




# Possible therapies to overcome resistance to MAPK inhibitors in mutant *BRAF* melanoma

Beatriz Barbosa Domingues

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# Possible therapies to overcome resistance to MAPK inhibitors in mutant *BRAF* melanoma

# Possíveis terapias para superar a resistência a inibidores da via MAPK em melanomas com mutações no gene *BRAF*

Beatriz Barbosa Domingues
MSc in Cell and Molecular Biology
Departament of Biology, Faculty of Sciences, University of Porto,
Rua do Campo Alegre 1021/1055, 4169-007 Porto
E-mail: up201602950@fc.up.pt

**Supervisor**
Helena Pópulo, PhD
Assistant Researcher of the Cancer Signalling and Metabolism group at Institute of Pathology and Immunology of the University of Porto/ Institute of Research and Innovation in Health of the University of Porto (IPATIMUP/i3S)
Rua Alfredo Allen, 208 | 4200-135 Porto, Portugal, Telf: +351 220 408 800
Affiliated professor at Faculty of Medicine of the University of Porto
Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal, Telf: 22 551 3600
E-mail: hpopulo@ipatimup.pt

**Co-supervisor**
Paula Soares, MSc, PhD
Assistant professor at Faculty of Medicine of the University of Porto
Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal, Telf: 22 551 3600
Group leader of the Cancer Signalling and Metabolism group at Institute of Pathology and Immunology of the University of Porto/ Institute of Research and Innovation in Health of the University of Porto (IPATIMUP/i3S)
Senior Investigator at IPATIMUP/i3S
Rua Alfredo Allen, 208 | 4200-135 Porto, Portugal, Telf: +351 220 408 800
E-mail: psoares@ipatimup.pt

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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Eu, Beatriz Barbosa Domingues, com o número up201602950, do Mestrado de Biologia Celular e Molecular da edição de 2016/2017, declaro por minha honra que sou a autora da totalidade do texto apresentado, não apresento texto plagiado, e tomei conhecimento das consequências de uma situação de plágio.

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*"O que distingue as pessoas é a sua capacidade de aprender."*

*Professor Manuel Sobrinho Simões*

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# Resumo

O melanoma representa apenas 1% de todos os tumores malignos da pele, sendo, no entanto, o cancro de pele mais letal. As atuais terapias para este tipo de tumor incluem cirurgia, quimioterapia, imunoterapias e terapias dirigidas, contudo, o melanoma continua a ser um dos cancros que está associado à maior perda de anos de vida.

O melanoma é a neoplasia maligna com o maior índice de mutações e a principal causa é a exposição à radiação ultravioleta. Considerando as mutações genéticas que originam esta doença, alguns fármacos foram desenvolvidos e aprovados para o tratamento de melanoma, como o vemurafenib, um inibidor oral de BRAF, usado para o tratamento de melanomas com a mutação $BRAF^{V600}$, com metástases ou sem possibilidade de resseção. Em resposta ao fármaco, esses pacientes podem sofrer alterações nas células de melanoma, o que origina mecanismos de resistência à terapia. Para superar esses mecanismos, que podem estar relacionados com desregulação das vias MAPK e PI3K/AKT/mTOR, a combinação dos fármacos vemurafenib e cobimetinib, um inibidor oral de MEK, foi aprovada para o tratamento de melanomas com a mutação $BRAF^{V600}$.

Apesar da recente aprovação de várias terapias, a sobrevida dos pacientes com melanoma não melhorou substancialmente e, assim, o desenvolvimento de novas terapias continua a ser fundamental. Tendo em consideração as alterações genéticas e moleculares e a presença do efeito de Warburg nas células de melanoma, de forma a superar a resistência a inibidores da via MAPK, neste projeto, foram testados os fármacos vemurafenib (inibidor de BRAF), cobimetinib (inibidor de MEK), everolimus (inibidor de mTOR) e dicloroacetato, um modulador metabólico, separadamente ou em combinação, numa linha celular de melanoma com a mutação $BRAF^{V600E}$ e com sensibilidade a vemurafenib, e numa linha celular de melanoma derivada da anterior, com resistência a vemurafenib.

Os resultados obtidos sugerem que a combinação de cobimetinib e everolimus é uma terapia mais adequada do que a terapia aprovada para pacientes com melanoma com $BRAF^{V600}$, vemurafenib com cobimetinib. Os nossos resultados indicam que direcionar a terapia para duas vias cruciais, as vias MAPK e PI3K, poderá ser mais eficiente do que usar dois inibidores para a via MAPK.

**Palavras-chave**

Melanoma, terapia, resistência, inibidores da via MAPK, inibidores de mTOR, dicloroacetato, metabolismo.

# Abstract

Melanoma accounts for only 1% of all skin malignant tumours, however it is the deadliest form of skin cancer. Current therapeutic approaches for melanoma include surgical resection, chemotherapies, immunotherapies and targeted therapies, however, melanoma remains one of the cancers with more years of productive life lost.

Melanoma has the highest mutation rate from all types of cancer and the main cause is the ultraviolet exposure. Considering the genetic mutations that originate this disease, some drugs were developed and approved for melanoma treatment, such as vemurafenib, an oral selective BRAF inhibitor, used for the treatment of unresectable or metastatic melanomas harbouring $BRAF^{V600}$ mutations. In $BRAF^{V600}$ melanoma patients, alterations in the melanoma cells can originate mechanisms of resistance to vemurafenib, which may be related with deregulation of MAPK and PI3K/AKT/mTOR pathways. To overcome this undesired outcome, the combination of vemurafenib and cobimetinib, an oral selective MEK inhibitor, was approved for melanomas harbouring $BRAF^{V600}$ mutations.

Despite the approved therapies for melanoma, the overall survival of patients did not change significantly, in the past years, and the development of new therapies is still crucial. Considering the genetic and molecular alterations and the presence of the Warburg effect in melanoma cells, and in order to overcome the resistance to MAPK inhibitors, in this project, a vemurafenib-sensitive melanoma cell line, with $BRAF^{V600E}$ mutation, and a derived vemurafenib-resistant melanoma cell line were tested in response to vemurafenib (BRAF inhibitor), cobimetinib (MEK inhibitor), everolimus (mTOR inhibitor) and dichloroacetate, a metabolic modulator, alone or in combination.

Our data suggests that the combination of cobimetinib and everolimus is a more appropriate therapy than the approved combination for $BRAF^{V600E}$ melanoma patients, vemurafenib and cobimetinib. Our results point that, in melanoma, targeting two crucial pathways, MAPK and PI3K pathways, is more effective than using two different inhibitors to target the MAPK pathway.

## Key-Words

Melanoma, therapy, resistance, MAPK inhibitors, mTOR inhibitors, dichloroacetate, metabolism.

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| | |
|---|---|
| **4E-BP1** | Eukaryotic translation initiation factor 4E-binding protein 1 |
| **A** | Adenine |
| **ACD** | Shelterin complex subunit and telomerase recruitment factor |
| **AJCC** | American Joint Committee on Cancer |
| **AKT/PKB** | Protein kinase B |
| **ALM** | Acral lentiginous melanoma |
| **AMPK** | 5' adenosine monophosphate-activated protein kinase |
| **ATP** | Adenosine triphosphate |
| **BAD** | Bcl-2-associated death promoter |
| **BAP1** | Breast cancer 1 associated protein 1 |
| **BCC** | Basal cell carcinomas |
| **BRAF** | v-RAF murine sarcoma viral oncogene homolog B |
| **BRCA1** | Breast cancer 1 |
| **BSA** | Bovine serum albumin |
| **C** | Cytosine |
| **CARs** | Chimeric antigen receptors |
| **CDK2** | Cyclin-dependent kinase 2 |
| **CDK4** | Cyclin-dependent kinase 4 |
| **CDKN2A** | Cyclin-dependent kinase inhibitor 2A |
| **c-Kit** | KIT proto-oncogene receptor tyrosine kinase |
| **CTLA-4** | Cytotoxic T lymphocyte-associated antigen 4 |
| **CTNNB1** | Catenin beta 1 gene |
| **CXCL1** | Melanoma growth-stimulating activity α |
| **CXCL3** | Melanoma growth-stimulating activity γ |
| **DCA** | Dichloroacetate |
| **$dH_2O$** | Distilled water |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **E** | Glutamic acid |
| **ECL** | Enhanced chemiluminescence |
| **ECT** | Electrochemotherapy |
| **eIF4E** | Eukaryotic translation initiation factor 4E |
| **ERK** | Extracellular regulated MAP kinase |
| **ESMO** | European Society for Medical Oncology |

| | |
|---|---|
| **ESOPE** | European Standard Operating Procedures of Electrochemotherapy |
| **EZH2** | Enhancer of zeste 2 polycomb repressive complex 2 subunit |
| **FBS** | Fetal Bovine Serum |
| **FDA** | Food and Drug Administration |
| **FGF** | Fibroblast growth factor |
| **FKBP12** | FK506 binding protein 12 |
| **G** | Guanine |
| **GNA11** | G protein subunit alpha 11 |
| **GNAQ** | G protein subunit alpha q gene |
| **GTP** | Guanosine triphosphate |
| **HGF** | Hepatocyte growth factor |
| **HIF1-α** | Hypoxia-inducible factor 1 α |
| **$IC_{50}$** | Half maximal inhibitory concentration |
| **IDH1** | Isocitrate dehydrogenase 1 |
| **IFN** | Interferon |
| **IGF-1R** | Insulin-like growth factor I receptor |
| **IL-2** | Interleukin-2 |
| **IL-8** | Interleukin 8 |
| **K** | Lysine |
| **KDR** | Kinase insert domain receptor |
| **LB** | Loading buffer |
| **LDH** | Lactate dehydrogenase |
| **LMM** | Lentigo maligna melanoma |
| **M** | Mitosis |
| **MAP** | Mitogen-activated protein |
| **MAP3K8** | Mitogen-activated protein kinase kinase kinase 8 |
| **MAPK** | Mitogen-activated protein kinase |
| **MC1R** | Melanocortin 1 Receptor |
| **MDM2** | Mouse double minute 2 homolog |
| **MEK** | Mitogen activated protein kinase kinase |
| **MHC** | Major histocompatibility complex |
| **miRNA** | microRNA |
| **MITF** | Microphthalmia-associated transcription factor |
| **MSH** | Melanocyte-stimulating hormone |
| **mTOR** | Mammalian target of rapamycin |
| **mTORC1** | mTOR complex 1 |

| | |
|---|---|
| **mTORC2** | mTOR complex 2 |
| **NAD** | Nicotinamide adenine dinucleotide |
| **NF1** | Neurofibromin type 1 |
| **NM** | Nodular melanoma |
| **p14ARF** | p14 alternate reading frame protein |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PB** | Presto Blue |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase chain reaction |
| **PD-1** | Programmed cell death protein 1 |
| **PDC** | Pyruvate dehydrogenase complex |
| **PDGFRA** | Platelet derived growth factor receptor A |
| **PDH** | Pyruvate dehydrogenase |
| **PDK** | Pyruvate dehydrogenase kinase |
| **PD-L1** | Programmed death ligand 1 |
| **PD-L2** | Programmed death ligand 2 |
| **PDP** | Pyruvate dehydrogenase phosphatase |
| **Peg-IFN** | Peginterferon α-2b |
| **PI** | Phosphatidylinositol |
| **PI3K** | Phosphoinositide 3-kinase |
| **PIP3** | Phosphatidylinositol-3 phosphate |
| **PKC-α** | Protein kinase C α |
| **POT1** | Protection of telomeres protein 1 |
| **PTEN** | Phosphatase and tensin homologue |
| **R** | Arginine |
| **RAF** | Proto-oncogene serine/threonine-protein kinase |
| **RAS** | Rat sarcoma virus oncogene |
| **RB** | Retinoblastoma protein |
| **RIPA** | Radioimmunoprecipitation |
| **ROS** | Reactive oxygen species |
| **RPMI** | Roswell Park Memorial Institute |
| **RTKs** | Receptors of tyrosine kinases |
| **S6** | 40S Ribosomal protein S6 |
| **S6K1** | S6 kinase |
| **SCC** | Squamous cell carcinomas |
| **SCF** | Stem-cell factor |

| | |
|---|---|
| **SD** | Standard deviation |
| **SDS** | Sodium dodecyl sulfate |
| **SSM** | Superficial spreading melanoma |
| **STK19** | Serine/threonine-protein kinase 19 |
| **STRs** | Short tandem repeats |
| **T** | Thymine |
| **TCA** | Tricarboxylic acid |
| **TCGA** | The Cancer Genome Atlas |
| **Teffs** | Effector T cells |
| **TERF2IP** | TERF2 interacting protein |
| **TERT** | Telomerase reverse transcriptase |
| **TILs** | Tumour infiltrating lymphocytes |
| **TME** | Tumour microenvironment |
| **TNM** | Tumor node metastases |
| **Tregs** | Regulatory T cells |
| **T-VEC** | Talimogene laherparepvec |
| **USA** | United States of America |
| **UV** | Ultraviolet |
| **V** | Valine |
| **VEGF** | Vascular endothelial growth factor |
| **WHO** | World Health Organization |
| **WT** | Wild-type |

# Chapter 1: Introduction

## 1.1 Skin cancer

Cancer is a group of neoplastic diseases that have several hallmarks in common. Cancer cells sustain proliferative signalling, evade growth suppressors, resist to cell death, enable replicative immortality, and reprogram the metabolism. These cells can evade immune destruction, induce angiogenesis and have the capacity to invade and create metastasis [1]. According to the World Health Organization (WHO), cancer is the second leading cause of death worldwide and it is considered a public health concern, due to the significant impact that causes in the society, including physical, psychological and economic effects.

Skin cancer is the most frequent cancer in Caucasians [2-4]. Skin cancer can be divided in non-melanoma skin cancer and malignant melanoma. Non-melanoma skin cancers, that include basal cell carcinomas (BCC), the most common form of skin cancer, and squamous cell carcinomas (SCC), the second most common form, are the least dangerous, especially if the cancer is detected and treated in an early stage [5-7]. Although melanoma accounts for only 1% of all skin malignant tumours, malignant melanoma represents the most aggressive and the deadliest form of skin cancer [2, 8, 9]. Malignant melanoma arises from complex genetic mutations that occur in melanocytes, which, beyond the skin (cutaneous melanoma), can also be found in the eye (uveal melanoma), and in the mucosal surfaces, as nasal passages and oral cavity (mucosal melanoma) [10-12]. Cutaneous melanoma accounts for more than 90% of all melanomas, uveal melanoma for 3%–5% of the cases, and mucosal melanoma for 1% of the cases [13, 14].

Over the past 30 years, the incidence of malignant melanoma has risen rapidly, being the 19$^{th}$ most common cancer worldwide and the 9$^{th}$ most common in Europe [2, 5, 8, 15-17]. Beyond ethnicity, incidence rates vary depending on sex, age and latitude of residence. In men, melanoma represents the disease whose incidence is increasing more rapidly and, in women, melanoma occurrences are increasing more rapidly than any other pathology, except for lung cancer [5, 18]. Overall, men are more susceptible to melanoma, accounting for about 60% of all new melanoma cases [19-21]. Before age 50, melanoma affects more women than men, however, this tendency is inverted by age 65 [2]. From birth to age 14, melanoma represents 3% of all the pediatric cancers [22], and between age 15 and 19, melanoma accounts for 5% of all the cancers diagnosed. Worldwide, the regions with the greatest melanoma incidence are Australasia, North

America and Europe [23]. In Europe, the highest rates of melanoma are registered in Denmark and the lowest in Portugal [4].

## 1.2 Cutaneous melanoma

The human skin is comprised by the dermis and the epidermis. The dermis has connective tissue, hair follicles and sweat glands. Above the dermis is the epidermis, the upper layer of the skin, that ensures the impermeable barrier function [24]. The epidermis is constituted by keratinocytes, the main structural cells that represent 90% of this layer, and melanocytes, the cells responsible for melanogenesis, the synthesis of the pigment melanin [25]. The pigmentation of the skin is determined by the ratio between the two forms of melanin, the eumelanin, the brown/black pigment, and the pheomelanin, the red/yellow pigment [26]. Melanin has an important photoprotective function [16, 27, 28]. After ultraviolet (UV) exposure, keratinocytes secrete factors that control melanocytes survival, differentiation, proliferation and motility, stimulating the melanin production that results in the tanning response [16]. Particularly, the eumelanin can reduce the photodamage in the deoxyribonucleic acid (DNA), proteins, and lipids, since it can act as a scavenger of reactive oxygen species (ROS), which are produced as a consequence to the UV exposure [27]. The pheomelanin form might be a pro-oxidant, lacking the photoprotective effect [29].

The Clark model explains the cutaneous melanoma tumour progression from nevi to metastatic melanoma [30] (Figure 1). Nevi are benign neoplasms constituted by melanocytes that appear on the skin, including palms, soles and nails. Nevi are very common, but the pigment colour may vary between individuals, depending on the natural pigmentation of the skin. A benign nevus, smaller than 6 mm, is symmetrical, with regular borders and homogenous colour [31]. Dysplastic nevi are less frequent and represent an intermediate state between a benign nevus and a malignant melanoma. Atypical/dysplastic nevus may appear from a pre-existing nevus or in a new location of the body. This type of pre-malignant lesion is usually greater than 5 mm, with irregular borders and heterogeneous colour [31]. Although normally melanocytes have a low proliferation potential [32, 33], these cells can develop the capacity to proliferate horizontally, leading to radial growth in the epidermis. In the course of time, the biochemical changes that occur in the malignant melanocytes, as the loss of E-cadherin and expression of N-cadherin, may allow these cells to have vertical growth as well, initially with the invasion of the dermis [31]. Due to the continuous propagation, cells can

reach the lymph nodes and other organs, such as lung, liver and brain, originating metastatic melanoma [31, 34, 35].

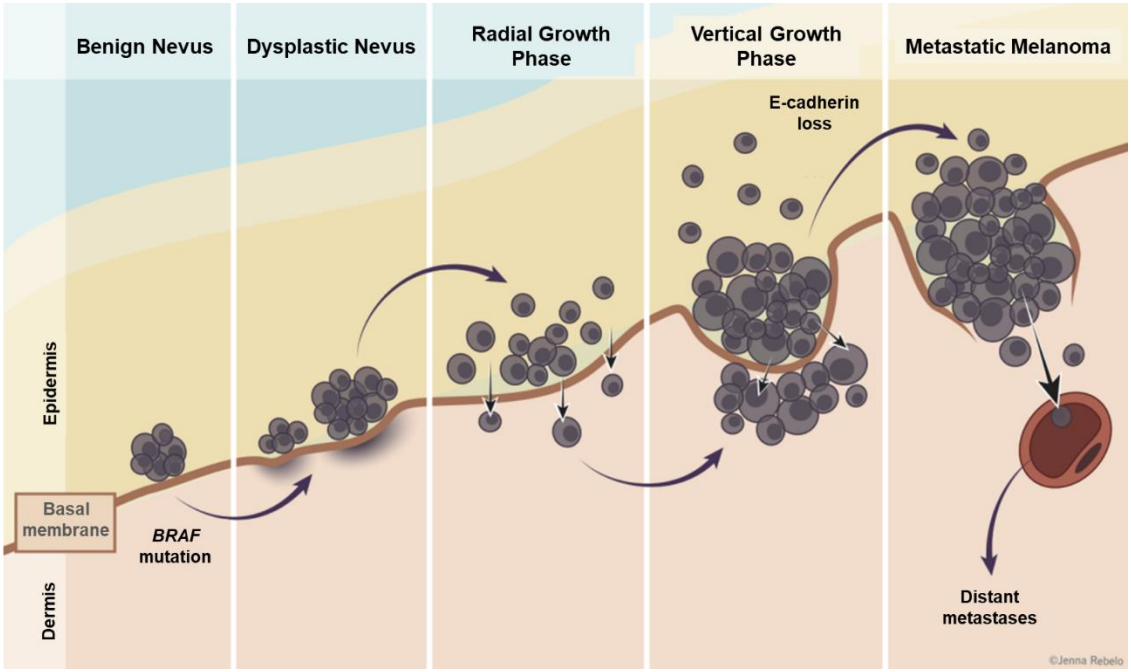



**Figure 1. The Clark model for the melanoma progression, from benign nevus to metastatic melanoma**. Nevi are benign neoplasms constituted by melanocytes that appear on the skin, including palms, soles and nails. Dysplastic nevi are less frequent and represent an intermediate state between a benign nevus and a malignant melanoma. Biochemical changes that occur in the malignant melanocytes contribute to initial radial growth in the epidermis. Posteriorly, the loss of E-cadherin leads to vertical growth, which contributes to initially the invasion of the dermis. Cells can reach lymph nodes and originate metastatic melanoma in other organs, such as lung, liver and brain. Adapted from [36].

Cutaneous melanoma can be divided in four different subtypes, depending on the clinical and histologic features, such as location, morphology, tumour progression, type of skin and UV exposure [37-40] (Table 1). The WHO classification recognizes superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM) as the subtypes of cutaneous melanoma [40]. However, this classification has no prognostic value [41] and melanoma is classified by the pathological staging according to the American Joint Committee on Cancer (AJCC) (Supplementary Table 1). The main clinical and histopathologic predictors of outcome, based on the tumour node metastases (TNM), are tumour thickness, mitotic rate, ulceration and extent of metastatic disease. Additionally, it could also be taking into account the lymphovascular and perineural invasion and the lactate dehydrogenase (LDH) levels. Mutational profile, gene expression, proteomics and microRNA (miRNA) are considered new and promising prognostic factors for melanoma [8].

In the United States of America (USA), patients with an early diagnosis have an estimated 5-year survival rate of 99% [2]. When the cancer extents to the lymph nodes (regional stage disease), this rate decrease to 63% [2]. Once it becomes metastatic (distant stage disease), the prognosis is very poor, with mortality rates even higher [42] and the 5-year survival rate decreases to 20% [2], with a median overall survival of 6–9 months [43]. Thus, the early diagnosis of melanoma, based on the ABCDE rules of melanoma recognition, that analyses the asymmetry, the borders, the colour, the diameter and the evolution of the moles, is crucial for the success of the treatment, since the melanoma misdiagnosis increases the probability of metastasis [43].

**Table 1. Cutaneous melanoma subtypes** [40].

| Subtype | Most common location | Features |
|---|---|---|
| **Superficial spreading melanoma (SSM)** | Trunk and legs | Most common subtype; Radial growth; Diffused borders. |
| **Nodular melanoma (NM)** | Trunk | Common subtype; Vertical growth; Sharp borders. |
| **Lentigo maligna melanoma (LMM)** | Head and neck | Arises from chronic sun exposure; Invades the dermis. |
| **Acral lentiginous melanoma (ALM)** | Acral regions, as palms, soles and nails | In people with naturally darker skin pigmentation is the most common subtype; Not associated with UV exposure. |

### 1.2.1 Cutaneous melanoma aetiology

The origin of cutaneous melanoma is associated with environmental, phenotypic and genetic risk factors [44]. UV exposure is the main cause for cutaneous melanoma since it can increase the number of the atypical nevi and trigger several oncogenic mutations [17, 31, 45, 46]. UV radiation promotes the formation of pyrimidine dimers between adjacent cytosine (C) and thymine (T) [31]. UV can also influence the immune system, specifically the inflammation and immunosuppression processes, generates photoproducts and oxidative stress, and promotes the melanogenesis, the synthesis of melanin [44]. Subsequently to studies that proved that UV light emitted from tanning beds increases the risk of melanoma [47], the WHO International Agency for Research on Cancer recognized this type of UV as a carcinogen [48]. As concerns sun exposure, ten or more sunburns duplicate the susceptibility of developing cutaneous melanoma [49]. The sporadic sun exposure is particularly dangerous for people with indoor jobs whose skin is not prepared to sun exposure [31].

Besides the major environmental stressor, risk factors for the development of cutaneous melanoma include also the phenotypic features, such as lighter pigmentation characteristics, fair complexion, blue or green eyes, blond or red hair, freckles, low tanning capacity and 100 or more melanocytic nevi or five or more atypical nevi [28, 31, 44, 50].

The combination of UV exposure and genetic susceptibility raises the mutations that inactivate tumour suppressor genes and activate oncogenes, reducing the DNA repair mechanisms and increasing the melanocytes capacity to proliferate [35]. Regarding the genetic susceptibility, patients with family history of melanoma represent 10% of all the melanoma cases [44, 51]. Melanoma genetic counselling is especially important in familial melanoma to better understand the disease and the prone individuals.

The most commonly mutated gene in melanoma families is the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) tumour suppressor gene, located in the 9p21 locus (Figure 2). *CDKN2A* is mutated in about 20% of melanoma-prone families [44], nonetheless, mutations in this gene also occur in 1% of sporadic melanoma patients [52]. *CDKN2A*, through differential splicing and alternative reading frames, encodes for the proteins p16INK4a (p16) and p14ARF (p14) [11, 53-56]. In normal conditions, p16INK4a inhibits the cell cycle progression, in the G1 phase, by inhibiting retinoblastoma protein (RB) phosphorylation through cyclin-dependent kinase 4 (CDK4) [50, 55, 57].The p14ARF induces cell cycle arrest or favours apoptosis, through the p53 pathway [58]. The loss of these functions leads to an uncontrolled cell cycle progression [11, 53-55].

Another melanoma high risk gene is *CDK4*, located in the 12q14 region. *CDK4* encodes for the kinase that is inhibited by p16INK4a, therefore controls cell cycle progression through the G1 phase [59]. Mutations on this oncogene are rare, less than 1% of melanoma families [60]. The most common mutation compromises the p16INK4a binding domain, which prevents the interaction between the two proteins and leads to the cell cycle progression [60].

Breast cancer 1 (BRCA1) associated protein 1 (*BAP1*), located in the 3p21 region, is also considered a high risk gene in familial melanoma [44, 61]. *BAP1* germline mutations have been associated with an increased incidence of cutaneous and uveal melanoma [62-64]. *BAP1* encodes for a deubiquitylase, and, besides the cleavage of ubiquitin, this protein plays a role in the cell cycle, cellular differentiation, cell death, gluconeogenesis and the DNA damage response [62].

Inherited copy number variations have also been associated with several human diseases, including melanoma [44, 65]. The duplication of the region 4q13 was identified in familial melanoma and comprises several genes that belong to the family of CXC chemokines, such as melanoma growth-stimulating activity α (CXCL1), melanoma growth-stimulating activity γ (CXCL3) and interleukin 8 (IL-8), which are associated with melanoma growth [44, 66].

A germline mutation was also found in the promoter of telomerase reverse transcriptase (*TERT*) in a family with melanoma history. *TERT*, located in the 5p15 region, encodes the catalytic subunit of the telomerase, which is a ribonucleoprotein polymerase that maintains telomere ends by adding the telomere repeat TTAGGG [67, 68]. *TERT* reactivation is associated with carcinogenesis, tumour aggressiveness and represents an important step towards tumour immortalization [69]. Telomerase activity, which is associated with worse prognostic features, such as, ulceration, vascular invasion, mitotic rate and thickness, varies depending on the cell stage, with increasing values from normal skin to benign nevi, dysplastic nevi and finally to melanoma [69]. Beyond UV radiation, telomere length can also be influenced by the high-risk melanoma susceptibility gene *CDKN2A* mutational status and pigmentation phenotype, and for these reasons cannot be considered a biomarker to predict melanoma risk per se [69].

In *CDKN2A* wild-type melanoma-prone families were found rare germline variants in the protection of telomeres protein 1 (*POT1*) gene. *POT1*, located in the 7q31 region, encodes a nuclear protein of the telomeric shelterin complex, which is crucial for the telomere maintenance [70]. POT1 loss of function influences melanomagenesis, by a direct effect on telomeres [71]. Germline mutations of two more genes that participate in the telomere maintenance, shelterin complex subunit and telomerase recruitment factor (*ACD*) and TERF2 interacting protein (*TERF2IP*), were identified in melanoma-

prone families [72]. Around 1% of familial melanoma patients present germline mutations in *TERT*, *POT1*, *ACD* and *TERF2IP* [44], which associates telomere dysregulation with melanoma susceptibility [72].

Melanocortin 1 receptor (*MC1R*), located in 16q24 region, encodes for α melanocyte-stimulating hormone (α-MSH) receptor 1 and it is a regulator of the ratio between eumelanin and pheomelanin. *MC1R* is considered a moderate risk gene in familial melanoma and it is highly polymorphic among the Caucasian population [73, 74].

Another moderate risk gene is microphthalmia-associated transcription factor (*MITF*), located in the 3p14 region. MITF, also known as master melanocyte transcription factor, regulates the transcriptional control of several critical genes, including the cyclin-dependent kinase 2 (*CDK2*) [75]. *MITF* mutations confer a genetic predisposition to melanoma, associated with a five-fold increased risk, and are associated with the development and differentiation of melanocytes and with the development and progression of melanoma [76-78].

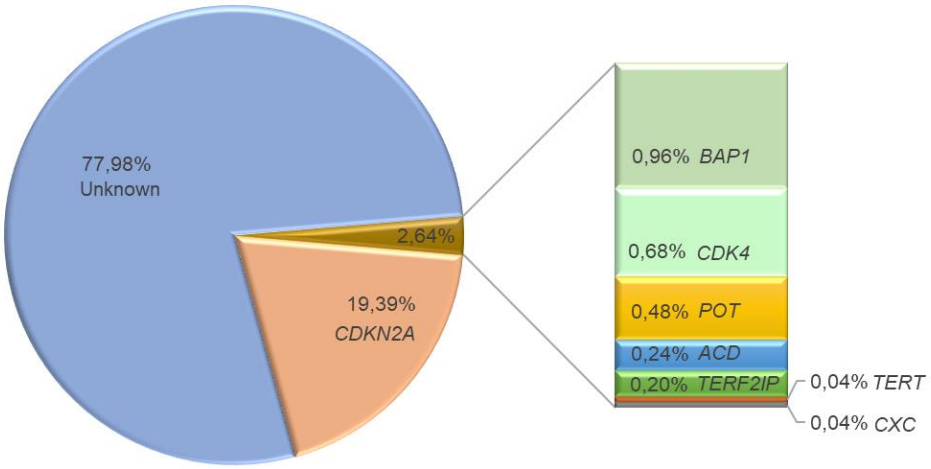



**Figure 2. Frequency of mutations in familial melanoma.** Graphic representation of the prevalence of high risk melanoma genes in melanoma families. Most of the melanoma-prone families have unknown mutation. Adapted from [44].

### 1.2.2 Signalling and metabolic pathways in cutaneous melanoma

Melanoma has the highest mutation rate reported for any cancer sequenced [79] and the main cause is the UV exposure [80]. In primary melanoma, the molecular analyses are limited since most or all of the primary tumour tissue is used for diagnosis, thus regional and distant metastatic sites are frequently used for the study of this solid tumour [79].

In normal conditions, the keratinocytes regulate the melanocytes, however, mutations in critical growth regulatory genes, the production of autocrine growth factors, and the loss of adhesion receptors allow melanocytes to escape this regulation [81]. This leads to proliferation and spread of the melanocytes, which can result in the formation of a nevus or a common mole [16]. The understanding of the biology underlying melanoma initiation and progression revealed that molecular alterations have a crucial role in the melanomagenesis.

The mitogen-activated protein kinase (MAPK) pathway (Figure 3) is one of the most important pathways in normal and cancer cells, due to its role in the cell growth, survival, proliferation, differentiation, migration and apoptosis [82]. The small G protein of Ras family (H-Ras, K-Ras, and N-Ras) activates Raf family (A-Raf, B-Raf and C-Raf), which in turn activates mitogen activated protein kinase kinase (MEK) and then the extracellular regulated MAP kinase (ERK), which creates a regulator signal to transcription in the nucleus [83]. In cutaneous melanoma, this pathway, also termed Ras/Raf/MEK/ERK pathway, is activated in more than 80% of the cases [83] and ERK is reported to be hyperactivated in more than 90% of melanomas [84]. In melanocytes, to achieve a sustained ERK activation is necessary the stimuli of several growth factors simultaneously, such as stem-cell factor (SCF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) [85].

*B-RAF*, v-RAF murine sarcoma viral oncogene homolog B, encodes for a key serine–threonine kinase of the MAPK pathway and *BRAF* mutations appear in about 50% of melanoma cases, causing tumour growth, proliferation and metastasis [79, 86, 87]. The most common *BRAF* mutation appears at amino acid 600, in which the normal valine (V) is substituted, in about 90% of the *BRAF*-mutated melanomas, by glutamic acid (E), resulting in $BRAF^{V600E}$ mutation, or, in some cases, it is substituted by lysine (K), resulting in $BRAF^{V600K}$, or by arginine (R), resulting in $BRAF^{V600R}$ [79, 88, 89]. The K601 residue was also reported as mutated in a few melanoma cases [79].

The Ras family is composed by small GTPases, proteins that hydrolyse guanosine triphosphate (GTP). The Ras proteins are involved in cellular signal

transduction and influence cell growth, differentiation and survival [90]. Almost 30% of melanoma patients harbour *NRAS* somatic mutations, which translates into functional consequences. Mutations can also occur in other members of the Ras family, as *KRAS* and *HRAS* [79].

*NF1* gene, located at 17q11.2, encodes for the tumour suppressor protein neurofibromin type 1, which is mutated in approximately 15% of melanoma patients, representing the third most frequently mutated gene in melanoma [79, 91, 92]. Neurofibromin has an intrinsic GTPase activity and usually downregulates the RAS activity [93]. The most common mutation causes loss of function of NF1 [94]. This type of mutation can trigger the activation of the MAPK signalling pathway, leading to abnormal cell growth [93].

Besides the activation of B-Raf, Ras can also activate the phosphoinositide 3-kinase (PI3K) pathway (Figure 3). In melanoma, activation of the PI3K signalling pathway is important for the carcinogenesis, since it modulates the extracellular signals that control cell growth, proliferation and apoptosis of the melanocytes [95]. PI3K catalyses the phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol-3 phosphate (PIP3), which activates the protein kinase B (AKT), which sequentially activates the mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR signalling pathway). Phosphatase and tensin homologue (PTEN) negatively regulates the PI3K pathway by dephosphorylating and inactivating PIP3. The inactivation of PTEN appears in 5% to 20% of late-stage melanomas [96] and prevents the inhibition of the PI3K pathway, resulting in the pathway activation [97-99]. The expression of PI3K and AKT was described to increase during the progression from benign nevi to early melanoma and to metastatic disease [100].

mTOR, a downstream effector of the PI3K/AKT/mTOR signalling pathway, plays a key role in tumour development and progression. mTOR forms two protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), that act differentially [101]. mTORC1 is activated by growth factors, nutrients, energy and stress signals, and signalling pathways, such as PI3K, MAPK and 5' adenosine monophosphate-activated protein kinase (AMPK) pathways. mTORC1 activates S6 kinase (S6K1), which activates 40S ribosomal protein (S6), and phosphorylates eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). The phosphorylation of 4E-BP1 leads to the dissociation from eukaryotic translation initiation factor 4E (eIF4E), which has a role in the formation of the translation initiation complex [101, 102]. mTORC2 is activated by growth factors and phosphorylates protein kinase C α (PKC-α), AKT (on Ser473) and paxillin (focal adhesion-associated adaptor protein). This complex regulates crucial cellular processes,

such as cell survival, migration and regulation of the actin cytoskeleton, by regulating the activity of the small GTPases Rac and Rho [101, 103-105].

*PI3K* amplification/mutation, loss of PTEN, the negative regulator of PI3K pathway, and AKT, S6K1, 4E-BP1 and eIF4E overexpression have been reported in melanoma and are associated with tumour progression [101].

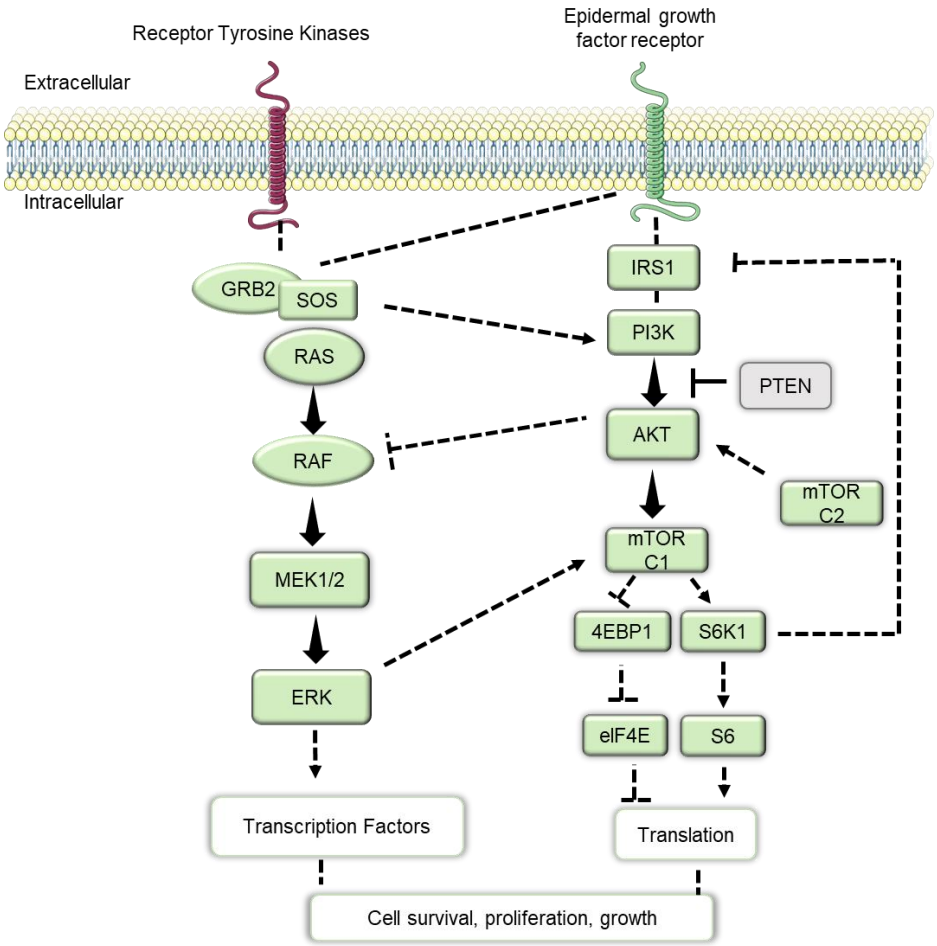



**Figure 3. MAPK and PI3K/AKT/mTOR pathways.** The MAPK and PI3K/AKT/mTOR pathways respond to extracellular and intracellular stimuli to control translation, cell survival, proliferation, motility, and metabolism. Mutations on key signalling oncogenes of MAPK and PI3/AKT/mTOR pathways can be associated with melanoma cell proliferation, cell-cycle progression and malignant phenotype. The cross-talk between these pathways suggests that targeting multiple pathways may be a valuable approach for melanoma treatment.

The different signalling pathways are connected in the cells and a single mutation can trigger multiple effects. The KIT proto-oncogene receptor tyrosine kinase (c-Kit) signalling activates the MAPK and PI3K pathways [106]. In cutaneous melanoma, *CKIT* mutations and amplifications have been described in 2% and 7% of the cases, respectively [107]. Both C*KIT* mutations or gene amplifications can lead to the

constitutive ligand-independent activation of this receptor and subsequently upregulation of the MAPK and PI3K pathway [108, 109].

Somatic mutations in the *TERT* promoter were found in a range of 29%-71% of cutaneous melanoma cases [67-69, 110-112]. These mutations can result from UV radiation and might be associated with a poorer prognosis in melanoma [112, 113].

Due to the high mutation rate, defining the driver mutations in melanoma is a complex task. The Cancer Genome Atlas (TCGA) Network defined four subtypes of cutaneous melanoma, based on the most significantly mutated genes: mutant *BRAF*, mutant *RAS*, mutant *NF1*, and triple wild-type (WT) [79]. This genomic classification provides comprehensive knowledge of the genetic diversity in cutaneous melanoma that can be helpful for the identification of predictive biomarkers and suitable targets for therapies [75, 79].

The mutant *BRAF* subtype, the most common subtype, is defined by *BRAF* hot-spot mutations [79, 86]. Although this subtype presents high frequency of C>T transitions (UV signature), epidemiological studies suggest that *BRAF* mutations are more frequent in younger subjects with many nevi than in the sun-exposed skin of older patients [114, 115]. In primary cutaneous melanomas with $BRAF^{V600E}$ mutation, the presence of *TERT* promoter mutations, found in about 75% of cases, was associated with worse prognostic features and shorter disease-free and overall survival [79, 111-113]. Patients in this subtype respond to BRAF and MEK inhibitors [79].

Mutant *RAS* subtype represents another major hot-spot for mutations and it is also associated with UV signature. These melanomas present *TERT* promoter mutations in about 70% of the cases and are associated with isocitrate dehydrogenase 1 (*IDH1*) mutations [79, 116]. The association with therapies revealed that this subtype responds to MEK inhibitors therapy [79].

Mutant *NF1* subtype is more common among older melanoma patients or in chronically sun-exposed skin [93]. In this subtype, melanomas are dependent on MAPK signalling pathway, therefore patients in this group respond to MAPK inhibitors therapy. This subgroup has UV signature and it is associated with *TERT* promoter mutation in 85% of cases and *CDKN2A* mutations in about 70% of cases [79, 117, 118].

Melanoma cases without mutations in the hot-spots *BRAF*, *N/H/KRAS* and *NF1* are called triple-WT and its malignant phenotype may be associated with a structural rearrangement of the genome. The possible driver mutations of this subtype include co-amplified receptors of tyrosine kinases (RTKs), such as platelet derived growth factor receptor A (*PDGFRA*) and kinase insert domain receptor (*KDR*), which encodes one of the two receptors of the vascular endothelial growth factor (VEGF).Other mutations can also influence the triple-WT subtype, as in the G protein subunit alpha q gene (*GNAQ*),

G protein subunit alpha 11 gene (*GNA11*), catenin beta 1 gene (*CTNNB1*), and enhancer of zeste 2 polycomb repressive complex 2 subunit gene (*EZH2*) [79]. Copy number changes are also presented in this subgroup, such as in cyclin D1, *CDK4*, mouse double minute 2 homolog (*MDM2*), C*KIT* and *TERT* [79, 119].

Besides activation of signalling pathways, metabolic alterations are also crucial for melanoma biology. Metabolic switch is an established hallmark in cancer cells [1]. In normal cells, glucose can be fully oxidized to carbon dioxide in the presence of oxygen, through mitochondrial oxidative phosphorylation, a very efficient energetic process, or, in hypoxic conditions, glucose can be partially metabolized to lactate through glycolysis. In cancer cells, the principal process happening is glycolysis, independently of the oxygen level. This switch is known by the Warburg effect [120], which describes the fermentative activity of cancer cells [121]. Aerobic glycolysis confers tumour growth advantage and is required for evolution of invasive tumours, suppling fast ATP and conferring apoptotic resistance through limited mitochondrial oxidative phosphorylation activity [122, 123]. The by-product of glycolysis, lactate, degrades the extracellular matrix enabling tumour expansion and metastasis, favouring invasive tumours [123]. In terms of environment, the melanocytes are located in a naturally mild-hypoxic environment (10% or less of oxygen), which could pre-adapt melanoma to hypoxia [124]. The poor vascularisation of the tumour tissue also contributes to low oxygen levels [125]. At low oxygen levels, hypoxia-inducible factor 1 α (HIF1-α ) induces the glycolytic metabolism [126, 127]. Besides these environmental conditions and the ROS production, overexpression of HIF1-α in cutaneous melanoma is associated with activation of mTOR [128-131]. The *BRAF* or *NRAS* mutations, frequently present in melanoma, may also affect cell metabolism, via activation of HIF1-α [124, 129]. Thus, consequently, *BRAF* mutations decrease the oxidative metabolism in melanoma [132]. However, metabolism is not strictly glycolytic, as the tricarboxylic acid (TCA) cycle remains functional, even under hypoxia [121].

## 1.3 Cutaneous melanoma therapy

In the past years, the increased knowledge of melanoma genetics and biology led to new approaches for melanoma treatment. The European Society for Medical Oncology (ESMO) clinical practice guidelines for cutaneous melanoma highlight the importance of a detailed diagnosis for the determination of the tumour stage [133]. Depending on the location, stage and genetic profile of the tumour, the therapeutic

approaches may be surgical resection, chemotherapy, radiotherapy, immunotherapy, or targeted therapy [134] (Appendix I – Paper).

Surgery is the primary treatment for patients with stage I-III stage melanoma and the excision includes safety margins of 0.5 cm for *in situ* melanomas, 1 cm for tumours with a thickness up to 2 mm, and 2 cm for tumours thicker than 2 mm [135, 136].

Chemotherapy was the earliest treatment option for advanced melanoma (Figure 4). Dacarbazine, an alkylating agent, was approved in 1974 by the Food and Drug Administration (FDA). Studies demonstrated that a complete response was achieved by less than 5% of the patients, and 5-year survival by 2–6% of patients [75]. Although these poor results, dacarbazine was, for many years, the standard of care because other single-agents or combinatorial chemotherapies did not show improvement in the overall survival of patients [137]. This drug is still in clinical trials in combination with immunotherapies, chemotherapies and targeted therapies [138]. More recently, electrochemotherapy (ECT), a technique that combines the use of drugs with high intensity electric pulses, proved to be effective for the treatment of cutaneous and subcutaneous lesions of melanoma [139, 140]. ECT facilitates the delivery of cytotoxic drugs, as bleomycin and cisplatin, into the cells. A two-years long study of the European Standard Operating Procedures of Electrochemotherapy (ESOPE) showed an overall response of 85%, with a complete response of 74%, and no major negative side effects were observed [139, 141].

Despite being an alternative to other types of cancer, radiotherapy is rarely indicated for the treatment of primary tumours, but it can be useful for the treatment of skin, bone and brain metastases [142].

As known for many types of cancer, complex interactions between the tumour and the immune system play a role in the metastatic spread to distant sites from the primary tumour [143]. Metastases are frequently related with mortality and a more accurate prognosis and better therapeutic approaches are crucial. Thus, the modulation of the antitumoral immune responses is a valuable approach to treat melanoma. T cells recognize the tumour-specific antigens, which allows T cell activation. Activated T cells can proliferate, differentiate and acquire the capacity to destroy cells that express the recognized antigens [144]. Additionally, tumour infiltrating lymphocytes (TILs), that result from the immune response of the host against cancer cells, have been considered in many studies as independent prognostic factors for lymph node metastasis. TILs are also associated with a positive outcome and improved survival in patients with malignant melanoma [9, 143]. Moreover, the immunogenic tumour microenvironment (TME) of each patient, with mediators and cellular effectors of inflammation, also influences the success of immunotherapies [145].

High-dose interferon (IFN) α-2b was approved by the FDA, in 1995, and nowadays it is used as adjuvant therapy for melanoma [146, 147]. IFNs are cytokines secreted by leukocytes and these signalling proteins are able to interfere with viral replication and play an important role in the immunomodulatory, antiangiogenic, anti-proliferative, and antitumor activities [146, 148-150]. In melanoma, IFN-α plays an immunomodulatory antitumor effect, being able to directly inhibit the proliferation of melanoma cells [151]. A recent meta-analysis showed that adjuvant IFN-α significantly reduces the risk of recurrence and improves survival [152]. However, only a minority of patients are sensitive to IFN, and ulceration of the primary tumour is the most important predictive factor for IFN-sensitivity [153]. Although newer and more efficient immunotherapies modalities have appeared in recent years, IFN is still in clinical trials in combination with other immunotherapies and chemotherapies [138].

In 1998, FDA approved high-dose interleukin-2 (IL-2) for metastatic melanoma treatment [154]. IL-2 is a cytokine capable of expanding effector T cells (Teffs) and regulatory T cells (Tregs). Before undergoing IL-2 treatment, patients need to be evaluated and some biomarkers have been studied, such as VEGF, but the complete response rate for this treatment is only 4% [155]. IL-2 is also in clinical trials in combination with other immunotherapies and chemotherapies [138].

In 1999, FDA approved ontak [156], a fusion of the IL-2 protein and diphtheria toxin [157] that selectively eliminates Tregs expressing IL-2 receptor, from the peripheral blood. A phase II trial in stage IV melanoma patients showed 17% of partial responses, 5% stable disease and 15% of mixed responses [158]. Clinical trials are ongoing with ontak alone or in combination with other immunotherapies [138].

In 2011, peginterferon α-2b (peg-IFN) was approved by FDA as adjuvant therapy for stage III melanoma [159]. This treatment is used in patients who undergone surgery to remove cancer that has spread to the lymph nodes. Clinical trials are still ongoing to compare peg-IFN with IFN-α 2b [138].

Ipilimumab, approved by the FDA for the treatment of advanced melanoma, in 2011, is an anticytotoxic T lymphocyte-associated antigen 4 (anti-CTLA-4) antibody. CTLA-4 is an inhibitory checkpoint receptor that blocks T cell activation and induce the immune tolerance of the patients [160, 161]. CTLA-4 antibodies act as an antagonist, blocking the inhibitory effect, enhancing pro-inflammatory T cell cytokine production [162] and increasing clonal T cell expansion and infiltration in responding tumours [163]. To undergo ipilimumab treatment, melanoma patients must be in accordance with immune-related response criteria, such as absence of autoimmune or immunodeficiency disease [164]. Several clinical trials are ongoing with ipilimumab alone or in combination with other immunotherapies and chemotherapies [138].

The programmed cell death protein 1 (PD-1) receptor binds to PD-1 ligands 1 and 2 (PD-L1, PD-L2) and acts as a T cell co-inhibitory molecule and suppresses T cell activation. Beyond being expressed on the antigen-presenting cells, ligands are also expressed on many human tumours and on cells within the TME, in response to inflammatory stimuli. In 2014, nivolumab, a high-affinity PD-1 monoclonal antibody, that inhibits the binding between the PD-1 receptor and its ligands PD-L1 and PD-L2 [165 ], was approved by FDA for the treatment of patients with metastatic melanoma [166]. The blockade of the interaction between PD-1 and its ligands mediates immune responses and induces the preclinical antitumor activity that reduces tumour progression [167]. Nivolumab proved to be more efficient than monotherapies with ipilimumab or chemotherapy, or than chemotherapy in ipilimumab pre-treated patients [167]. In 2015, pembrolizumab, also an anti-PD-1 antibody, was approved by the FDA for the treatment of advanced melanoma and can be a new standard of care for the treatment of ipilimumab refractory melanoma [166, 168, 169]. PD-L1 expression was suggested to be a prognosis factor and a predictive biomarker for anti-PD-1 treatment, however its utility remains unclear [170-174]. Clinical trials are using these anti-PD-1 antibodies alone or in combination with other immunotherapies, chemotherapies or targeted therapies [138].

In 2015, FDA approved the first oncolytic virus for the treatment of melanoma, talimogene laherparepvec (T-VEC), a genetically modified herpes simplex virus type 1 [175-177]. This engineered non-pathogenic viral strain is injected directly into a metastatic melanoma lesion and, while it enters both normal and malignant cells, it only replicates in the melanoma cells, leading to tumour cell lysis and consecutively release of tumour-specific antigens [176]. These antigens are recognized, activating melanoma-specific T cell responses. Patients with refractory stage IV or unresectable stage III melanoma were treated with T-VEC and, in a phase II clinical trial, it was observed an objective clinical response of 28% [176]. Clinical trials are testing T-VEC alone or in combination with immunotherapies [138].

Based on the interactions between melanoma cells and the immune system, and taking into account that patients with advanced melanoma already showed durable complete responses, immunotherapy represents a more promising treatment option for patients with advanced stage (metastatic) malignant melanoma than the previous standard therapies [9, 145, 178]. Some immunotherapies, such as adoptive T-cell therapy with T-cells chimeric antigen receptors (CARs), are promising, yet unapproved, approaches for solid tumours, as melanoma [179].

Considering the genetic alterations mentioned above, new molecular approaches have been developed to target melanoma harbouring mutations. In 2011, vemurafenib, a selective oral *BRAF* mutant inhibitor, was approved by the FDA for the treatment of

unresectable or metastatic melanoma harbouring activating mutations in $BRAF^{V600}$ [108, 180]. Vemurafenib is a selective ATP-competitive BRAF inhibitor and acts by limiting the ATP binding, thereby reducing activation of the MAPK pathway. This drug inhibits tumour growth, triggers apoptosis and induces G1 cell cycle arrest [181-183]. A phase III randomized clinical trial compared vemurafenib with dacarbazine and showed that vemurafenib can achieve better overall survival (84% vs 64%) and better progression-free survival (5.3 vs 1.6 months) [180]. Studies have demonstrated that 90% of patients who received vemurafenib showed tumour regression [180]. Dabrafenib is also a selective BRAF inhibitor, with a similar mechanism of action of vemurafenib, approved by the FDA, in 2013, for the treatment of unresectable or metastatic melanoma harbouring *BRAF* activating mutations [108, 184]. Targeting the tyrosine kinases has led to remarkable response rates with superior overall survival rates in clinical melanoma trials [108]. However, the clinical benefit of these therapies is limited, due to the rapid development of multiple mechanisms of resistance [185-187]. Being a targeted therapy, it is possible to select the patients who will benefit from this treatment, based on the mutational profile of the tumour. Only the patients with tumours harbouring *BRAF* mutations can undergo treatment with a BRAF inhibitor and patients with known *RAS* mutations do not receive this treatment [108, 119, 188]. Several clinical melanoma trails are ongoing with BRAF inhibitors alone or in combination with other immunotherapies and targeted therapies [138].

Targeting signalling effectors downstream of driver oncogenes is a valid strategy to overcome resistance to BRAF inhibitors [189]. MEK, a downstream effector of B-Raf, is an important threonine-tyrosine kinase from MAPK pathway and its activation is required for the transmission of growth-promoting signals [190]. MEK inhibitors showed activity in *NRAS*-mutant melanoma [191]. In 2013, trametinib, a pharmacological MEK1/2 inhibitor with antitumoral activity, was approved as a single-agent, by the FDA, for the treatment of unresectable or metastatic malignant melanoma with *BRAF* mutations [108, 192]. The blocking of MEK1/2 results in the inhibition of growth factor-mediated cell signalling and cellular proliferation in tumour cells. Combinatorial therapy of dabrafenib (BRAF inhibitor) and trametinib showed durable objective responses in a randomized, multicentre, open-label study [193]. This combinatorial therapy was approved, by FDA, in 2014, for patients with unresectable or metastatic malignant melanoma with *BRAF* mutations. Patients previously submitted to surgery to remove melanoma that has spread to the lymph nodes can also receive the combination of trametinib and dabrafenib. After that, in 2015, FDA accepted the combination of vemurafenib, a BRAF inhibitor, with cobimetinib, an oral potent and highly selective inhibitor of MEK1/2, for the treatment of melanoma, harbouring *BRAF* mutation, that

cannot be removed by surgery or has metastasized [194, 195]. Cobimetinib binds to and inhibits the catalytic activity of MEK. This binding inhibits ERK phosphorylation and activation, decreases tumour growth and induces G1 arrest [196, 197]. This combination achieved improved clinical outcomes in *BRAF* mutated patients, with a progression-free survival of 10 months, compared to only 6 months for the treatment with vemurafenib [198]. More recently, in 2018, combination of encorafenib, a BRAF inhibitor, and binimetinib, a MEK1/2 inhibitor, was approved to treat melanoma patients with $BRAF^{V600E/K}$ mutation. MEK inhibitors are in clinical melanoma trails, either alone or in combination with other immunotherapies and targeted therapies [138].

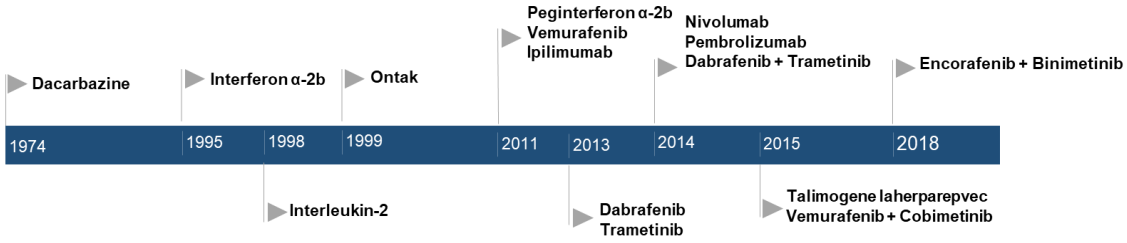



**Figure 4. FDA-approved drugs for melanoma treatment.** Dacarbazine was the first drug approved, in 1974, followed by interferon α-2b, interleukin-2, and ontak in the 1990s. Between 2011 and 2018, 11 therapies were approved, including selective inhibitors, antibodies, and combined targeted therapies.

## 1.4 Possible new therapies for mutant *BRAF* melanoma

Considering the incidence and the mortality rate of cutaneous melanoma worldwide, the relevance of the development of new therapeutic becomes clear. With the increased molecular knowledge and the better understanding of the signalling pathways in melanoma, the characterization of the tumour at the diagnosis stage became more accurate, which allowed patients to received suitable therapies for each type of tumour.

As mentioned above, most patients have mutant *BRAF* cutaneous melanoma and vemurafenib, a BRAF inhibitor, is widely used to treat patients [199, 200]. This therapy is associated with several adverse effects, such as arthralgia, rash, fatigue, alopecia, photosensitivity, cutaneous SCC, hyperkeratotic lesions, Grover's disease, keratosis pilaris-like reactions, peripheral oedema, headache, nausea, vomiting, diarrhoea and fever [180, 199, 201, 202]. Additionally, these patients frequently develop resistance to vemurafenib, thus the current therapeutic approaches for this cutaneous melanoma subtype need to be improved [185].

Due to their mutational and epigenomic profile, melanoma cells are highly heterogeneous, which affects their response to therapy and their intrinsic mechanisms [185-187]. In order to create new treatments to overcome the multiple mechanisms of resistance to vemurafenib, and avoid transient responses and the recurrence of the tumour, is necessary to understand the molecular pathways involved in these mechanisms [187, 203].

The resistance to vemurafenib, that appears after 5-7 months of treatment [204], can be divided in two subtypes, primary and secondary resistance [186]. Mechanisms of primary resistance include dysregulation of CDK4, activation of PI3K via the loss of PTEN, loss of NF1 and amplification of cyclin D gene [186, 187]. The acquired resistance, or secondary resistance, is associated with alternative splicing of *BRAF*, *BRAF* copy number amplification and reactivation of the PI3K/AKT/mTOR pathway, by compensatory feedback. Resistant cells may present an elevated expression of the kinases A-Raf and C-Raf, that, as B-Raf, are capable to induce proliferation, through MAPK pathway activation [187]. Thus, the mechanisms of secondary resistance include also reactivation of the MAPK pathway in a BRAF-independent manner [187], as mitogen-activated protein kinase kinase kinase 8 (MAP3K8) expression, *MEK* mutations and activated mutations in *NRAS*, which active transduction signals through C-Raf [186]. The secondary resistance can also be linked to the activation of several RTKs, in particular insulin-like growth factor I receptor (IGF-1R) and PDGFR, suggesting a possible interplay between *BRAF* and RTKs [187]. Additionally, the resistance to vemurafenib may be caused by factors secreted by the TME, particularly the stromal cell secretion of HGF that results in the activation of the HGF receptor, and, consequently, reactivation of MAPK and PI3K/AKT/mTOR pathways [205].

A synergy between strategies appears to be a suitable approach to overcome resistance and achieve better therapeutic results, as demonstrated by the FDA approved combinations of trametinib with dabrafenib, vemurafenib with cobimetinib, and encorafenib with binimetinib [198, 206]. The most common adverse effects for the combination of BRAF and MEK inhibitors are pyrexia, chills, fatigue, photosensitivity, nausea, vomiting, and diarrhoea [108, 207]. More serious side effects may include risk of new skin cancers, cardiac, eye, gastrointestinal, musculoskeletal, hepatobiliary, nervous-systems respiratory and renal disorders, and infections [207]. Regarding targeted therapies, studies suggested that combined approaches targeting multiple pathways could be more efficient [200, 208, 209].

Vemurafenib and cobimetinib are two inhibitors of the MAPK pathway and resistance to BRAF or MEK inhibitors can be associated with reactivation of MAPK pathway, *MEK1* mutations and PI3K/AKT/mTOR pathway activation [210-212]. The

cross-talk between MAPK and PI3K pathways includes cross-inhibition, as AKT negatively regulates ERK activation [213], cross-activation, since Ras-GTP can directly bind and allosterically activate PI3K [214], and pathway convergence, for instance when both MEK and PI3K inhibition is required to release Bcl-2-associated death promoter (BAD), a pro-apoptotic protein [215]. Thus, taking into account this cross-talk, besides MAPK pathway, targeting the PI3K/AKT/mTOR pathway, which is crucial to the cell growth, proliferation and apoptosis [101], may be a valuable approach to revert resistance to treatment in melanoma [212, 216].

The serine/threonine kinase mTOR, an effector of PI3K pathway, plays an important role in tumour progression and therefore, over the years, therapies have been developed to downregulate this protein [217]. The mTOR inhibitor rapamycin, isolated from the bacterium *Streptomyces hygroscopicus*, has immunosuppressive and antitumoral properties [218, 219] and, in 1999, FDA approved this fungicide for prevention of renal allograft rejection [220]. In melanoma cells, this mTOR inhibitor increases apoptosis and chemosensitivity [221, 222]. Rapamycin analogues, such as everolimus, were developed in order to achieved better pharmacokinetic and solubility features. Rapamycin and the analogues form a complex with the intracellular receptor FK506 binding protein 12 (FKBP12), that binds to mTOR and inhibits mTORC1 downstream signalling [223, 224]. Both mTOR complexes respond differently to this inhibitor, as mTORC1 is sensitive to the macrolide fungicide rapamycin, while mTORC2 is deemed resistant to this compound [101]. Everolimus was approved by the FDA for the treatment of renal cell carcinoma, brain tumour, breast cancer and neuroendocrine tumours of pancreatic origin [225]. Everolimus, with the same mechanism of action of rapamycin, inhibits the downstream effectors of PI3K/AKT/mTOR pathway, triggers the blockage in the cell transition from G1 into S phase, and, subsequently, induces cell growth arrest and apoptosis. Everolimus inhibits also the expression of HIF1-α and downregulates the tumour cell proliferation, angiogenesis, and glucose uptake [101, 226]. A phase II trial showed that this drug is well tolerated and has antitumoral activity in melanoma [227]. In cutaneous melanoma, mTOR activation and *BRAF* mutations are correlated with poor prognosis [95], thus PI3K/AKT/mTOR and MAPK signalling pathways may cooperate for the development and progression of the tumour [95, 203]. Cutaneous melanoma cell lines harbouring $BRAF^{V600E}$ mutation were reported to have higher sensitivity to everolimus [203], therefore, $BRAF^{V600E}$ melanoma patients may receive vemurafenib in combination with everolimus to overcome the resistance and achieve better clinical outcomes [11, 203]. The common adverse effects of everolimus include hypertriglyceridemia, hypercholesterolaemia, opportunistic infections, thrombocytopenia and leukocytopenia [228].

In addition to signalling pathways alterations, melanoma metabolism is also altered, as mentioned above, which influences the cells behaviour, and can be used to create different therapies. In the mitochondria, the pyruvate dehydrogenase complex (PDC), which includes the pyruvate dehydrogenase (PDH), converts pyruvate to acetyl coenzyme A, carbon dioxide and NADH. PDH is activated, by dephosphorylation, by the pyruvate dehydrogenase phosphatase (PDP), and it is inhibited, through phosphorylation, by the pyruvate dehydrogenase kinase (PDK), an enzyme upregulated by HIF1-α [229, 230]. Dichloroacetate (DCA) inhibits the PDK [231] (Figure 5), and has been used for the treatment of mitochondrial dysfunction, such as lactic acidosis, a congenital mitochondrial disease [232].

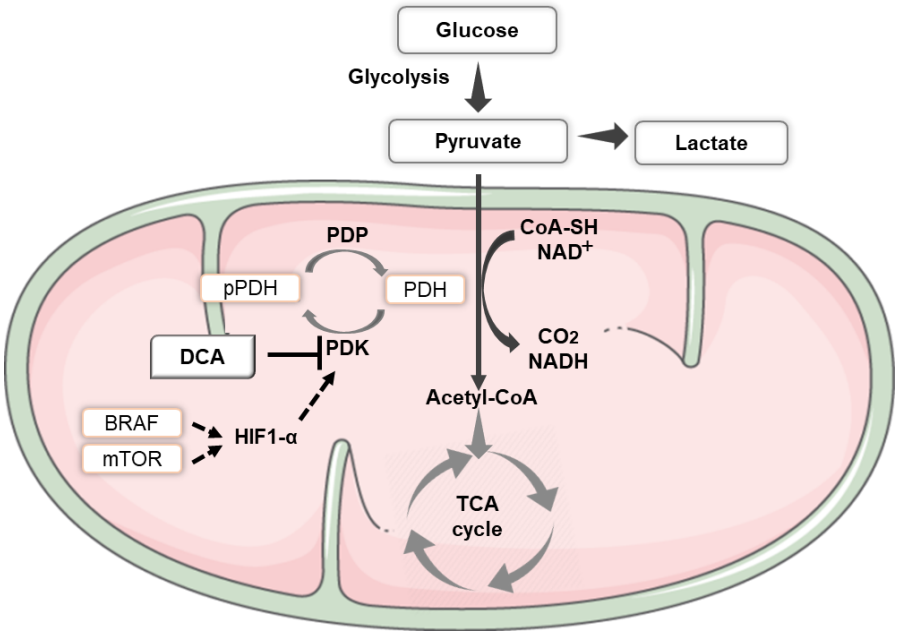



**Figure 5. Schematic representation of DCA interactions in cell metabolism.** PDK is activated by HIF1-α, present in melanoma cells, and inhibited by DCA. After the treatment with DCA, the phosphorylation and inactivation of PDH by PDK is inhibited and the activated form of PDH allows the conversion of pyruvate into acetyl-CoA, which participates in the TCA cycle. Therefore, DCA treatment decreases the conversion of pyruvate into lactate and favours the oxidative phosphorylation, leading to a switch in the metabolism. Adapted from Populo et al. [127]

Blocking PDK by DCA, which has a very similar structure to pyruvate, allows a higher amount of pyruvate to enter the mitochondria, in order to participate in the TCA cycle. In cancer, DCA treatment, that favours the metabolic profile switch from glycolysis to oxidative phosphorylation, is associated with increased production of mitochondrial ROS and decreased mitochondrial membrane potential, which leads to apoptosis [233]. Different cancer models were tested with DCA and an increase in apoptosis and a decrease in cell growth, glucose oxidation, mitochondrial membrane depolarization, and angiogenesis, through indirect HIF1-α inhibition, were observed [234]. In *in vitro* studies

in melanoma cells, DCA downregulated tumour cell proliferation, induced mTOR pathway downregulation, and promoted an increase of apoptosis [127, 232, 235, 236]. It was reported that $BRAF^{V600E}$ melanoma cells resistant to vemurafenib maintain sensitivity to DCA and, therefore, this drug could be use as possible adjuvant therapy to overcome BRAF inhibitors resistance [236, 237]. Since it has been proven the safety of the drug in humans, in addition to adjuvant therapy, DCA could also be tested as monotherapy [236].The adverse effects associated with this drug are considered moderate and age-dependent, and may include neurotoxicity (peripheral neuropathy), confusion, which is reversible, and, rarely, digestive disorders [238, 239].

The treatment of patients with mutant *BRAF* cutaneous melanoma remains a clinical challenging problem and, in order to improve the therapeutic outcomes, it is crucial to connect the comprehensive knowledge of the genetic diversity in melanoma.

# Chapter 2: Objectives

In 2011, FDA approved vemurafenib, a selective oral BRAF inhibitor, for the treatment of unresectable or metastatic melanomas harbouring $BRAF^{V600}$ mutations. This therapy presents limitations due to the rapidly acquirement of resistance and in order to overcome this resistance, in 2015, FDA approved the combination of vemurafenib with cobimetinib, an oral selective MEK inhibitor, for the treatment of melanomas harbouring *BRAF* mutations, which cannot be surgically removed or display metastization. However, the survival of melanoma patients did not alter significantly, and new therapeutic modalities need to be developed.

Considering that the mechanisms of resistance to MAPK inhibitors may be related with the deregulation of MAPK and PI3K/AKT/mTOR pathways and that the presence of the Warburg effect in melanoma cells may influence the response to therapy, we hypothesized that resistance may be overcome with different combinations of a BRAF inhibitor (vemurafenib), MEK inhibitor (cobimetinib), mTOR inhibitor (everolimus) and a metabolic modulator (DCA).

Since cancer cell lines represent an *in vitro* model commonly accepted to study tumorigenic molecular processes and to understand the role of signalling pathways in cancer [240], to evaluate this hypothesis, a vemurafenib-sensitive melanoma cell line, with $BRAF^{V600E}$ mutation, and a derived vemurafenib-resistant melanoma cell line, were tested. The main objective of this study was to determine the effects of the treatments with vemurafenib, cobimetinib, everolimus and DCA, either alone or in combination, in the two cell lines, in order to compare approved therapies with new possible therapies.

# Chapter 3: Materials and Methods

## 3.1 Cell lines and culture conditions

In this project, two melanoma cell lines were tested, ED013 and ED013R2. ED013 is a vemurafenib-sensitive melanoma cell line, with $BRAF^{V600E}$ mutation. ED013R2 was generated from ED013 and is a vemurafenib-resistant melanoma cell line, harbouring the same *BRAF* mutation. The acquired resistance to vemurafenib was induced by culturing ED013 cells with increasing concentrations of vemurafenib, until they grew in a concentration above the half maximal inhibitory concentration ($IC_{50}$) [237]. These cell lines were provided by Professor Per Guldberg, from the Danish Cancer Society Research Center, Copenhagen, Denmark. The cell lines were maintained at 37°C, in a humidified atmosphere (5% $CO_2$) and cultured as a monolayer. ED013 cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco/BRL – Invitrogen, Glasgow, United Kingdom), supplemented with 10% of fetal bovine serum (FBS) (Fetal Bovine Serum, qualified, heat inactivated, South America Origin, Gibco™, Darmstadt, Germany), 100 U/mL of penicillin and 100 ug/mL of streptomycin (Penicillin-Streptomycin 10000 U/mL, Gibco™, Darmstadt, Germany) and 1.25 µg/mL of amphotericin B and 1.025 µg/mL of sodium deoxycholate (Amphotericin B, Gibco™, Darmstadt, Germany). ED013R2 cells were maintained in the same supplemented medium with additional 1 µM of vemurafenib (Absource Diagnostics GmbH, München, Deutschland).

## 3.2 Genotypic profile of melanoma cell lines

The genotypic profiles of ED013 and ED013R2 melanoma cell lines, based on short tandem repeats (STRs) analysis, were provided by IPATIMUP's Cell Bank Lines Service. The exact number of repeting short sequences on a locus was measured by performing a polymerase chain reaction (PCR), with probes to specfic regions. Using a Promega Powerplex® System 16 and Identifiler®, 15 STRs (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, FGA) and a marker for gender identification (the Amelogenin) were analysed in ED013 and ED013R2 melanoma cell lines.

## 3.3 Treatment of melanoma cell lines using vemurafenib, cobimetinib, everolimus and DCA

Vemurafenib (Absource Diagnostics GmbH, München, Deutschland), molecular weight 489.92 g/mol, cobimetinib (Genentech, Roche Group, San Francisco, California, USA), molecular weight 531.32 g/mol, and everolimus (Novartis Pharma AG, Basel, Switzerland), molecular weight 958.22 g/mol, were dissolved in dimethyl sulfoxide (DMSO). DCA (Sodium dichloroacetate, Sigma-Aldrich, St. Louis, Missouri, USA), molecular weight 128.94 g/mol, was dissolved in distilled water ($dH_2O$) and filtered, with a 0.22 µm filter. To treat melanoma cell lines, drugs were added to the respective culture medium and incubated during 48 and 72 h. For control, both cell lines were incubated with the respetive culture medium and culture medium with DMSO and/or $dH_2O$, according to the drug solvent.

## 3.4 Cell viability assay

The cell viability assays were performed in ED013 and ED013R2 cells, using vemurafenib, cobimetinib, everolimus and DCA as treatments. The assays were performed in 96-well plates and ED013 and ED013R2 cells were seeded at a density of $5x10^3$ and $7.5x10^3$ cells/well, respectively, in 100 µL of the respective medium, using Multidrop™ Combi Reagent Dispenser (Thermo Fisher Scientific, Darmstadt, Germany). After 24 h, the medium was replaced by media containing different treatment concentrations. For vemurafenib, ED013 cells were treated with 500, 1000, 2000, 3000, 4000 and 5000 nM and ED013R2 cells were treated with 1000, 2500, 4000, 5000, 6000, 7500 and 10000 nM. For cobimetinib, ED013 cells were treated with 10, 20, 30, 40, 50, 100 and 200 nM and ED013R2 cells were treated with 1000, 2500, 4000, 5000, 6000, 7500 and 10000 nM. For DCA, both melanoma cell lines were treated with 5, 10, 20 and 40 mM. After treatment, cells were incubated for 48 and 72 h, in the culture conditions described above. At each time point, the medium was removed and cells were washed twice with 50 µL of medium without supplements. Posteriorly, cells were incubated, during 45 min, with the respective culture medium with 10% of PrestoBlue™ (PB) Reagent, according to the manufacturer's instructions. The metabolically active cells reduce the PB reagent, which allowed the quantitative measure of viability. Using a robotic-compatible microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski, Vermont, USA), fluorescence was read using 560

nm, as excitation wavelength, and 590 nm, as emission wavelength. Wells without cells seeded containing only medium and medium with DMSO/$dH_2O$, according with the drug tested, served as background fluorescence control. Each experimental condition was evaluated with triplicates. Using GraphPadPrism 6.0 (GraphPad Software, Inc., La Jolla, California, USA) the $IC_{50}$ for vemurafenib was estimated in ED013 cells, the $IC_{50}$ for cobimetinib was also estimated in ED013 cells and the $IC_{50}$ for DCA was estimated in both melanoma cell lines.

Following the same theoretical and experimental principles of PB reagent, ED013 and ED013R2 cells were tested with each drug alone and with drug combinations. For vemurafenib and cobimetinib, ED013 and ED013R2 cells were treated with half of the $IC_{50}$ and with the $IC_{50}$, determined previously for ED013 cells (900 and 1800 nM of vemurafenib and 20 and 40 nM of cobimetinib). For everolimus, 10 and 20 nM were used in both cell lines, being half of the recommended concentration and the recommended concentration by the manufacture. For DCA, cells were treated with the $IC_{50}$, 20 mM for ED013 and 14 mM for ED013R2 cells. All single-agent and drug combinations treatments are described in Table 2, for ED013 and ED013R2 cells. Each experimental condition was evaluated with triplicates and cell viability was analysed using GraphPadPrism 6.0 (GraphPad Software, Inc., La Jolla, California, USA).

## 3.5 Cell proliferation assay

The cell proliferation assays were performed in 6-well plates and ED013 and ED013R2 cells were seeded at a density of $1.8x10^5$ and $2x10^5$ cells/well, respectively. After 24 h, cells were incubated with the treatments described in the Table 2, which included treatments with a single-agent and combinations of drugs. After 72 h, cells were collected and diluted in isoton II diluent (Beckman Coulter, Brea, California, USA), with a dilution factor of 200. To measure the cell number in each condition, a Beckman Coulter Z series (Beckman Coulter, Brea, California, USA) was used. The cells in the isoton II, a conductive diluent, produce an alteration in the electrical resistance, which is detected and measured by the equipment that counts the number of cells by the number of pulses produced. Each experimental condition was performed three times.

**Table 2. Treatments applied to ED013 and ED013R2 cells.**

| | | ED013 | ED013R2 |
|---|---|---|---|
| **Single-drug treatments** | Vemurafenib | 900 nM<br>1800 nM | |
| | Cobimetinib | 20 nM<br>40 nM | |
| | Everolimus | 10 nM<br>20 nM | |
| | DCA | 20 mM | 14 mM |
| **Two-drug treatments** | Vemurafenib and cobimetinib | 900 nM vemurafenib + 20 nM cobimetinib<br>900 nM vemurafenib + 40 nM cobimetinib<br>1800 nM vemurafenib + 20 nM cobimetinib<br>1800 nM vemurafenib + 40 nM cobimetinib | |
| | Vemurafenib and everolimus | 900 nM vemurafenib + 20 nM everolimus<br>1800 nM vemurafenib + 20 nM everolimus | |
| | Vemurafenib and DCA | 900 nM vemurafenib + 20 mM DCA<br><br>1800 nM vemurafenib + 20 mM DCA | 900 nM vemurafenib + 14 mM DCA<br><br>1800 nM vemurafenib + 14 mM DCA |
| | Cobimetinib and everolimus | 40 nM cobimetinib + 20 nM everolimus | |
| | Cobimetinib and DCA | 40 nM cobimetinib + 20 mM DCA | 40 nM cobimetinib + 14 mM DCA |
| | Everolimus and DCA | 10 nM everolimus + 20 mM DCA<br><br>20 nM everolimus + 20 mM DCA | 10 nM everolimus + 14 mM DCA<br><br>20 nM everolimus + 14 mM DCA |
| **Three-drug treatments** | Vemurafenib, cobimetinib and everolimus | 1800 nM vemurafenib + 40 nM cobimetinib + 20 nM everolimus | |
| | Vemurafenib, cobimetinib and DCA | 1800 nM vemurafenib + 40 nM cobimetinib + 20 mM DCA | 1800 nM vemurafenib + 40 nM cobimetinib + 14 mM DCA |

## 3.6 Cell cycle and apoptosis analyses

ED013 and ED013R2 cells were seeded, in 6-well plates, at a density of $1.8x10^5$ and $2x10^5$ cells/well, respectively, and incubated in the culture conditions described above for 24 h. The treatments mentioned before (Table 2) were applied to cells during 72 h.

For cell cycle analysis, the harvested cells were fixed overnight in cold 70% ethanol, and then, using a DNA staining solution, containing phosphate buffered saline (PBS, composed by 0.01 M $Na_2HPO_4$, 0.0018 M $KH_2PO_4$, 0.1370 M NaCl, 0.0027 KCl, pH 7.4) 1x, 0.01 mg/mL of RNase A and 5 µg/mL of propidium iodine, cells were resuspended and analysed by flow cytometry, plotting at least 20000 events per sample. Each experiment was performed three times and the results obtained were analysed using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, USA).

Considering that annexin V binds to phosphatidylserine present on the surface of apoptotic cells and that propidium iodine, which binds to DNA, is an impermeant dye to live cells, for apoptosis analysis the Annexin-V FITC Apoptosis Kit (Clontech Laboratories, Inc., Saint-Germain-en-Laye, France) was used, according to the manufacturer's instructions, being added to the harvested cells 2.5% of anexin V and 2.5 µg/mL of propidium iodine. Cells were analysed by flow cytometry in a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA), plotting at least 20000 events per sample. The cells autofluorescence was measured, at each condition, plotting at least 10000 events per sample. Each experiment was performed three times and the results obtained were analysed using the BD Accuri C6 Software (BD Biosciences, Franklin Lakes, New Jersey, USA).

## 3.7 Protein expression analysis

For protein expression analysis, ED013 and ED013R2 cells were seeded, in 6-well plates, at a density of $1.8x10^5$ and $2x10^5$ cells/well, respectively, incubated for 24 h and subsequently the treatments mentioned before (Table 2) were applied. After 72 h, cells were subjected to a reagent-based cell lysis using radioimmunoprecipitation (RIPA) buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA and 1% NP-40, pH=7.5) with 1% phosphatase inhibitors (Sigma-Aldrich, Darmstadt, Germany) and 4% protease inhibitors (Roche Applied Science, Penzberg, Germany). The protein extracts obtained from each sample were quantified by the Bradford method (Bio-Rad, Hercules, California, USA).

To analyse the protein extracts in a gel, a loading buffer (LB) was prepared containing 5% β-mercaptoethanol and 5% bromophenol blue in Laemmli 4x with 250 mM Tris-HCl, 8% sodium dodecyl sulfate (SDS) and 40% glycerol. In order to identify proteins, samples containing 50 µg of protein in LB 4x, and, if needed, $dH_2O$, were firstly denatured for 5 min at 95ºC and then separated in 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), by molecular masses. Afterwards, the proteins were transferred onto a nitrocellulose membrane using iBlot 2 Dry Blotting System (Thermo Fisher Scientific, Darmstadt, Germany), according to the manufacturer's instructions, at 20 V for 1 min, 23 V for 4 min and 25 V for 2 min. Alternatively, the proteins were transferred onto a nitrocellulose membrane in a wet transfer system, with a transfer buffer composed by 25 mM Tris, 192 mM glycine and 20% methanol, for 2 h, at 100 V, on ice. Independently of the transfer system, membranes were stained with Ponceau S. Membranes were then blocked for 1 h at room temperature in 4% bovine serum albumin (BSA) or 5% low-fat dry milk, depending on the dilution of the primary antibodies, in PBS 1x.

To analyse the effects of the drugs tested, the primary antibodies anti-ERK 1/2, anti-phospho-ERK1/2 Thr202/Tyr204, anti-AKT (pan), anti-phospho-AKT Ser473, anti-mTOR, anti-phospho-mTOR Ser2448, anti-S6, anti-phospho-S6 Ser235/236, anti-4E-BP1, anti-phospo-4E-BP1 Thr37/4 (all 1:1000; Cell Signaling Technology, Danvers, Massachusetts, USA), and anti-PDH and anti-phospho-PDH Ser293 (1:2000, Abcam, Cambridge, United Kingdom) were incubated overnight at 4ºC. For loading control, anti-actin (1:2000; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-vinculin (1:1000, Sigma-Aldrich, St. Louis, Missouri, USA) antibodies were used. The secondary antibodies, linked to peroxidase, were used according to the host animal species in which the primary antibody was produced (1:2000, GE Healthcare, Munich, Germany or 1:3000, Santa Cruz Heidelberg, Germany). Between the incubation with primary and secondary antibodies, the membranes were washed in PBS 1x with 0.05% Tween-20. The proteins were detected using enhanced chemiluminescence (ECL), mixing equal volumes of the Enhanced Luminol Reagent and the Oxiding Reagent (PerkinElmer, Waltham Massachusetts, USA), and X-ray films (Amersham Hyperfilm ECL, GE Healthcare, Munich, Germany). In order to remove the primary and secondary antibodies, two protocols with different harshness were followed. In the mild stripping, membranes stayed at room temperature in a solution containing 0.2 M glycine, 1% of SDS 10% and 1% Tween-20, with pH adjusted to 2.2. In the harsh stripping, the membranes stood, in the fumehood, for 20 min, at 55°C, in a buffer composed by 20% of SDS 10%, 12.5% of Tris-HCl pH 6.8 and 0.8% β-mercaptoethanol. The quantification

of protein expression was performed with the Bio-Rad Quantity One 1-D Analysis software (4.6.9 version).

## 3.8 Statistical analysis

The data obtained was analysed by the unpaired and paired Student's t-test using STAT VIEW-J 5.0 (SAS Institute, Inc., Cary, North Carolina, USA). Statistically, a p-value between 0.0001 and 0.001 was considered extremely significant, between 0.001 and 0.01 was considered very significant, and between 0.01 and 0.05 was considered significant. A p-value equal or superior to 0.05 was considered non-significant. The data is presented with mean ± standard deviation (SD).

# Chapter 4: Results

## 4.1 Genomic characterization of ED013 and ED013R2 melanoma cell lines

ED013 and ED013R2 melanoma cell lines were analysed, using a Powerplex 16 HS kit. The DNA typing compared 15 STRs and the Amelogenin marker for gender determination. The obtained results (Table 3) showed that both melanoma cell lines present a similar genotypic pattern, confirming the derivation of ED013R2 cell line from the ED013 cell line.

**Table 3. Autosomal STR DNA profile of ED013 and ED013R2 melanoma cell lines, using Powerplex 16 HS kit.**

| | | ED013 | ED013R2 |
|---|---|---|---|
| STRs | **D3S1358** | 14 | 14 |
| | **TH01** | 5-7 | 5-7$^{(-)}$ |
| | **D21S11** | 28 | 28 |
| | **D18S51** | 13-17 | 13-17 |
| | **Penta E** | 7-12 | 7-12$^{(-)}$ |
| | **D5S818** | 10-11$^{(-)}$ | 10-11$^{(-)}$ |
| | **D13S317** | 11 | 11 |
| | **D7S820** | 8-10 | 8-10 |
| | **D16S539** | 12-13 | 12-13 |
| | **CSF1PO** | 10-12 | 10$^{(-)}$-12 |
| | **Penta D** | 9-12 | 12 |
| | **Amelogenin** | XY | XY |
| | **vWA** | 17 (+18) | 17 |
| | **D8S1179** | 12-14 | 12-14 |
| | **TPOX** | 9-11$^{(-)}$ | 9-11 |
| | **FGA** | 24 | 24 (+23) |

## 4.2 ED013 and ED013R2 melanoma cell lines viability

Vemurafenib, cobimetinib, everolimus and DCA are already approved to treat human diseases. In order to establish the impact of the drugs, as single-agents and in combination, the viability of ED013 and ED013R2 cells was evaluated, by PB assay, after the exposure to the treatments. The viability of untreated cells was taking into account to determinate the viability of cells exposed to the treatments.

### 4.2.1 Determination of the $IC_{50}$ of vemurafenib, cobimetinib and DCA in ED013 and ED013R2 melanoma cell lines

ED013 and ED013R2 cell lines, harbouring the $BRAF^{V600E}$ mutation, were treated with increasing concentrations of vemurafenib, a $BRAF^{V600}$ inhibitor, for 48 and 72 h, in order to estimate the $IC_{50}$. In the ED013 cells, treated with 500 to 5000 nM of vemurafenib (Figure 6A), the cell viability decreased in a dose-dependent manner, since the highest doses induced the most evident effects. In these cells, vemurafenib triggered also a decrease in cell viability in a time-dependent manner, since the same drug concentration is associated with lower percentage of cell viability in the 72 h of treatment. The $IC_{50}$ was estimated as 1800 ± 2.16 nM, after 48 h of treatment. In the ED013R2 cells, from 1000 to 10000 nM of vemurafenib (Figure 6B), a pronounced reduction on cell viability was not observed, comparing with the untreated cells in control, in both time points. The treatments could not induce 50% of loss of cell viability and, therefore, the $IC_{50}$ could not be determined.

Cobimetinib, a MEK inhibitor, from 10 to 200 nM, induced a decrease on ED013 cell viability (Figure 6C), in a dose- and time-dependent manner. The $IC_{50}$ was estimated as 40 ± 2.63 nM, after 48 h of treatment. Similar to vemurafenib, the ED013 and ED013R2 cells had distinct sensitivity to cobimetinib, and ED013R2 cells, treated with 1000 to 10000 nM of cobimetinib, did not reach the $IC_{50}$ (Figure 6D).

DCA, the metabolic modulator, induced evident effects in both melanoma cell lines. With 5 to 40 mM of DCA, the cell viability was reduced in a dose-dependent manner and the $IC_{50}$, after 48 h of treatment, was 20 ± 1.83 mM for ED013 cells (Figure 6E) and 14 ± 2.03 mM for ED013R2 cells (Figure 6F).

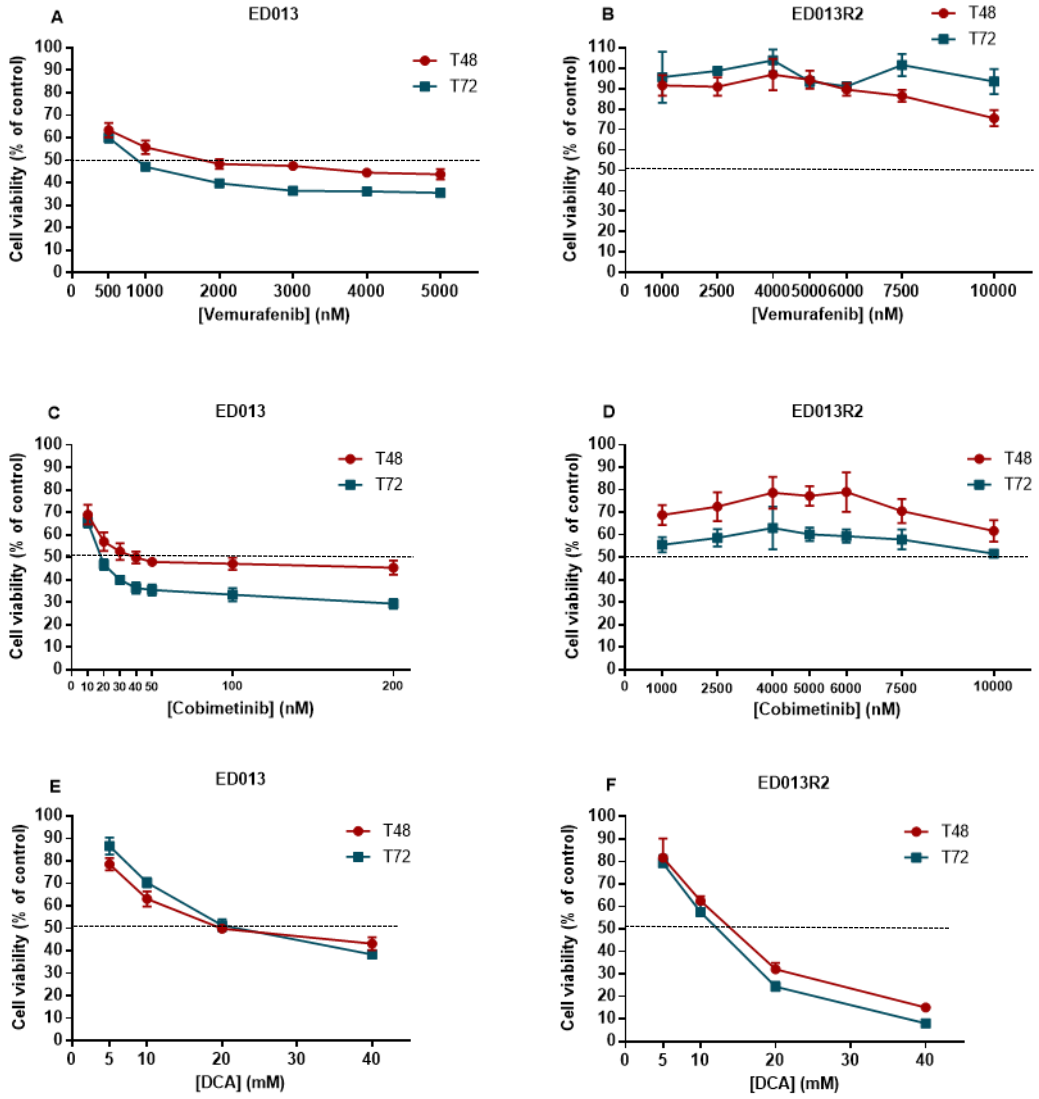



**Figure 6. Effects of vemurafenib, cobimetinib and DCA in ED013 and ED013R2 melanoma cell lines viability.** Graphic representation of the percentage of viable ED013 cells (A, C and E) and ED013R2 cells (B, D and F), after treatment with vemurafenib, cobimetinib and DCA, for 48 and 72 h, determined by Presto Blue assay, relatively to the cell viability in the control (untreated cells). The dashed line indicates the 50% of cell viability. **A:** ED013 cells were treated with increasing concentrations of vemurafenib, between 500 nM and 5000 nM, and the $IC_{50}$ value was estimated as 1800 ± 2.16 nM, after 48 h of treatment. **B:** ED013R2 cells were treated with increasing concentrations of vemurafenib, between 1000 nM and 10000 nM, and the $IC_{50}$ value was not reached. **C:** ED013 cells were treated with increasing concentrations of cobimetinib, between 10 nM and 200 nM, and the $IC_{50}$ value was estimated as 40 ± 2.63 nM, after 48 h of treatment. **D:** ED013R2 cells were treated with increasing concentrations of cobimetinib, between 1 000 nM and 10 000 nM, and the $IC_{50}$ value was not reached. **E:** ED013 cells were treated with increasing concentrations of DCA, between 5 mM and 40 mM, and the $IC_{50}$ value was estimated as 20 ± 1.83 mM, after 48 h of treatment. **F:** ED013R2 cells were treated with increasing concentrations of DCA, between 5 mM and 40 mM, and the $IC_{50}$ value was estimated as 14 ± 2.03 mM, after 48 h of treatment. The data are presented as mean ± SD.

### 4.2.2 Effects of treatments on ED013 and ED013R2 melanoma cell lines viability

To evaluate ED013 cells viability, the targeted drugs associated with more adverse effects were used at half of the $IC_{50}$ and at the $IC_{50}$, determined previously (900 and 1800 nM of vemurafenib, and 20 and 40 nM of cobimetinib). Similarly, everolimus was used at half of the recommended concentration by the manufacture and at the recommended concentration by the manufacture, 10 and 20 nM. DCA, which is associated with mild to moderate side effects, was used at the $IC_{50}$, 20 mM. ED013 cells were treated for 48 and 72 h (Figure 7A and B, respectively).

For ED013 cells, comparing to the control, treatments decreased the cell viability, with extremely significant differences, at both time points ($p < 0.0001$). Within treatments, the drug combinations induced a more pronounced decrease on cell viability than the single-agent treatments, with extremely significant differences between effects, at both time points ($p < 0.0001$). ED013 cells responded in a time-dependent manner to treatments, reflected in a higher decrease on cell viability at 72 h of treatment.

Considering the treatments with only one drug, the higher doses of vemurafenib and cobimetinib induced a more pronounced decrease on ED013 cell viability, comparing with the respective lower doses, at both time points ($p= 0.0002$ to $0.0048$). In response to MAPK inhibitors as single-agent treatments, for the 48 h of treatment, the reduction of ED013 cell viability was superior to 40% and, for the 72 h of treatment, the reduction was superior to 50%. For everolimus, although the higher dose induced more effects than the lower dose, the difference was not significant, and the inhibition of viability was around 20%, at both time points. The metabolism modulator, DCA, induced a decrease on cell viability, in a time-dependent manner, with significant differences between time points ($p = 0.0162$), with a reduction superior to 40 and 50%, for the 48 and 72 h treatments, respectively.

Treatments with two drugs included 12 different combinations. In general, two-drug treatments induced a loss of ED013 cell viability superior to 50%, for the 48 h treatment, and superior to 70% in response to the majority of treatments, in the 72 h treatment. Among this class of treatments, the association of 40 nM of cobimetinib with 20 nM of everolimus, the only two-drug treatment combining cobimetinib and everolimus, caused the most significant loss of viability, at both time points, in ED013 cells ($29.07 \pm 1.46\%$ and $15.03 \pm 0.26\%$, for 48 and 72 h of treatment, respectively). Extremely or very significant differences on cell viability were observed comparing the effects from this treatment with all the other treatments, after 48 h ($p < 0.0001$ to $0.0073$) and 72 h of treatment ($p < 0.0001$ to $0.0015$). Excepting 40 nM of cobimetinib with 20 nM of

everolimus treatment, the two-drug combinations that contained vemurafenib induced a more pronounced decrease on cell viability than the other two-drug combinations, after 48 h ($p < 0.0001$ to 0.0285) and 72 h of treatment ($p < 0.0001$ to 0.0137).

From all the treatments tested, the three-drug combination of vemurafenib, cobimetinib and everolimus triggered the most evident damage on ED013 cell viability, at both time points (25.89 ± 0.64% and 14.23 ± 0.13%, for 48 and 72 h of treatment, respectively).

The combinations of vemurafenib with cobimetinib induced loss of ED013 cell viability around 40 and 70%, for the 48 and 72 h of treatment, respectively. The treatments that consistently caused more pronounced effects on cell viability comparing to these combinations, at both time points, were 40 nM of cobimetinib with 20 nM of everolimus and the three-drug combination of vemurafenib, cobimetinib and everolimus ($p < 0.0001$ to 0.0295, for 48 h of treatment, and $p < 0.0001$, for 72 h of treatment).

The ED013R2 cells showed to be resistant to both vemurafenib and cobimetinib, since the $IC_{50\,s}$ for each agent was above 10000 nM. Therefore, the $IC_{50\,s}$ estimated for these drugs in the ED013 cells were used in the ED013R2 cells, and the rationale mentioned above was used to create the 21 treatments, using 900 and 1800 nM of vemurafenib, 20 and 40 nM of cobimetinib, 10 and 20 nM of everolimus and the $IC_{50}$ of DCA, 14 mM. ED013R2 cells were treated for 48 and 72 h (Figure 7C and D, respectively).

Similar to ED013 cells, in the ED013R2 cells the treatments reduced the cell viability relatively to control, with extremely significant differences between effects, at both time points ($p < 0.0001$). The combinations reduced the cell viability more effectively than the single-agent treatments, inducing effects with extremely significant differences, at both time points ($p < 0.0001$).

For the single-agent treatments, the higher doses induced no significant differences on cell viability comparing to the lower doses for vemurafenib, cobimetinib and everolimus, during the 48 h of exposure, and each drug induced a reduction of around 5%, 15%, and 30%, respectively. Despite the resistance observed, ED013R2 cells responded in a time-dependent manner to 900 nM of vemurafenib treatment (93.30 ± 1.89% and 87.85 ± 2.22%, for 48 and 72 h of treatment, respectively, $p = 0.0331$), to 1800 nM of vemurafenib treatment (93.42 ± 2.01% and 84.63 ± 4.07%, for 48 and 72 h of treatment, respectively, $p = 0.0064$) and to 20 nM of cobimetinib treatment (74.87 ± 1.95 and 67.35 ± 1.45, for 48 and 72 h of treatment, respectively, $p = 0.0041$).

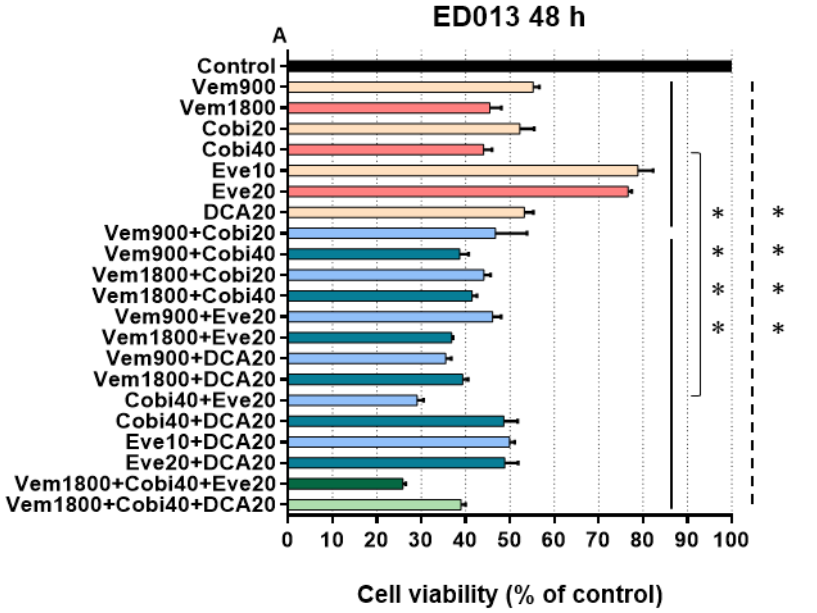
In ED013R2 cells, the only two-drug treatment capable to induce a reduction on cell viability consistently superior to 50%, at both time points, was 40 nM of cobimetinib

with 20 nM of everolimus (31.87 ± 0.95% and 28.70 ± 1.34%, for 48 and 72 h of treatment, respectively).

For the three-drug approaches, in ED013R2 cells, combining vemurafenib, cobimetinib and everolimus (36.64 ± 0.76% and 32.28 ± 1.26%, for 48 and 72 h of treatment, respectively) was more effective than combining the two melanoma approved drugs with DCA (40.56 ± 1.45% and 47.36 ± 1.81%, for 48 and 72 h of treatment, respectively).

The four combinations between vemurafenib and cobimetinib exhibited similar effects, between treatments and also between time points. Comparing to the approved combination for melanoma, the treatments that achieved a more significant reduction on ED013R2 cell viability, at both time points, were 1800 nM of vemurafenib with 20 nM of everolimus, 40 nM of cobimetinib with 20 nM of everolimus, 20 nM of everolimus with DCA, and both three-drug combinations ($p < 0.0001$ to 0.0051, for 48 h of treatment, and $p < 0.0001$ to 0.0083, for 72 h of treatment).

Analysing the same treatment in both melanoma cell lines, at 48 h, the treatments more efficient in the resistant cells were the 10 nM of everolimus (78.88 ± 3.45% in ED013 cells and 64.90 ± 2.17% in ED013R2 cells, $p = 0.0051$) and 20 nM of everolimus (76.62 ± 0.86% in ED013 cells and 68.04 ± 1.70% in ED013R2 cells, $p = 0.0066$), with very significant differences between ED013 and ED013R2 cell viability. At 72 h of treatment, only 10 nM of everolimus managed to induce more efficient effects in ED013R2 cells (66.30 ± 0.47%) with extremely significant differences from the effect in the ED013 cells viability (85.46 ± 1.89%, $p = 0.0005$).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ED013 48 h
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Cell viability (% of control)
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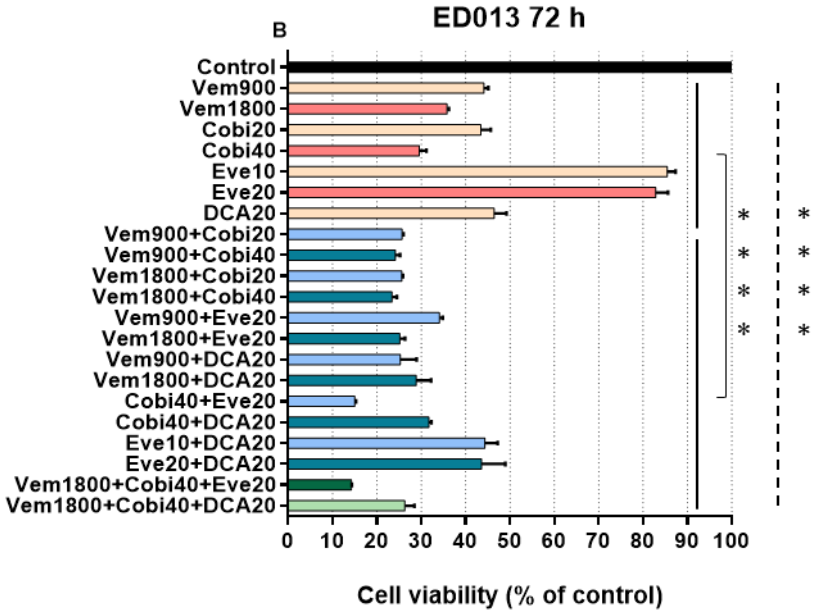
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ED013 72 h
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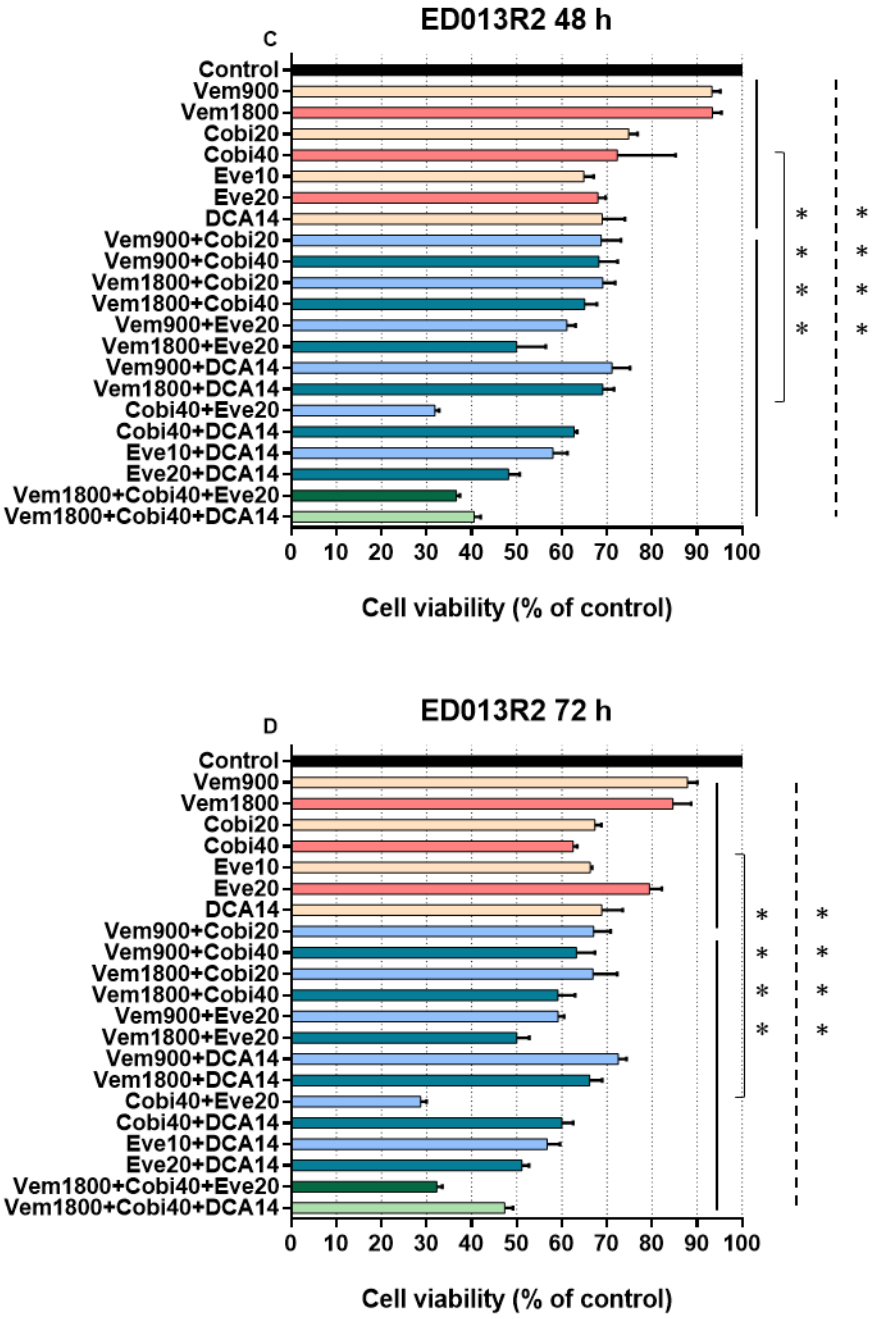



**Figure 7. Effects of treatments on ED013 and ED013R2 melanoma cell lines viability.** Graphic representation of the percentage of viable ED013 cells treated for 48 and 72 h (A and B, respectively) and ED013R2 cells treated for 48 and 72 h (C and D, respectively) with vemurafenib, cobimetinib, everolimus, DCA and combined treatments. The viability was determined by PB assay, relatively to cell viability in the control (untreated cells). ED013 cells were treated with vemurafenib and cobimetinib at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40). ED013R2 cells, the resistant cells to vemurafenib and cobimetinib, were treated with the concentrations described above for ED013 cells. In both cell lines, for everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20) for EDO013 cells and 14 mM of DCA (DCA14) for ED013R2 cells. Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. Single-agent treatments are represented in pink bars, two-drug combinations are represented in blue bars and three-drug combinations are represented in green bars. The data are presented as mean ± SD. The dashed line represents the statistical difference between all the treatments and the control. The continuous lines represent the statistical difference between single-agent treatments and drug combinations. **** represents $p < 0.0001$.

## 4.3 Effects of treatments on ED013 and ED013R2 melanoma cell lines proliferation

The cancer cells proliferation depends on several crucial pathways, which are frequently the target of antitumoral drugs. The effects of the drugs on the cell proliferation were evaluated after 72 h of treatment and the number of cells was counted and normalized in relation to number of untreated cells in the control.

For the ED013 cells (Figure 8A), all the treatments induced an extremely significant decrease in the number of cells, comparing to the control ($p < 0.0001$). The results showed a reduction of at least 40% in all the treatments. The effects induced by combinatorial treatments caused more damage in the cell proliferation than the single-agent treatments, with very significant differences between effects ($p = 0.0027$).

Among the single-agent treatments, the $IC_{50}$ of vemurafenib, cobimetinib and DCA induced similar effects on the proliferation of the ED013 cells (0.45 ± 0.06, 0.43 ± 0.09 and 0.46 ± 0.06, respectively). Everolimus induced effects in a dose-dependent manner, although without statistical differences, inducing a reduction superior to 40% in the number of cells.

The association of the two unapproved drugs for melanoma, 10 nM everolimus and DCA, induced the most evident reduction in the number of ED013 cells among the two-drug treatments (0.38 ± 0.07), although statistically the effects do not differ from 12 other treatments, such as the two-drug treatment of 40 nM of cobimetinib with 20 nM of everolimus (0.43 ± 0.11) and the three-drug treatment of vemurafenib, cobimetinib and everolimus (0.48 ± 0.06).

Regarding the four combinations of vemurafenib with cobimetinib, the number of cells counted is statistically similar, with a reduction near 50% in ED013 cell proliferation. The treatments capable to surpass the effects induced by the combinations of vemurafenib and cobimetinib, with statistical differences, were 10 nM of everolimus with DCA and the three-drug treatment of vemurafenib, cobimetinib and DCA ($p = 0.0008$ to $p = 0.0175$).

In ED013R2 (Figure 8B), the treatments also induced an extremely significant reduction in the number of cells, comparing to the control ($p < 0.0001$). The combinations of two and three drugs induced an extremely significant loss of cells relatively to treatments with only one drug ($p < 0.0001$).

Vemurafenib and cobimetinib as single-agent treatments induced effects in a dose-dependent manner, although without statistical differences between doses. These treatments induced a reduction on ED013R2 cell proliferation, with extremely to very significant differences from the control ($p < 0.0001$ to 0.007), with a reduction around

10% in response to vemurafenib treatments and around 30% in reponse to cobimetinib treatments. Everolimus induced similar effects between doses, reducing around 30% the number of cells comparing to control ($p = 0.0003$ and 0.0009, for 10 and 20 nM of everolimus, respectively). DCA caused a reduction around 50% in ED013R2 cell proliferation, with extremely significant differences from the control l ($0.59 \pm 0.08$, $p < 0.0001$).

All the two-drug treatments involving vemurafenib with cobimetinib and vemurafenib with everolimus are statistically similar, with a normalized cell number between $0.58 \pm 0.06$ and $0.65 \pm 0.06$.

The treatments that most prevented ED013R2 cell proliferation were the combination of two unapproved drugs for melanoma, 20 nM of everolimus with DCA, and both three-drugs treatments ($p < 0.0001$, relatively to control). In addition to those, only three more treatments were capable to induce a reduction on the proliferation superior to 50%, relative to control, 1800 nM of vemurafenib with DCA ($0.47 \pm 0.05$, $p < 0.0001$), 40 nM of cobimetinib with DCA ($0.48 \pm 0.10$, $p < 0.0001$) and 10 nM of everolimus with DCA ($0.49 \pm 0.07$, $p < 0.0001$).

Comparing to the drug combination of vemurafenib with cobimetinib, only the three-drug treatment of vemurafenib, cobimetinib and everolimus ($0.44 \pm 0.06$) induced a more pronounced decrease on ED013R2 cell proliferation, with statistically significant differences ($p = 0.0028$ to $p = 0.0296$).

Generally, the effects of the treatments were more evident on the ED013 cells than on the ED013R2 cells, however, statistically, most of the treatments (15 of 21 treatments) induced similar effects in both cell lines. The only approach that was capable to decrease the proliferation more efficiently, although without statistical diferences, in ED013R2 cells than in ED013 cells, was the three-drug combination with everolimus ($0.48 \pm 0.06$ and $0.44 \pm 0.06$, for ED013 and ED013R2 cells, respectively).

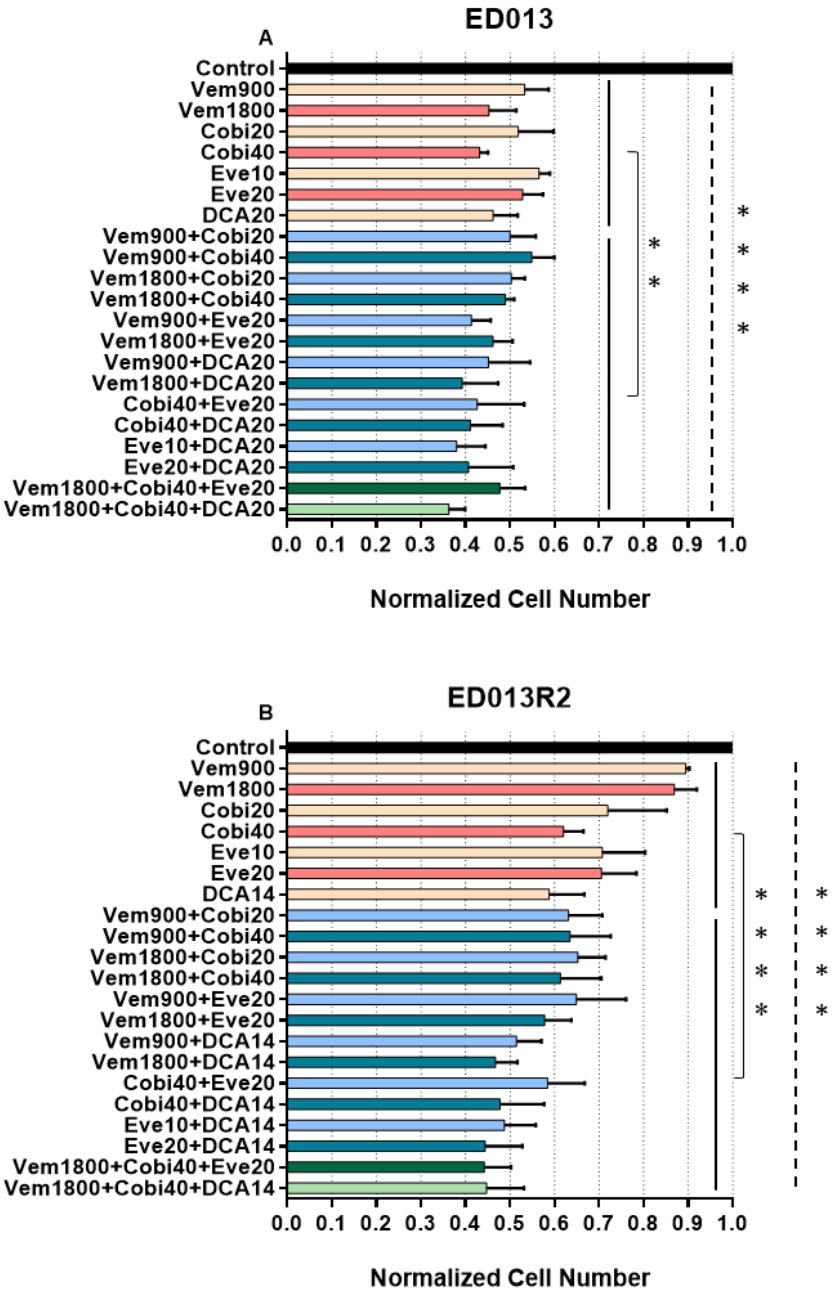



**Figure 8. Effects of treatments on ED013 and ED013R2 melanoma cell lines proliferation.** Graphic representation of the number of ED013 (A) and ED013R2 (B) cells, after normalization in relation to the number of untreated cells of the control. Number of cells were counted after 72 h of treatment with vemurafenib, cobimetinib, everolimus, DCA and combined treatments. Vemurafenib and cobimetinib at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40). ED013R2 cells, the resistant cells to vemurafenib and cobimetinib, were treated with the concentrations described above for ED013 cells. In both cell lines, for everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20) for EDO013 cells and 14 mM of DCA (DCA14) for ED013R2 cells. Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. Single-agent treatments are represented in pink bars, two-drug combinations are represented in blue bars and three-drug combinations are represented in green bars. The data are presented as mean ± SD. The dashed line represents the statistical difference between all the treatments and the control. The continuous lines represent the statistical difference between single-agent treatments and drug combinations. ** represents very significant differences ($p = 0.0027$) and **** represents extremely significant differences ($p < 0.0001$).

## 4.4 Effects of treatments on the cell cycle of ED013 and ED013R2 melanoma cell lines

As eukaryotic cells, melanoma cells growth and division depend on the cell cycle. After 72 h of treatment, ED013 and ED013R2 cells were analysed by flow cytometry and, using a DNA staining solution, it was possible to distinguish G0/G1, S and G2/mitosis (M) phases (Figure 9). Untreated cells were used as control to evaluate the effects of the treatments in the cells.

In general, and comparing to control, treatments increased the percentage of cells in G0/G1 phase and decreased the percentage of cells in S and G2/M phases, in both cell lines, although not all the differences were statistically significant.

Most of ED013 cells were in G0/G1 phase, with, approximately, 60 to 80% of cells in this phase (Figure 9A). Comparing to the control (66.70 ± 6.40%), in general, the percentage of ED013 cells in G0/G1 phase increased in response to the single-drug treatments, although without significant differences. Among two-drug treatments, a significant increase of the percentage of ED013 cells was observed in response to 1800 nM of vemurafenib with 40 nM of cobimetinib treatment (77.69 ± 3.85%, p = 0.0353) and 1800 nM of vemurafenib with 20 nM of everolimus treatment (78.38 ± 2.08%, p = 0.0171). The highest percentages of ED013 cells in the G0/G1 phase were observed in response to the 40 nM of cobimetinib with 20 nM of everolimus treatment (80.90 ± 2.58%, p = 0.0092) and to the three-drug treatment of vemurafenib, cobimetinib and everolimus (80.86 ± 2.75%, p = 0.0098), with very significant differences comparing with the control.

The frequency of ED013 cells in the S phase varied between 10 and 30% of total cells, approximately. Comparing to the control (22.56 ± 5.77%), the percentage of ED013 cells in replicative phase decreased significantly in response to single-treatment of 20 nM of cobimetinib (7.80 ± 3.86%, p = 0.0211). Among combinations, the 900 nM of vemurafenib with 20 nM of everolimus (14.65 ± 1.30%, p = 0.0408), the 40 nM of cobimetinib with DCA (12.31 ± 2.11%, p = 0.0444) and the three-drug of vemurafenib, cobimetinib and DCA (10.76 ±3.18%, p = 0.0361) induced a decrease on percentage of ED013 cells, with significant differences relatively to the control. The lowest percentages of ED013 cells in the S phase were observed in response to 40 nM of cobimetinib with 20 nM of everolimus treatment (10.37 ± 0.36%, p = 0.0072) and to the three-drug treatment of vemurafenib, cobimetinib and everolimus (9.11 ± 2.31%, p = 0.0075), with very significant differences comparing with the control.

The percentage of ED013 cells in G2/M phase varied between 5 to 10% of the total cells. Comparing to the control (9.08 ± 0.89%), a significant decrease was observed in response to 1800 nM of vemurafenib treatment (6.09 ± 1.05, p =0.0191). Among two-

drug treatments, a decrease in the percentage of ED013 cells in G2/M phase was observed in response to the combinations with vemurafenib and cobimetinib ($p = 0.0048$ to $p = 0.0202$), with very significant to significant differences comparing with the control. Analogously to the other phases, the 40 nM of cobimetinib with 20 nM of everolimus ($4.49 \pm 1.70\%$, $p = 0.0084$) and the three-drug treatment of vemurafenib, cobimetinib and everolimus ($5.27 \pm 1.08\%$, $p = 0.0042$) induced a very significant decrease in the percentage of ED013 cells in G2/M phase.

In the ED013 cells, comparing to the approved combinations of vemurafenib and cobimetinib, the treatment that was capable to consistently induce more significant effects, in all the phases, was 40 nM of cobimetinib with 20 nM of everolimus ($p < 0.0001$ to $0.0328$), which was associated with a very significant increase in the percentage of ED013 cells in the G0/G1 phase ($p = 0.001$ to $0.006$), a significant decrease in the S phase ($p < 0.0001$ to $0.0281$), and a decrease in the G2/M phase, without statistical differences.

In agreement with ED013 cells, ED013R2 cells were mostly in the first phase of the interphase, with a percentage of cells between 60 and 80%, approximately (Figure 9B). In the G0/G1 phase, comparing to the control ($59.05 \pm 5.01\%$), the single-agent treatment of 40 nM of cobimetinib induced a very significant increase of the percentage of ED013R2 cells ($75.65 \pm 2.01\%$, $p = 0.0016$). Within two-drug treatments, all the two-drug combination with vemurafenib and cobimetinib presented a percentage of cells significantly higher than the control ($p = 0.0177$ to $0.0359$). The combination of 1800 nM of vemurafenib with 20 nM of everolimus ($76.78 \pm 3.94\%$, $p = 0.0085$) and the combination of DCA with 1800 nM of vemurafenib ($69.65 \pm 1.22\%$, $p = 0.0235$), or with cobimetinib ($71.76 \pm 0.92\%$, $p = 0.0124$), or with 10 nM of everolimus ($67.11 \pm 2.53\%$, $p = 0.0365$) were also associated with a higher percentage of cells, with very significant to significant differences from the control. Identically to ED013 cells, 40 nM of cobimetinib with 20 nM of everolimus treatment ($76.80 \pm 3.83\%$, $p = 0.0031$) and the three-drug treatment vemurafenib, cobimetinib and everolimus ($73.46 \pm 1.43\%$, $p = 0.0087$) were associated with a higher percentage of cells in G0/G1 phase, with very significant differences from the percentage of cells in the control.

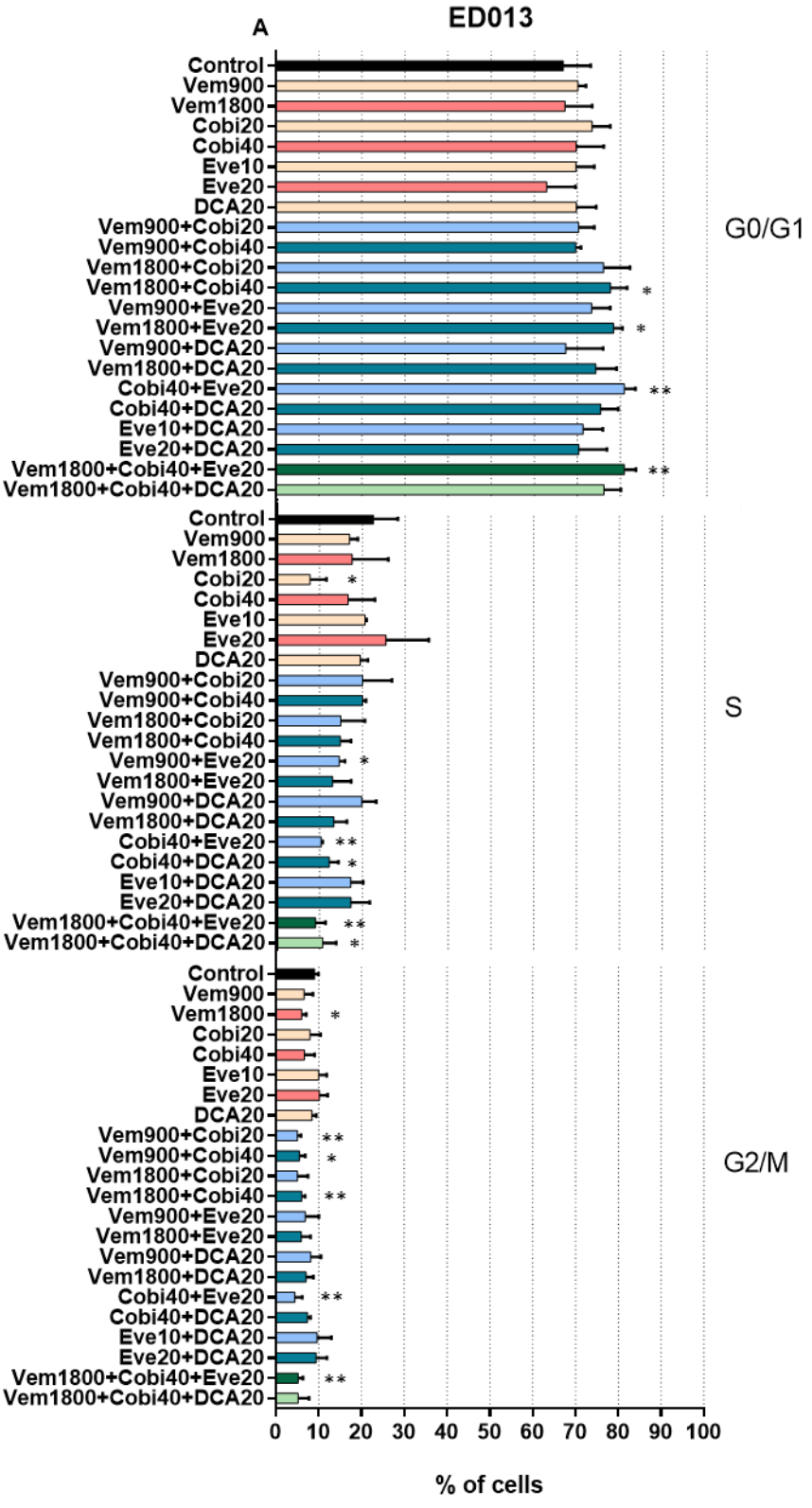
In the replicative phase, the frequency of ED013R2 cells ranged between 10 and 30% of total cells, approximately. Comparing to the control ($26.91 \pm 2.29\%$), the single-agent treatments of 20 nM of cobimetinib ($19.63 \pm 2.02\%$, $p = 0.0144$) and 40 nM of cobimetinib ($14.92 \pm 2.87\%$, $p = 0.0020$) were associated with the lowest percentages of cells in the S phase, with significant and very significant differences, respectively, comparing with the control. Within the combinations, several treatments induced significant statistical differences from the control, including 900 nM of vemurafenib with

40 nM of cobimetinib, 1800 nM of vemurafenib with 40 nM of cobimetinib, 1800 nM of vemurafenib with 20 nM of everolimus, 900 nM of vemurafenib with DCA, 40 nM of cobimetinib with DCA and the three-drug treatment containing DCA ($p = 0.0144$ to 0.0388). The 900 of vemurafenib with everolimus (19.01 ± 1.01%), 1800 nM of vemurafenib with DCA (19.38 ± 1.37%), 10 nM of everolimus with DCA (19.50 ± 2.25%), and the three-drug treatment of vemurafenib, cobimetinib and everolimus (14.12 ± 3.29%) decreased the percentage of ED013R2 cells in the S phase, with very significant statistical differences from the control ($p = 0.0052$ to 0.0081). The 40 nM of cobimetinib and 20 nM of everolimus treatment (11.36 ± 1.58%, $p = 0.0001$) decreased the percentage of ED013R2 cells in the S phase, with extremely significant differences from the control.

In the G2/M phase, the frequency of ED013R2 cells varied between 7 and 14%. The observed decrease of the percentage of cells in G2/M phase after treatments was not statistically significant different from the percentage of ED013R2 cells in G2/M phase in the control (13.68 ± 5.17%).

In the ED013R2 cells, the treatment that consistently surpass the effects induced by the approved combination for melanoma, in all the phases, was 40 nM of cobimetinib with 20 nM of everolimus, that was associated with a significant increase in the percentage of ED013R2 cells in the G1/G0 phase ($p = 0.0353$ to 0.0492), a significant decrease in the S phase ($p = 0.0037$ to 0.0219) and a decrease in the G2/M phase, without statistical differences.

Several treatments affected the cell cycle differently, comparing the two melanoma cells lines, and in generally, the effects of the treatments were more evident on the ED013 cells than on the ED013R2 cells.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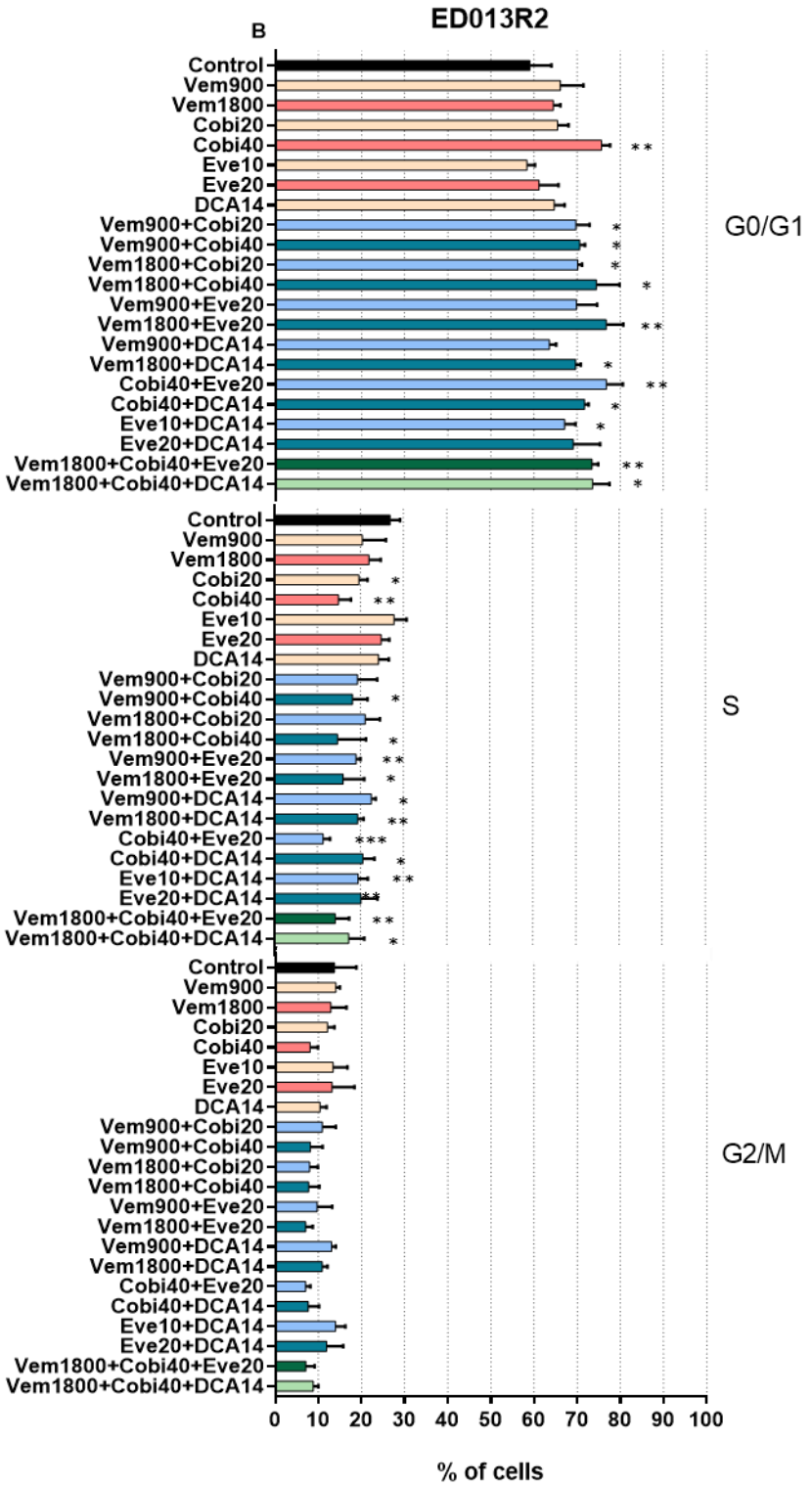



**Figure 9. Effects of treatments on the cell cycle of ED013 and ED013R2 melanoma cell lines.** Graphic representation of percentage of ED013 (A) and ED013R2 (B) cells per phase of the cell cycle (G0/G1, S and G2/M phases), determined by flow cytometry, after 72 h of treatment with vemurafenib, cobimetinib, everolimus, DCA and combined treatments. Vemurafenib and cobimetinib at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40). ED013R2 cells, the resistant cells to vemurafenib and cobimetinib, were treated with the concentrations described above for ED013 cells. In both cell lines, for everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20) for EDO013 cells and 14 mM of DCA (DCA14) for ED013R2 cells. Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. Single-agent treatments are

represented in pink bars, two-drug combinations are represented in blue bars and three-drug combinations are represented in green bars. The data are presented as mean ± SD. * represents significant differences (0.01≤ p < 0.05), ** represents very significant differences (0.001≤ p < 0.01), *** represents extremely significant differences (0.0001≤ p < 0.001) and **** represents extremely significant differences (p < 0.0001) comparing treatments with control.

## 4.5 Effects of treatments on ED013 and ED013R2 melanoma cell lines apoptosis

After 72 h of treatment, the cells were analysed, by flow cytometry, and categorized as viable, apoptotic or necrotic cells, considering the externalization of phosphatidylserine and the membrane integrity. The treatments tested were able to promote early and late apoptosis in the ED013 and ED013R2 cells. Untreated cells were used as control to evaluate the effects of the treatments in the cells.

In ED013 cells (Figure 10A), the treatments induced a significant increase on the percentage of apoptotic cells, comparing with the control (p = 0.0156), and the drug combinations were associated with higher percentage of apoptotic cells comparing with single-agent treatments, with extremely significant differences (p < 0.0001).

The single-treatments of 1800 nM of vemurafenib (14.29 ± 1.44%), 40 nM of cobimetinib (16.48 ± 2.74%) and DCA (15.13 ± 0.71%) induced a similar increase of apoptosis in ED013 cells, with extremely to very significant differences comparing to the control (5.26 ± 0.05, p < 0.0001 to 0.0021). The single-agent treatments with everolimus induced less than 10% of apoptosis, with very significant and significant differences comparing to the control (p = 0.0053 and 0.0334, for 10 and 20 nM of everolimus, respectively).

Among two-drug treatments, in ED013 cells, the combinations with vemurafenib and DCA were more efficient than the combinations with vemurafenib and everolimus, (p = 0.020 to 0.0364). Distinctly, it was observed that the combination of 40 nM of cobimetinib with 20 nM of everolimus (36.07 ± 5.48%) was more efficient than the combination of cobimetinib with DCA (24.33 ± 1.78%, p = 0.0092).

The three-drug treatment combining vemurafenib, cobimetinib and everolimus was the most efficient, inducing apoptosis in 48.58 ± 7.75% of ED013 cells (p = 0.0005, relatively to control). Similar effects were observed in the two-drug combinations of 900 nM of vemurafenib with 40 nM of cobimetinib (37.08 ± 4.62%), 1800 nM of vemurafenib with 40 nM of cobimetinib (38.29 ± 6.16%) and 40 nM of cobimetinib with 20 nM of everolimus (36.07 ± 5.48%), in ED013 cells. All treatments mentioned had in common 40 nM of cobimetinib, the single-agent that induced the higher percentage of apoptosis (16.48 ± 2.74%).

In terms of apoptotic effects, ED013 cells showed no significant differences between the four different combinations with vemurafenib and cobimetinib, achieving 30 to 40% of apoptotic cells. These combinations were the most effective among two-drug combinations, and only the three-drug treatment with vemurafenib, cobimetinib and everolimus induced a higher percentage of apoptosis in ED013 cells ($p = 0.0124$ to 0.0160).

In ED013R2 cells (Figure 10B), the treatments induced a non-significant increase on the percentage of apoptotic cells, comparing to the control. The combinatorial treatments led to an extremely significant increase of the percentage of apoptotic cells, comparing with single-drug treatments ($p < 0.0001$).

The maximum percentage of apoptosis among the single-agent treatments was achieved by DCA treatment (10.32 ± 1.14%), with very significant differences comparing with the control (5.81± 0.65%, $p = 0.0040$). The other single-agent treatments induced less than 10% of ED013R2 apoptotic cells, without statistical differences between treatments.

The most efficient treatment was 40 nM of cobimetinib with 20 nM of everolimus (16.17 ± 2.26%), however seven other combinations achieved effects without statistical differences (900 nM of vemurafenib with 40 nM of cobimetinib, 1800 nM of vemurafenib with 40 nM of cobimetinib, 1800 nM of vemurafenib with DCA, 40 nM of cobimetinib with DCA, 20 nM of everolimus with DCA, and both three-drug treatments).

Similar to ED013 cells, in the ED013R2 cells the combinations of vemurafenib and cobimetinib induced statistically identical effects between each other, around 10% of apoptotic cells. Four combinations were more efficient at inducing apoptosis in ED013R2 cells than the approved combination (40 nM of cobimetinib with 20 nM of everolimus, 40 nM of cobimetinib with DCA and both three-drug combinations), with very significant to significant differences between effects ($p = 0.0034$ to $p = 0.0171$).

Globally, the treatments induced less apoptosis in the ED013R2 cells than in the ED013 cells, except for 10 nM of everolimus treatment, although without statistical differences.

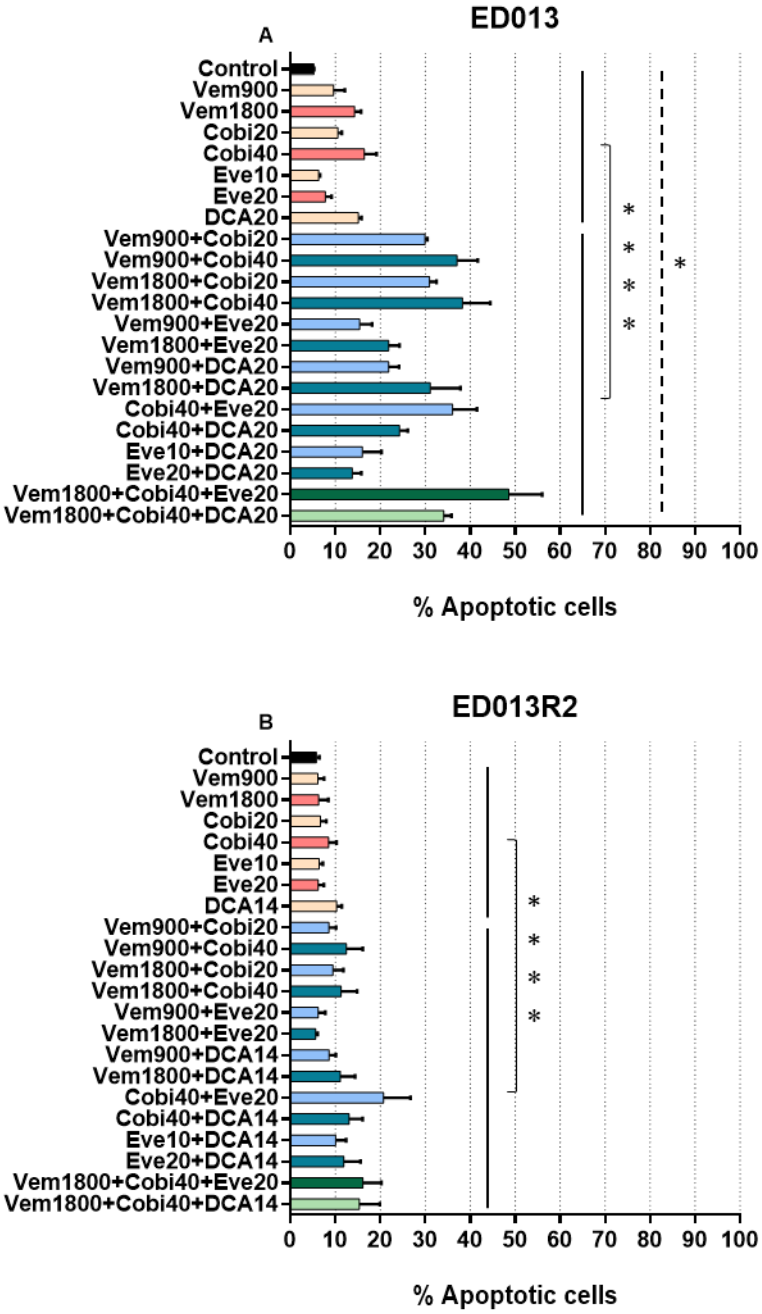



**Figure 10. Effects of treatments on ED013 and ED013R2 melanoma cell lines apoptosis.** Graphic representation of percentage of apoptotic ED013 (A) and ED013R2 (B) cells, determined by flow cytometry, after 72 h of treatment with vemurafenib, cobimetinib, everolimus, DCA and combined treatments. Vemurafenib and cobimetinib at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40). ED013R2 cells, the resistant cells to vemurafenib and cobimetinib, were treated with the concentrations described above for ED013 cells. In both cell lines, for everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20) for EDO013 cells and 14 mM of DCA (DCA14) for ED013R2 cells. Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. Single-agent treatments are represented in pink bars, two-drug combinations are represented in blue bars and three-drug combinations are represented in green bars. The data are presented as mean ± SD. The dashed line represents the statistical difference between all the treatments and the control. The continuous lines represent the statistical difference between single-agent treatments and drug combinations. * represents a significant difference ($p = 0.0156$) and **** represents extremely significant differences ($p < 0.0001$).

## 4.6 Effects of treatments on protein expression of ED013 and ED013R2 melanoma cell lines

All drugs used in this project, vemurafenib, cobimetinib, everolimus and DCA, are inhibitors of important proteins in melanoma cells, B-Raf, MEK, mTOR and PDK, respectively. The inhibition of those proteins influences signalling pathways and the metabolism of melanoma cells. To analyse the efficacy of the treatments, the expression of proteins in the PI3K/AKT/mTOR pathway and the phosphorylated form of PDH was evaluated. The expression of proteins in the MAPK pathway were tested, however, no unanswerable results were obtained. The expression was evaluated and normalized in relation to the expression of each protein in the untreated cells, used as control. The presented results correspond to an experience, with two replicates, thus the statistical analysis was not sustainable.

For ED013 cells, the expression of phosphorylated AKT, phosphorylated mTOR, phosphorylated S6, phosphorylated 4E-BP1, and phosphorylated PDH was evaluated in response to the 21 treatments (Figure 11A-E). The supplementary figure 1 shows a representative western blot of the observed protein expression in ED013 cells.

The AKT is one of the first intermediates in the PI3K pathway. In the ED013 cells, the expression of phosphorylated AKT decreased in response to vemurafenib and cobimetinib, as single-agent treatment or in combination, comparing to the control, and increased in response to treatments with everolimus, alone or in combination with the MAPK inhibitors, comparing to the control (Figure 11A). The DCA treatment, alone or in combination with MAPK inhibitors, also induced an increase in the phosphorylated AKT expression.

In the PI3K pathway, the downstream of AKT is mTOR. In general, in ED013 cells, the expression of phosphorylated mTOR decreased in response to treatments with everolimus (Figure 11B).

The downstream effectors of mTOR are S6K1, that phosphorylates S6, and 4E-BP1 proteins. The expression of phosphorylated S6 decreased, comparing to the control, in response to treatments with everolimus, as single-agent or in combination with the other three drugs, being the most evident decrease associated with the three-drug treatment containing everolimus (Figure 11C). Considering the treatments without everolimus, in general, the expression of phosphorylated S6 increased in response to treatments with MAPK inhibitors. The expression of phosphorylated 4E-BP1, in the ED013 cells, increased in response to DCA treatments (Figure 11D).

Relatively to the phosphorylated PDH, a decrease of the expression was observed in response to DCA, as single-agent or in combination, comparing with the

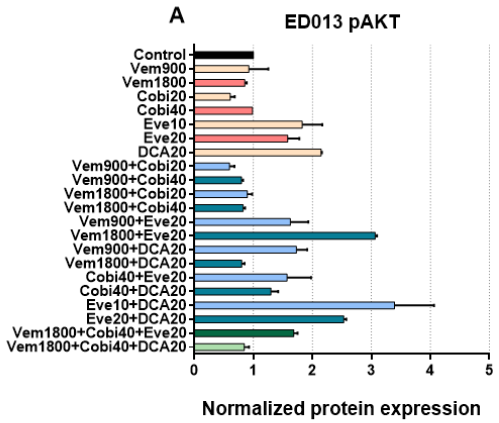
control (Figure 11E). The most evident decrease in the expression of this protein was observed in response to the three-drug treatment containing DCA.

For ED013R2 cells, the expression of phosphorylated AKT, phosphorylated S6, phosphorylated 4E-BP1, and phosphorylated PDH were evaluated in response to the 21 treatments (Figure 11F-I).

In general, the expression of phosphorylated AKT, in the ED013R2 cells, increased in response to treatments, comparing to control (Figure 11F). The most evident increase of the expression was achieved by both three-drug treatments.

The expression of the phosphorylated form of the downstream effectors of mTOR, S6 and 4E-BP1, was evaluated. Comparing to the control, in ED013R2 cells, the expression of the phosphorylated S6 decreased in response to the treatments with everolimus, and increased in response to the combination of MAPK inhibitors (Figure 6G). The expression of phosphorylated 4E-BP1, comparing to control, increased in response to combinations of the MAPK inhibitors and in response to the three-drug treatments (Figure 11H).

In the ED013R2 cells, the expression of phosphorylated PDH decreased, relatively to control, in response to all the approaches containing DCA, highlining the combination of 1800 nM of vemurafenib with DCA that induced the most evident reduction in the expression of this protein (Figure 11I).


A
ED013 pAKT
Control
Vem900
Vem1800
Cobi20
Cobi40
Eve10
Eve20
DCA20
Vem900+Cobi20
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Vem1800+Cobi20
Vem1800+Cobi40
Vem900+Eve20
Vem1800+Eve20
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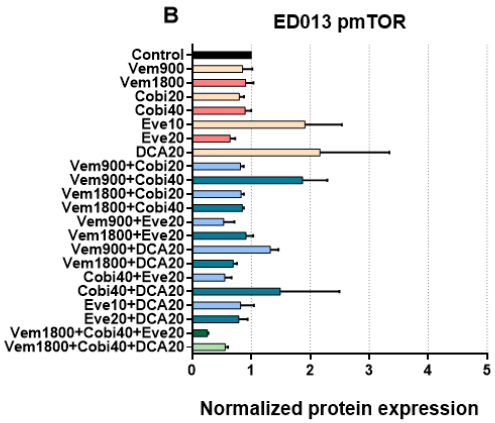
Normalized protein expression


B
ED013 pmTOR
Control
Vem900
Vem1800
Cobi20
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Eve20
DCA20
Vem900+Cobi20
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Vem1800+Cobi20
Vem1800+Cobi40
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Vem1800+Eve20
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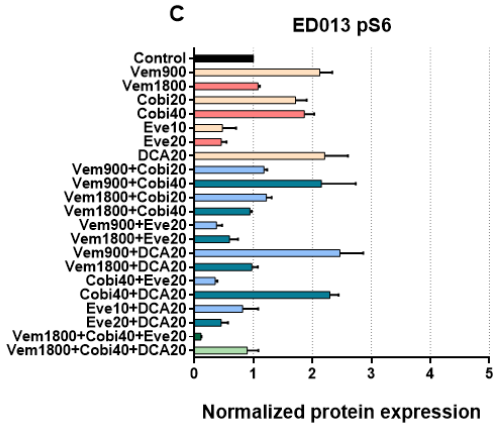
Normalized protein expression


C
ED013 pS6
Control
Vem900
Vem1800
Cobi20
Cobi40
Eve10
Eve20
DCA20
Vem900+Cobi20
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Vem1800+Cobi20
Vem1800+Cobi40
Vem900+Eve20
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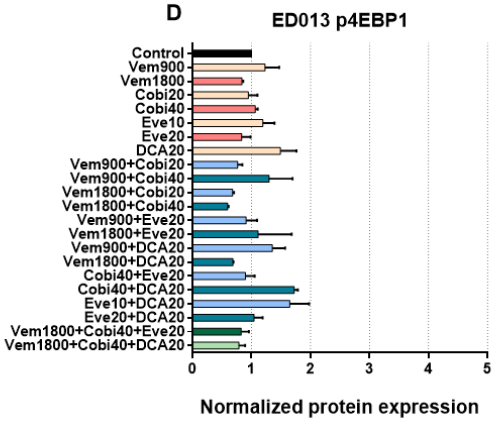
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ED013 p4EBP1
Control
Vem900
Vem1800
Cobi20
Cobi40
Eve10
Eve20
DCA20
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Vem900+Cobi40
Vem1800+Cobi20
Vem1800+Cobi40
Vem900+Eve20
Vem1800+Eve20
Vem900+DCA20
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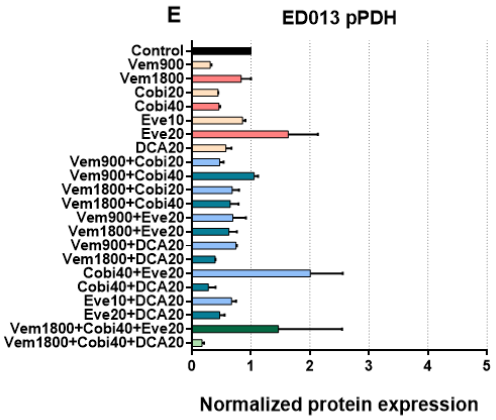
E
ED013 pPDH
Control
Vem900
Vem1800
Cobi20
Cobi40
Eve10
Eve20
DCA20
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Normalized protein expression

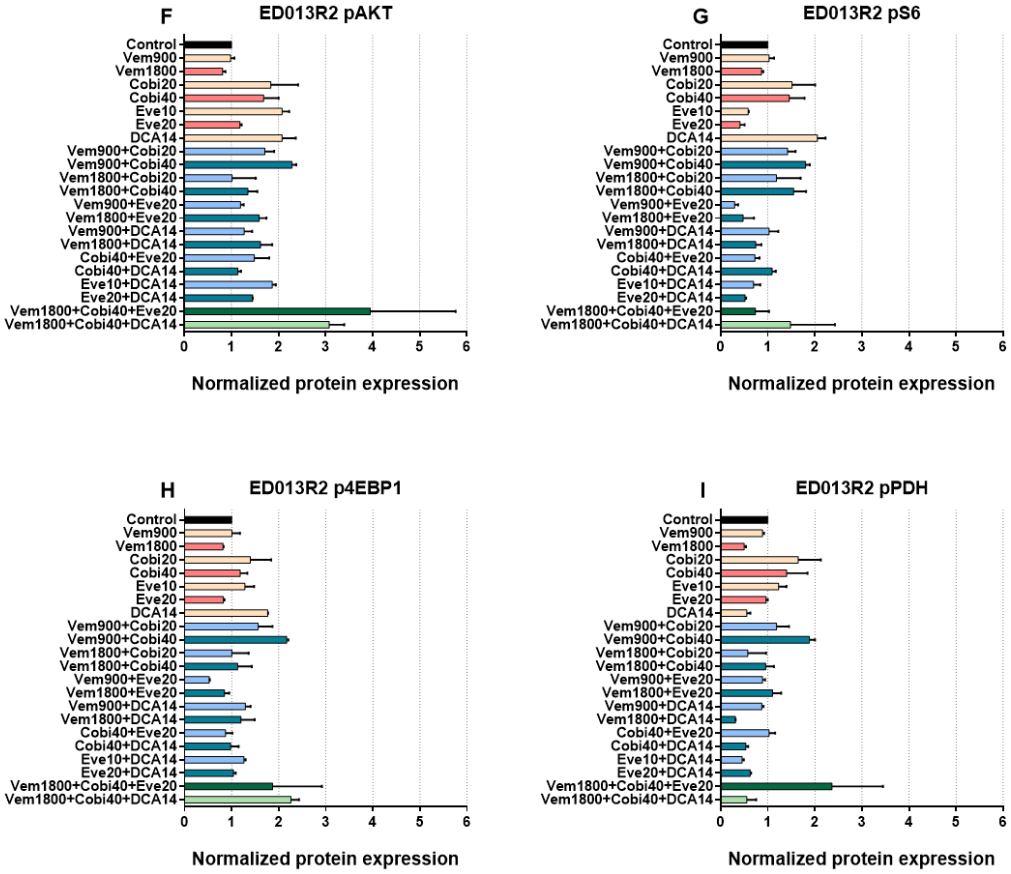


**Figure 11. Effects of treatments on protein expression of ED013 and ED013R2 melanoma cell lines.** Graphic representation of the protein expression, normalized in relation to the protein expression of the untreated cells in the control, of phosphorylated AKT (A), phosphorylated mTOR (B), phosphorylated S6 (C), phosphorylated 4E-BP1 (D), and phosphorylated PDH (E) in ED013 cells, and phosphorylated AKT (F), phosphorylated S6 (G), phosphorylated 4E-BP1 (H), and phosphorylated PDH (I) in ED013R2 cells, after 72 h of treatment with vemurafenib, cobimetinib, everolimus, DCA and combined treatments. Vemurafenib and cobimetinib at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40) were used. ED013R2 cells, the resistant cells to vemurafenib and cobimetinib, were treated with the concentrations described above for ED013 cells. In both cell lines, for everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20) for EDO013 cells and 14 mM of DCA (DCA14) for ED013R2 cells. Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. Single-agent treatments are represented in pink bars, two-drug combinations are represented in blue bars and three-drug combinations are represented in green bars. The data are presented as mean ± SD.

# Chapter 5: Discussion

Cutaneous melanoma patients, harbouring *BRAF*$^{V600E}$ mutation, present initial tumour response to vemurafenib, a BRAF inhibitor, approved by the FDA. However, after this treatment, due to alterations in melanoma cell population, patients can develop mechanisms of resistance to the BRAF inhibitor. To overcome this unintended outcome, the understanding of the molecular mechanisms of resistance and the identification of melanoma features, as the Warburg effect, need to be considered to improve melanoma therapies.

ED013 and ED013R2 melanoma cell lines have similar genotypic pattern, based on the analysis of 15 STRs. These cells harbour a *BRAF*$^{V600E}$ mutation, the most common *BRAF* mutation in melanoma patients [79]. ED013R2 cell line derived from ED013 cell line and acquired the resistance to vemurafenib *in vitro*. The selective pressure from the therapy can select pre-existing resistant clones or induce alterations, as an evolving process during treatment. Despite the heterogenous profile within melanoma cells, with numerous genetic aberrations, the fact that ED013 cell viability exhibited a pronounced decrease in response to treatments with vemurafenib, confirms the features of oncogene addiction of *BRAF*$^{V600E}$ [241].

The allele status analysis between cell lines determined that the increased *BRAF*$^{V600E}$-to-*BRAF*$^{WT}$ ratio in the ED013R2 cells, in relation to ED013 cells, may be associated with a copy number gain [237]. This alteration in *BRAF*$^{V600E}$, in the ED013R2 cells, can contribute to the activation of the MAPK pathway and, consequently, can lead to the resistance to vemurafenib, through reactivation of ERK, independently of RAS [237, 242]. Since MEK is an effector downstream of B-Raf and upstream of ERK, *BRAF* mutant melanomas depend on MEK activity to upregulate this pathway [197]. Therefore, MEK inhibitors could be used to prevent the activation of the pathway, and FDA approved for *BRAF*$^{V600}$ mutant melanoma patients the administration of 960 mg of the BRAF inhibitor, vemurafenib, twice a day, in the 28-day cycle, and 60 mg of the MEK inhibitor, cobimetinib, once a day for 21 days, followed by 7 days off, to complete the cycle [198, 243].

Since, melanoma cases harbouring *BRAF* mutation are associated with PTEN inactivation, and consequent activation of the PI3K pathway [244], in this study, the MAPK inhibitors, vemurafenib and cobimetinib, were combined with a mTOR inhibitor, everolimus. Considering that DCA can change the metabolic profile of cutaneous melanoma [127], synergetic combinations were also tested in melanoma cells, in combination with the melanoma approved drugs and everolimus.

The evaluation of cell viability showed a differential sensitivity of ED013 and ED013R2 cells to the MEK inhibitor, suggesting that both *BRAF* mutant cell lines depend differently from the signalling mechanisms that involve MEK. As cell lines with $IC_{50}$ above 10000 nM are considered resistant to the drug [212], ED013R2 cell viability evaluation showed that these cells, besides the resistance to BRAF inhibitor vemurafenib, also presented resistance to cobimetinib, a MEK inhibitor. The cross-resistance to MAPK inhibitors in *BRAF* mutant melanoma was already described in others *in vitro* and *in vivo* models [245]. This cross-resistance to MAPK inhibitors in the ED013R2 melanoma cells suggests that the acquired resistance *in vitro* may be associated with an activation of other important pathways, as continuous PI3/AKT/mTOR pathway activation, rather than the activation of MAPK pathway by secondary oncogenic events, as *NRAS* mutations or elevated expression of the kinases A-Raf and C-Raf.

To maintain homeostasis, cell viability and proliferation, cell cycle and apoptosis must be regulated. The main purpose of therapies is to induce cytostatic effects, inhibiting cell growth and proliferation, and cytocidal effects, leading to cell death. In the present study, synergetic combinations were more efficient than the single-agent treatments, which corroborates previous studies [245]. Different combinations achieved better results *in vitro* than the approved therapy for *BRAF* mutant melanomas, vemurafenib with cobimetinib. Regarding cell viability, comparing with the approved treatment, the combinations that induced a more pronounced reduction in the percentage of viable ED013 and ED013R2 cells contained everolimus (cobimetinib with everolimus and the three-drug combination containing everolimus in both cell lines, plus vemurafenib with everolimus, and everolimus with DCA, in the ED013R2 cells). These results support the previous studies from our group, which highlighted the importance of the PI3/AKT/mTOR pathway in melanoma cell lines and its role in cell survival [101].

Considering cell proliferation, a cancer hallmark, the MAPK and PI3K/AKT/mTOR pathways and the metabolic profile of melanoma cells revealed to be crucial for the process to occur, since MAPK inhibitors, everolimus and the metabolic modulator, DCA, influenced substantially the proliferation of both cell lines. In ED013 cells, the combination of everolimus with DCA and the three-drug combination containing DCA were the most efficient treatments, surpassing the effects of the FDA approved combination. In ED013R2, the three-drug combination containing everolimus surpassed the effects of the approved combination. The pathway inhibitors are considered strong anti-proliferative drugs for melanoma cells, due to the disruption of important pathways signalling [246]. As already known, melanoma cells exhibit the Warburg effect, in which the aerobic glycolysis provides fast ATP and the carbon intermediates to anabolic pathways, which leads to an increase in cellular biomass and drives cancer cell

proliferation [247]. Previous findings of our group showed that the bioenergetic modulation by DCA induces the switch of metabolism [127], which prevents ATP availability and downregulates cell proliferation. The present study points that, besides the differential sensitivity presented by ED013 and ED013R2 cell lines to DCA, translated by different $IC_{50s}$, this metabolism modulator is capable to reduce proliferation in both melanoma cells.

Regarding apoptosis, genes that are involved in the cell cycle progression also regulate the programmed cell death. Therefore, alterations in the cell cycle can induce or prevent apoptosis [248]. In cancer cells, the known mutations in survival signalling pathways, as *BRAF* mutations, and the loss of PTEN contribute to the resistance to apoptosis, another cancer hallmark [249]. Antitumoral drugs must be capable to induce cell cycle arrest, which prevents cell proliferation and induces apoptosis. In this project, it was observed that the combination of cobimetinib with everolimus and the three-drug treatment containing everolimus induced the most pronounced G0/G1 arrest, greater than the approved combination of vemurafenib with cobimetinib, in both cell lines. The treatments capable to cause G0/G1 arrest also led to a more pronounced induction of apoptosis, as expected, surpassing the effects of the approved therapy in both melanoma cell lines.

Our results are in accordance with previous reports that showed that the drugs used in this project induce cell cycle arrest, apoptosis and alteration in pathways. Vemurafenib is described as pro-apoptotic in the sensitive cell lines, as ED013 cells, and causes growth inhibition, G0/G1 arrest, and apoptosis [182, 183, 250]. Vemurafenib, as cobimetinib, downregulates MAPK pathway, prevents the activation of ERK, and blocks the consequent activation of transcription factors that promote growth and mitosis [251, 252]. Studies with cobimetinib suggested that *BRAF* mutant cell lines presented cytocidal and cytostatic effects in response to MEK inhibition [197] and displayed downregulation of cyclin D1, that regulates G1/S transition [252]. In this project, the evaluation of the protein expression showed that these MAPK inhibitors were also associated with effects on PI3/AKT/mTOR pathway. In response to MAPK inhibitors, a decrease on phosphorylated AKT in the ED013 cells, and an activation of AKT in the ED013R2 cells, was observed. These differential effects of MAPK inhibitors in the melanoma cells lines was not expected, since other authors reported that MAPK pathway inhibition induces AKT activation, through a compensatory feedback loop between these two pathways [212, 253-256]. Therefore, the activation of AKT, in the ED013R2 cells, may be related with the mechanisms of resistance to MAPK inhibitors, which can include the activation of the epidermal growth factor that induces AKT activation, the deregulation on the PI3K/AKT/mTOR pathway effectors or alterations in the regulators of this pathway [216,

257]. Our data suggest that, besides activation of AKT, other PI3/AKT/mTOR pathway effectors also respond to the combination of the MAPK inhibitors treatment. In the ED013R2 cells, the phosphorylated form of S6 and phosphorylated form of 4E-BP1 increased in response to vemurafenib with cobimetinib, which is in accordance with the compensatory feedback between these pathways. The existence of several crosstalk points between the MAPK and PI3/AKT/mTOR pathways validates the potential of synergetic therapies for melanoma treatment.

Everolimus, through the inhibition of mTORC1, blocks the signals to the two downstream effectors, S6K1 and 4E-BP1, which control cell cycle progression from G1 to S phase, leading to cell cycle arrest [258, 259]. As expected, in response to treatments containing everolimus a decrease of phosphorylated mTOR, in ED013 cells, and phosphorylated S6, in ED013 and in ED013R2 cells, was observed in this work. The mTOR inhibitor causes the inhibition of S6K1, and therefore the transfer γ-phosphate from an ATP molecule to the S6 is limited [216]. The phosphorylated AKT increased in response to the mTOR inhibitor, in both cell lines, which corroborates with previous studies that demonstrated that the mTOR pathway inhibition with everolimus induces insulin receptor substrate-1 expression, that prevents the downregulation of the pathway, leading to AKT activation [260]. The AKT activation, in response to mTORC1 inhibition, may be also associated with the compensatory activation feedback, mediated by mTORC2 [261].

The present results demonstrate that everolimus treatments induced a greater reduction on ED013R2 cell viability and proliferation, and a greater induction of apoptosis in ED013R2 cells, comparing with ED013 cells. These results suggest that the isogenic cell lines have differential sensitivity to the mTORC1 inhibitor, and ED013R2 cells may have a maintained dependence on the PI3K/AKT/mTOR pathway in order to survive. This observation is in agreement with other studies that demonstrated differences in this pathway activation between vemurafenib-sensitive and vemurafenib-resistant cell lines [212].

DCA, through PDK inhibition, decreased phosphorylated PDH, as observed in both cell lines. The resistance to apoptosis is associated with high mitochondrial membrane potential and low expression of the $K^+$ channel, and DCA induces the decrease of mitochondrial membrane potential, increases mitochondrial $H_2O_2$, and activates the $K^+$ channels [233]. As observed previously by our group, in melanoma cells, and in the present study, in the ED013R2 cells, the shift in the metabolism is also associated with G0/G1 arrest [127, 262].

The evaluation of cell viability, proliferation, cell cycle and apoptosis in response to treatments consistently demonstrated that the combination of cobimetinib

with everolimus and the three-drug treatment of vemurafenib, cobimetinib and everolimus induced a pronounced decreased on cell viability and proliferation, caused G0/G1 arrest, and led to a higher percentage of apoptotic cells, in both cells lines. This suggests that targeting multiple pathways is a suitable approach to treat melanomas harbouring $BRAF^{V600E}$, including those with resistance to MAPK inhibitors. Our data corroborate previous studies that demonstrate the benefit of synergetic drug combinations, particularly the concomitant inhibition of MAPK and PI3K/AKT/mTOR pathways, that may overcome the mechanisms of resistance developed frequently in *BRAF* mutant melanomas.

# Chapter 6: Conclusion

Our data suggest that isogenic cell lines with different sensitivity to a BRAF inhibitor also have differential sensitivity to MEK inhibition, mTORC1 inhibition and DCA. The present work suggests that the cross-resistance to MAPK inhibitors, vemurafenib and cobimetinib, in melanoma cells harbouring $BRAF^{V600E}$, can be reversed by the synergic approach of a MEK inhibitor with a mTOR inhibitor. This concomitant treatment inhibits the signals from the MAPK pathway, targeting a downstream effector of B-Raf through the MEK inhibition with cobimetinib, and blocks the MEK-independent survival pathways, through inhibition of PI3K/AKT/mTOR pathway, by the mTORC1 inhibitor, everolimus.

The assessment of cell viability, proliferation, cell cycle, and apoptosis suggests that the combination of cobimetinib and everolimus, that targets MAPK and PI3/AKT/mTOR crucial pathways, is a more appropriate therapy than the FDA approved combination for $BRAF^{V600}$ melanoma patients, vemurafenib and cobimetinib, which targets two kinases in the MAPK pathway. Considering the general features of melanoma cells, which includes MAPK and PI3K/AKT/mTOR signalling pathways activation and metabolic alterations, the approved therapy achieved better results when combined with everolimus or with DCA.

The enhanced knowledge of the biochemical processes happening in melanoma cells, from the melanomagenesis to the mechanisms of intrinsic and acquired resistance, may be helpful to create more suitable approaches for $BRAF^{V600E}$ melanoma patients. Although the validation of the treatments in a two-dimension model *in vitro* is important, the translation of these findings into the clinic will be fundamental to validate the discussed approaches.

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# Chapter 8: Appendices

## Supplementary Table

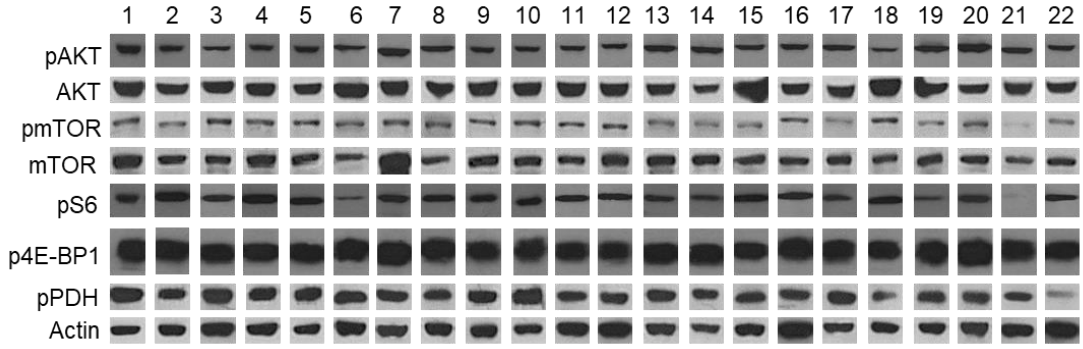
**Supplementary Table 1. Cutaneous melanoma staging** [263].

| Stage | T | N | M | Clinical-Histopathological Features |
|---|---|---|---|---|
| **0** | Tis | N0 | M0 | *In situ* melanoma (intraepithelial) |
| **IA** | T1a | N0 | M0 | ≤1 mm without ulceration |
| **IB** | T1b | N0 | M0 | ≤1 mm with ulceration |
| | T2a | N0 | M0 | 1.01-2 mm without ulceration |
| **IIA** | T2b | N0 | M0 | 1.01-2 mm with ulceration |
| | T3a | N0 | M0 | 2.01-4 mm without ulceration |
| **IIB** | T3b | N0 | M0 | 2.01-4 mm with ulceration |
| | T4a | N0 | M0 | ≥4 mm without ulceration |
| **IIC** | T4b | N0 | M0 | >4 mm with ulceration |
| **IIIA** | T1-4a | N1a | M0 | Single regional nodal micrometastasis, without ulceration |
| | T1-4a | N2a | M0 | 2-3 microscopic positive regional nodes, without ulceration |
| **IIIB** | T1-4b | N1a | M0 | Single regional nodal micrometastasis, with ulceration |
| | T1-4b | N2a | M0 | 2-3 microscopic positive regional nodes, with ulceration |
| | T1-4a | N1b | M0 | Single regional nodal macrometastasis, without ulceration |
| | T1-4a | N2b | M0 | 2-3 macroscopic regional nodes, without ulceration |
| | T1-4a/b | N2c | M0 | In-transit met(s)/ satellite lesion(s) without metastatic lymph nodes |
| **IIIC** | T1-4b | N1b | M0 | Single regional nodal macrometastasis, with ulceration |
| | T1-4b | N2b | M0 | 2-3 macroscopic regional nodes, with ulceration |
| | Any T | N3 | M0 | 4 or more metastatic nodes, matted nodes, or in-transit met(s)/satellite lesion(s) with metastatic nodes |

| | | | | |
|---|---|---|---|---|
| **IV** | Any T | Any N | Any M1 | M1a: Distant skin, subcutaneous, or nodal mets with normal LDH levels |
| | | | | M1b: Lung metastases with normal LDH |
| | | | | M1c: All other visceral metastases with normal LDH or any distant metastasis with elevated LDH |

Legend: T=tumour size; N=node status; M=metastasis; Ta=without ulceration; Tb=with ulceration

## Supplementary Figure




**Supplementary Figure 1. Representative western blot analysis.** Representative western blots of pAKT, AKT, pmTOR, mTOR, pS6, p4EBP1, pPDH and actin expression in ED013 cells in response to vemurafenib, cobimetinib, everolimus and DCA, alone and in combination, for 72 h, compared to untreated cells. Vemurafenib and cobimetinib were used at half of the $IC_{50}$ and at the $IC_{50}$, 900 and 1800 nM of vemurafenib (Vem900 and Vem1800) and 20 and 40 nM of cobimetinib (Cobi20 and Cobi40). For everolimus, half of the recommended concentration, 10 nM, and the recommended concentration by the manufacture, 20 nM, were used (Eve10 and Eve20). For DCA, the $IC_{50}$ concentration was used, 20 mM of DCA (DCA20). Drug combinations of vemurafenib+cobimetinib, vemurafenib+everolimus, vemurafenib+DCA, cobimetinib+everolimus, cobimetinib+DCA, everolimus+DCA, vemurafenib+cobimetinib+everolimus and vemurafenib+cobimetinib+DCA were used with the concentrations described above. The numbers represent conditions. 1 – Control, 2 - Vem900, 3 -Vem1800, 4 – Cobi20, 5 – Cobi40, 6 – Eve10, 7 – Eve20, 8 – DCA20, 9 - Vem900+Cobi20, 10 - Vem900+Cobi40, 11 - Vem1800+Cobi20, 12 - Vem1800+Cobi40, 13 - Vem900+Eve20, 14 - Vem1800 + Eve20, 15 - Vem900+DCA20, 16 - Vem1800+DCA20, 17 - Cobi40+Eve20, 18 - Cobi40+DCA20, 19 - Eve10+DCA20, 20 - Eve20+DCA20, 21 - Vem1800+Cobi40+Eve20, 22 - Vem1800+Cobi40+DCA20**.**

# Appendix I – Paper: Melanoma treatment in review

ImmunoTargets and Therapy

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REVIEW

# Melanoma treatment in review



This article was published in the following Dove Press journal:
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Beatriz Domingues[1–3]
José Manuel Lopes[1,2,4,5]
Paula Soares[1,2,5]
Helena Pópulo[1,2]

[1]Institute of Molecular Pathology and Immunology, University of Porto (IPATIMUP), Porto, Portugal; [2]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal; [3]Faculty of Sciences, University of Porto, Porto, Portugal; [4]Department of Pathology, Hospital S João, Porto, Portugal; [5]Department of Pathology, Medical Faculty, University of Porto, Porto, Portugal

Correspondence: Helena Pópulo
Institute of Molecular Pathology and Immunology, University of Porto (IPATIMUP), Rua Júlio Amaral de Carvalho 45, 4200-135 Porto, Portugal
Tel +351 22 557 0700
Fax +351 22 557 0799
Email hpopulo@ipatimup.pt

**Abstract:** Melanoma represents the most aggressive and the deadliest form of skin cancer. Current therapeutic approaches include surgical resection, chemotherapy, photodynamic therapy, immunotherapy, biochemotherapy, and targeted therapy. The therapeutic strategy can include single agents or combined therapies, depending on the patient's health, stage, and location of the tumor. The efficiency of these treatments can be decreased due to the development of diverse resistance mechanisms. New therapeutic targets have emerged from studies of the genetic profile of melanocytes and from the identification of molecular factors involved in the pathogenesis of the malignant transformation. In this review, we aim to survey therapies approved and under evaluation for melanoma treatment and relevant research on the molecular mechanisms underlying melanomagenesis.

**Keywords:** cancer, melanoma, therapy, targets

## Introduction

Melanoma arises from the occurrence of genetic mutations in melanocytes, the pigment producing cells, which can be found in the skin, eye, inner ear, and leptomeninges.[1–4] Although melanoma accounts for about 1% of all skin malignant tumors, cutaneous malignant melanoma represents the most aggressive and the deadliest form of skin cancer.[5] This disease affects mostly the Caucasian population of both genders,[6] and once it becomes metastatic, the prognosis is very poor.[7,8] Therefore, early identification of this cancer is crucial for the success of patient treatment. The European Society for Medical Oncology clinical practice guidelines for cutaneous melanoma highlight the importance of a detailed diagnosis for the establishment of the tumor stage and, in some tumors, a mutation test is also required.[9] Over the past years, several therapies have been approved by the US Food and Drug Administration (FDA) (Figure 1). Depending on the features of the tumor (location, stage, and genetic profile), the therapeutic options may be surgical resection, chemotherapy, radiotherapy, photodynamic therapy (PDT), immunotherapy, or targeted therapy. For patients with stage I–IIIB melanoma, surgery is the primary treatment.[10–12] The surgery procedures differ according to the clinic-pathologic features of the tumor. Excision includes safety margins of 0.5 cm for in situ melanomas, 1 cm for tumors with a thickness of up to 2 mm, and 2 cm for tumors thicker than 2 mm.[11] To improve survival, adjuvant therapies, such as targeted therapy and immunotherapy, are recommended.[11,13] For patients with a solitary melanoma metastasis, metastasectomy is part of the standard of care and, in some

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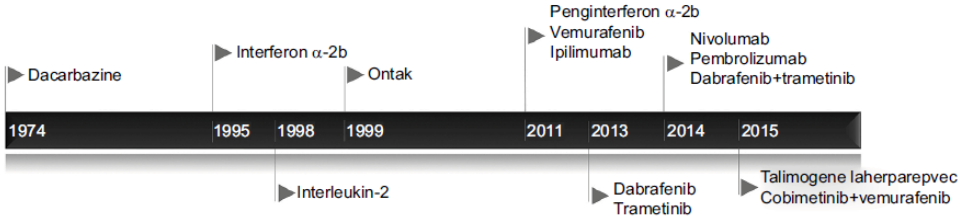


**Figure 1** FDA-approved drugs for melanoma treatment. Dacarbazine was the first drug approved, in 1974, followed by interferon α-2b, interleukin-2, and ontak in the 1990s. Between 2011 and 2015, 10 therapies were approved, including selective inhibitors, antibodies, and combined targeted therapies.
**Abbreviation:** FDA, US Food and Drug Administration.



metastatic melanoma cases, chemotherapy treatment may also be considered.[12,13] Despite being rarely indicated for primary tumor treatment, radiotherapy can be useful for the treatment of skin, bone, and brain metastases.[14]

Two types of limitations are relevant in melanoma therapy: 1) adverse events (AEs), which can lead to skin and gastrointestinal toxicity, can be high and usually related to immune reactions and lack of specificity for tumor cells[15–17] and 2) reduced efficiency, which can occur due to resistance to immune, chemo/targeted and intralesional therapies.[13] Recently, new therapeutic targets have emerged from studies of the genetic profile of melanocytes and from the identification of molecular factors involved in the pathogenesis of the malignant transformation of the melanocytic cells.[1,18] In this review, we will present melanoma therapies, approved and under evaluation, which resulted from the studies of melanocyte biology and malignant transformation.

## Chemotherapy

Chemotherapy was the earliest treatment option for advanced melanoma. Chemotherapy combinations have been evaluated to improve the clinical responses, but the overall survival (OS) did not show improvement.[19] Resistance to apoptosis is probably the major cause of chemotherapy drug resistance in melanoma.[20] Although it has been replaced by additional options, chemotherapy remains important in the palliative treatment of refractory, progressive, and relapsed melanomas.[19]

### Dacarbazine

Dacarbazine, an alkylating agent approved in 1974 by FDA, is the standard chemotherapy medication for metastatic melanoma. Studies reported that a complete response was achieved in <5% and 5-year survival in 2%–6% of patients.[21] Despite these results, dacarbazine was the standard of care because other single agents or combination chemotherapies did not reveal improvements in the OS of patients.[21] Still, several clinical trials are ongoing, using only dacarbazine as comparison or in combination with other chemotherapies, immunotherapies, and targeted therapies.[22]

### Temozolomide (TMZ)

TMZ, an oral prodrug of the active metabolite of dacarbazine, has been used in advanced melanoma.[21] Compared to dacarbazine, TMZ showed a reduced improvement in median progression-free survival (PFS), but no differences were observed in OS or objective response rates.[23]

### Electrochemotherapy (ECT)

ECT is a technique that combines the use of cytotoxic drugs, bleomycin and cisplatin, with high-intensity electric pulses, which facilitates drug delivery into the cells.[24,25] ECT was reported to be effective for the treatment of cutaneous and subcutaneous nodules of melanoma.[26,27] A study of the European Standard Operating Procedures of Electrochemotherapy reported an overall response of 85% and no major negative AEs were observed.[27] Another remarkable aspect of ECT is that usually the treated nodules do not recur in the treated area, possibly because the treatment destroys the lymphatic stream; however, more studies are needed in this regard.[24]

## PDT

Light-based therapy is a promising adjuvant therapy and may be a suitable palliative treatment option for patients with stage III/IV cutaneous metastatic melanomas.[13,28] PDT is a minimally invasive procedure[28–30] that requires a photosensitizer (PS), which is better absorbed in metabolically active tissues, and light of a defined wavelength, to activate the PS.[13] Both these elements are non-toxic, but create reactive oxygen species (ROS) when combined with oxygen, through a photochemical reaction.[30] ROS unleash irreversible damage to tumor cells[31,32] and tumor-associated blood

vessels, also activating antitumor, immune, and inflammatory responses.[33–35]

Acai oil in nanoemulsion has been used as a novel PS in melanoma cells lines and in in vivo experimental models. The results showed 85% of melanoma cell death by late apoptosis/necrosis, preserving high viability in normal cells.[36]

Although PDT can be used for the treatment of nonmalignant and malignant disease, studies reported that PDT alone has limited efficiency in melanomas.[37,38] To improve PDT results in melanoma, protective mechanisms, such as pigmentation and oxidative stress resistance, have to be overcome.[28] Combined therapies have been studied and, specifically, the combination of PDT and chemotherapy (dacarbazine) was reported to be an efficient treatment to reduce resistance in pigmented and unpigmented metastatic melanomas.[39] Combination of PDT with immunostimulatory therapies may be more efficient in the eradication of the initial tumor and micrometastases and additionally may also decrease melanoma recurrences.[28] A new clinical trial with PDT is underway in melanomas (NCT02685592).

## Immunotherapy

In the 19th century, it was proposed, for the first time, that cancer and immune system are associated, and this observation was based on the frequent appearance of tumors at the sites of chronic inflammation and on the presence of immune cells in tumor tissues.[40] In antitumoral responses, T-cells recognize tumor-specific antigens, becoming activated and then proliferate and differentiate, acquiring the capacity to destroy cells that express tumor-specific antigens. In addition to the stimulatory and inhibitory signaling pathways that limit T-cell antitumoral responses, cancer cells can escape T-cell detection, as usually they do not express B7 molecules.[41]

It is known in many types of cancer that complex interactions between the tumor and the immune system play a role in the metastatic spread to distant sites.[42] Metastases are the main cause of cancer death and more accurate prognostic markers are warranted.[42] Tumor infiltrating lymphocytes (TILs) have been considered in many studies as independent markers for the occurrence of lymph node metastasis.[43] TILs can mediate immune responses of the host against cancer cells, being associated with a positive outcome and improved survival in patients with malignant melanomas.[43,44] Based on these interactions, immunotherapy appears to be a promising treatment option for patients with advanced stage (metastatic) malignant melanomas, when compared to previous standard therapies, showing durable complete responses in selected patients with advanced melanomas.[43,45–47] The immunogenic tumor microenvironment (TME), with mediators and cellular effectors of inflammation, influences the success of immunotherapies.[45] The molecular pathways involved in this cancer-related inflammation are now being clarified, in order to establish new target molecules that may lead to improvements in the diagnosis and treatment of cancer.[44] Despite positive results, recurrence of cancers and variable success among different cancers are not uncommon. Even in responsive cancers, the immune checkpoint inhibitor success rate is often <50%.[48] Primary and acquired resistance to immunotherapy is common and may be due to the lack of recognition by T-cells, as described above. Also, it can involve various components of the cancer immune cycle (including regulatory T-cells [Tregs], myeloid-derived suppressor cells [MDSCs], and M2 macrophages), and interactions between multiple signaling molecules and pathways that prevent immune cell infiltration or function within the TME.[49,50]

In recent years, improved knowledge of the pathophysiology and a better understanding of the role of the immune system in tumor evolution have led to the development and approval of several immunotherapies (Figure 2).

### Interferon (IFN) α-2b

IFNs are cytokines secreted by leukocytes. These signaling proteins are able to interfere with viral replication and play an important role in the immunomodulatory, antiangiogenic, antiproliferative, and antitumor activities.[51–54] IFNs activate multiple cell types of the immune system, such as T-cells, B lymphocytes, natural killer cells, and dendritic cells, and inhibit other negative elements, such as Tregs and MDSCs. Beyond the known IFN effects, the complex network of different cells involved and the biologic variability of each patient influence the response to the therapy.[51]

IFNs offer opportunities of synergism with conventional treatments, and high-dose IFN α-2b was approved by the FDA in 1995 as adjuvant therapy for the treatment of resected stage IIB/III melanoma.[54,55] In melanoma, IFN-α demonstrates an immunomodulatory antitumor effect, inducing a stimulatory effect on major histocompatibility complex class I expression of melanoma and immune cells, being able to inhibit the proliferation of melanoma cells, with a dose-dependent proapoptotic effect.[56] A recent meta-analysis reported that adjuvant IFN-α significantly reduces the risk of recurrence and improves survival of melanoma patients.[57] However, only a minority of patients respond to IFNs and ulceration of the primary tumor is the most important predictive factor for IFN sensitivity.[58] Although newer and more efficient immunotherapies have emerged in recent years, IFNs persist

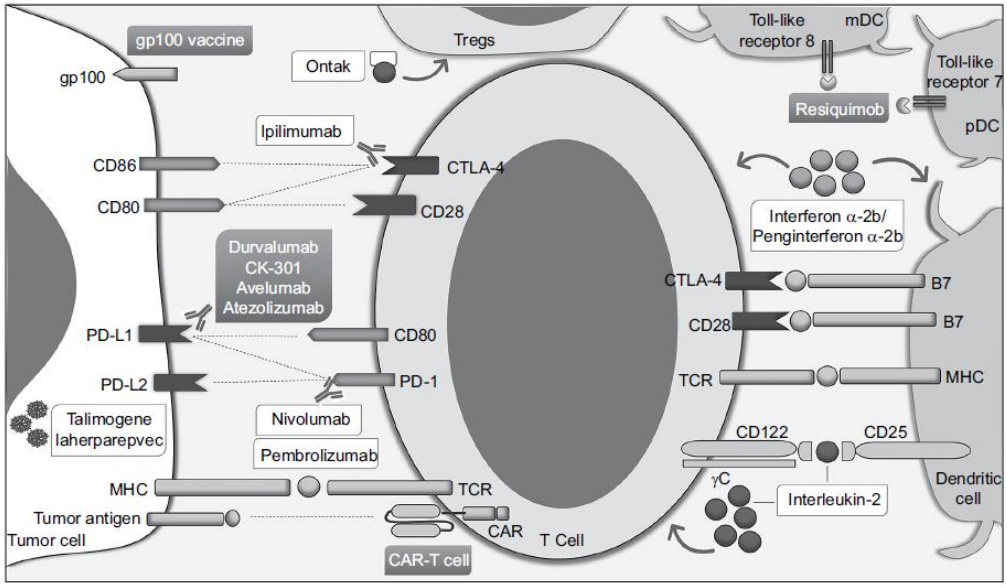


**Figure 2** Immunotherapies approved by FDA (in white – interferon α-2b/peginterferon α-2b, interleukin-2, ontak, ipilimumab, nivolumab, pembrolizumab, and talimogene laherparepvec) or in trials (in gray – durvalumab, CK-301, avelumab, atezolizumab, gp100 vaccine, resiquimod, and CAR-T cells) for cutaneous melanoma treatment. Immunotherapy induces antitumor immune responses by altering metabolites, growth factors, and cytokines, such as interferon α-2b and interleukin-2, in the TME. Ipilimumab, an anti-CTLA-4 antibody, induces pro-inflammatory T-cell cytokine production, and increases clonal T-cell expansion and infiltration. The anti-PD-1 antibodies, nivolumab and pembrolizumab, block the interaction between PD-1 and PD-L1/PD-L2, similar to the effect of durvalumab, CK-301, avelumab, atezolizumab, which are anti-PDL-1 antibodies. Other immunotherapies can activate the immune system at the TME, such as resiquimod and CAR-T cells, or suppress Tregs, as ontak. Oncolytic virus therapy with talimogene laherparepvec also interferes with the immune system, inducing melanoma cell lysis and consecutively release of tumor-specific antigens.
**Abbreviations:** FDA, US Food and Drug Administration; TME, tumor microenvironment; PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand; CTLA, cytotoxic T lymphocyte-associated antigen; CAR, chimeric antigen receptor; MHC, major histocompatibility complex; TCR, T-cell receptor; Treg, regulatory T-cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell.

in clinical trials in combination with other immunotherapies and targeted therapies.[22]

## Peginterferon α-2b (Peg-IFN)

Peg-IFN was approved by the FDA (2011) as adjuvant therapy for stage III melanomas.[59] Peginterferon is the combination of IFN α-2b with the molecule polyethylene glycol (Peg). It was reported that this molecule allows the compound to stay longer in the blood, thus improving its therapeutic effect.[60]

The most common AEs observed were grade 1 liver toxicities, neutropenia, skin rash, and anemia. The only grade 3/4 toxicities were lymphopenia and hyponatremia.[61]

## Interleukin-2 (IL-2)

IL-2 is a cytokine capable of expanding effector T-cells (Teffs) and Tregs. Studies showed that high-dose IL-2 has antitumoral activity and the FDA approved (1998) this treatment for metastatic melanomas.[62] A recent meta-analysis reported that the complete response rate for IL-2 treatment was 4.0%, partial response 12.5%, and overall response 19.7%. High and intermediate dose showed no complete response differences, and thus the therapeutic dose should be reconsidered.[63]

Before undergoing IL-2 treatment, patients need to be evaluated, and some biomarkers have been studied, such as serum vascular endothelial growth factor (VEGF) and fibronectin levels.[64] AEs can include hypotension, tachycardia, peripheral edema, reversible multisystem organ failure, and cardiac arrhythmias.[65] Like IFNs, IL-2 is still included in clinical trials, in combination with chemotherapy, radiotherapy, other immunotherapies, and targeted therapies.[22]

## Treg inhibition

Tregs suppress activated Teffs and can inhibit antitumoral immune responses.[66,67] In melanomas, Tregs appear in peripheral circulation and in the TME and seem to be associated with poor clinical outcome.[68] The therapeutic strategy consists in the suppression of Tregs, thus increasing the antitumoral immunity. Ontak, approved by the FDA in 1999,[69] is the

fusion of IL-2 protein with diphtheria toxin that selectively eliminates Tregs expressing IL-2 receptor from the peripheral blood.[70] A Phase II trial in stage IV melanoma patients showed 16.7% of partial responses, 5.0% stable disease, and 15.0% mixed responses.[71] Conversely, another study reported that metastatic melanoma patients administered with ontak showed no objective clinical response, no regression of the disease, and no elimination of regulatory T lymphocytes.[72]

## Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade

Ipilimumab, approved by the FDA for the treatment of advanced melanomas in 2011, is an anti-CTLA-4 antibody. CTLA-4 is a inhibitory checkpoint receptor that blocks T-cell activation and induces immune tolerance.[73,74] Anti-CTLA-4 antibodies act as antagonists, blocking the inhibitory effect, enhancing pro-inflammatory T-cell cytokine production,[75] and increasing clonal T-cell expansion and infiltration in responding tumors.[76]

Combined therapies have been studied and ipilimumab with IL-2 showed similar AEs compared to the respective monotherapies, but did not show improvement in the efficacy over ipilimumab monotherapy.[77] Combination of ipilimumab and Peg-IFN showed an overall response rate of 40% and median PFS of 5.9 months.[78] Also, in a Phase III trial, patients with advanced melanomas were administered randomly with ipilimumab monotherapy, ipilimumab combined with gp100 peptide vaccine, or vaccine monotherapy. Ipilimumab monotherapy showed the best response rate, with a median OS of 10.1 months, followed by ipilimumab plus gp100, with a median OS of 10.0 months. These results indicate that ipilimumab is a valuable approach due to the better OS rates compared to vaccine monotherapy, which presented a median OS of 6.4 months.[79]

To undergo ipilimumab treatment, melanoma patients must comply with immune-related response criteria. The AEs of this treatment include autoimmune alterations, such as dermatitis, colitis, drug-related hepatitis, endocrinopathies, and rarely neuritis, which are more frequent with high doses.[80] Corticosteroids, and occasionally more intense immunosuppressive medication, may control these AEs.[12] Several clinical trials are ongoing with ipilimumab in combination with chemotherapy, radiotherapy, other immunotherapies, and targeted therapies.[22] Tremelimumab, another anti-CTLA-4 antibody, is also used in clinical trials in monotherapy (NCT00378482) and in combination with other immunotherapies (NCT01103635/NCT02535078/NCT02643303).

## Programmed cell death protein 1 (PD-1)/PD-1 ligand (PD-L1) blockade

The PD-1 receptor binds to PD-L1 and PD-L2, acts as a T-cell co-inhibitory molecule, and suppresses T-cell activation. Further than being expressed on the antigen-presenting cells, ligands are also expressed in many human tumors and in cells within the TME, in response to inflammatory stimuli. Yet, the utility of PD-L1 immunostaining as a predictive biomarker for anti-PD-1 treatment remains unclear.[81]

Nivolumab is a high-affinity anti-PD-1 monoclonal antibody that inhibits the binding between the PD-1 receptor and its ligands PD-L1 and PD-L2.[82] Nivolumab was approved (2014) by the FDA for the treatment of patients with metastatic melanoma.[83] The blockade of the interaction between PD-1 and its ligands mediates immune responses and induces (preclinical) antitumor activity that reduces tumor progression.[84] Nivolumab, with a PFS of 6.9 months, seems to be more efficient than monotherapies with ipilimumab, which display a median PFS of 2.9 months, or chemotherapy, with a median PFS of 2.2 months.[84] The combination of nivolumab and ipilimumab achieved a median PFS of 11.5 months, superior than monotherapies, especially in patients with PD-L1 negative tumors.[84,85]

Pembrolizumab, an anti-PD-1 antibody, was approved by the FDA in 2015 for the treatment of advanced melanomas and may turn a new standard for the treatment of ipilimumab refractory melanomas.[86–88] The antitumor activity of pembrolizumab led to a prolonged PFS and OS of patients with advanced melanomas, with less high-grade toxicity than ipilimumab.[86] The combination of pembrolizumab and Peg-IFN is also well tolerated and clinically active, especially in patients with advanced melanoma that cannot be removed by surgery.[61] The AEs related with this therapy are fatigue, infusion reactions, diarrhea, arthralgia, rash, nausea, pruritus, and headaches.[12] More studies are needed to identify suitable prognostic biomarkers for this treatment.[84]

Several clinical trials are ongoing, using nivolumab and pembrolizumab in monotherapy or in combination with chemotherapy, radiotherapy, other immunotherapies, and targeted therapies.[22] Other anti-PD-1 molecules are being used in trials, such as JS100 monotherapy (NCT03013101) and in combination with targeted therapies (NCT03086174), REGN2810 (NCT03002376), and PDR001 monotherapy (NCT02404441) and in combination with other immunotherapies (NCT02608268) and targeted therapies (NCT02607813/NCT02967692).

Other anti-PD-L1 molecules are used in Phase I/II trials, such as durvalumab in combination with other immunotherapies (NCT02535078) and targeted therapies (NCT02027961), CK-301 (NCT03212404), avelumab in combination with other immunotherapies (NCT03167177), and atezolizumab in combination with other immunotherapies (NCT03138889) and targeted therapies (NCT03178851/NCT01656642).

## Oncolytic virus therapy

FDA approved (2015) the first oncolytic virus for the treatment of melanomas, talimogene laherparepvec (T-VEC), a genetically modified herpes simplex virus type 1.[85,89,90] This engineered nonpathogenic viral strain is injected directly into a metastatic melanoma nodule and while it enters into both normal and malignant cells, it only replicates in the melanoma cells, leading to tumor cell lysis and the release of tumor-specific antigens.[89] These antigens are recognized by antigen-presenting cells, activating melanoma-specific T-cell responses.

Patients with refractory stage IV or unresectable stage III melanomas were treated with T-VEC and, in a Phase II clinical trial, an objective clinical response of 28% was observed.[89] This strategy is safe and the AEs reported are not severe, including fatigue, chills, pyrexia, nausea, influenza-like illness, and injection site pain.[85] Clinical trials are still ongoing, using T-VEC alone or in combination with chemotherapy, radiotherapy, other immunotherapies, and targeted therapies.[22]

Coxsackievirus (CVA21) or CAVATAK is an oncolytic virus in late-stage clinical development that presented lytic activity against melanomas in in vitro cultures and in vivo.[90–92] Ongoing clinical trials are testing CAVATAK with pembrolizumab in advanced melanomas and CAVATAK with ipilimumab in unresectable stage III–IV melanomas.[91]

Other oncolytic viruses are being used in trials, such as HF10 in combination with other immunotherapies (NCT03259425/NCT02272955/NCT03153085), and GL-ONC1 monotherapy, prior to surgery (NCT002714374).

## gp100 Peptide vaccine

gp100 is a glycoprotein expressed only by melanoma cells and, with the exception of healthy epidermal melanocytes and retina, is not expressed in healthy tissues.[93,94] gp100 is recognized by cytotoxic T lymphocytes (CTLs), and administration of gp100 epitopes can enhance CTLs reactivity, being an appealing therapy option. Preclinical models showed that gp100 peptide monotherapy had unsatisfactory clinical benefits; therefore, it might be used as adjuvant therapy.[93] Combination of gp100 peptide vaccine with IL-2 showed significant improvement in the overall clinical response and longer PFS, with a complete response of 5%.[63] The median OS was longer in the combined treatment compared to IL-2 monotherapy.[95] Other clinical trials are ongoing, using gp100 in monotherapy (NCT01744171/NCT02889861) or in combination with other immunotherapies (NCT00960752/NCT00470015/NCT01176461/NCT01176474/NCT02535078).

## Toll-like receptor (TLR) agonists

TLRs are type I membrane glycoproteins that belong to the IL-1R superfamily and are able to induce the production of local cytokines, such as IFN-α and IL-12, that improve local immune responses.[96] Moreover, TLRs may also enhance antitumor immunity.[97] TLR agonists may be a potent adjuvant for vaccines and can activate the immune system in the TME.[98] Resiquimod is a TLR 7/8 agonist that can activate both myeloid (mDC, TLR 8) and plasmacytoid (pDC, TLR 7) dendritic cells in patients with advanced stage melanomas.[98] Patients treated with resiquimod as an adjuvant therapy to the gp100 vaccination displayed upregulation of type I IFN and IFN-γ at the peptide vaccination site, by activation of pDC/mDC, and improvement of the antitumor response with regression of in-transit melanoma metastases.[98] Clinical trials are ongoing, using TLR agonists in combination with chemotherapy (NCT02650635) and other immunotherapies (NCT00960752/NCT02320305).

## Adoptive T-cell therapy

In adoptive cell transfer (ACT) therapy, patients are infused with a large number of melanoma-specific T-cells, but the generation of these cells is difficult and time consuming.[99] The antitumoral activity of ACT is not fully understood, but may include suppression of Tregs, removal of cytokine sinks, and eradication of host tumor immunosuppressive factors.[99] T-cells must be able to proliferate, to complete effector functions and to form long-lived memory T-cells that are crucial for a suitable immune response.[100] In fact, in in vitro studies, the more-differentiated Teffs had enhanced antitumoral properties, but in vivo these T-cells are less effective.[101] Modulation of T-cells metabolism may be a valuable method to induce the formation of memory T-cells instead of more-differentiated Teffs. Since memory T-cells exhibit a limited glucose uptake, inhibiting the glycolytic metabolism induces the formation of memory precursor cells and therefore improves antitumoral functions.[102]

In metastatic melanomas, this immunotherapy seems to be associated with complete and durable responses as well as partial responses and prolonged disease stabilization.[103] ACT led to durable complete regression (24% of the studied patients) of metastatic melanomas, with a reported median survival of >3 years.[104] Combined therapies are efficient, and studies reported that metastasectomy in patients with progressive melanomas, undergoing ACT therapy, showed a PFS of 11 months and 5-year OS of 57%.[103] The AEs of this therapy include autoimmune alterations, such as the destruction of normal melanocytes in the eyes and skin, and immunosuppression is sometimes required to control these AEs.[105] Several clinical trials are ongoing with ACT in combination with chemotherapy, radiotherapy, other immunotherapies, and targeted therapies.[22]

ACT with T-cells chimeric antigen receptors (CARs) is a new therapeutic approach for solid tumors, including melanomas.[106] CARs comprise an extracellular domain, which is an antibody single-chain variable fragment that recognizes a specific antigen (lipid, protein, or carbohydrate antigens), a transmembrane domain, and an intracellular signaling domain, which is frequently the CD3 zeta chain of the T-cell receptor that stimulates T-cells in order to destroy the tumor cells.[107,108] For this therapy, patients are infused with previously isolated T-cells that are activated and genetically modified with retroviral or plasmid vectors to generate CAR-T cells.[107,109] A recent study reported that Cas9-based gene editing technique can enhance CAR-T cells efficacy.[110] The selection of the target antigen must have as criterion the maximal effect in tumor cells and the minimal effect in normal cells.[107,111] Although the studies in melanomas are limited, ganglioside GD2 is an example of a molecule that is highly expressed in melanoma cells and can be targeted by CARs.[111,112] A Phase I trial of T-cells expressing an anti-GD2 CAR was performed in children and young adults with melanomas (NCT02107963), but no conclusive results were reported. A dose-escalation Phase I CAR-T cells trial against the antigen VEGFR2 in solid tumors, including melanomas, showed one partial response (4%) in 24 patients.[111] Nevertheless, CAR-T cells are also exposed to inhibitory immune checkpoint signals of the TME. Thus, combined therapies of CAR-T cells with a PD-1 antibody or a CTLA-4 antibody might overcome the TME features.[107,111,113] Other clinical trials are ongoing, using CAR-T cells expressing cMET (NCT03060356) and CAR-T cells with anti-CD70 (NCT02830724).

## Biochemotherapy (BCT)

BCT is the combination of chemotherapy and immunotherapy. Certain conventional chemotherapies may act in part through immune-stimulatory mechanisms.[114] The most common BCT approach uses as chemotherapy a combination of dacarbazine, cisplatin (an antitumoral agent that induces DNA damage and apoptotic signals[115]), and vinblastine (a microtubule targeting agent[116]), with IL-2 and IFN α-2b as immunotherapy. Compared to chemotherapy monotherapy, BCT showed a higher response rate and an improvement in the median PFS. However, BCT did not show improvement in OS and it was associated with severe toxicity and risk of brain metastases development.[117]

## Targeted therapy

About 70% of patients with cutaneous melanoma harbor mutations in genes of key signaling pathways. These oncogenic mutations may be associated with melanoma cell proliferation and a malignant phenotype.[118] The targeted therapy approach uses small molecule inhibitors or antibodies that affect these mutated proteins, which are important for the progression of the disease (Figure 3).

### BRAF inhibitors

In the past few years, new molecular approaches have been developed to target melanoma harboring mutations. BRAF is a key serine–threonine kinase from the mitogen-activated protein kinase (MAPK) signaling pathway and 50% of cutaneous melanomas without association with chronic sun damage harbor a *BRAF* mutation.[119] Mutations in the *BRAF* gene are associated with activation of the MAPK signaling pathway and with increased growth and proliferation of cancer cells.[120] The most common *BRAF* mutation occurs at amino acid 600, in which the normal valine is substituted, in most cases, by glutamic acid ($BRAF^{V600E}$), or, less commonly, it is substituted by lysine ($BRAF^{V600K}$).[12]

Vemurafenib, a selective oral BRAF-mutant inhibitor, was approved (2011) by the FDA for the treatment of unresectable or metastatic melanomas harboring activating $BRAF^{V600E}$ mutations.[121,122] Compared to chemotherapy, in $BRAF^{V600E/K}$ mutation-positive melanomas, vemurafenib improved clinical response rates, PFS, and OS of metastatic melanoma patients. Studies have reported that 90% of patients who received vemurafenib showed tumor regression.[121] Several clinical trials are ongoing with vemurafenib in monotherapy and in combination with chemotherapy, immunotherapies, and other targeted therapies.[22]

Dabrafenib is also a selective BRAF-mutant inhibitor approved (2013) by the FDA for the treatment of unresectable or metastatic melanomas harboring $BRAF^{V600E}$ mutations.[120,122] Several clinical trials are ongoing with dabrafenib in monotherapy and in combination with radiotherapy, immunotherapies, and other targeted therapies.[22]

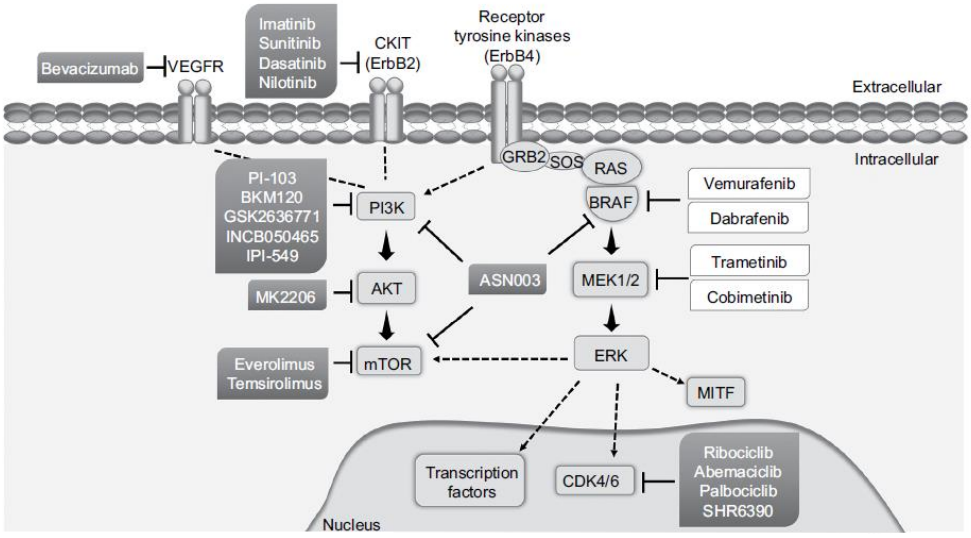


**Figure 3** Targeted therapies approved by FDA (in white – vemurafenib, dabrafenib, trametinib, and cobimetinib) or in trials (in gray – imatinib, sunitinib, dasatinib, nilotinib, bevacizumab, PI-103, BKM120, GSK2636771, INCB050465, IPI-549, MK2206, everolimus, temsirolimus, ribociclib, abemaciclib, palbociclib, SHR6390, and ASN003) for cutaneous melanoma treatment. Mutations on key signaling oncogenes, used as targets for melanoma therapy, are associated with melanoma cell proliferation, cell-cycle progression, and malignant phenotype. Melanoma patients may benefit from combined therapies, using two different targeted therapies or targeted therapy with adjuvant immune therapy or chemotherapy.

**Abbreviations:** FDA, US Food and Drug Administration; mTOR, mammalian target of rapamycin; VEGFR, vascular endothelial growth factor receptor; ERK, extracellular signal-regulated kinase; MITF, microphthalmia-associated transcription factor; CDK, cyclin-dependent kinase; PI3K, phosphatidylinositol-3-OH kinase.

Encorafenib, another BRAF-mutant inhibitor, has also been used in trials in monotherapy (NCT01436656), in combination with other targeted therapies (NCT02159066/NCT01909453) and with targeted therapies plus immunotherapies (NCT02902042/NCT03235245/NCT02631447).

Targeting the tyrosine kinases has led to remarkable response rates with better OS rates in melanoma clinical trials.[122] However, the clinical benefit of these therapies is limited, due to the rapid development of multiple mechanisms of resistance, such as elevated expression of the kinases CRAF, COT1, or mutant *BRAF*, activated mutations in *N-RAS*, *MEK1*, or *AKT1*, aberrant splicing of *BRAF*, activation of phosphatidylinositol-3-OH kinase (PI3K) via the loss of phosphatase and tensin homolog (PTEN), and persistent activation of receptor tyrosine kinases.[123] Combined therapies seem to be an adequate strategy for melanoma patients, in order to overcome these resistance mechanisms.[122] In in vitro studies, dichloroacetate (DCA), which reverts the metabolic profile of cancer cells from glycolysis to oxidative phosphorylation, induced mammalian target of rapamycin (mTOR) inhibition and an increase of apoptosis in melanoma cells.[124] Furthermore, melanoma cells resistant to vemurafenib maintained sensitivity to DCA, suggesting a possible combination therapy to overcome BRAF inhibitors resistance.[125]

Being a targeted therapy, it is possible to select the patients who will benefit from this treatment, based on the mutational profile of the tumor. Only patients with tumors harboring *BRAF* mutations should undergo treatment with a BRAF inhibitor, and patients with known *RAS*-mutant should not receive this treatment.[122,126,127] The AEs of this treatment appear in 90% of the patients. The duration of the treatment is dependent on the toleration to toxicity. In patients with grade 1 and tolerable grade 2 toxicities, treatment can be continued at the usual dosage, but the treatment should be suspended for higher grades. Frequent noncutaneous AEs include arthralgia, fatigue, nausea, diarrhea, and headache. Cutaneous AEs include pyrexia, rash, photosensitivity, pruritus, acneiform eruptions, erythematous hyperkeratotic follicular papules, granulomatous eruption, hyperkeratosis, warts, milia, keratoacanthoma, cutaneous squamous cell carcinoma, and basal cell carcinoma.[122,128]

## MEK inhibitors

Targeting signaling effectors downstream of driver oncogenes is a valid strategy to overcome resistance to BRAF inhibitors.[129] MEK is a downstream target of BRAF and, in contrast to the BRAF inhibitors, MEK inhibitors showed activity in *NRAS*-mutant melanomas.[130]

Trametinib, a pharmacological MEK1/2 inhibitor with antitumoral activity, was approved (2013) as a monotherapy by the FDA for the treatment of unresectable or metastatic malignant melanomas with *BRAF* mutations.[122,131] The blocking of MEK1/2 results in the inhibition of growth factors-mediated cell signaling and decrease of tumor cells proliferation. In metastatic melanoma patients with activating *BRAF* mutations not previously treated with selective BRAF inhibitors, trametinib was reported to improve clinical response rate, PFS, and OS, compared to chemotherapy.[129] The most common general AEs of MEK inhibitors are diarrhea, peripheral edema, fatigue, nausea, and vomiting.[122] A clinical trial to test the effect of trametinib in patients with *BRAF* non-V600 mutation is ongoing (NCT02296112). Combined therapy of trametinib and dabrafenib (BRAF-mutant inhibitor) showed durable objective responses in a randomized, multicenter, open-label study,[132] and the combination was approved (2014) by the FDA for the treatment of unresectable and metastatic melanomas harboring *BRAF* mutations.[133] Several clinical trials are ongoing with the combination of trametinib and dabrafenib and the two drugs in combination with radiotherapy, immunotherapies, and other targeted therapies.[22]

In 2015, the combination of cobimetinib, an oral selective MEK inhibitor, and vemurafenib (BRAF-mutant inhibitor) was approved for the treatment of melanomas, harboring *BRAF* mutations, which cannot be surgically removed or display metastization.[133,134] This approach was reported to achieve significant improvement in the PFS of melanoma patients.[135] Several clinical trials are ongoing with the combination of cobimetinib and vemurafenib and the two drugs in combination with chemotherapy, immunotherapies, and other targeted therapies.[22] The most frequent AEs for the combination of BRAF and MEK inhibitors are pyrexia, chills, fatigue, nausea, vomiting, and diarrhea.[122]

## CKIT inhibitors

In melanoma, *CKIT* mutations have been described in 39% of mucosal melanoma, 36% of acral lentiginous melanoma, 28% of cutaneous melanomas arising in areas of chronic sun-damaged skin, and none in melanomas of skin without chronic sun damage.[136,137] *CKIT* mutations or gene amplifications can lead to the constitutive ligand-independent activation of this receptor and upregulation of the MAPK and PI3K/AKT pathway.[122,138] *CKIT* mutations have been reported across several exons and were associated with the development of drug resistance.[139]

Imatinib is an oral CKIT inhibitor that reveals significant activity in patients with metastatic melanoma harboring *CKIT* aberrations, with a response rate of 30%, but with a median PFS of 3–4 months.[140–142] Two clinical trials with imatinib are ongoing, in combination with chemotherapy (NCT00667953) and with immunotherapies (NCT02812693).

Other multikinase inhibitors, such as sunitinib, dasatinib, and nilotinib, may have activity in patients with melanoma harboring *KIT* mutations. Clinical trials with these drugs are ongoing, in combination with chemotherapy (NCT01005472) and with immunotherapies (NCT01876212). The known AEs are myelosuppression, fatigue, and fluid retention.[122]

## VEGF inhibitors

Melanomas express high levels of VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 which are associated with poor prognosis, immune suppression, and growth of tumor neovasculature.[143,144] The angiogenesis promoted by the VEGF is crucial for cancer progression.[145] Therefore, VEGF blockade may be a useful approach for melanoma therapy.

Bevacizumab is an anti-VEGF monoclonal antibody that can target and neutralize VEGF and inhibit tumor growth.[146] In a single-arm Phase II clinical trial, patients with previously untreated metastatic melanomas were treated with a combined therapy of TMZ and bevacizumab.[147] The objective response rate was 16%, the overall disease control rate was 52%, the median PFS was 4.2 months, the OS was 9.6 months, and an improvement in OS in patients with $BRAF^{V600E}$-mutated melanoma was observed. In another single-arm Phase II clinical trial, bevacizumab was administered in combination with IFN α-2b. The median progression-free rate was 4.8 months and OS rate was 17 months. These studies indicate the potential of VEGF as a target, but failed to validate this therapy for melanomas. Other clinical trials are ongoing, using bevacizumab in combination with chemotherapy (NCT03175432/NCT03175432) and with immunotherapies (NCT02681549/NCT03167177/NCT00790010/NCT01950390/NCT02158520).

## PI3K-AKT-mTOR pathway inhibitors

mTOR plays a key role in tumor development and progression, and therapies have been developed to downregulate its pathway.[4,148] mTOR forms two protein complexes, mTOR

complex 1, which is activated by the PI3K/AKT pathway, and mTOR complex 2.[148] Activation of the mTOR pathway was described in cutaneous melanoma, associated with the presence of *BRAF* mutations and with poor prognosis.[149]

The combination of PI-103, a PI3K inhibitor, with the mTOR inhibitor rapamycin may effectively block the growth of melanoma cells and induce autophagy compared to both single agents separately.[4,150] Studies showed that PI3K-AKT pathway inhibitors led to a higher increase of the apoptosis rates compared to BRAF or MEK inhibitors.[151] Other PI3K inhibitors are being used in clinical trials, such as BKM120 in combination with other targeted therapies (NCT02159066), GSK2636771 in combination with immunotherapies (NCT03131908), INCB050465 in combination with other targeted therapies and with immunotherapies (NCT02646748), and IPI-549 monotherapy compared to the combination with immunotherapies (NCT002637531). An AKT inhibitor, MK2206, is also being used in a trial, in combination with chemotherapy (NCT01480154).

The antitumor effects of mTOR inhibition may be enhanced when combined with MAPK pathway inhibitors. An increase in mTOR pathway activation was observed in cells transfected with BRAF vectors and *BRAF*-mutated melanoma cell lines were reported to be more sensitive to mTOR inhibition.[152] Moreover, inhibition of AKT or mTOR and combined inhibition of PI3K and mTOR were reported to be alternative strategies to overcome BRAF inhibitors resistance.[153,154] A clinical trial is ongoing to test the effect of two mTOR inhibitors, everolimus or temsirolimus, in combination with a BRAF inhibitor (NCT01596140), and another trial is testing ASN003, a BRAF inhibitor with additional selective activity against PI3K and mTOR kinases (NCT02961283). Combination of low-dose mTOR inhibitors with immunotherapy needs clinical validation, as mTOR inhibition can result in either immunosuppression or immune activation, depending on the dose, timing, and sequencing of administration.[155]

## Cyclin-dependent kinase (CDK) inhibitors

In familial melanomas, 2% are associated with germline mutations in *CDK4*.[156] *CDK4* is an oncogene that controls cellular proliferation and it is inhibited by p16.[12,157–159] Beyond CDK4, CDK6 and cyclins (D1, D2, or D3) also control the point in G1.[160] CDK4/cyclin D kinase hyperactivation, associated with the mutation of *CDK4*, amplification of *cyclin D*, or complete deletion of *p16INK4a*, leads to an increased risk of developing melanomas.[161]

A new generation of selective CDK4/6 inhibitors, including ribociclib, abemaciclib, and palbociclib, has enabled tumors to be targeted with improved effectiveness and fewer AEs.[159] Abemaciclib has also been reported to induce growth regression in vemurafenib-resistant melanoma models, in which high levels of cyclin D1 expression and MAPK-pathway reactivation were observed.[162] The appropriate selection of patients harboring *CDK4* mutations seems crucial for the success of the therapy.[159] CDK4/6 inhibitors are in use in clinical trials for melanoma, such as ribociclib in combination with targeted therapies (NCT01781572/NCT02159066), abemaciclib monotherapy (NCT02308020), in combination with chemotherapy (NCT02857270) and with immunotherapies (NCT02791334), palbociclib monotherapy (NCT01037790) and in combination with targeted therapies (NCT02202200), and SHR6390 monotherapy (NCT02671513).

## ErbB4 inhibitor

ErbB4 belongs to the ErbB family of tyrosine kinase receptors. *ErbB4* mutations were identified in melanomas and are associated with increased kinase activity and transformation ability. Thus, the development of selective inhibitors of ErbB4 may help in the treatment of melanomas. It was reported that melanoma cells expressing mutant *ErbB4* which were submitted to shRNA-mediated knockdown of *ErbB4* or treatment with the ErbB inhibitor lapatinib displayed reduced cell growth.[163]

# Conclusion

The understanding of melanoma pathogenesis was crucial for the development of new therapeutic modalities. Characterization of oncogenic signaling pathways and interactions allowed the identification of novel targets for clinically effective treatments, such as pathways inhibitors and immune checkpoint antibodies. Although it represents an advancement for melanomas treatment, these types of approaches face several challenges. The comprehensive features of patients that will benefit from each strategy aim to establish biomarkers (eg, specific mutations) for the best (eg, targeted) therapy in advanced melanomas. The clinical tolerated doses are also an important issue, as it must comply with an acceptable tumor inhibition with minimal AEs. Moreover, patient and tumor heterogeneity are associated with different mechanisms of resistance, which influence negatively clinical outcomes in melanomas. To overcome these resistance mechanisms, a synergy between strategies (chemotherapy, immunotherapy, and targeted therapy) appears to be a suitable approach, targeting distinct pathways. Recently, the number of approved immunotherapies has been increasing. Favorable therapy

results, in melanoma and in other types of cancer, can be explained by the immune response triggered that produces a T-cell repertoire, which adapts to heterogeneous tumors, and generates memory T-cells that guarantee efficient responses against recurrent tumor.

Depending on the molecular features of the patients and tumors, as well as the responses to therapy, personalized treatment should be considered for melanoma patients, in order to achieve better clinical benefits. Further research is necessary to explore oncogenic pathways and the TME potential in the treatment of melanomas.

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## Disclosure

The authors report no conflicts of interest in this work.

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