conditions applied in individual PCR of *BRAF* and *NRAS* genes and *TERTp.*

BRAF, NRAS and *TERT promoter* analysis was performed with the pair of primers which are presented in the Table 3. All PCR reactions were conducted in the SimpliAmp Thermal Cycler System (California, USA).

Table 3 - Primers Forward and Reverse utilized in the PCR and sequencing reactions.

Gene	Primers (5`- 3`) Forward	Reverse	Fragment size (bp)
BRAF	TTCCTTTACTTACTACACCTCAG	CATCCACAAAATGGATCCAGAC	133
NRAS	CAGAAAACAAGTGGTTATAGATG	GTCCTCATGTATTGGTCTCTCA	110
TERTp	CAGCGCTGCCTGAAACTC	GTCCTGCCCCTTCACCTT	166

3.8- Electrophoresis for PCR products evaluation

To evaluate the efficacy of the DNA amplification, 2.5 µL of products amplified by PCR, mixed with 1 µL of Loading Buffer (LB) containing Gel Red, were separate in 1-2% agarose gel electrophoresis (GRSvAgarose LE, GriSP, Porto, Portugal) in 0.5 X concentrated SGTB buffer (20 X SGTBvagarose electrophoresis buffer, GriSP, Porto, Portugal). A DNA Ladder (Invitrogen, CA, USA) was used to assess the size of the PCR products. Gel development was performed by UV light transillumination using the ChemiDoc™ XRS+ System, BIORAD (Model: Universal Hood II, Hercules, CA, USA – 50/60 Hz).

3.9- PCR product purification

PCR products were then purified using 1.5 µL of Exonuclease I (2000 U, 20 U/µL; Thermo Fisher Scientific, Lithuania) and Fast AP thermosensitive alkaline phosphatase (1,000 U, 1 U/µL; Thermo Fisher Scientific, Lithuania). The reaction lasts for 45 minutes, where in the first 30 minutes at 37ºC there is enzymatic activation, and in the last 15 minutes at 85ºC there is enzymatic inactivation (Figure 7).

In this step, the enzymes will remove excess primers, dNTPs and single-stranded DNA that were not integrated in the PCR reaction, which in turn may interfere with the sequencing reaction. All samples were preserved at 15ºC until the beginning of the Sanger sequencing protocol. The reaction was carried out on the SimpliAmp Thermal Cycler (California, USA).

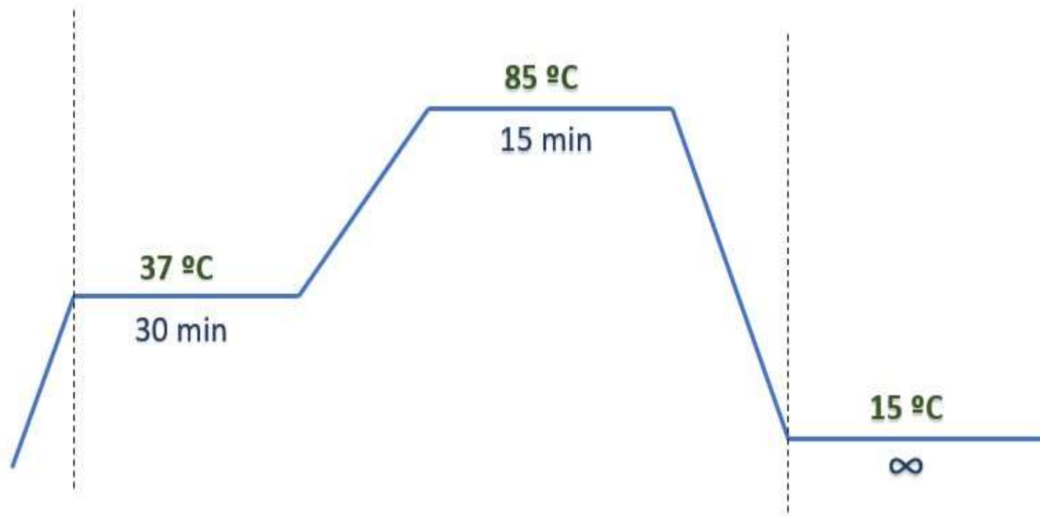


Figure 7 - Thermocycler conditions applied in DNA purification reaction.

3.10- Sanger sequencing

All PCR products were sequenced by Sanger sequencing using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Warrington, UK). The final sequencing reaction mix consists of 0.4 µL BigDye, 3.4 µL Sequencing Buffer (Big Dye Terminator v1.1, v1.3, Applied Biosystems, UK), 0.3 µL of the primer of interest, 3.5 µL of DNase and RNase free water and 0.5 – 3 µL of purified PCR product. *TERT* promoter, *BRAF* and *NRAS* genes analysis was performed with the primers presented in the Table 3. Reverse primers were used for *TERT* promoter, and forward primers for *BRAF* and *NRAS*. Samples were amplified on the SimpliAmp Thermal Cycler (California, USA). The reaction used is shown in figure 8.

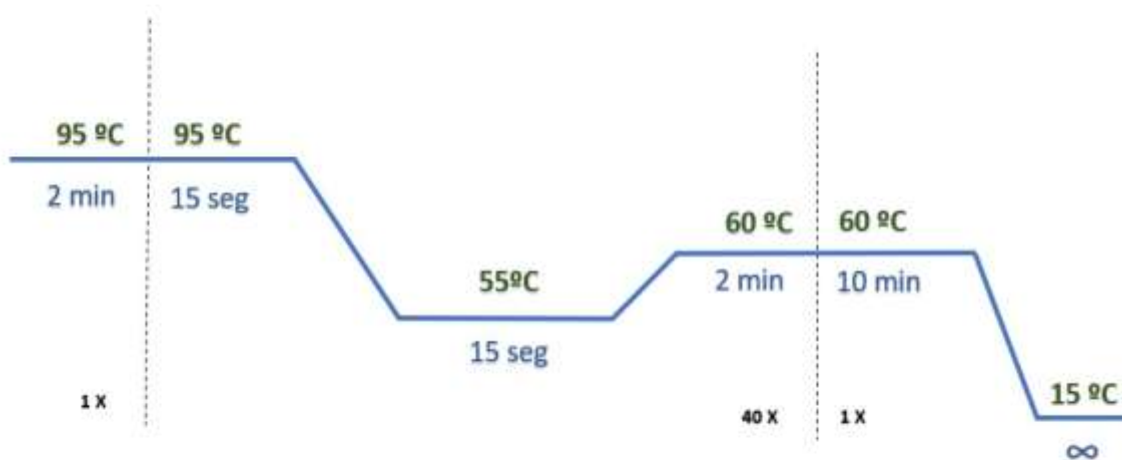


Figure 8 - Thermocycler conditions for the sequencing reaction.

3.11- Sanger sequencing products purification and precipitation

All products resulting from the sequencing reaction were purified using thin columns of Sephadex TM G-50 resin (GE Healthcare Life Sciences, Little Chalfont, UK), to eliminate all dNTPs and ddNTPs that were not incorporated into the DNA strands and that could interfere with the reading signal. The samples were loaded vertically on top of each column, and then centrifuged at 3200 rpm (1100G) for 4 minutes. 15 µL of Hi-Di™ Formamide (Applied Biosystems ®, California, USA) was added to each sample to keep the DNA denatured. All samples resulting from purification were analyzed by capillary electrophoresis using the Applied Biosystems 3130/3130xl Genetic Analyzers (California, USA). Products with suspected mutations were reassessed by performing additional individual Polymerase chain reaction.

3.12-Statistical Analysis

Statistical analysis was performed using STAT VIEW-J5.0 (SAS Institute, Cary, NC). The relationship between mutational status and clinicopathological characteristics was evaluated by Fisher's exact test, except for the mean age and thickness that were evaluated by ANOVA. The Freeman-Halton extension of the Fisher's exact test for two-

rows by three-columns and five-columns contingency tables was also used. Pearson's correlation coefficient was used to measure the correlation between the molecular status of the 3 genes. Survival data was evaluated with the Kaplan-Meier method and the log-rank test. The prognostic value of covariates, concerning DFS and OS, was assessed in univariate analyses using the Cox regression model. A p value < 0.05 was considered statistically significant.

4 – Results

4.1- Optimization

Before the molecular analysis, the optimization of the standard protocols for the multiplex PCR and individual PCR was performed.

4.1.1-Optimization of the multiplex PCR reaction

It was necessary to perform hybridization temperature optimizations, in order to increase the amplification efficient of the three genes tested. For this, a temperature gradient with different temperatures ranging between 55ºC and 63ºC was performed. After several attempts, the temperature that proved to be more efficient for amplification of the three genes at the same time was 62.4ºC (Figure 9).

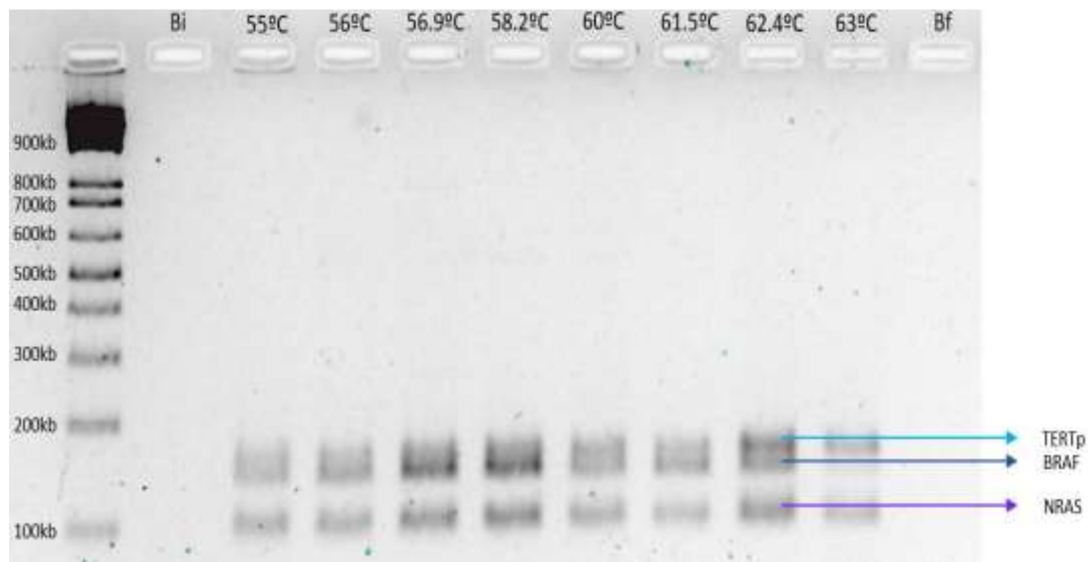


Figure 9 - Temperature gradient electrophoresis gel. The three genes analyzed in this study (*BRAF, NRAS* and *TERTp*) were visibly amplified at the hybridization temperature of 62.4ºC. At lower temperatures, *TERTp* is not amplified. We used two blank samples, without DNA (Bi and Bf) to ensure that the reagents were not contaminated, and that no contamination occurred during the procedure.

4.1.2- Optimization of the individual PCR reaction

For samples that did not amplify in the multiplex PCR, it was necessary to optimize the individual PCR for *BRAF, NRAS* and *TERTp*. For this, a gradient with two temperatures was performed, using 58ºC and 60ºC for *BRAF* and *NRAS* genes (Figure 10) and 62ºC and 64ºC for *TERT*p mutation (Figure 11), After the first attempt, the temperature that proved to be the most efficient for amplification of *BRAF* and *NRAS* was 58ºC and for *TERTp* was 62ºC.

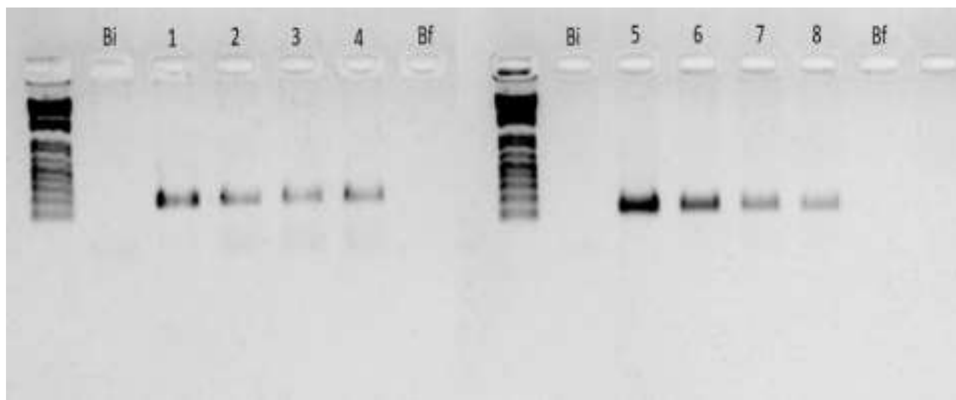


Figure 10 - Temperature gradient electrophoresis gel for *BRAF* and *NRAS* genes. The genes, *BRAF* (1-4) and *NRAS* (5-8) were visibly amplified at the hybridization temperature of 58°C. We used two blank samples, without DNA (Bi and Bf) to ensure that the reagents were not contaminated, and that no contamination occurred during the procedure. 1, 5 - thyroid control samples, 2-4 and 6-8 - cutaneous melanoma samples.

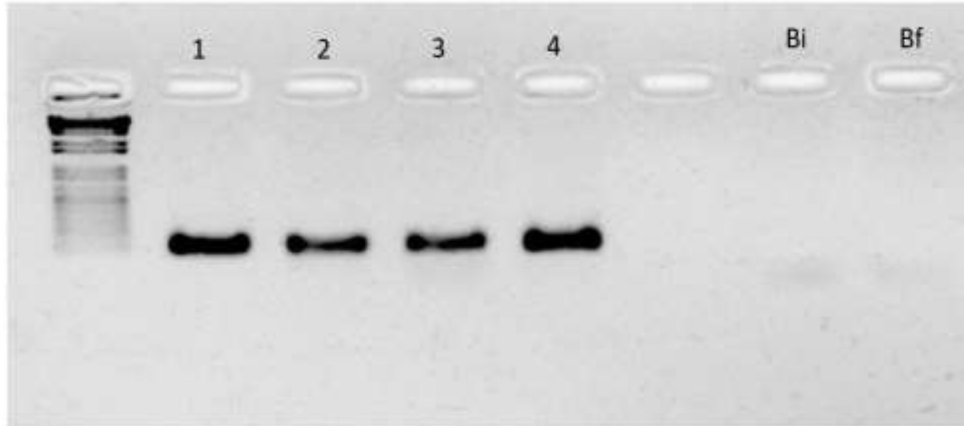


Figure 11 - Temperature gradient electrophoresis gel for *TERT* promotor. *TERTp*, was visibly amplified at the hybridization temperature of 60°C. We used two blank samples, without DNA (Bi and Bf) to ensure that the reagents were not contaminated, and that no contamination occurred during the procedure. 1- thyroid control sample, 2-4 cutaneous melanoma samples.

4.2- Database preparation

The clinical-pathological information of all patients in the study were obtained through clinical reports, provided by the clinicians responsible for the project at Hospital Distrital de Santarém and Hospital dos Capuchos. However, HC did not provide data on vascular invasion, the presence of microsatellites and follow-up, and therefore this data was only included for HDS patients. These cases were carefully evaluated and considered in the construction of the database and in the statistical analysis.

4.3- Molecular Analysis

4.3.1 - Clinicopathologic characteristics

Tumor samples from 128 patients were tested for *BRAF, NRAS* and *TERTp* mutational status.

Several clinicopathological parameters were evaluated, being the series constitute by 54% of male patients and 46% of female patients. The mean age of patients at diagnosis was 65 years, ranging from 22 to 97 years. The series had 64% of superficial spreading melanomas (SSM), 18% of nodular melanomas (NM), 15% of malignant acral melanomas (ALM), 2% of lentiginous malignant melanomas (LMM) and 1% of in situ melanomas. The majority of the tumors were located in the limbs (50%) and 9% in the head and neck. Only 53% of the patients are still alive (Table 4).

Table 4 - Clinicopathological characteristics of the studied series.

Clinicopathological characteristics	Total melanomas
Mean Age (±SD)	65 (16.6)
Gender [n (%)]	
Female	58 (46)
Male	69 (54)
Sample Distribution [n (%)]	
Primary Tumors	117 (91)
Recurrences	1 (1)
Metastases	4 (3)
Sentinel Lymph Nodes	6 (5)
Location [n (%)]	
Head and Neck	11 (9)
Upper and Lower Limbs	61 (50)
Trunk	49 (41)
Histological Subtype [n (%)]	
SSM	75 (64)
Nodular	21 (18)
ALM	18 (15)
LMM	2 (2)
In Situ	1 (1)
Clark Level [n (%)]	
I-III	36 (30)
IV-V	83 (70)
Breslow Index [n (%)]	
≤1mm	32 (28)
>1mm	84 (72)
Growth Stage [n (%)]	
Radial	29 (25)
Vertical	89 (75)
Ulceration [n (%)]	
Present	40 (34)
Absent	79 (66)
Sun exposure [n (%)]	
Chronic	11 (9)
Intermittent	90 (76)
No exposure	18 (15)

4.3.2- Genetic Alterations

4.3.2.1- *BRAF* gene analysis

Tumor samples from 120 patients were successfully amplified for *BRAF gene*. *BRAF* gene was mutated in 43% of the cases. Two alterations were identified. Of the 120 cases amplified for the *BRAF* mutation, 43 mutations (36%) were V600E: T>A and only 9 alterations (7%) were V600K: GT>AA substitutions (Figure 12).

Figure 12 - Illustration and distribution of the two mutational subtypes of the *BRAF* gene, found in melanoma human samples.

No significant correlations were found between *BRAF* status and the clinical-pathological characteristics of the patients. Although not statistically significant, it is possible to see a three years difference in the mean age at diagnosis when comparing *BRAF WT* and *BRAF* mutated tumors. Regarding gender, there is a predominance of males (29/65) when compared to female patients (23/54). Tumors with *BRAF* mutation were found more frequent in the trunk (23/47), being less frequent in the head/neck (4/10) and limbs (21/56). *BRAF* mutations were more frequent in superficial spreading melanoma (31/69), being less frequent in acral melanoma (5/17) (Table 6). *BRAF* mutations were also more frequently found in cases with higher Clark level (35/80) and a mitotic index bigger than 1 mm^2 (34/72) (Table 5, Figure 13).

Table 5 - Clinicopathological features and their relationship with *BRAF* mutational status.

Clinicopathological characteristics	Total melanomas	*BRAF* WT	*BRAF* Mutated	p- value
Number of cases (%)	**120**	**68 (57)**	**52 (43)**	**-**
Mean Age (± SD)	66 ± 16.6	68 ± 15.4	64 ± 17.4	0.26
Gender [n (%)]				
Female	54 (45)	31 (46)	23 (44)	0.85
male	65 (55)	36 (54)	29 (56)	
Localization [n (%)]				
Head and Neck	10 (9)	6 (9)	4 (8)	
Limbs	56 (49)	35 (54)	21 (44)	0.50
Trunk	47(42)	24 (37)	23 (48)	
Histological Subtype [n (%)]				
SSM	69 (63)	38 (60)	31 (67)	
Nodular	21 (19)	12 (19)	9 (20)	
ALM	17 (15)	12 (19)	5 (11)	0.64
LMM	2 (2)	1 (1)	1 (2)	
In Situ	1 (1)	1 (1)	0 (0)	
Clark Level				
I-III	31 (28)	18 (29)	13 (27)	>0.99
IV-V	80 (72)	45 (71)	35 (73)	
Breslow Index [n (%)]				
≤ 1 mm	26 (24)	12 (20)	14(30)	0.26
> 1 mm	82 (76)	49 (80)	33 (70)	
Growth Stage [n (%)]				
Radial	23 (20)	9 (15)	14 (29)	0.10
Vertical	87 (80)	53 (85)	34 (71)	
Ulceration [n (%)]				
Absent	71 (64)	40 (63)	31 (65)	>0.99
Present	40 (36)	23 (17)	17 (35)	
Mitotic Index [n (%)]				
< 1 mm^2	32 (29)	19 (30)	13 (28)	0.83
≥ 1 mm^2	75 (71)	44 (70)	34 (72)	
Lymphoid Infiltrate [n (%)]				
Absent	20 (18)	12 (19)	8 (17)	0.80
Present	90 (82)	50 (81)	40 (83)	

Tumour Regression [n (%)]				
Absent	90 (81)	55 (87)	35 (73)	**0.09**
Present	21 (19)	8 (13)	13 (27)	
Vascular Invasion [n (%)]				
Absent	37 (90)	24 (92)	13 (87)	0.61
Present	4 (10)	2 (8)	2 (13)	
Perivascular Invasion [n (%)]				
Absent	95 (86)	54 (87)	41 (85)	>0.99
Present	15 (14)	8 (13)	7 (15)	
Perineural Invasion [n (%)]				
Absent	100 (91)	57 (92)	43 (90)	0.74
Present	10 (9)	5 (8)	5 (10)	
Microsatellites [n (%)]				
Absent	43 (93)	25 (89)	18 (100)	0.27
Present	3 (7)	3 (11)	0 ()	
pTNM Stage [n (%)]				
pT1/2	57 (51)	32 (50)	25 (52)	0.85
pT3/4	55 (49)	32(50)	23 (48)	
Sun exposure [n (%)]				
Chronic	10 (9)	6 (9)	4 (9)	
Intermittent	84 (76)	46 (72)	38 (81)	0.49
No exposure	17 (15)	12 (19)	5 (10)	

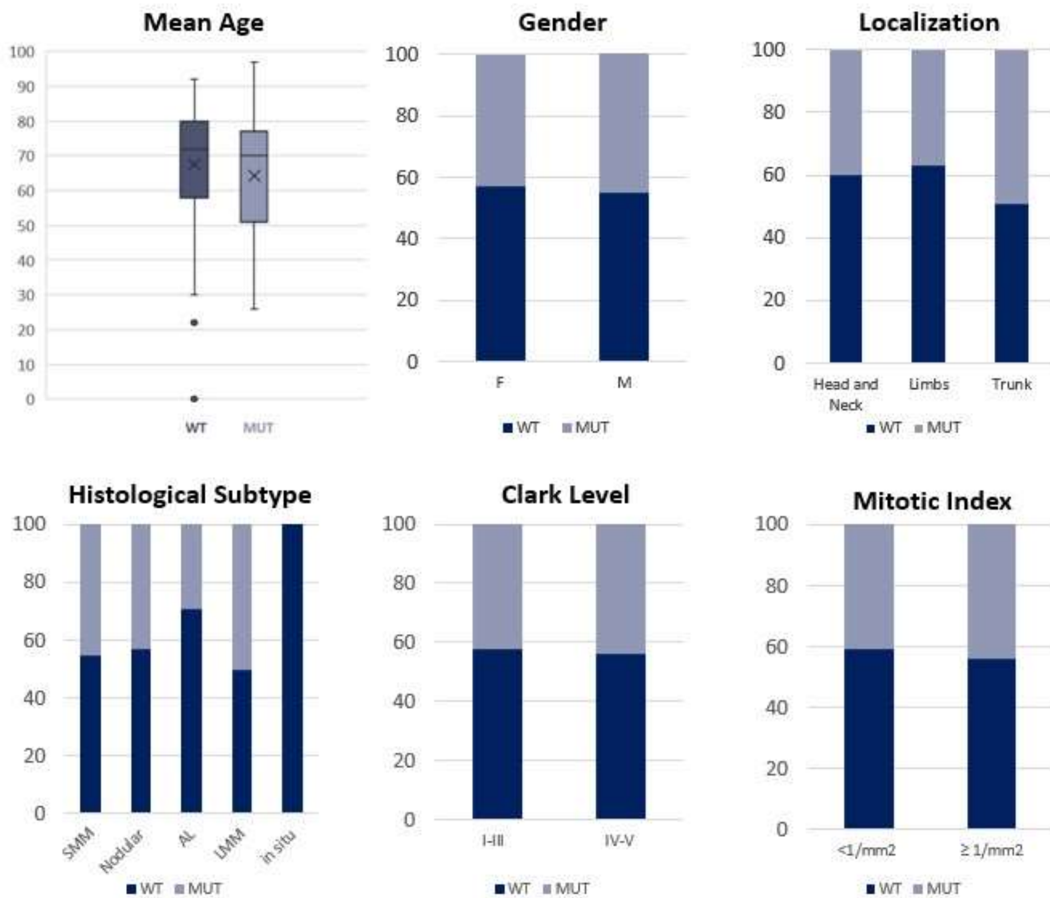


Figure 13 - Graphic representation of *BRAF* mutational status according to mean age, gender, localization, histological subtype, Clark level, and mitotic index.

The correlation between the presence of *BRAF* mutations and overall survival was assessed by Kaplan-Meier curves (Figure 14). Although not statically significant, patients with *BRAF* mutated tumors were found to have a higher OS when compared with patients with *BRAF* non-mutated tumors (91 months, SE ± 23.93, range 44 - 138 vs 76 months, SE ± 9, range 57 – 95, p=0.94).

This association was further tested with univariate Cox regression analysis (Table 8). A HR = 1.02 (95% CI (0.5- 2.3) and p= 0.95) was detected for *BRAF* gene. 61 % of the patients with tumors harboring these mutations died from melanoma, and 39% of the patients with *BRAF* non-mutated tumors had a related death.

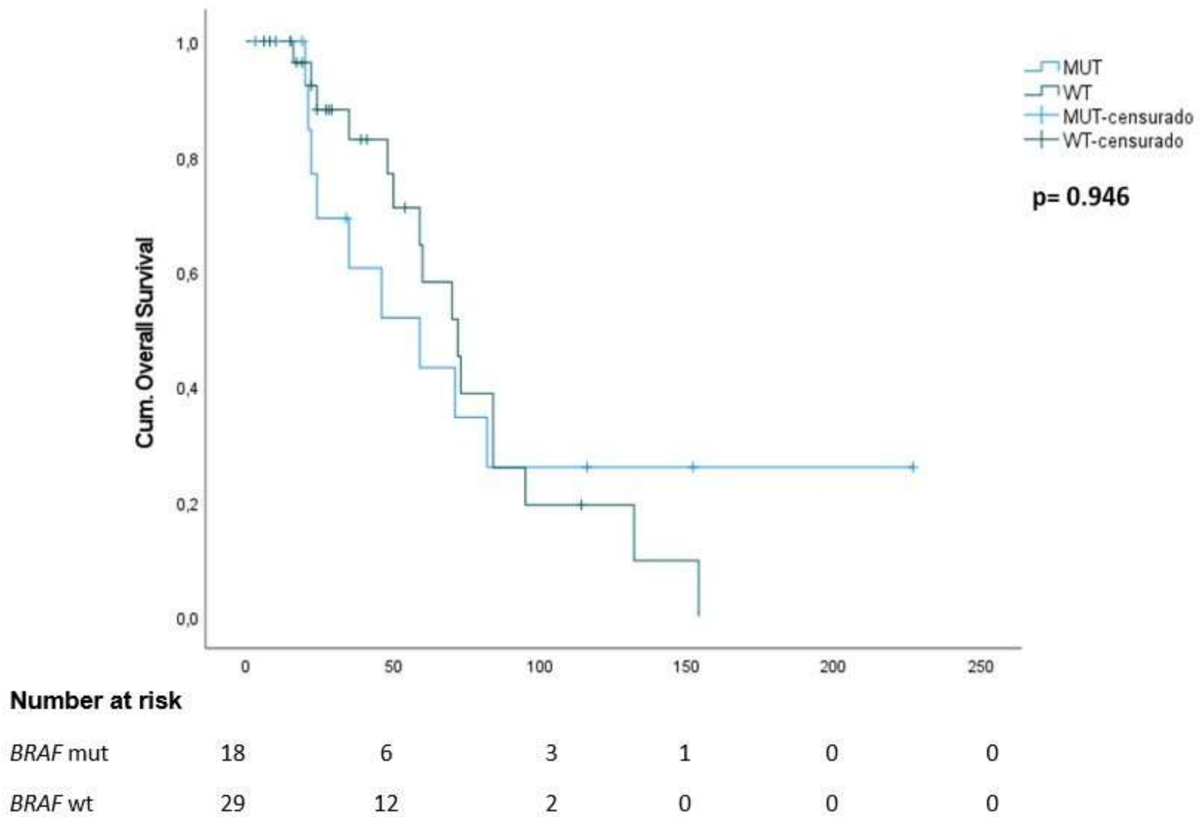


Figure 14 - Kaplan- Meier curves for overall survival of cutaneous melanoma patients. Kaplan -Meier curves evaluating the correlation between the presence of BRAF mutations and overall survival of melanoma patients. BRAF mutated cases (blue line) and BRAF wilt ty pe cases (green line).

4.3.2.2- *NRAS* gene analysis

Tumor samples from 125 patients were successfully amplified for *NRAS. NRAS* was mutated in 18% of the cases. It was observed that 21 alterations (17%) were Q61R: A>G and only 1 alteration (1%) was Q61L: A>T substitution (Figure 15).

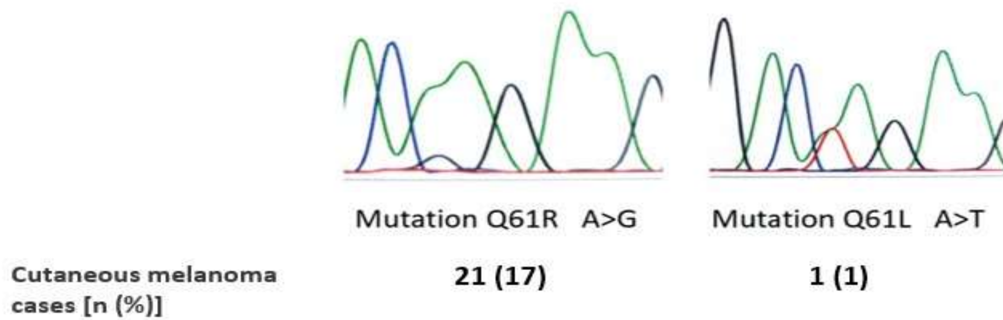


Figure 15 - Illustration and distribution of the two mutational subtypes of *NRAS* gene, found in melanoma human samples.

The status of the *NRAS* gene was evaluated and it was observed that tumors with *NRAS* mutation have low Breslow index (p=0.01). *NRAS* mutated melanomas also have lower mitotic index (12/35), when compared to the *NRAS* WT melanomas (8/80) (p= <0.01) (Table 6).

Although not statically significant, the mean age at diagnosis for tumors with NRAS mutation has a higher value (67 ± 15.8) when compared to *NRAS* WT patients (65 ± 16.9) (Table 6). Females patients (10/58) tend to have more mutations when compared with male patients (12/66). CM with *NRAS* mutation were found more frequent in trunk (9/47) and are less frequent in the limbs (9/60) and head/neck (2/11). Mutations were found more frequent in acral melanoma (4/17), being less frequent in nodular melanoma (1/21). *NRAS* mutations were more frequently detected in melanomas with lower Clark level (10/36) (p=0.06) and in radial growth phase (6/27) (Table 6). Most *NRAS* mutation tumors do not present perivascular invasion (19/100) and perineural invasion (20/105).

Table 6 - Clinicopathological features and their relationship with *NRAS* mutational status.

Clinicopathological Characteristics	Total melanoma	*NRAS* WT	*NRAS* Mutated	p- value
Number of cases (%)	**125**	103 (82)	22 (18)	**-**
Mean Age (± SD)	65 ± 16.6	65 ± 16.9	67 ± 15.8	0.49
Gender [n (%)]				
Female	58 (47)	48 (47)	10 (45)	>0.99
male	66 (53)	54 (53)	12 (55)	
Localization [n (%)]				
Head and Neck	11 (9)	9 (9)	2 (10)	
Limbs	60 (51)	51 (52)	9 (45)	0.85
Trunk	47 (40)	38 (39)	9 (45)	
Histological Subtype [n (%)]				
SSM	73 (64)	61(64)	12(66)	
Nodular	21 (18)	20 (20)	1 (6)	
ALM	17 (15)	13 (14)	4 (22)	0.30
LMM	2 (2)	1 (1)	1 (6)	
In Situ	1 (1)	1 (1)	0 (0)	
Clark Level [n (%)]				
I-III	36 (31)	26 (27)	10 (50)	**0.06**
IV-V	80 (69)	70 (73)	10 (50)	
Breslow Index [n (%)]				
≤ 1 mm	30 (27)	20 (22)	10 (50)	**0.01**
> 1 mm	83 (73)	73 (78)	10 (50)	
Growth Stage [n (%)]				
Radial	27 (23)	21 (22)	6 (30)	0.56
Vertical	74 (77)	74 (78)	14 (70)	
Ulceration [n (%)]				
Absent	76 (66)	61 (64)	15 (75)	0.44
Present	35 (34)	35 (36)	5 (25)	
Mitotic Index [n (%)]				
< 1 mm^2	35 (30)	23 (24)	12 (60)	**<0.01**
≥ 1mm^2	80(70)	72 (76)	8 (40)	
Lymphoid Infiltrate [n (%)]				
Absent	20 (17)	16 (17)	4 (20)	0.75
Present	95 (83)	79 (83)	16 (80)	
Tumour Regression [n (%)]				
Absent	94 (81)	78 (81)	16 (80)	>0.99
Present	22 (19)	18 (19)	4 (20)	
Vascular Invasion [n (%)]				

Absent	39 (91)	32 (91)	7 (88)	>0.99
Present	4 (9)	3 (9)	1 (12)	
Perivascular Invasion [n (%)]				
Absent	100 (87)	81 (85)	19 (95)	0.46
Present	15 (13)	14 (15)	1 (5)	
Perineural Invasion [n (%)]				
Absent	105 (91)	85 (89)	20 (100)	0.21
Present	10 (9)	10 (11)	0 (0)	
Microsatellites [n (%)]				
Absent	45 (94)	37 (95)	8 (89)	0.47
present	3 (6)	2 (5)	1 (11)	
pTNM Stage [n (%)]				
pT1/2	62 (47)	48 (50)	14 (67)	0.23
pT3/4	55 (53)	48 (50)	7 (33)	
Sun exposure [n (%)]				
Chronic	11 (9)	8 (8)	3 (14)	
Intermittent	88 (76)	74 (78)	14 (67)	0.50
No exposure	17 (15)	13 (14)	4 (19)	

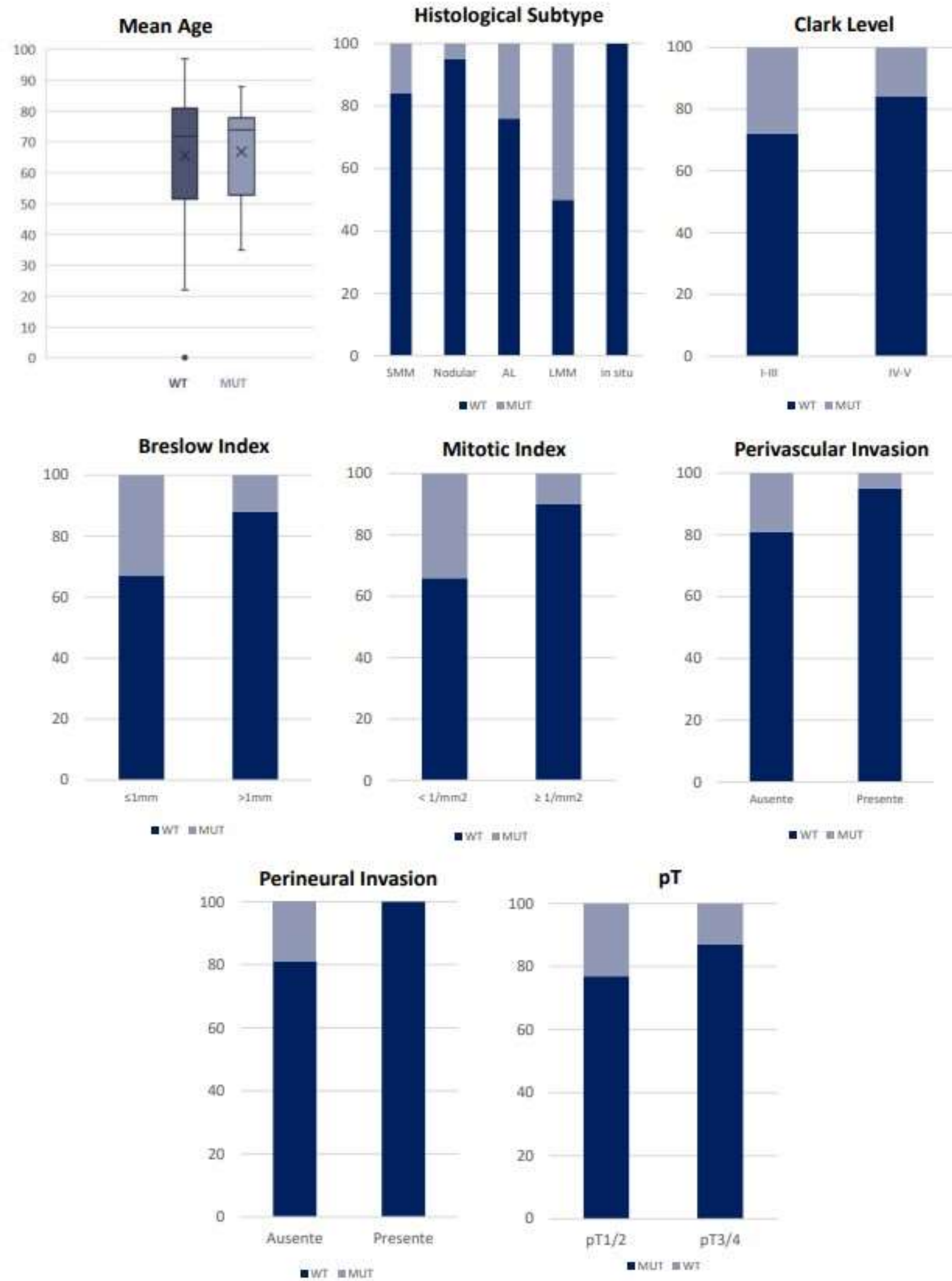


Figure 16 - Graphic representation of *NRAS* mutational status according to mean age, histological subtype,Clark level, Breslow index, mitotic index, perivascular invasion, perineural invasion and pT.

The correlation between the presence of *NRAS* mutations and overall survival was assessed by Kaplan-Meier curves (Figure 17). Although not statically significant, patients with *NRAS* mutated tumors were found to have reduce OS when compared with patients WT (79 months, SE ± 13.90, range 52 - 106 vs 85 months, SE ± 13, range 60 – 110, p=0.91).

This association was further tested with univariate Cox regression analysis (Table 8). A HR = 1.05 (95% CI (0.5- 2.6), p= 0.91). was detected for *NRAS*. 78% of the patients harboring these mutations died from melanoma, and 22% of the patients with *NRAS* WT tumors had a related death.

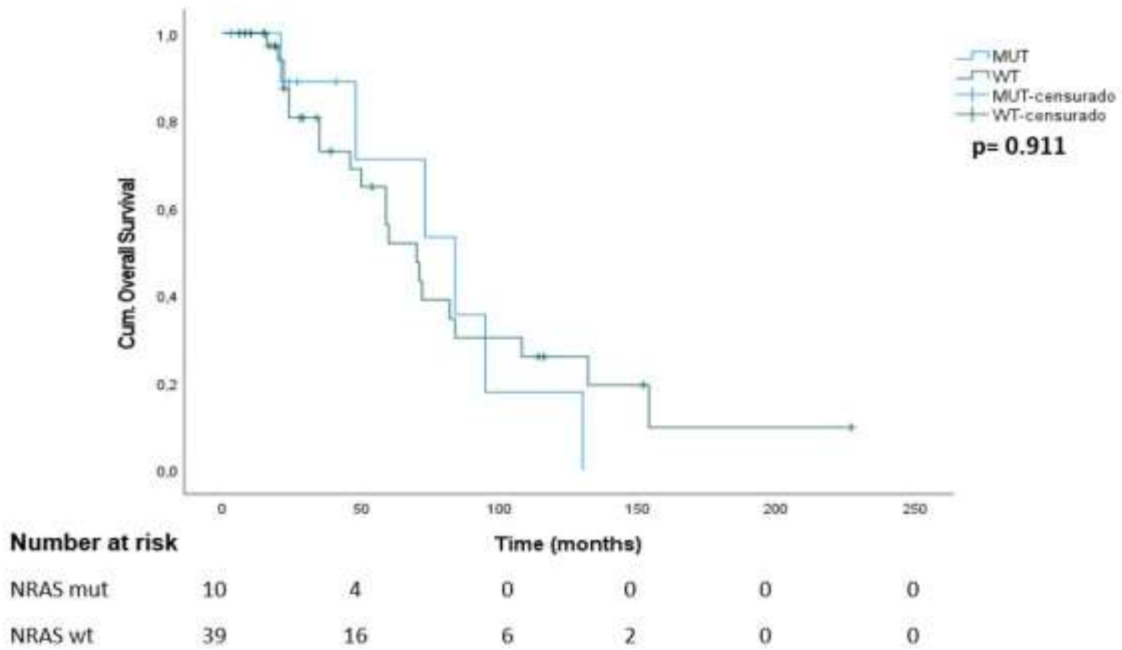


Figure 17 - Kaplan- Meier curves for overall survival of cutaneous melanoma patients. Kaplan -Meier curves evaluating the correlation between the presence of *NRAS* mutations and overall survival of melanoma patients. *NRAS* mutated cases (blue line) and *NRAS* wilt type cases (green line).

4.3.2.1- *TERT* promoter analysis

Tumor samples from 99 patients were successfully amplified for *TERTp*. *TERTp* was mutated in 30% of the cases. was observed that 11 alterations (11%) were 124: G>A, 2 (2%) were -124/-125: GG>AA and 17 changes (17%) were -146: G>A substitutions (Figure 18).

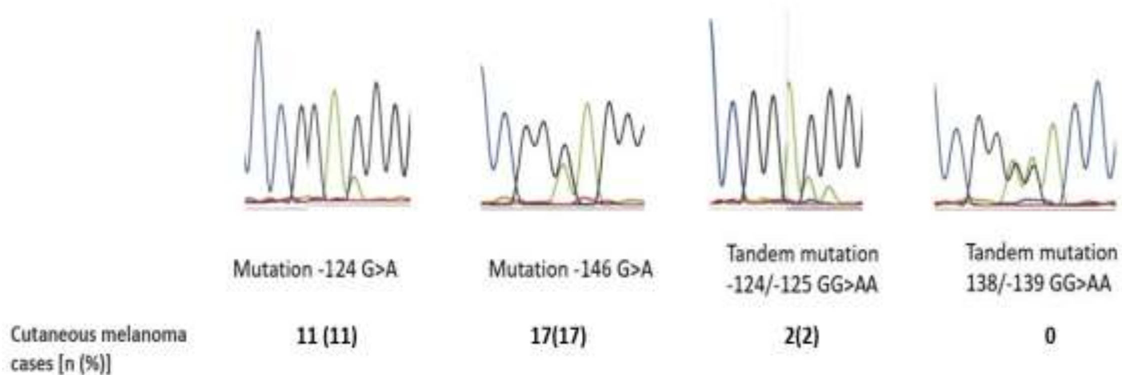


Figure 18 - Illustration and distribution of the four mutational subtypes of *TERT* promoter found in melanoma human samples.

Although not statistically significant, the mean age at diagnosis for tumors with *TERTp* mutation was lower (63.5 ± 20.0) when compared to WT patients (67.9 ± 15.7). Female patients (14/44) have more mutations when compared to male patients (15/54) (Table 5). *TERTp* mutations were found more frequent in superficial spreading subtype (20/59) and nodular melanoma (5/18), being rare in acral lentiginous melanomas (1/12). *TERTp* mutations were found more frequent in melanomas with higher Clark level (24/68), ulceration (15/38) and higher mitotic rate (22/64) (Table 7, Figure 19).

Table 7 - Clinicopathological features and their relationship with *TERTp* mutational status.

Clinicopathological characteristics	**Total melanomas**	***TERT*p WT**	***TERT*p Mutated**	**p- value**
Number of cases (%)	**99**	**69 (70)**	**30 (30)**	**-**
Mean Age (± SD)	66 ± 16.6	68 ± 15.7	64 ± 20.0	0.25
Gender [n (%)]				
Female	44 (45)	30 (43)	14 (48)	0.82
Male	54 (55)	39 (57)	15 (52)	
Location [n (%)]				
Head and Neck	10 (11)	7 (11)	3 (11)	
Limbs	44 (48)	31 (48)	13 (48)	0.99
Trunk	38(41)	27 (41)	11 (41)	
Histological Subtype [n (%)]				
SSM	59(65)	39 (61)	20 (74)	
Nodular	18(20)	13 (20)	5 (18)	
ALM	12(13)	11 (17)	1 (4)	0.14
LMM	1(1)	0 (0)	1 (4)	
In Situ	1(1)	1 (2)	0 (0)	
Clark Level [n (%)]				
I-III	24(26)	18 (29)	6 (20)	0.45
IV-V	68(74)	44 (71)	24 (80)	
Breslow Index [n (%)]				
≤ 1 mm	21(23)	13 (22)	8 (27)	0.61
> 1 mm	69(77)	47 (78)	22 (73)	
Growth Phase [n (%)]				
Radial	17 (19)	10 (16)	7 (23)	0.57
Vertical	74 (81)	51 (84)	23 (77)	
Ulceration [n (%)]				
Absent	54 (59)	39 (63)	15 (50)	0.26
Present	38 (41)	23 (37)	15 (50)	
Mitotic Index [n (%)]				
< 1 mm^2	26 (29)	18 (30)	8 (27)	0.80
≥ 1 mm^2	64 (71)	42 (70)	22 (73)	
Lymphoid Infiltrate [n (%)]				
Absent	16 (17)	11 (18)	5 (17)	>0.99
Present	76 (83)	51 (82)	25 (83)	
Tumor Regression [n (%)]				
Absent	75 (82)	48 (77)	27 (90)	0.17
Present	17 (18)	14 (23)	3 (10)	
Vascular Invasion [n (%)]				

Absent	35 (90)	12 (86)	23 (92)	0.60
Present	4 (10)	2 (14)	2 (8)	
Perivascular Invasion [n (%)]				
Absent	78 (86)	52 (85)	26 (87)	>0.99
Present	13 (14)	9 (15)	4 (13)	
Perineural Invasion [n (%)]				
Absent	83 (91)	56 (92)	27 (90)	>0.99
Present	8 (9)	5 (8)	3 (10)	
Microsatellites [n (%)]				
Absent	40 (93)	24 (89)	16 (100)	0.28
present	3 (7)	3 (11)	0 (0)	
pTNM Stage [n (%)]				
pT1/2	44 (47)	30 (48)	14 (47)	>0.99
pT3/4	49 (53)	3 (52)	16 (53)	
Sun exposure [n (%)]				
Chronic	10 (11)	6 (10)	4 (13)	
Intermittent	71 (76)	46 (73)	25 (83)	0.16
No exposure	12 (13)	11 (17)	1 (4)	

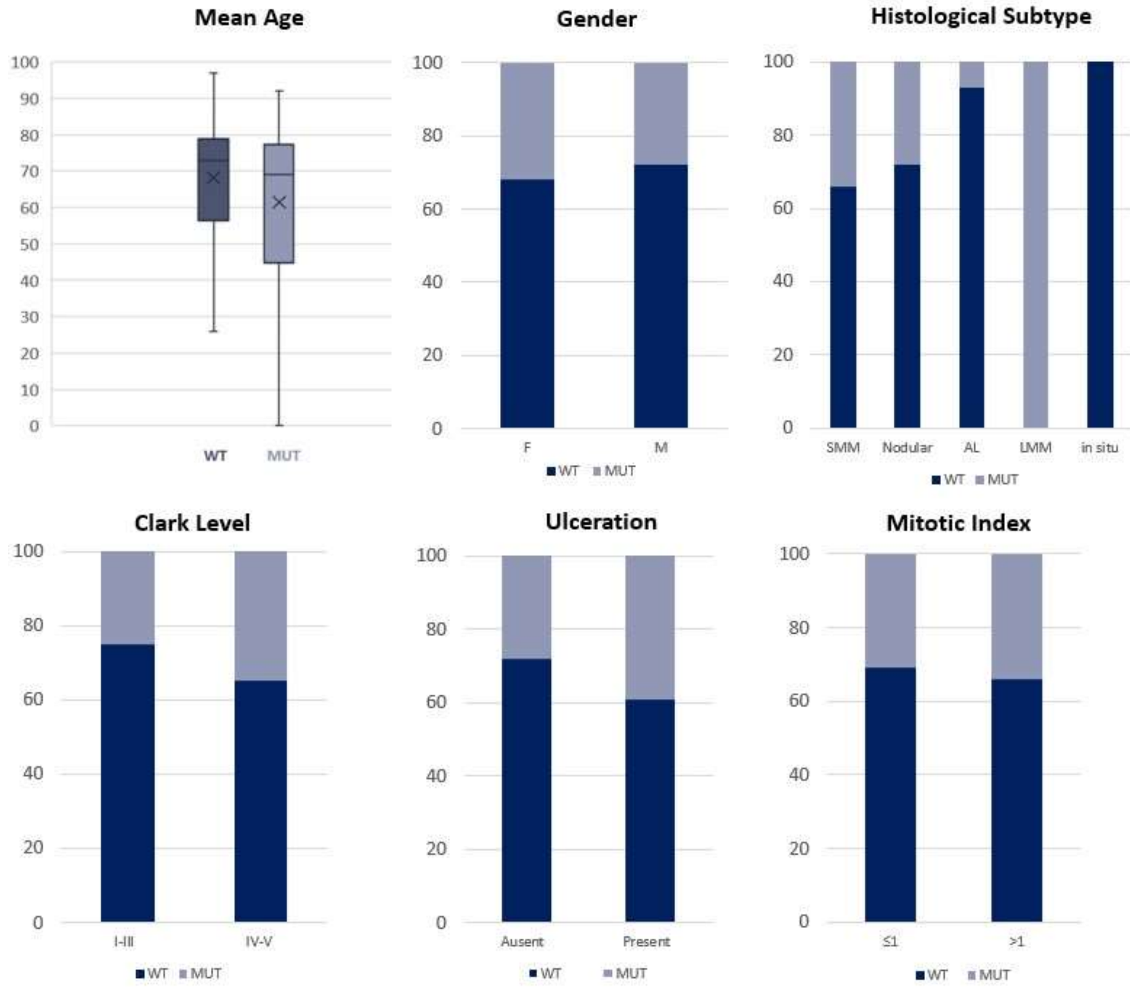
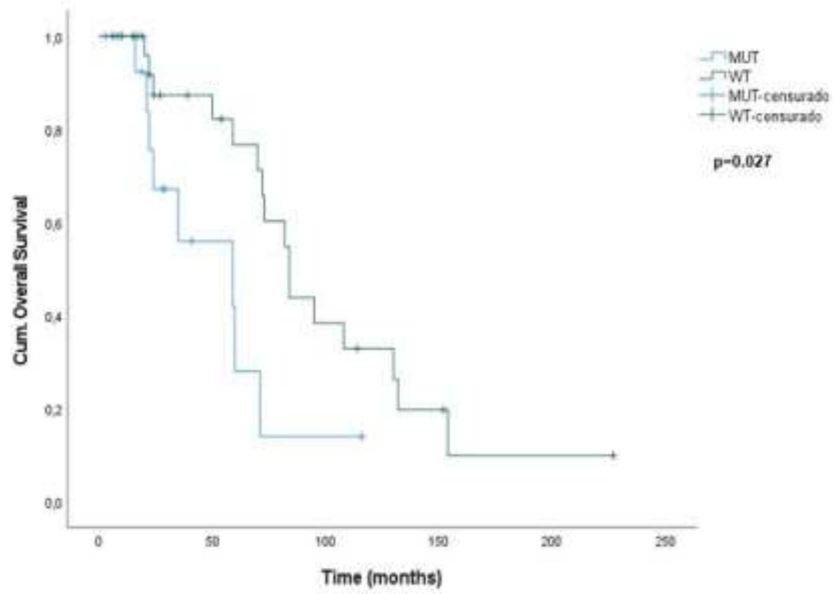


Figure 19 - Graphic representation of *TERTp* mutational status according to mean age, gender, histological subtype, Clark level, ulceration, and mitotic index.

The correlation between the presence of *TERTp* mutations and overall survival was assessed by Kaplan-Meier curves (Figure 20). Patients with *TERTp* mutated tumors were found to have reduce OS when compared with patients with *TERTp* non-mutated tumors (54 months, SE ± 10.18, range 34 - 74 vs 100 months, SE ± 13, range 74 – 126, p= 0.03).

This association was further tested with univariate Cox regression analysis, which also included other classical prognostic features, such as Breslow index, ulceration, mitotic index and pTNM (Table 8). The higher HR was detected for the molecular status of *TERTp* (HR = 2.7, 95% CI (1- 7), p= 0.03). 68% of the patients with tumors harboring these mutations died from melanoma, and only 32% of the patients with WT tumours had a related death.



Number at risk

TERT mut	14	3	1	0	0	0
TERT wt	31	16	6	2	0	0

Figure 20 - Kaplan- Meier curves for overall survival of cutaneous melanoma patients. Kaplan -Meier curves evaluating the correlation between the presence of *TERT* promoter mutations and overall survival of melanoma patients. *TERT* mutated cases (blue line) and *TERT* wild type cases (green line).

Table 8 - Overall survival univariate Cox regression analysis.

Clinicopathological features	Number of events (%)	HR	95% CI	p-value
Breslow Index				
≤ 1mm	11 (28)	1.24	0.46 – 3.30	0.66
>1 mm	28 (72)			
Ulceration				
Absent	20 (49)	0.48	0.17 – 1.30	0.15
Present	21 (51)			
Mitotic Index				
< 1/mm^2	11 (27)	0.74	0.30 – 1.84	0.52
≥ 1/mm^2	30 (73)			
pTNM				
pT1/2	16 (38)	0.85	0.36 – 1.99	0.70
pT3/4	26 (62)			
BRAF Status				
WT	19 (39)	1.02	0.45 – 2.33	0.95
Mutated	30 (61)			
NRAS Status				
WT	11 (22)	1.05	0.41 – 2.65	0.91
Mutated	40 (78)			
TERTp Status				
WT	15 (32)	2.70	1.08 – 6.80	**0.03**
Mutated	32 (68)			

4.3.2.4- Relationship between the mutational status of *TERT* promoter, *BRAF* and *NRAS* genes

Fourteen samples were found to be mutated simultaneously for *TERTp* and *BRAF* genes (positive correlation, p= <0.01), and 5 samples were found to be mutated simultaneously for *TERTp* and *NRAS* genes (negative correlation, not significant) (Table 9).

Table 9 - Pearson's Correlation Analysis. The correlation is positive if the value is between 0 - 1, and negative if the value is less than -1. When the value is equal to 1, it indicates that the correlation is perfect between the two variables.

	Pearson's Correlation	P-value
mutTERT – mutBRAF	0.421	**0.004**
mutTERT - mutNRAS	-0.021	0.887
mutNRAS – mutBRAF	-0.091	0.533

Moreover, comparing the median survival time, patients with *TERTp* mutated tumors (54 months) presented the lowest survival, when compared with patients with *NRAS* mutated tumors (79 months), and the highest median survival time was observed in patients with *BRAF* mutated tumors (91 months) (Figure 21).

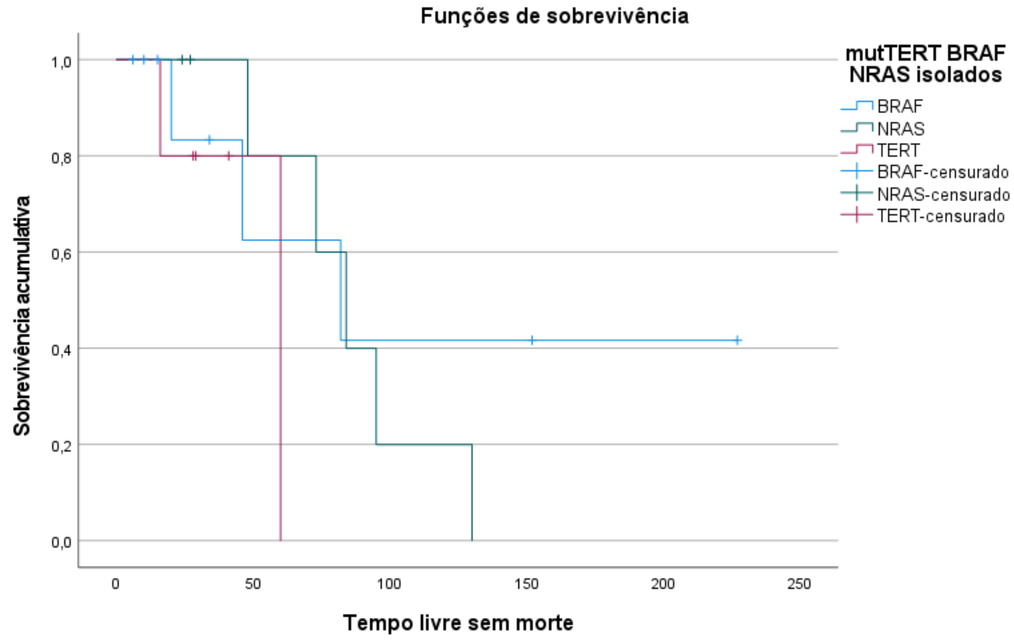


Figure 21 - Kaplan- Meier curves for overall survival of cutaneous melanoma patients. Kaplan -Meier curves evaluating the correlation between the presence of *BRAF, NRAS* and *TERT* promoter mutations and overall survival of melanoma patients. *BRAF* mutated cases (blue line), NRAS mutated cases (green line) and *TERT* mutated cases (red line).

5- Discussion

In this study, we evaluated and correlated the mutational status of *BRAF* and *NRAS* genes and *TERTp, with* the clinicopathological characteristics.

Correlating the presence of MAPK pathway mutations and the clinicopathological characteristics of patients with melanoma, some trends were verified in relation to the presence of the mutations in the *BRAF* and *NRAS* genes, which are the two most relevant genes in the pathway. Mutations belonging to this signaling pathway have frequently appeared in cutaneous melanoma. Over the past few years, *BRAF* and *NRAS* mutations have shown a high frequency in cutaneous melanoma [78] however, there is still not much information about the relationship between the two and the proliferation of tumor cells or progression of cutaneous melanomas. In our study, 43% of the cases presented *BRAF* mutations in exon 15. The mutational frequency of *BRAF* gene mutations found in our study was similar to previously published studies, with rates ranging from 30% to 60% [68, 80]. *NRAS* mutations were detected in 18% of the studied cutaneous melanomas. A mutational rate between 15% and 20% was reported in previous studies [108], which is in agreement with the data of our work.

With regard to gender, although the values were not significant, there were more *BRAF* mutated tumors in male patients and *NRAS* in female patients. Similar to previously published studies, we found that *BRAF* mutated cutaneous melanomas were more frequently found in the trunk [97] and *NRAS* mutated melanomas were associated with old age and acral-subtype patients [124].

In our study, it is possible to observe that melanoma with *BRAF* mutation tend to have a higher level of Clark and higher mitotic index, as already described by other authors, that suggested an association between the presence of BRAF mutations and poor prognosis [78, 125]. In contrast, *NRAS* mutated melanomas seem to have low Breslow level, low Clark level and low mitotic index, presenting in this way characteristic that are associated with a good prognosis. This is not in agreement with previously published studies, which demonstrate that cutaneous melanomas with *NRAS* mutations have a high Breslow level [96].

In contradiction, patients with the *BRAF* mutation have a longer survival, and in patients with the *NRAS* mutation the survival values are approximately the same

comparing with patients without the mutation. Our results are not in agreement with previous studies, that associated the presence of *BRAF* mutations with a shorter survival [126]. Previous studies analyzing the survival of patients with NRAS mutations reported discordant results. A published study found no difference between the survival of patients with NRAS mutations and NRAS WT patients [97]. Another study reported a lower survival for patients with NRAS mutation [96].

Numerous studies mention the presence of sequence variations of the *TERT* promoter in human malignant tumors, with somatic mutations being frequent in several cancers that arise in tissues with low rates of self-renewal, evidencing their importance in human carcinogenesis [116]. Mutations in the *TERT* promoter have been frequently found in nervous system tumors [106, 117], hepatocellular carcinoma [106], bladder cancer [118], thyroid cancer [119], skin cancer [104, 109, 117, 120], among others [121]. In the present series, *TERTp* was mutated in 30% of the analyzed cases. Concordantly, other studies reveal different rates of *TERTp* mutation in cutaneous melanoma, ranging from 29%- 71% [68, 103].

Heidenreich *et al.*, studied the molecular status of the *TERTp* promoter in 287 primary melanoma tumors and found a mutation frequency of 38% and correlation between the presence of the mutation and parameters such as thickness, ulceration and the presence of regional and distant metastases [122]. In this study there was no statistically significant association found between *TERTp* promoter status and clinical-pathological characteristics such as age, gender, and tumor location. Similar results were obtained on previous studies that also found no significant correlation between mutations and different characteristics [109]. Although it does not present significant values, in our study *TERT* mutations tend to appear more frequently in women, at a lower mean age and are often of the SSM and NM subtypes.

In addition, in our study, *TERTp* mutations were found more frequent in melanomas with higher Clark level, the presence of ulceration and higher mitotic rate. Regarding, clinicopathological features, *TERTp* mutations were previously associated with increase thickness and mitotic rate, which are well-established features of worse prognostic [109, 120]. Moreover, Populo *et al.*, also reported correlation between the presence of *TERT* promoter mutations and increased tumor thickness, ulceration and mitotic index, demonstrating that these markers may play an important role in the clinical prognostics of melanoma [109].

Through Kaplan-Meier and Cox regression analysis, we observed that patients with cutaneous melanoma with *TERT* promoter mutations have a significantly reduced

OS when compared with patients with *TERT*p non-mutated tumors, which is in agreement with previous studies that associate the presence of *TERT*p mutation with low overall survival [103, 123].

It is important to emphasize that the series under study has a bias as it is constituted mainly by aggressive cutaneous melanomas.

TERTp mutations were significantly associated with the presence of *BRAF* mutations, as described in other studies [109, 127]. Although we could not compare cases with only one mutation and cases with two mutations, because of the small number of samples, the simultaneous occurrence of *TERT* and *BRAF* mutations in cutaneous melanoma has been reported in previous studies to be associated with worse prognosis and reduced survival [103, 124, 128].

6- Conclusion

One of the key factors in reducing mortality from cutaneous melanoma is the early detection that is very important to act faster with a more localized treatment. However, cutaneous melanoma has advantage over other types of cancer, due to it is location that allows early detection through non-invasive approaches.

The overall aim of this project was to study of genetic alterations found in patients with melanomas and correlate them with the clinical and pathological data of the patients. For that, we created a detailed database, including data from two hospitals, Hospital Distrital de Santarém and Hospital dos Capuchos, where it was possible to perform a comparative analysis. It is important to highlight that this is a retrospective molecular analysis and a series mainly constitute with aggressive cutaneous melanoma, which may result in bias in the analysis.

In our study it was possible to confirm a high rate of *BRAF* mutations, followed by *TERT* promotor and *NRAS* mutations in cutaneous melanoma. It was also found that the presence of *TERTp* mutations associated with low patients' survival. In contrast, the presence of *NRAS* mutations seemed to have an association with good prognostic features. In addition, in our work, we observed that mutations of *TERTp* and *BRAF* genes tend to occur simultaneously.

As science and technology advance, biomarkers are essential tools in disease management, thus providing crucial information about tumor aggressiveness and the response to therapies. In a future perspective, more research can be focused on choosing more exact criteria to assess the mutational status of *TERTp*, as this mutation may be associated with poor prognosis. The search can be extended to other mutational status of more genes. This may lead to the identification of new and more effective biomarkers in cutaneous melanoma.

7- References

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8- Appendices

8.1- Thesis results presented in an oral communication at IJUP meeting

Certifica-se que **Luísa Gonçalves** participou no **IJUP'22 – 15º Encontro de Investigação Jovem da Universidade do Porto**, que decorreu nos dias 4, 5 e 6 de maio de 2022, tendo apresentado a comunicação oral com o título ***"Genetic profiling of primary and metastatic cutaneous melanoma"***.

8.2- Thesis results presented in a poster at Porto Cancer Meeting

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PORTO CANCER MEETING
12-13 MAY 2022

Certificate

This is to certify that
Luísa Martins Gonçalves
attended to the 28th Porto Cancer Meeting - Extracellular Vesicles, Cell Communication and Cancer: Time to Prick the Bubble held on 12-13 May 2022 at i3S - Instituto de Investigação e Inovação em Saúde in Porto, Portugal, and online.

André Albergaria | Jorge Lima | Sónia Melo