UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Unraveling the relevance of ARL1 GTPase in cutaneous melanoma aggressiveness

Inês Isabel Rodrigues Saraiva

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
Doutora Marta Sofia Pojo Sousa
Prof.ª Doutora Rita Maria Pulido Garcia Zilhão Aranha Moreira

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ABSTRACT

Cutaneous melanoma (CM) is the skin cancer with the highest mortality rates due to its great potential for metastasis. The incidence of CM continues to increase worldwide, and its biology remains poorly understood. In addition, not all patients respond to current therapies. Therefore, it is crucial to better understand the molecular mechanisms responsible for this disease and find precise biomarkers that help in early diagnosis, prognosis and development of new therapies. In this context, we hypothesized that ARL proteins could be CM biomarkers and aggressiveness key targets. Furthermore, ARLs play an essencial role in several cellular processes (e.g. cell migration, actin remodeling and vesicle transport) that may impact cancer progression. In particular, the involvement of ARL1 in membrane traffic and innate immunity was only described once in CM. In a bioinformatics study performed by our group, high expression of *ARL1* was correlated with prolonged overall survival of CM patients and higher infiltration of immune cells.

Here, we investigated *ARL1* expression in the Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG) CM cohort and assessed the relevance of *ARL1* in CM aggressiveness *in vitro*.

ARL1 expression was evaluated in surgical samples from CM patients by RT-qPCR, and the correlation of *ARL1* expression with various immune subsets was detected by flow cytometry. Additionally, transwell migration and invasion assays were performed with A375 and WM115 CM cell lines to determine the impact of *ARL1* on CM aggressiveness. Western blot evaluated the interaction of ARL1 with the MAPK and PI3K pathways, and RT-qPCR evaluated the relationship of *ARL1* knockdown with different genes involved in cellular metabolism.

Thus far, our results showed no differences in *ARL1* expression between CM samples and normal skin, as well as according to the stage, sample type and *BRAF* mutational status. We found a positive correlation between *ARL1* expression in the tumor and infiltration of helper T lymphocytes, cytotoxic T lymphocytes and CCR4 expression in regulatory T cells. Regarding the *in vitro* results, *ARL1* knockdown did not affect the cell viability of A375 and WM115 cell lines. However, it decreased the A375 CM cell lines's cell migration, while harboring no effect on WM115 migration capability. No significant differences were verified in cell invasion for both CM cell lines. Our results indicate that lower levels of ARL1 decreases and increases the activation of the MAPK pathway in A375 and WM115 cell lines, respectively. In addition, ARL1 knockdown appears to decrease AKT protein levels in the PI3K pathway in A375 cell line. Finally, *in vitro ARL1* does not seem to interfere with expression of genes linked to lactate production (*LDHA*, *LDHC*, *MCT1* and *MCT4*), pentose phosphate pathway (*G6PD*) and amino acid synthesis (*GLS1*, *SNAT1*, *SNAT2*, *XCT* and *EAAT3*).

Here, we could characterize *ARL1* expression in a CM cohort from IPOLFG, and we have not seen differences between CM and normal skin. ARL1 has been shown to have an immunosuppressive and antitumor role. Our data further indicate that ARL1 knockdown altered A375 cell migration, suggesting that high levels of ARL1 may increase CM aggressiveness, but we found no differences in invasiveness in two distinct cell lines. We verified that ARL1 is involved in the MAPK and PI3K pathways in both CM cell lines, but we found no relationship between *ARL1* silencing and CM metabolism.

Key words: Cutaneous melanoma; aggressiveness; small GTPases; Arls; ARL1.

RESUMO

O Melanoma Cutâneo (MC) é um tumor que resulta de alterações genéticas nos melanócitos, sendo o cancro de pele menos comum, mas com a taxa de mortalidade mais alta devido ao seu grande potencial de metástase. A incidência do MC continua a aumentar em todo o mundo, representando um dos cancros com maior incidência em jovens adultos. O principal comportamento de risco associado ao desenvolvimento desta doença é a exposição à radiação ultravioleta, mas outros fatores como, fotótipo de pele, presença de nevos e história pessoal e familiar de MC também estão associados. No MC esporádico, as vias de sinalização MAPK e PI3K encontram-se frequentemente alteradas. A causa mais comum de desregulação da via MAPK são mutações no gene *BRAF*, que representa a maioria dos casos de MC, seguidas de mutações em *NRAS* e *NF1*. Mutações nestes genes resultam na ativação aberrante destas vias de sinalização, potenciando o crescimento celular, proliferação, migração, invasão e redução da apoptose, contribuindo para o desenvolvimento de MC. Nos últimos anos a utilização de terapias direcionadas a genes da via MAPK, como as terapias baseadas em inibidores BRAF e MEK, revolucionaram o tratamento dos doentes com MC. Além disso, o MC é conhecido como um tumor muito imunogénico devido à sua alta carga mutacional, e assim as imunoterapias baseadas em inibidores de checkpoint imunológico, como anticorpos anti-CTLA-4 e anticorpos anti-PD-1 conferiram altas taxas de sucesso no tratamento do MC. No entanto, apesar das terapias direcionadas e imunoterapias baseadas em inibidores de checkpoint imunológico terem melhorado a sobrevida dos doentes com MC, alguns acabam por desenvolver resistência às terapias. Assim, é crucial perceber melhor os mecanismos moleculares responsáveis por esta doença e encontrar biomarcadores precisos que auxiliem no diagnóstico precoce e no desenvolvimento de novas terapias.

Considerando que o funcionamento de vias de sinalização depende da ação de GTPases, que funcionam como interruptores moleculares, controlando redes de tráfego e sinalização das células, é importante explorar estas proteínas como biomarcadores em cancro, incluindo em MC. As pequenas GTPases compreendem 5 subfamílias de proteínas (RAS, RHO, RAB, RAN e ARF), que coordenam inúmeros processos celulares como, ciclo celular, proliferação celular, remodelação do citoesqueleto, tráfego membranar e migração. Sabe-se que muitas destas proteínas quando desreguladas contribuem para a progressão de diversos cancros, por exemplo, o gene *RAS* é um oncogene frequentemente mutado em cancros humanos e a segunda mutação mais frequente em melanoma, nomeadamente a sua isoforma *NRAS*.

Dentro da família das ARFs, as proteínas ARL desempenham funções importantes em diversos processos celulares, tais como, migração celular, remodelação de actina e transporte vesicular, processos que podem estar associados com o desenvolvimento de cancro. De facto, a desregulação destas proteínas tem vindo a ser descrita em diversos cancros. Neste contexto, hipotetizámos que as proteínas ARL poderão ser biomarcadores precisos e alvos chave de agressividade em MC. Assim, começámos por realizar uma análise bioinformática, que mostrou que todas as ARLs se encontravam desreguladas em MC, sendo que 3 ARLs mostraram ser fatores prognósticos independentes em MC, entre as quais ARL1, ARL11 e ARL15. A expressão elevada de *ARL1* mostrou estar correlacionada com sobrevida global prolongada em doentes com MC. Além disso, constatámos uma correlação significativa entre a expressão de *ARL1* e a infiltração de células imunes. Sabe-se que a proteína ARL1 modula funções ao nível do complexo de Golgi e está envolvida no tráfego membranar e imunidade inata. Esta proteína foi apenas descrita uma vez em MC, nomeadamente no estudo de bioinformática do nosso grupo.

Neste trabalho, investigámos se a expressão de *ARL1* tem impacto na agressividade do MC. Assim sendo, os objetivos deste trabalho passaram por, avaliar a expressão de *ARL1* na *cohort* de MC

do IPOLFG, perceber se *ARL1* está associada com o sistema imunitário e compreender a relevância de *ARL1* na agressividade do MC *in vitro* ao nível da migração celular, invasão e metabolismo.

A expressão de *ARL1* foi avaliada em amostras cirúrgicas de doentes com MC por RT-qPCR, comparando: 1) pele normal e MC; 2) estágio; 3) tipo de amostra; 4) estado mutacional de *BRAF*. Além disso, as amostras cirúrgicas, bem como amostras de sangue de doentes com MC foram analisadas para avaliar a correlação da expressão de *ARL1* com diversas populações imunes (linfócitos T citotóxicos, linfócitos T auxiliares, linfócitos T reguladores, monócitos e macrófagos) infiltradas no tumor e em circulação por citometria de fluxo. Para os ensaios *in vitro*, silenciámos *ARL1* nas linhas celulares de MC, A375 e WM115. A confirmação do silenciamento foi realizada ao nível do mRNA por RT-qPCR e ao nível da proteína por western blot. Posteriormente, verificámos se o silenciamento de *ARL1* afetou a viabilidade celular através de dois ensaios, trypan blue e CCK8. Para determinar o impacto de *ARL1* na agressividade do MC, avaliámos a capacidade migratória e invasiva das linhas celulares A375 e WM115 silenciadas para *ARL1* através de ensaios de migração e invasão. A interação de ARL1 com as vias de sinalização MAPK e PI3K foi investigada por western blot. Por fim, avaliámos se *ARL1* é capaz de influenciar o metabolismo celular do melanoma através de um painel de diferentes genes envolvidos em vias metabólicas por RT-qPCR.

Até ao momento, os nossos resultados mostram que não existem diferenças na expressão de *ARL1* entre amostras de MC e pele normal, nem com o estágio, tipo de amostra e o estado mutacional de *BRAF*. Encontrámos uma correlação positiva entre a expressão de *ARL1* e a infiltração de linfócitos T auxiliares, linfócitos T citotóxicos e expressão de CCR4 em células T reguladoras. Relativamente aos resultados *in vitro*, o silenciamento de *ARL1* não afetou a viabilidade celular das linhas celulares A375 e WM115. No entanto, levou a uma diminuição na migração celular na linha A375, mas sem afetar a capacidade de migração da linha WM115. Não foram verificadas diferenças significativas na invasão celular para ambas as linhas de MC. Os nossos resultados indicam que níveis mais baixos de ARL1 diminuem e aumentam a ativação da via MAPK nas células A375 e WM115, respectivamente. O silenciamento de ARL1 parece diminuir os níveis de proteína AKT na via PI3K nas células A375, mas não vimos diferenças na linha WM115. Finalmente, *in vitro ARL1* não parece interferir com a expressão de genes ligados à produção de lactato (*LDHA*, *LDHC*, *MCT1* e *MCT4*), via das pentoses fosfato (*G6PD*) e síntese de aminoácidos (*GLS1*, *SNAT1*, *SNAT2*, *XCT* e *EAAT3*).

Neste trabalho, conseguimos caracterizar o efeito da expressão de *ARL1* na *cohort* de MC do IPOLFG, não tendo detectado diferenças entre MC e pele normal ao nível da expressão de *ARL1*. *ARL1* parece ser capaz de modular o sistema imune através de uma atividade imunossupressora e antitumoral. Os nossos dados indicaram ainda, que o silenciamento de *ARL1* não afetou a viabilidade celular, nem a invasão celular para ambas as linhas de MC. A migração celular diminuiu na linha celular A375 silenciada para *ARL1* comparativamente ao controlo. Assim, altos níveis de *ARL1* parecem aumentar a agressividade do MC. Estes resultados foram apoiados pela diminuição da ativação da via MAPK. No entanto, não encontrámos diferenças significativas na migração celular da linha WM115, mas o silenciamento de *ARL1* parece aumentar a ativação da via MAPK. Portanto, *ARL1* poderá desempenhar um papel controverso. Não encontrámos relação entre o silenciamento de *ARL1* e o metabolismo do MC.

Palavras-chave: Melanoma Cutâneo; agressividade; pequenas GTPases; Arls; ARL1.

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LIST OF ABREVIATIONS

AKT Protein kinase B

ARF ADP ribosylation factor

ARL ADP ribosylation factor like GTPase

BCA Bicinchoninic acid

BRAF B-Raf proto-oncogene, serine/threonine kinase

BSA Bovine serum albumin

CCK8 Cell counting kit-8

CDK4 Cyclin-dependent kinase 4

CDKN2A Cyclin-dependent kinase inhibitor 2

cDNA Complementary deoxyribonucleic acid

CM Cutaneous melanoma

CTLA-4 Cytotoxic T-lymphocyte-associated protein 4

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco's modified eagle's medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

EAAT3 Excitatory amino acid transporter 3

ERK1/2 Extracellular signal regulated kinase 1/2

ERK Extracellular signal regulated kinase

FBS Fetal bovine serum

G6PD Glucose-6-phosphate dehydrogenase

GAPs GTPase activating proteins

GDP Guanosine diphosphate

GEFs Guanine nucleotide exchange factors

GLS1 Glutaminase 1

GTEx Genotype tissue expression

GTP Guanosine triphosphate

HGF/c-MET Hepatocyte growth factor/c-mesenchymal-epithelial transition factor

HIF1α Hypoxia inducible factor 1α

HLA Human leukocyte antigen

HLA-DR Human leukocyte antigen DR

HPRT1 Hypoxanthine phosphoribosyltransferase 1

HRAS HRas proto-oncogene

IPOLFG Instituto Português de Oncologia de Lisboa Francisco Gentil

KIT KIT proto-oncogene, receptor tyrosine kinase

KRAS KRAS proto-oncogene

LDHs Lactate dehydrogenases

LNM Lymph node metastasis

mAbs Mononuclear antibodies

MAPK Mitogen-activated protein kinase

MC1R Melanocortin 1 receptor

MCTs Monocarboxylate transporters

MEK Mitogen-activated protein kinase kinase

MET MET proto-oncogene, receptor tyrosine kinase

MHC Major histocompatibility complex

MMPs Matrix metallopeptidases

mTOR Mammalian target of rapamycin

MYC Myelocytomatosis oncogene homolog

NF1 Neurofibromin 1

NMSC Nonmelanoma skin cancer

NRAS NRAS proto-oncogene

NS Normal skin

OS Overall survival

P/S Penicillin/streptomycin

PBMCs Peripheral blood mononuclear cells

PBS Phosphate-buffered saline

PD-1 Programmed cell death protein 1

PD1L1/2 Programmed cell death 1 ligand 1/2

PFS Progression free survival

PI3K Phosphatidylinositol-3-kinase

PM Primary melanoma

PTEN Phosphatase and tensin homolog

Rab Ras-like proteins in the brain

RAC1 Rac family small GTPase 1

Ran Ras-like nuclear

Ras Rat sarcoma virus

Rho Ras homologous

RNA Ribonucleic acid

RT-qPCR Reverse transcriptase quantitative polymerase chain reaction

RTKs Receptor tyrosine kinases

shRNA short hairpin RNA

SM Skin metastasis

SNAT1 Sodium-coupled neutral amino acid transporter 1

SNAT2 Sodium-coupled neutral amino acid transporter 2

TCGA The cancer genome atlas

TERT Telomerase reverse transcriptase

TIM-3 T-cell immunoglobulin and mucin domain 3

TIMER Tumor immune estimation resource

TNFα Tumor necrosis factor alpha

TP53 Tumor protein p53

UVR Ultraviolet radiation

V600E Substitution of valine for glutamic acid at amino acid 600

WT Wildtype

XCT Cystine glutamate transporter

1. INTRODUCTION

1.1 CARCINOGENESIS

Cancer is a heterogeneous and complex disease, a malignant neoplasm that involves the progressive accumulation of multiple alterations in Deoxyribonucleic acid (DNA) and gene expression. Consequently, it leads to the uncontrolled proliferation of genetically altered cells and their ability to invade other tissues[1]. The alterations in DNA can be inherited and the individual has susceptibility to develop cancer (germline mutations) or can occur in somatic cells due to internal agents (e.g. byproducts of normal intracellular metabolism - reactive oxygen intermediates) or by external agents (e.g. tobacco and alcohol components, ultraviolet light, diet, chemicals, and infectious agents)[1-3]. However, cells have DNA repair mechanisms that detect DNA damage and cause cell cycle arrest, including so-called cell cycle checkpoints - decreased accuracy of DNA damage repair results in several mechanisms that condition the development of cancer[2]. In a primary stage of carcinogenesis, an initial cell that has acquired mutations will undergo cell division, originating a population of genetically modified tumor-initiating cells, resulting in increased cell proliferation, and a pre-neoplastic lesion. This lesion will undergo genotoxic changes (DNA or chromosomal damage), progressing to neoplasia[1].

Major genetic alterations in cancer result from mutations in proto-oncogenes and tumor suppressor genes. Oncogenes result from mutations in genes that regulate proliferation, survival, and invasion (proto-oncogenes). The activation of oncogenes (e.g. genes from the Ras family) can occur due chromosome structural abnormalities (e.g. amplification and translocation) or point mutations, which potentiates the upregulation of cell proliferation, survival and invasion. Tumor suppressor genes inhibit cell proliferation, survival, and invasion. Deletion mutation can result in inactivation of tumor suppressor genes (e.g. p53 and Rb1) leads to an inability to inhibit cell proliferation and survival, decreased accuracy of cell cycle control and evasion of cell apoptosis[1,2,4].

However, the development and progression of cancer is not just about these genetic alterations mentioned, but also the interaction of the tumor with different molecules, immune cells, fibroblasts, blood vessels and extracellular matrix. In 2000, Hanahan and Weinberg described six cellular processes related to cancer progression (hallmarks of cancer), which include, increased cell proliferation, growth suppressors evasion, apoptosis resistance, induction of angiogenesis, immortalization by telomerase and invasive and metastatic potential. In 2011 and 2022, more hallmarks of cancer were added, including genomic instability, reprogramming cellular metabolism, evading immune destruction, inflammation, unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, polymorphic microbiomes and senescent cells[5-7].

Malignant neoplasms are classified according to the type of altered cells in carcinoma (epithelial cells), sarcoma (mesenchymal cells), lymphoma (lymphocytes), melanoma (melanocytes), among others. Neoplasms can be further classified as to their histogenesis, for example, carcinomas can be adenocarcinomas (e.g. colon cells) and squamous cell carcinomas (skin cells)[1,3].

1.2 SKIN AND SKIN CANCER

The human skin is the body's biggest organ, with several functions such as water loss control, thermoregulation, vitamin D production, the barrier against pathogens, and Ultraviolet radiation (UVR), which is essencial in immune responses and sensory function. The skin consists of three layers: first is the epidermis, followed by the dermis, and lastly, by a subcutaneous or hypodermis layer. The epidermis is formed mainly by keratinocytes and in a lesser amount, by melanocytes. The

dermis comprises fibroblasts, blood vessels, immune cells, nerves and glands. Finally, the subcutaneous layer contains subcutaneous fat and connective tissue[8,9].

Skin cancer represents a group of diseases characterized by the abnormal growth of skin cells, being the third most common cancer in the world[10,11]. According to its origin, skin cancer can be classified into Nonmelanoma skin cancer (NMSC) and Cutaneous melanoma (CM). Within NMSCs, the most frequent is basal cell carcinoma, which comprises malignant basal cells, cells present in the basal layer of the skin, followed by squamous cell carcinoma, derived from the malignant transformation of keratinocytes (or squamous cells), cells in the upper part of the epidermis[11-14]. In addition to these two types of NMSCs, Sebaceous gland carcinoma (sebaceous glands), Merkel cell carcinoma (neuroendocrine cells) and angiosarcoma (vascular endothelial cells) are other forms of NMSC, but rarer[14]. Finally, another type of skin cancer is CM, characterized by genetic alterations in melanocytes, representing about 4% of malignant skin tumors cases (Figure 1.1)[13,15]. Melanocytes are responsible for melanin synthesis, the pigment that gives skin color and acts as a barrier against UVR, thus preventing alterations in DNA[9,13].

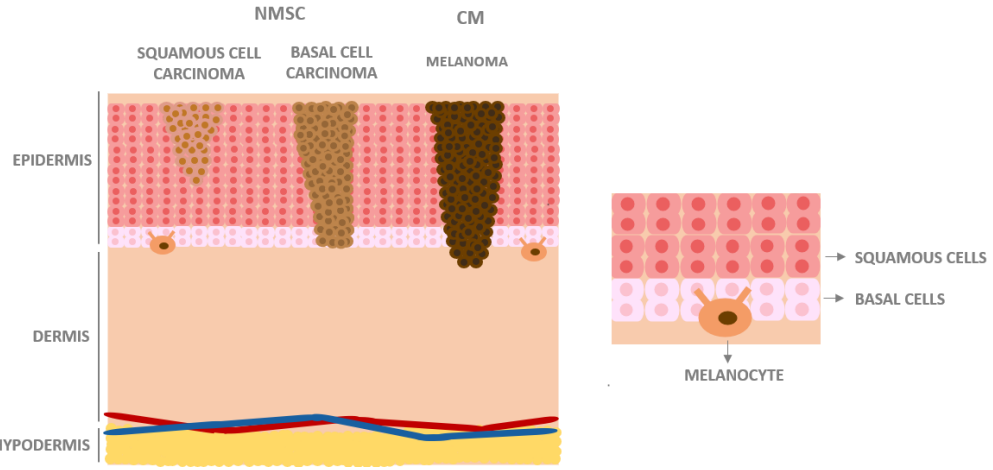


Figure 1.1 Layers of human skin. Non-melanoma Skin Cancer (NMSC) and Cutaneous melanoma (CM). The three layers of skin are the epidermis (the top layer), dermis (the middle layer) and hypodermis (the bottom layer). Skin cancer is divided according to cell type altered in NMSC, such as squamous cell carcinoma (squamous cells) and basal cell carcinoma (basal cells), and CM (melanocytes).

1.3 CUTANEOUS MELANOMA

CM is the most aggressive and deadly cutaneous malignancy, representing about 70-75% of deaths from malignant skin tumors[13,16]. CM has a high metastatic potential, especially to the lungs, abdominal organs, brain, bones, skin and distant lymph nodes, which is reflected in a poor prognosis[16]. Furthermore, from 1990 to 2019 it was one of the cancers with the highest increase in incident cases (about 170%) worldwide[15]. CM can be classified according to clinical and histopathological characteristics into four main types. The most common is the superficial spreading melanoma, which is characterized by irregular and pigmented spots. Nodular melanoma is the second most common and the most aggressive, appearing as a black or brown nodule. Lentigo melanoma is the subtype with the lowest risk of metastasis and arises as a large, flat, pigmented spot, mainly located on the face. Lastly, acral lentiginous is a very aggressive type of melanoma that appears as a nodule on the palms, soles of the feet or beneath the nail beds[13,17]. Although about 90% of melanomas affect the skin, there are rarer forms that can arise as a result of malignant transformation of melanocytes on mucosal surfaces (mucosal melanoma) and of melanocytes in the eyes (uveal melanoma)[13,18].

1.3.1 EPIDEMIOLOGY AND RISK FACTORS

According to the Global Cancer Statistics, in 2020, there were an estimated 19.3 million new cases and 10 million deaths from cancer worldwide, of which 324635 cases and 57043 deaths were due to CM, the 19th most common cancer in the world[10]. In 2020, in the USA, estimates pointed to CM as the third most common cancer in young adults (20 to 39 years)[19]. Currently, CM is more frequent in women aged up to 50 years than in men of the same age; but between 65 and 80 years, the scenario is reversed, with males presenting higher incidence rates and the sex with the lowest survival[16,20]. This last fact may be due sex hormones in women (e.g. estrogen) might increase immune response, and men have less tolerance to oxidative stress (men express lower amounts of antioxidant enzymes, resulting in an increase in reactive oxygen species, which stimulates the occurrence of metastases)[16,21,22].

In addition to gender, other factors are related to the development of CM, like UVR exposure, geographic variation, skin phototype, presence of melanocytic nevi, family and personal history of CM and socioeconomic status[11,23-39].

UVR from sunlight is the main risk factor directly related with CM[11]. Furthermore, sunburns and UVR from artificial sources (e.g. tanning beds) are also intimately linked to the risk of CM[23-25]. UVR potentiates DNA alterations caused by oxidative damage to base pairs, DNA single strand breaks and the formation of cyclobutane pyrimidine dimers[26]. One of the events leading to UVR-induced cutaneous tumorigenesis is the induction of mutations in p53, which is involved in DNA repair and apoptosis. Once mutated, it will be unable to correct UVR damage to DNA[27]. UVR also affects the Mitogen-activated protein kinase (MAPK) signaling pathway that controls cell proliferation and survival[28]. UVR makes CM one of the cancers with the highest mutational load and can lead to immune suppression affecting the immune system's ability to attack malignant melanocytes. Immunosuppression caused by UVR may result from the formation of pyrimidine dimers that decrease the ability to present antigens and increase the production of immunosuppressive cytokines[26-31]. Furthermore, UVR exposure intensity is directly related with the incidence of CM in the world. In Australia, the estimated standardized incidence rates of CM are the highest in the world. GLOBOCAN 2020 estimates have indicated CM as the third most common cancer in Australia in 2020[32-34].

Skin phototype is an endogenous factor that predisposes to the development of CM. Fair skin people have a higher risk of developing CM than dark skin people since they produce less eumelanin, a type of melanin[35]. Melanin is synthesized in melanocytes, specifically in melanosomes that transfer melanin to keratinocytes. There are two types of melanin responsible for skin pigmentation, eumelanin (as mentioned above) and pheomelanin. Eumelanin is produced in higher quantities in dark skin individuals, while fair skin individuals produce more pheomelanin. Eumelanin offers some degree of photoprotection and eliminates free radicals, while pheomelanin can lead to the production of reactive oxygen species[35]. UVR exposure induces the secretion of alpha-melanocyte stimulating hormone by keratinocytes, which bind and activate signaling molecules on the surface of melanocytes (e.g. Melanocortin 1 receptor (*MC1R*)), consequently leading to the increased expression of genes necessary to produce eumelanin. Normally, fair skin people have polymorphisms in the *MCR1* gene that cause its inactivation, and therefore less production of eumelanin and more of pheomelanin. Thus, fair skin people are more likely to develop CM than dark skin people[29,35]. In addition to fair skin people having a higher risk of CM, individuals with more than 100 common nevi have a 7-fold risk of developing CM, whereas the presence of 5 atypical nevi increases the risk in more than 6 times[36]. Although most cases of CM are due to somatic alterations, around 10% of cases are hereditary. The first two genes identified associated with CM susceptibility were Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), followed by Cyclin-dependent kinase 4 (*CDK4*). *CDKN2A* is a tumor suppressor gene

that encodes two proteins, p16INK4A and p14ARF. p16INK4A induces the arrest of the cell cycle in the G1 phase and p14ARF is also involved in cell cycle arrest via p53 and can promote apoptosis. *CDK4* is an oncogene that encodes a protein involved in cell cycle progression through the G1 phase[37]. So, when these two genes are mutated, the cell cycle progresses without correction of any DNA damage. Mutations in these two genes can be found, for example, in families with familial atypical multiple mole melanoma syndrome and melanoma-astrocytoma syndrome, and there is therefore a high risk of developing CM[38]. Familial retinoblastomas, Lynch syndrome type II, Li-Fraumeni cancer syndrome and xeroderma pigmentosum are hereditary diseases that also predispose to melanoma[38]. For example, individuals with xeroderma pigmentosum have defects in nucleotide excision repair, a mechanism that allows the removal of UVR-induced DNA damage[14]. Additionally, personal history of melanoma increases the risk of developing a second melanoma[13].

Finally, another risk factor is socioeconomic status. CM cases are being more common in people with higher socioeconomic status, essentially due to more frequent holidays, which implies greater exposure to the sun[39].

1.3.2 MOLECULAR BIOLOGY

CM, like other sporadic cancers, results in the accumulation of genetic mutations that consequently lead to the dysregulation of signaling pathways that play a crucial role in various cellular processes, including processes linked to tumorigenesis. The aberrant activation of MAPK and Phosphatidylinositol-3-kinase (PI3K) signaling pathways, responsible for controlling growth, cell proliferation, survival, apoptosis, migration and invasion are described in CM[38,40]. The MAPK pathway is the main pathway altered in CM and mutations in B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) are the most common, being present in 37-50% of melanoma cases[38,41]. The main *BRAF* mutation in CM involves the Substitution of valine for glutamic acid at amino acid 600 (V600E), which results in the constitutive activation of BRAF. This increased protein kinase function favors cell growth and inhibition of apoptosis through interaction with different components of the MAPK pathway, namely Mitogen-activated protein kinase kinase (MEK) and Extracellular signal regulated kinase (ERK). About 80% of benign nevi have *BRAF* mutations; however, this is not enough to lead to tumorigenesis, and therefore other additional mutations in genes such as Telomerase reverse transcriptase (*TERT*) or *CDKN2A* are common[38,41]. Mutations in NRAS proto-oncogene (*NRAS*) represent 15-30% of melanomas[38,41]. Most of these mutations (missense mutations in codon 61) promote its active state linked to Guanosine triphosphate (GTP), contributing to the dysregulation of the MAPK and PI3K pathways, favoring cell growth, cell proliferation and cell cycle dysregulation[41]. One of the mechanisms responsible for *NRAS* dysregulation is the loss of the Neurofibromin 1 gene (*NF1*), mutation found in about 10-15% of melanoma patients[38,41]. NF1 protein is a negative regulator of the Rat sarcoma virus (Ras) family by inactivation of the on state of Ras. Thus, NF1 loss of function potentiates the increase of NRAS protein function and consequently hyperactivation of the MAPK and PI3K pathways. Mutations in *NF1* can result in a truncated protein due to nonsense mutations, insertions, or deletions. Furthermore, in melanomas with *BRAF*, *NRAS* or *NF1* mutations can also be found mutations in Tumor protein p53 (*TP53*), important in cell cycle control, apoptosis and DNA repair. Mutations in Rac family small GTPase 1 (*RAC1*), normally present with *BRAF* or *NRAS* mutations, lead to cell proliferation and migration. Additionally, mutations in the KIT proto-oncogene, receptor tyrosine kinase (*KIT*), although uncommon in CM, also affect the PI3K and MAPK pathways, favoring the proliferation, cell migration and survival of melanoma. Lastly, deletions in the Phosphatase and tensin homolog (*PTEN*) are responsible for the increased activation of the PI3K pathway[38,41].

1.3.3 STAGING AND TREATMENT

CM is divided into five stages (0, I, II, III and IV) according to tumor extension, lymph node involvement and presence of distant metastases. Stage 0, also referred melanoma *in situ*, is characterized by lesions restricted to the epidermis. Stage I includes primary tumors, without evidence of regional or distant metastases and may or may not have ulceration. At stage II, the primary tumor has not yet spread to the lymph nodes or metastasized, but the risk of ulceration is greater than at stage I[42,43,44]. When CM is diagnosed at stages I and II, patients have a 5-year survival rate of 99,4% and 82,6%, respectively[45]. At stage III, when tumor cells are found in regional lymph nodes, the 5-year survival rate drops to 69,3%. If the tumor has spread to other parts of the body (stage IV, distant metastases), it is of only 23%[43,45].

Treatment options for CM vary according to the stage of the disease. Wide local excision is the standard treatment for primary tumors; however, when the surgery involves disfigurement, as in lentigo melanoma, radiotherapy is recommended. For locoregional disease, sentinel lymph node biopsy is recommended when the thickness of the tumor is greater than 0.8 mm or in cases where the thickness is thinner, but with ulceration[46,47]. Complete lymph node dissection was performed for sentinel lymph node-positive patients, but some studies have not found significant benefits with this procedure, and therefore complete lymph node dissection can no longer be recommended, with intense follow-up being recommended instead[47]. If locoregional lymph node metastases are detected (stage III), patients may benefit from therapeutic lymph node dissection along with adjuvant therapy, such as targeted therapy or immunotherapy. In cases of inoperable regional metastases, targeted therapy or immunotherapy can be an option[46,47]. In stage IV, surgical excision of distant metastases, preferentially combined with adjuvant therapies (targeted therapy or immunotherapy), is indicated, but when the surgical approach is of high-risk complications, as in the case of brain metastases, radiotherapy can be used as an alternative. Additionally, when metastases are inoperable, targeted therapy or immunotherapy can be indicated for first-line treatment (palliative). Targeted therapy is chosen in *BRAF* mutant patients, while immunotherapy based on checkpoint inhibitors is considered in patients with Wildtype (WT) *BRAF* tumors, but also in *BRAF* mutant tumors. Chemotherapy is a last-line option in case of resistance to targeted therapies and immunotherapy[28,46,47].

CM treatment has undergone a revolution since 2011 with Food and Drug Administration's approval of targeted therapies and immune checkpoint inhibitors for advanced stages (III and IV)[28,48].

Targeted therapies based on BRAF and MEK inhibitors have been shown to be beneficial in *BRAF*-mutated advanced CM patients[49-54]. Vemurafenib was the first BRAF inhibitor approved for CM treatment[28,48]. Melanoma patients positive for *BRAF* mutation showed a median disease-free survival of 23.1 months when treated with Vemurafenib, but only 15.4 months with placebo[49]. After the approval of Vemurafenib, another BRAF inhibitor, Dabrafenib, showed to improve median Progression free survival (PFS) (PFS, 5.1 months) comparing with chemotherapy (PFS, 2.7 months) in *BRAF*-mutated metastatic melanoma[50]. Analysis of real-world data demonstrated a 12 month survival probability of 40.5% and 60.7% for CM patients treated with Vemurafenib and Dabrafenib, respectively[51]. Within the MEK inhibitors, Trametinib and Cobimetinib combined with BRAF inhibitors were also approved by the Food and Drug Administration to be used in CM therapy[18]. Melanoma patients who received Dabrafenib plus Trametinib had an Overall survival (OS) of 86% at 3 years and a estimated 3-year rate of relapse-free survival of 58%, higher than in patients who received placebo[52]. A 2020 study showed that 52% of patients with stage III of melanoma who received Dabrafenib plus Trametinib were alive without relapse at 5 years, compared with 36% of those on the placebo group[53]. Advanced melanoma patients that received Cobimetinib plus Vemurafenib showed a median OS of 22.5 months and 5-year OS rates of 31% versus 17.4 months of

median OS and 26% of 5-year OS rates with placebo plus Vermurafenib[54]. Despite the effectiveness of BRAF and MEK inhibitors, responses are short-lived. 20% of patients have intrinsic resistance to therapy and patients who respond to therapy may develop acquired resistance due to genetic and epigenetic mechanisms[55,56]. Some mechanisms that can lead to intrinsic resistance are: 1) loss of *PTEN*, leading to apoptosis evasion by melanoma cells; 2) overexpression of Cyclin D1 and *CDK4*, promoting cell proliferation; 3) stromal secretion of Hepatocyte growth factor/c-mesenchymal-epithelial transition factor (HGF/c-MET), which leads to PI3K pathway activation; 4) *NF1* loss with consequent activation of PI3K and MAPK pathways. Acquired resistance can consist, for example, in RAS overexpression, *BRAF* amplification and splicing that results in MEK-ERK activation, and hyperactivation of Receptor tyrosine kinases (RTKs). Initially the strategy to overcome resistance to BRAF inhibitors was the combination of BRAF and MEK inhibitors; however, patients still develop resistance[55]. Resistance to MEK inhibitors may be related to mutations in MEK that lead to overactivation of MEK or the inability of inhibitors to bind to MEK, reactivation of RTKs; tumor cells may resort to other pathways to sustain their growth (e.g. activation of the PI3K pathway) and may also activate metabolic pathways to favor tumor growth[57]. Furthermore, patients with other mutations, such as *NRAS* mutations, still do not benefit from targeted therapies[56,58]. Moreover, targeting NRAS is a difficult task due to the interaction of NRAS with GTP and Guanosine diphosphate (GDP), as well as the high presence of GTP in the cell[59]. Therefore, additional approaches have been studied, such as the combination of BRAF and MEK inhibitors with other targeted therapies (e.g. ERK, PI3K and CDK inhibitors and anti-MET therapies) or even a combination of targeted therapy with immunotherapy[55,56,60]. In fact, both MEK and BRAF inhibitors appear to enhance immune responses (e.g. increased T-cell infiltration and decreased immunosuppressive cytokines), raising interest in the combination of targeted therapy with immunotherapy[56,57].

Immunotherapy approved to treat unresectable or metastatic melanoma is based on immune checkpoint inhibitors, such as the anti-Cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) antibody, Ipilimumab and the anti-Programmed cell death protein 1 (anti-PD-1) antibody, Nivolumab or Pembrolizumab. CTLA-4 and PD-1 are receptors expressed on T cells that inactivate the immune response by binding to receptors B7-1/2 on antigen-presenting cell and Programmed cell death 1 ligand 1/2 (PD-L1/2) on melanoma cells or on antigen-presenting cell, respectivaly[28,48]. Thus, anti-CTLA-4 and anti-PD-1 antibodies function as keys to stimulate effector T cells in tumor elimination[28]. Patients who received Ipilimumab had good outcomes, however Nivolumab and Pembrolizumab showed better responses[61]. Nivolumab improved OS at 1 year (72.9%) compare with chemotherapy (42,1%)[62]. Pembrolizumab showed a survival of 68.4% while Ipilimumab had only 58.2%[63]. Combination of immunotherapies seems more promising. Nivolumab plus Ipilimumab revealed 52% of OS at 5 years, against only 44% and 26% in patients who receive Nivolumab and Ipilimumab, respectively[64]. However, immunotherapies alone or even together lead to toxicity complications or adverse immune reactions that affect the skin and other organs[61,65]. In addition, like targeted therapy, some patients do not respond, and others develop resistance. The resistance observed is mainly associated with mechanisms that interfere with the recruitment and activity of T cells, increase of molecules with immunosuppressive activity, immune evasion and loss of the antigen presentation by the tumor[66]. Other potential candidates for immunotherapy in cancer, including in CM, are the immune checkpoint receptors, Lymphocyte activation gene 3 and T-cell immunoglobulin and mucin domain 3 (TIM-3), expressing in T cells, and the enzyme, indoleamine 2,3-dioxygenase[67-71].

1.4 IMMUNE SYSTEM AND MELANOMA

The tumor is surrounded by an environment comprising the extracellular matrix, blood vessels, stromal cells (e.g. endothelial cells and cancer associated fibroblasts) and immune cells. This environment supports tumor survival through the secretion of several factors (e.g. growth factors, enzymes and cytokines) that stimulate cell proliferation, angiogenesis, migration, invasion and metastasis[72].

The immune cells in tumor microenvironment play an important role in the elimination of cancer cells. Natural killer cells, macrophages, neutrophils and dendritic cells are the first line (innate immunity) of defense against the tumor. Cytotoxic or memory T cells (CD8+ T cells) and T helper cells (CD4+ T cells) participate in adaptive immunity, that is, in a specific response against the tumor, being able to destroy the malignant cells and to develop a long-term memory response[30,72]. First, the immune response involves the antigen-presenting cells (e.g. dendritic cells) that through Major histocompatibility complex (MHC) molecules, capture tumor antigens that in the lymph nodes are recognized and eliminated by T cells (e.g. CD8+ T cells)[73]. However, tumor cells can go unnoticed and escape the immune response[30]. Before the tumor evades the immune system, it goes through two stages: elimination and equilibrium. The elimination stage, tumor cells are eliminated by the immune system. However, during the equilibrium stage, there are tumor cells resistant to suppression by the immune system that proliferate and form clones, while other more susceptible tumor cells keep being eliminated. When the immune system starts to become progressively less able to attack tumor cells, we have the stage called escape, where tumor cells gain an advantage over immune cells, leading to an immunosuppressive microenvironment[74].

The high mutational load present in melanoma makes it one of the most immunogenic cancers[30]. Even though melanoma cells express extremely immunogenic proteins (tumor-associated antigens), their high plasticity contributes to an escape from detection by the immune system, through mechanisms such as: 1) downregulation of tumor-associated antigens; 2) negative regulation of MHC molecules, therefore reducing tumor antigen presentation; 3) dysfunctional dendritic cells through the secretion of vascular endothelial growth factor, interleukins and transforming growth factor β, leading to inhibition of T cells; 4) high expression of receptors (e.g. PD-L1 and B7-1/2) that turn off T cell activity; 5) exhaustion of CD8+ T cells due to high antigen load in the tumor microenvironment; 6) pro-tumoral behavior of macrophages (M2-like phenotype); 7) immune suppression by cancer-associated fibroblasts; 8) presence of regulatory T cells, which secrete immunosuppressive cytokines and chemokines (e.g. interleukins-10 and transforming growth factor β)[30,75].

1.5 CELL METABOLISM AND MELANOMA

Cellular metabolism consists of the production of energy, mainly through glucose, allowing cellular homeostasis to be maintained. The high proliferative capacity of cancer cells is accompanied by a high need for energy supply. In order to satisfy the energy demand, even in an oxygen-poor environment, cancer cells have the ability to modulate their metabolism, converting glucose into lactate, a process known as the Warburg effect or aerobic glycolsis[76]. The lactate production allows to maintain high levels of glycolysis without causing cellular stress and generate energy more quickly. Lactate production is controlled by Lactate dehydrogenases (LDHs) and Monocarboxylate transporters (MCTs), which are normally overexpressed in a tumor context[76,77]. LDHA is responsible for converting pyruvate to lactate and LDHC by the reverse reaction. MCT1 and MCT4 are associated with lactate export and import, respectively[78]. In parallel with glycolysis, the phosphate pentose pathway, responsible for the synthesis of amino acids and energy, is vital for the survival of cancer

cells, being Glucose-6-phosphate dehydrogenase (G6PD) an enzyme that regulates this pathway[77]. Additionally, the synthesis of certain amino acids has been described in the metabolic remodulation of cancer. Glutamine, when converted into glutamate by the action of Glutaminase 1 (GLS1), is used as a carbon source for tumor supply and is also responsible for maintaining the redox state of cancer cells. High levels of glutamine transporters, Sodium-coupled neutral amino acid transporter 1 and 2 (SNAT1 and SNAT2) are found in cancer[77,78]. Cysteine is an amino acid also used as a source of carbon and energy by malignant cells. Furthermore, it is a scavenger of free radicals. Uptake of cysteine is regulated by Cystine glutamate transporter (XCT) and Excitatory amino acid transporter 3 (EAAT3), which are upregulated in cancer[77,78].

In melanoma cells, 60-80% and about 90% of all glucose is converted to lactate under normal oxygen conditions and under hypoxic conditions, respectively[79]. Activation of the MAPK and PI3K pathways influence the metabolic reprogramming of melanoma and consequently its survival, as these pathways regulate several genes involved in cellular metabolism. For example: 1) through the avian viral Myelocytomatosis oncogene homolog (MYC) there can be increased glucose uptake through the activation of glucose transporters and LDH; 2) activation of Protein kinase B (AKT) results in stimulation of glucose transporters too; 3) Mammalian target of rapamycin (mTOR) can inhibit the activity of genes, which when downregulated lead to a decrease mitochondrial oxidative metabolism; 4) mTOR can activate Hypoxia inducible factor 1α (HIF1α), which consequently induces inactivation of pyruvate dehydrogenase, decreasing mitochondrial respiration and oxidative phosphorylation; 5) increase of MCT4 levels due to overexpression of MYC and HIF1α; 6) lactate secretion via MCT1 activates HIF1α and immunosuppressive interleukins with upregulation vascular endothelial growth factor[77].

1.6 CELL SIGNALING

Cell survival depends on the detection of extracellular signals and the response to these signals to ensure vital cell functions (e.g. cell growth, proliferation, apoptosis and differentiation). The cellular response to these signals is transduced through intracellular signaling pathways[80,81]. Activation of one or more signaling pathways firstly involves the binding of an extracellular signal molecule with receptor proteins (normally present on the cell surface), which can be ion channel receptors, G protein-coupled receptors and enzyme-coupled receptors. After interaction between signal molecule and receptor proteins, there is transmission of the signal to intracellular proteins present in the signaling pathway. The intracellular signaling proteins can work like molecular switches, namely, proteins that change between an on and off stage. There are two types of molecular switches, proteins that by phosphorylation are turned on or off and GTP-binding proteins. The latter are divided into two types, trimeric GTP-binding proteins (relay signals from G-protein-coupled receptors) and small monomeric GTPases (relay signals from several receptor proteins). Lastly, the intracellular signaling proteins distribute information to specific targets, called effector proteins, resulting in changes in cell behavior. Effector proteins can be metabolic enzymes (metabolic alterations), transcription regulatory proteins (dysregulation of gene expression) and cytoskeletal proteins (cell shape)[81].

When something goes wrong with the components that regulate signaling pathways it can result in cancer. In fact, cell signaling pathways in cancer are overactivated or suppressed, which consequently results in changes in different cellular processes, such as increased cell proliferation or evasion of apoptosis. Several signaling pathways are described as dysregulated in cancer, as already mentioned, such as, the MAPK and PI3K pathways[82].

1.7 SMALL GTPASES

Small GTPase are a family of enzymes that turn on or off various cellular processes. This on or off state is mediated by Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs stimulate the release of GDP for GTP to bind, allowing the activation of GTPase. On the other hand, GAPs lead to the hydrolysis of GTP to GDP and as a result, the proteins remain in a switched off state. Within this superfamily there are 5 subfamilies, Ras, Ras homologous (Rho), Ras-like proteins in the brain (Rab), Ras-like nuclear (Ran) and ADP ribosylation factor (Arf), being known about 150 members (Figure 1.2). Considering the role of GTPases as signal transducers in signaling pathways, controlling a spectrum of cellular processes, their contribution to tumorigenesis must be considered[83].

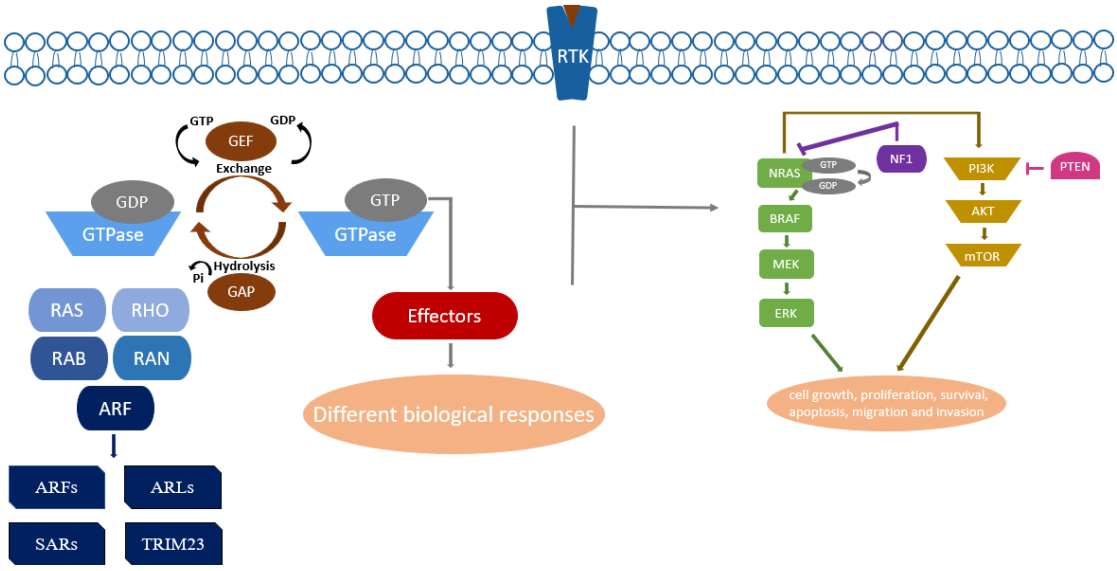


Figure 1.2 Small GTPases. Small GTPases comprise 5 protein subfamilies (RAS, RHO, RAB, RAN and ARF). The activation and deactivation of these proteins happens due to the presence of Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. The on state of GTPase allows the activation of different biological responses. For example, the interaction between an extracellular signal molecule and a receptor tyrosine kinase (RTK) leads to NRAS activity (RAS subfamily), which stimulates the activation of MAPK and PI3K pathways, resulting in the activation of cell growth, proliferation, survival, apoptosis, migration and invasion.

The Ras family is the most diversified, being important in mediating immunity, proliferation, differentiation and survival[83]. The isoforms *NRAS*, HRas proto-oncogene (*HRAS*) and KRAS proto-oncogene (*KRAS*) belong to the Ras family and are well known as oncogenes. Ras interacts with effector proteins such as BRAF and PI3K, which consequently stimulates downstream targets, allowing the activation of biological responses like cell survival and proliferation[83,84]. Mutation in the *RAS* oncogene is the most frequent in human cancers (e.g. pancreatic, colorectal and lung), being *KRAS* mutation the most common[85,86]. As previously mentioned, mutations in *NRAS* are the second most frequent in CM, blocking the hydrolysis of GTP by GAPs. In addition to mutations in *NRAS*, mutations in *HRAS* and *KRAS* have already been identified in CM, but in a low percentage[87,88]. The presence of *NRAS* activating mutations has been associated with thicker melanomas, higher mitotic rates and presence of lymph node metastasis[87].

The Arf family comprises 30 members in mammals, of which 6 are ARFs and 21 are ADP ribosylation factor like GTPase (ARL)[89]. ARFs are involved in the regulation of vesicle budding, tethering, actin cytoskeleton remodeling and integrin trafficking[87,90]. ARFs have already been shown to play a role in the malignancy of several tumors, specifically high *ARF1* expression in prostate cancer, upregulation of *ARF3* in breast cancer, upregulation of *ARF4* in ovarian cancer and high levels of

ARF6 in hepatocellular carcinoma[91-94]. Yoo et al. showed that *ARF6* activation *in vivo* potentiates melanoma metastasis and *ARF6* overexpression in melanoma cells stimulates PI3K and AKT activation, suggesting the role of *ARF6* in the interaction with the PI3K/AKT pathway. Furthermore, according to The cancer genome atlas (TCGA) data, the same authors suggest that ARF6 hyperactivation in melanoma is associated with metastatic progression[95]. In another study, the authors showed a possible role between ARF6 and the occurrence of lymphangiogenesis. In fact, ARF6 appears to regulate VEGF-associated cell migration and consequent vascular network formation in melanoma due to β1-integrin internalization[96]. Finally, Tague et al. demonstrated that the ability to invade melanoma cells may be associated with the interaction between ARF6 and ERK[97].

1.8 ARL PROTEINS

Within the family of ARFs, ARLs have been shown to be associated with cell communication, migration and invasion, being involved in actin remodeling, tubulin assembly, lysosome positioning and motility, cilia-associated signaling pathways, vesicle-mediated transport and immune function[89,98].

Considering the biological relevance of mechanisms about ARLs, their involvement in carcinogenesis, especially in the ability to migrate and invade, resulting in metastasis, is understandable[98]. For cells to migrate and invade they must lose adhesion, in other words, lose contact with each other and establish new contacts with the surrounding extracellular matrix. This process may depend on changes in the dynamics of cytoskeletal proteins, such as actin and tubulin, responsible for cell shape, movement and division[90,99,100]. Actin and tubulin dysregulation appears to be an important process in the development of cancer. During actin remodeling, protrusions are formed which assist in the metastasis of tumor cells[99,100]. It is also known that alterations in microtubules due to deregulation in the expression of tubulin isotypes and tubulin post-translational modifications in cancer contribute to cell survival under conditions of metabolic, oxidative and hypoxic stress[101]. Furthermore, actin and tubulin deregulation are able to promote the epithelial-to-mesenchymal transition, and as consequence metastasis[99-101]. Additionally, the biological processes of lysosomes are related to cancer progression: 1) Autophagic-lysosomal process helps cancer cells survive by supplying them with nutrients and energy; 2) the increase in lysosome synthesis maintains the proliferation of tumor cells; 3) lysosomes influence the immune response, for example, through the transport of immune checkpoint inhibitors such as CTLA-4; 4) help cancer cells overcome the acidic microenvironment by controlling pH; 5) lysosomes release enzymes that degrade the extracellular matrix, promoting angiogenesis, invasion and metastasis[102]. Indeed, an association between defects in cilia and the dysregulation of the cell cycle, activation of signaling pathways that induce malignancy (e.g. Hedgehog and Wnt pathways) and metabolic reprogramming had been reported[103]. Finally, vesicular trafficking is necessary for cellular functionality, responsible for transporting molecules between organelles within the cell, as well as between the cell and the outside. Phenomena related to vesicular transport interfere with the development of cancer, for example, autophagy induction, transport of proteins to the cell membrane, such as Matrix metallopeptidases (MMPs) and integrins, modulation of receptors in the cell membrane and transport of growth factors[104,105].

The knowledge about ARLs is still limited. However, the dysregulation (up or down) of ARL proteins in cancer has been described in several studies and associated to cellular processes that contribute to tumor malignancy, such as proliferation, migration and invasion[106-121]. For example, *ARL2* downregulation in glioma[106] and breast cancer[107], and *ARL2* upregulation in colon[108], cervical[109] and bladder cancer[110]; *ARL3* downregulation in glioma[111]; *ARL4C* downregulation in ovarian cancer[112], and *ARL4C* upregulation in colorectal[113,114], lung[113] and gastric cancer[115]. *ARL5* upregulation in

colorectal cancer[116]; *ARL6* upregulation in rhabdomyosarcoma[117]; *ARL8B* upregulation in prostate cancer[118]; *ARL13B* upregulation in gastric[119] and breast cancer[120]; *ARL14* upregulation in lung[121].

To the best of our knowledge, only three studies explored the role of ARLs in CM[98,122,123]. A bioinformatic study performed by our group, which aimed to investigate the expression of ARLs, their impact on the prognosis of CM and their role on the immune microenvironment, showed that all ARLs are differentially expressed in CM. *ARL1, ARL11* and *ARL15* showed to represent independent prognostic factors in CM. The upregulation of *ARL1* and *ARL11* was found in CM samples, while *ARL15* was downregulated. High levels of *ARL1* and *ARL11* was correlated with good prognosis and infiltration of immune cells. In contrast, high levels of *ARL15* revealed poor prognosis and was also associated with immune system[98]. In patients with familial melanoma, genetic polymorphisms in *ARL11* were associated with the risk of developing melanoma and *ARL11* was suggested as a low-penetrance tumor suppressor gene[122,123].

1.9 ARL1 PROTEIN

ARL1 (12q23.2) is a protein that plays a role in the Golgi complex and is present in several human tissues (e.g. colon, lung, liver, kidney, pancreas, and skin)[124,125]. The Golgi is a very important organelle as it is the center for transport of proteins and lipids to a certain destination in the cell[126]. Its function depends on golgin proteins, including golgin-97, golgin-245, GCC185 and GCC88. The interaction of ARL1 with golgin proteins (e.g. golgin-97 and golgin-245) is described in vesicular trafficking at the trans-Golgi network being involved in cell polarity, innate immunity, lipid droplet and influences the secretion of MMPs[125]. So far, in addition to our group's bioinformatic analysis[98], other studies described ARL1 in cancer[127-130]. A case of non-small cell lung cancer with an ARL1-MET proto-oncogene, receptor tyrosine kinase (MET) fusion was identified for the first time. This novel ARL1-MET fusion protein contained exon 1 of ARL1 and part of exon 14 and exon 15-21 of MET, preserving the complete tyrosine kinase domain of MET[127]. After treatment with a MET inhibitor, the tumor shrunk, being this fusion identified by authors as a therapeutic target. Ong et al. studied hypoxia-inducible protein in triple-negative breast cancer. The increased expression of hypoxia-inducible protein was associated with lower survival and correlated with the differential expression of several genes, including *ARL1*[128]. An older study identified novel gene fusions in sarcomas, one of which included ARL1. However, this fusion was not considered of great importance because it resulted in an out-of-frame transcript[129]. Low levels of *ARL1* were found in endometrial cancer patients[130]. Additionally, it is known that ARL1 is altered in diseases such as Cholera[131].

2. OBJECTIVE

Considering the possible potential role of ARLs in CM, this study focuses on evaluating the impact of *ARL1* on CM aggressiveness. To achieve our goal, we intend to: 1) evaluate *ARL1* expression in tumor samples from Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG) patients; 2) understand if there is a correlation between *ARL1* expression and different immune populations; 3) analyze the impact of *ARL1* knockdown on melanoma cells aggressiveness through *in vitro* assays (cell viability, migration and invasion) and with different genes involved in cellular metabolism.

3. MATERIALS AND METHODS

3.1 BIOLOGICAL SAMPLES

The study cohort includes surgical fragments of primary tumor, cutaneous metastases, and lymph node metastases (n=21) and peripheral blood samples (n=9) of CM patients (male and female) with stages II, III and IV from IPOLFG. These CM patients were diagnosed from 2016 to 2021. Diagnosis and staging were performed according to the American Joint Committee on Cancer 8th edition guidelines[42]. The presence of *BRAF* mutation was also assessed. Skin samples without tumor from patients with CM were used as control samples. This study was previously approved by the IPOLFG Ethics Board Committee (UIC/1310), and written informed consent was obtained from patients.

3.2 SURGICAL TISSUE AND PERIPHERAL BLOOD PROCESSING

Surgical tissue was processed immediately after the surgery. In a petri dish and with tweezers and scalpel, the sample underwent mechanical digestion and was washed with Phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, USA). Then the sample was divided in cryovials containing 900 µL of Fetal bovine serum (FBS) (PAN-Biotech, Germany) and 100 µL of Dimethyl sulfoxide (DMSO) (Honeywell, USA) for flow cytometry analysis, and for Ribonucleic acid (RNA) extraction in cryovials with 500 µL RNA later (Qiagen, USA).

Peripheral blood mononuclear cells (PBMCs) extraction was achieved by density gradient centrifugation. Total blood was diluted (1:1) in PBS. Diluted blood was carefully overlaid in Lymphoprep™ in proportion 1:1. Then, gradients were centrifuged at 800 g for 30 minutes, resulting in a mononuclear cell layer (PBMCs). This layer was transferred to a 50 mL Falcon tube and washed with PBS until reaching a final volume of 20 mL, and then centrifuged at 650 g for 10 minutes. To remove erythrocytes, cells were resuspended in 1 mL Red Blood Cell Lysis Buffer 1x (BioLegend, USA) per 5 mL of initial total peripheral blood, for 15 minutes, in the dark. Next, a second wash with PBS was performed (final volume of 20 mL), and then centrifuged at 250 g for 10 minutes. The pellet (isolated PBMCs) was resuspended in FBS. Cells were counted on a hemocytometer with trypan blue (1:2 dilution). Lastly, cells were frozen in FBS and in 10% of DMSO at -80 ºC in a concentration of 2×10^6 cells/mL.

3.3 FLOW CYTOMETRY

Surgical fragments and PBMCs were stained and analyzed for flow cytometry using BD FACS Canto II with FACSDiva Software v8.0.1 (BD Biosciences). Tumor samples were processed with Medicon, washed with 1 mL PBS 1x to obtain a single cell suspension. The surface Mononuclear antibodies (mAbs) used for staining were anti-Human leukocyte antigen DR (HLA-DR)-APC (L243), anti-CD163-PE (GHI/61), anti-CD80-FITC (2D10), anti-CD3-PerCP (HIT3a), anti-CD206-APC/Cyanine7 (15-2), anti-CCR4-BV421 (L291H4), anti-CD14-FITC (63D3), anti-CD4-FITC (OKT4), anti-CD25-PE (BC96), anti-CD127-PE/Cy7 (A019D5), anti-CD8a-PE (HIT8a) and anti-CD45-PerCP (HI30). The intracellular mAb used was anti-CD68-APC (Y1/82A). All antibodies were acquired from BioLegend. The immune populations studied were defined as follows: Helper T lymphocytes (Th, CD45+/CD3+/CD4+), Cytotoxic T lymphocytes (CTL, CD45+/CD3+/CD8+), Regulatory T lymphocytes (Tregs, CD45+/CD3+/CD4+/CD25high/CD127low), Macrophages (CD45+/CD68+), Monocytes (CD45+/CD14+), classically activated monocytes/macrophages (M1, CD45+/CD68+/CD80+) and alternatively activated monocytes/macrophages (M2, CD45+/CD68+/CD163+/CD206+). Additionally, relevant markers (CCR4 and HLA-DR) in specific populations were also evaluated.

Approximately $5x10^5$ cells were used for each staining panel (panel 1, 2, 3 and 4 - Table A.1). Cells were centrifuged at 1100 g for 5 minutes and resuspended in 400 µL PBS 1x. Of those 400 µL, 100 uL were distributed to each 1.5 mL-microcentrifuge tube containing 2 µL of the respective surface mAbs. Samples were incubated in the dark for 15 minutes at room temperature. Then, samples were washed with 1 mL of PBS 1x and centrifuged at 300 g for 5 minutes. The pellet was resuspended in 200 µL of PBS 1x for further analysis. For intracellular staining, samples were firstly permeabilized with 100 µL of eBioscience™ Fixation/Permeabilization Concentrate (Invitrogen, Thermo Fisher Scientific, USA) for 30 minutes, in the dark. This was followed by a wash with 1 mL of eBioscience™ Permeabilization Buffer 1x (Invitrogen, ThermoFisher Scientific, USA). Cells were resuspended in 100 µL Permeabilization Buffer 1x and 2 µL intracellular mAb were added to the samples, followed by incubation in the dark for 30 minutes. Finally, a wash with PBS 1x was performed and the pellet was resuspended in 200 µL of PBS 1x for analysis. Data were analyzed using FlowJo software vX.0.7.

3.4 CM CELL LINES

Human melanoma cell lines A375 (American Type Cell Collection – ATCC, No. CRL-1619, $BRAF^{V600E}$ mutated, USA) and WM115 (Rockland code WM115-01-0001, $BRAF^{V600D}$ mutated, USA) were grown Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS and 1% Penicillin/streptomycin (P/S) (Gibco, Thermo Fisher Scientific, USA). All cell lines were maintained at 37ºC with 5% CO_2.

3.5 TRANSDUCTION

Cells were transduced with short hairpin RNAs (shRNA) to knockdown *ARL1*. Five different shRNA sequences (shRNA1, shRNA2, shRNA3, shRNA4 and shRNA5) were cloned into a lentiviral expression construct. The shRNA sequences were kindly donated by the Membrane Traffic in Disease group at the Chronic Diseases Research Center, Nova Medical School, led by Professor Duarte Barral. The shRNAs targeting *ARL1* were obtained from the Broad Institute RNAi Consortium (Cambridge, MA, USA). Cells were also transduced with empty vector (without the gene of interest), a negative control, obtained from the human shRNA libraries (Sigma-Aldrich). Transformed *Escherichia coli* glycerol stocks were used to produce all vectors. The shRNA sequences used are listed in Table A.2. The production of lentivirus was carried out through HEKStarRdPro packaging cells, cultured at $1.5x10^5$ cells/well in DMEM, with 10% FBS, 2 mM GlutaMAX and 15 mM HEPES. The cells were transfected with 900 ng of 2nd generation packaging plasmid (psPAX2), 100 ng of envelope plasmid (pMD2.G) and 1 µg of target expression vector pLKO.1 in 90 µL of Opti-MEM (Gibco) with 6 µL of FuGENE 6 (Promega, Madison, WI, USA). After 20 hours of transfection, the medium was changed to DMEM supplemented with 30% FBS. Subsequently, after 40 and 64 hours of transfection, the lentiviral particles were collected and stored at -80ºC until use. A375 and WM115 cell lines were transduced in 1400 µL medium DMEM with 10% FBS and 8 µg/mL polybrene with 400 µL of lentivirus construct and selected with puromycin for 3 weeks.

3.6 RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS AND REVERSE TRANSCRIPTASE QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

RNA extraction from cells was executed with RNeasy Mini Kit (Qiagen, USA), and RNA extraction of tumor samples was performed by the Trizol RNA extraction (Invitrogen, Thermo Fisher Scientific, USA). RNA extraction from cells was performed in RNeasy Spin Column. First, cells were

lysed in a mix of Buffer RLT (350 µL) and β-mercaptoethanol mix (3.5 µL). Then, 350 µL of 70% ethanol was added to the RNA to bind to the membrane of column. The sample was centrifuged at 12000 rcf for 30 seconds. To remove contaminants, the sample was washed with two wash buffers, once with 700 µL RW1 buffer and twice with 500 µL RPE buffer, all washes for 30 seconds at 12000 rcf. Finally, the RNA was eluted in 40 µL of Rnase-free water. Tumor samples were homogenized with tweezers and scalpel. After mechanical digestion, 1 mL of Trizol and 200 µL of chloroform were added to separate RNA from DNA and proteins. Then, the samples were centrifuged at 12000 g for 15 minutes at 4ºC resulting into two phases (RNA in the upper aqueous phase). The aqueous phase was transferred to a new microtube. RNA was precipitated with 500 µL isopropanol and centrifuged at 12000 rcf for 5 minutes at 4ºC. RNA pellet was then washed with 75% ethanol and centrifuged at 7500 rcf for 10 minutes at 4ºC. Lastly, isolated RNA was dissolved in 20 µL RNase-free water. RNA quantification was performed with Nanodrop 2000 (Thermo Fisher, USA).

Complementary deoxyribonucleic acid (cDNA) was obtained from 1 µg of total RNA and retro-transcribed by SuperScript™ II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, USA) according with manufacturer's instruction.

RT-qPCR was necessary to evaluate *ARL1* expression in tumor samples, to validate *ARL1* mRNA expression in cell culture and to investigate metabolism genes. For *ARL1* expression in tumor samples and CM cells, RT-qPCR was run in a final volume of 20 µL: 2 µL of cDNA and 18 µL of a mix of 12 µL of RNase-free water, 5 µL of SYBR Green 5X (Applied Biosystems, Thermo Fisher Scientific, USA), 0.5 µL of forward and reverse primers (100 µM). The reaction was performed in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). The gene Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used as the housekeeping gene (endogenous control). The forward and reverse sequences of *HPRT1* and *ARL1* primers are listed in Table A.3. The RT-qPCR program used consisted of a first step at 50 ºC for 2 minutes, followed by 95 ºC for 10 minutes, 41 cycles of denaturing at 95 ºC for 15 seconds and annealing/extension at 60 ºC for 1 minute and a final denaturation at 95 ºC for 15 seconds. For metabolism genes, RT-qPCR was performed in a final volume of 8.3 µL: 1 µL of cDNA and 7.3 µL of a mix of 3 µL of RNase-free water, 4 µL of SYBR Green 5X, 0.15 µL of forward and reverse primer (100 µM). RT-qPCR occurred during 40 cycles in Lightcycler 480 System instrument (Roche, Basel, Switzerland), consisting of the following program: denaturation at 95 ºC, 10 minutes, amplification cycles at 95 ºC, 15 seconds and 60 ºC, 1 minute. The primer sequences used for genes (*LDHA*, *LDHC*, *MCT1*, *MCT4*, *G6PD*, *GLS1*, *SNAT1*, *SNAT2*, *XCT* and *EAAT3*) are listed in Table A.3. The *HPRT1* was selected as the housekeeping gene (endogenous control). The ΔΔCt method was used for relative quantification of the expression of all the mentioned genes[132].

3.7 PROTEIN EXTRACTION AND WESTERN BLOT

For protein extraction, A375 and WM115 cells were seeded at a density of $6.5x10^5$ cells/T75 flask and $10.0x10^5$ cells/T75 flask, respectively. Cells were removed using a plastic cell scraper and lysed with 300 µL of ice-cold radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, USA) and 3 µL of protease and phosphatase inhibitor cocktail (Sigma, Merk, dilution 1:100). The cell suspension was transferred to a tube followed by vortex for 30 seconds every 10 minutes. Then, samples were centrifuged for 20 minutes at 12000 g at 4°C. Protein concentration of cell lysate was determined through a colorimetric method, the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA), following manufacturer's instructions. To determine the protein concentration, 200 µL of BCA working reagent (50 parts of BCA reagent A with 1 part of BCA Reagent B) and 25 µL of protein samples were added to a 96-well plate. Then the plate was incubated

at 37°C for 30 minutes in the dark. Through iMark™ Microplate Absorbance Reader (BioRad, USA) absorbance was measured at 595 nm. For each sample, Laemmli Buffer (250 mM Tris-HCL, 10% sodium dodecyl sulfate, 0.5% Bromophenol Blue and 50% Glycerol) was added (1:5 dilution) along with β-mercaptoethanol at a 1:20 dilution. Finally, the samples were kept at 100°C for 5 minutes in a Dri-Block DB 2A (BIO-Techne, USA).

Confirmation of ARL1 protein silencing, as well as the investigation of ARL1 impact on MAPK and PI3K pathways were done by Western Blot. Proteins were resolved on 12% (ARL1 knockdown validation) and 10% (involvement of the MAPK and PI3K pathways) sodium dodecyl sulphate–polyacrylamide gels, separated by electrophoresis and transferred from the gel to polyvinylidene difluoride membranes (BioRad, USA) through the Trans-Blot Turbo Transfer System (BioRad, USA). Membrane blocking was performed for 2 hours (ARL1 knockdown validation) or 1 hour (involvement of the MAPK and PI3K pathways) with PBS-Bovine serum albumin (BSA) 5% Tween 0.1%. Membranes incubated overnight at 4ºC with the following primary antibodies diluted in PBS-BSA 3% Tween 0.1%: anti-ARL1 (1:5000 dilution, PA5-64479, Invitrogen, USA), anti-Extracellular signal regulated kinase 1/2 (ERK1/2) (1:500 dilution, 168-10069, Raybiotech, USA), anti-phospho-ERK1/2 (pT202/pY204) clone AW39 (1:500 dilution, 612358, BD Biosciences, USA), anti-AKT (1:1000 dilution, 4691 Cell Signaling Technology) and anti-phospho-AKT (Thr308) (1:1000 dilution, 13038 Cell Signaling Technology). Subsequently, secondary goat anti-rabbit (1:10000 dilution, 31460, ThermoFisher, USA) or anti-mouse antibodies (1:10000 dilution, 31430, ThermoFisher, USA) conjugated with horseradish peroxidase was added for 1 h for detection of the primary antibodies. The endogenous control used for ARL1 knockdown validation and involvement of the PI3K pathway was anti-β-actin antibody, clone AC-15 (1:5000 dilution, A5441, Merck, Germany), incubated for 1 hour. To assess the involvement of the MAPK pathway, anti-α-tubulin antibody, clone B-5-1-2 (dilution 1:4000, T5168, Merck KGaA, Germany) was used as control, incubated for 1 hour. Membranes were revealed with Clarity Max Western ECL Substrate (BioRad, USA) in ChemiDoc XRS+ (BioRad, USA).

3.8 CELL VIABILITY, MIGRATION AND INVASION ASSAYS

Cell viability of transduced cells was evaluated through Cell counting kit-8 (CCK8) (Dojindo, Japan) and trypan blue exclusion assays (Gibco, Thermo Fisher Scientific, USA). CCK8 is a metabolic assay that allows determining cell viability by reducing water-soluble tetrazolium (WST-8) salt into an orange formazan dye through dehydrogenases. Trypan blue exclusion assay indicates the number of live cells, which are cells with an intact membrane that exclude trypan blue dye. For CCK8, cells were seeded in 24-well plates in triplicates at $1.5x10^4$ cells/well. On days 0, 3 and 6, 300 µL of CCK8 solution (dilution 1:10) was added to the wells and cells were incubated for 30 minutes in the dark at 37ºC with 5% CO_2. Absorption was read at 450 nm using a microplate absorbance reader (iMark Microplate Reader, BioRad, USA). For trypan blue assay, $3.0x10^4$ cells/well (A375) and $5.0x10^4$ cells/well (WM115) were seeded in 6-well plates. Cells were incubated for 3, 6 and 10 days. Then, trypan blue was added to cells and number of viable cells were counted with a hemocytometer under the microscope.

Cell migration was performed in Falcon Permeable Cell Culture Inserts for 24-well plate with 8.0 µm Transparent Pet Membrane (Corning, USA). Cell invasion was assessed using Corning Matrigel Invasion Chambers for 24-well Plate with 8.0 µm Transparent Polyethylene Terephthalate Membrane (Corning, USA). A375 cells were seeded in inserts with a density of $5.0x10^4$ cells/well in DMEM with 5% FBS and 1% P/S. For WM115, $6x10^4$ cells/well were resuspended in DMEM with 5% FBS and 1% P/S. The bottom chambers contained 500 µL of DMEM with 10% FBS, 1% P/S and

20 ng/mL of epidermal growth factor. Then, cells were incubated at 37 ºC with 5% CO_2 for 24 hours. After 24 hours, non-migrating and invading cells were removed using a cotton swab and cells that migrated and invaded were fixed with cold metanol for 5 minutes, stained with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, USA) and visualized by inverted fluorescence microscopy (Olympus IX53). The total number of cells that migrated and invaded was quantified with ImageJ 1.53e.

3.9 STATISTICAL ANALYSIS

Comparison of *ARL1* expression in Normal skin (NS) versus CM samples and *BRAF* status were done using Mann Whitney test. Differences in *ARL1* expression according to stage and sample type were verified using Kruskal-Wallis test. PFS and OS of patients were analyzed through Kaplan Meir curves and Log-rank test. For flow cytometry, a significance level of correlation between *ARL1* expression and distinct immune population was evaluated by Spearman's rank correlation. *ARL1* knockdown validation was assessed by One-way ANOVA. The results of cell viability were evaluated using Two-way ANOVA and migration and invasion by One-way ANOVA and Unpaired t-test. Correlation between *ARL1* and cellular metabolism were assessed through Kruskal-Wallis and Mann-Whitney tests. GraphPad Prism version 8.0.1 was chosen for statistical analysis and graphical representations. All statistical tests were two-sided, with a significance level of 5%. Data are represented by mean ± standard deviation (SD).

4. RESULTS

4.1 EVALUATION OF *ARL1* EXPRESSION IN TUMOR SAMPLES

In this study we sought to understand the relevance of ARL1 in CM to find a useful biomarker. ARLs have been described as dysregulated in different types of cancers,[98,106,111-116,120,121] including ARL1[98,128,130] with prognostic value. Recently, bioinformatics analysis published by our group showed that 3 ARLs (ARL1, ARL11 and ARL15) might have a role in CM aggressiveness[98]. Thus, in this work we to explore the role of ARL1 in CM using tumor samples and *in vitro* analysis. One of the aims of this study was to assess *ARL1* expression in surgical fragments from IPOLFG CM patients through RT-qPCR. We compared *ARL1* expression between NS and CM samples (Figure 4.1 A). In addition, we analyze the differences between: a) stages (II, III and IV) (Figure 4.2 B), b) type of sample (Primary melanoma (PM), Skin metastasis (SM) and Lymph node metastasis (LNM)) (Figure 4.2 C), and c) *BRAF* WT and *BRAF* mutated (Figure 4.2 D). We found no significant differences in *ARL1* expression between NS and CM samples, regarding stage, sample type and *BRAF* status. Therefore, these results are not following those found in bioinformatics analysis, where *ARL1* is upregulated in CM samples. However, it is crucial to highlight the number of samples included in the previous study, 556 NS, 102 primary CM and 366 metastatic CM, compared to the samples analyzed in this study, 8 NS, 1 PM and 20 metastatic CM, and therefore it is necessary to increase our cohort of patients in order to corroborate the data *in silico*[98].

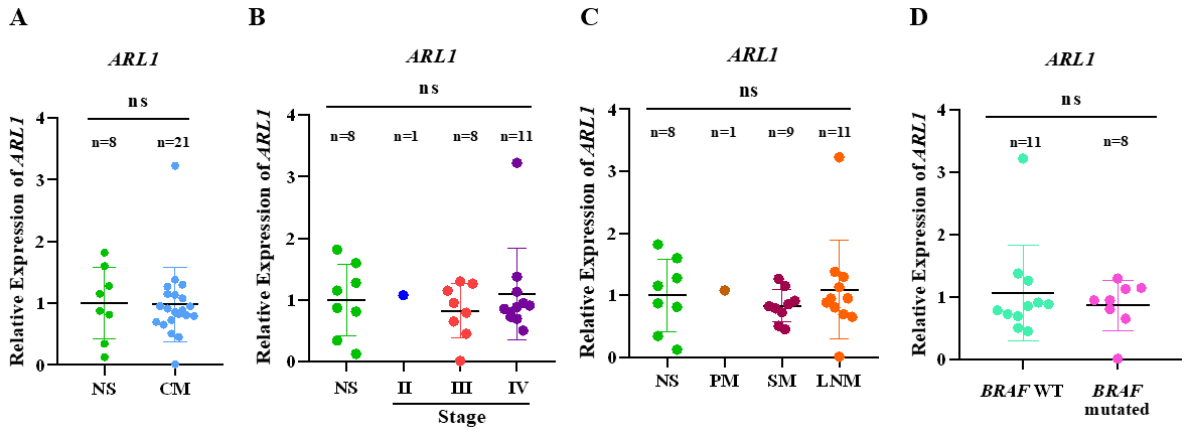


Figure 4.1 Evaluation of *ARL1* expression in Cutaneous melanoma (CM) patients. (A) Assessment of *ARL1* expression in Normal Skin (NS) and CM samples. Differences in expression were also assessed according with **(B)** stage (II, III and IV), **(C)** sample type, Primary melanoma (PM), Skin metastasis (SM) and Lymph node metastasis (LNM), and **(D)** *BRAF* status. Mann Whitney test was used to compare *ARL1* expression between NS and CM, as well as according to *BRAF* status, whilst Kruskal-Wallis test examined stage and sample type. Ns, -not statistically significant.

4.2 *ARL1* EXPRESSION AND PROGNOSTIC VALUE

Several studies have described the impact of ARL genes deregulation on cancer patient outcomes[98,106,111-116,120,121,128,130]. Additionally our previous bioinformatic study showed that *ARL1*, *ARL11* and *ARL15* could have prognostic value in CM. Specifically, high levels of *ARL1* were positively correlated with OS[98]. Here using another cohort of CM patients, we investigated the role of *ARL1* expression in PFS and OS. Kaplan Meier curves were used to analyze PFS and OS considering low and high *ARL1* expression, determined by the median as a cut-off point (Figure 4.2 A and B). The results seem to show a trend for PFS to be higher in patients with low *ARL1* expression compared to high *ARL1* expression (Figure 4.2 A). However, these data still do not prove to be statistically significant (p-value=0.4882, HR=1.501). No statistically significant differences were also found for OS (Figure 4.2 B, p-value=0.6820, HR=1.344). Therefore, more samples are needed to corroborate these data and corroborate what was observed *in silico*[98].

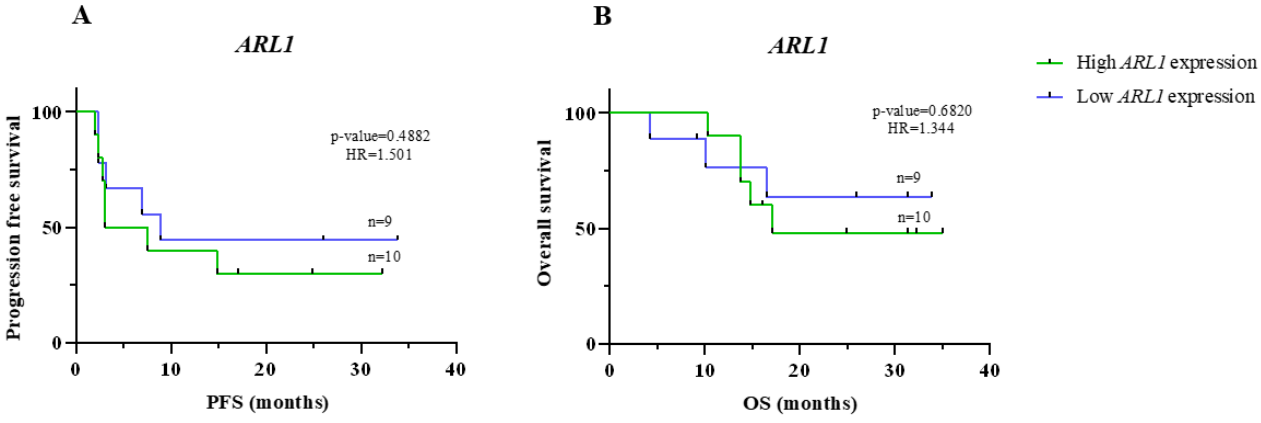
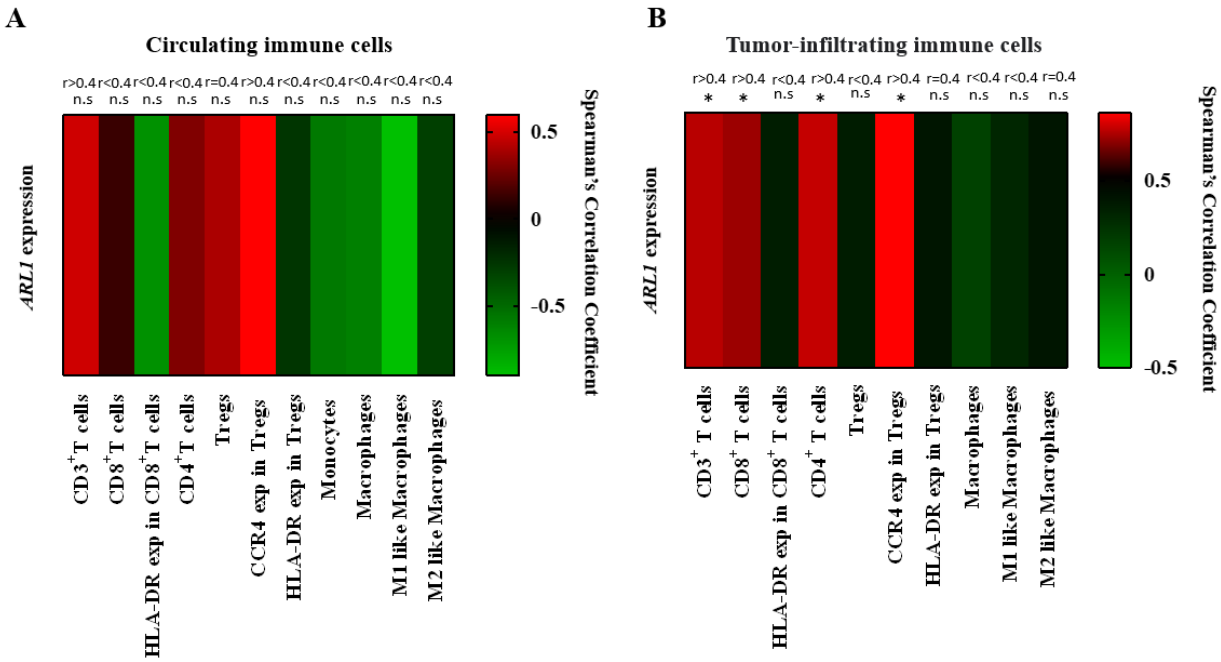


Figure 4.2 Prognostic value of *ARL1* in CM patients. Progression free survival (PFS) and Overall survival (OS) was analyzed by Kaplan Meir curves considering the median of *ARL1* expression as a cut-off point. Two curves were evaluated, high *ARL1* expression (green line) and low *ARL1* expression (blue line). Log-rank test was used to assess the differences between high *ARL1* expression and low *ARL1* expression, according to **(A)** PFS (p-value=0.4882, HR=1.501) and **(B)** OS (p-value=0.6820, HR=1.344).

4.3 ASSOCIATION BETWEEN *ARL1* EXPRESSION IN CM AND IMMUNE POPULATIONS

Immune microenvironment has an essential role in tumor development and progression. Cancer cells can modulate immune responses to gain survival advantages. Antitumor or immunosuppressive activity of several immune cell populations have been described in cancer[30,133-136]. CM develope several mechanisms to circumvent immune response, resulting in immune suppression, tumor progression and aggressiveness. The known high immunogenicity of CM is responsible for good success rates of immunotherapy in CM treatment[30]. The bioinformatics analysis done previously by our group showed that *ARL1* expression was negatively correlated with CD4+ Th1 cells and positively correlated with CD4+ Th2 cells, neutrophils, and common lymphoid progenitor cells in CM[98]. In this context, we investigated whether the expression of *ARL1* in the tumor was correlated with distinct immune population frequencies in circulation at the time of surgery, as well as with the infiltration of immune cells in the tumor. To achieve our goals, we analyzed several immune populations in peripheral blood and tumor tissue of CM patients by flow cytometry. Some of the subsets of immune cells assessed are known to have antitumor activity, such as CD8+ T cells, CD4+ T cells and M1-like macrophages, whilst others are known for their immunosuppressive potential, such as Tregs and M2-like macrophages[30,133-136]. We also determined the expression of molecular receptors, HLA-DR receptor in CD8+ T cells and Tregs and CCR4 expression in Tregs. The results showed no statistical differences between *ARL1* expression and circulating immune populations (Figure 4.3 A). However, a significant positive correlations between *ARL1* expression and several immune subsets infiltrated in the tumor was observed (Figure 4.3 B), namely CD8+ T cells (Figure 4.3 C), CD4+ T cells (Figure 4.3 D), and Tregs expressing CCR4 (Figure 4.3 E). These data indicate that *ARL1* might interfere with immune modulation of CM.



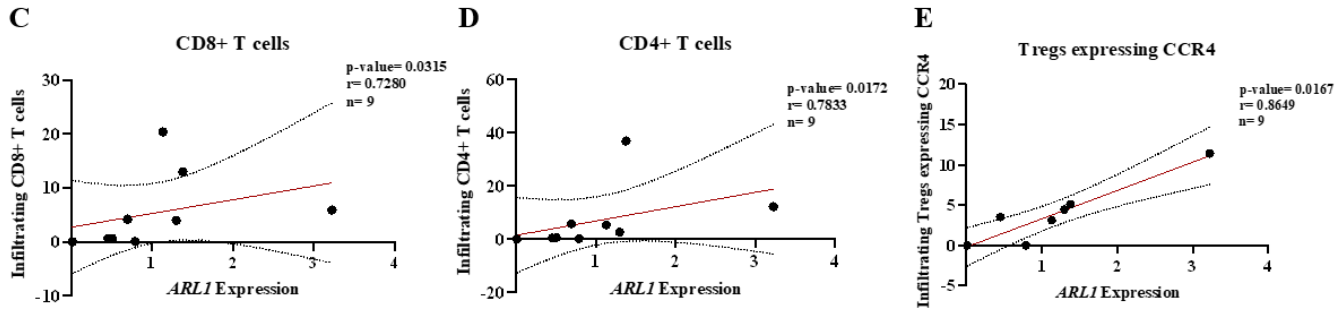


Figure 4.3 Correlation between *ARL1* expression and immune population levels. Heat map based on Spearman's correlation coefficients indicates **(A)** association between *ARL1* expression and immune cells in circulation, and **(B)** correlation between *ARL1* expression and immune cells recruited to the tumor. A cutoff of r=0.4 was used to differentiate weak from strong correlations. *p-value< 0.05; ns, -not statistically significant. Graphic representation of subsets of tumor-infiltrated immune cells shows correlations between **(C)** *ARL1* expression and CD8+ T cells (p-value=0.0315, r=0.7280, n=9), **(D)** *ARL1* expression and CD4+ T cells (p-value=0.0172, r=0.7833, n=9), **(E)** *ARL1* expression and Tregs expressing CCR4 (p-value=0.0167, r=0.8649, n=9). The percentage of immune cells were determined by flow cytometry and *ARL1* expression by RT-qPCR.

4.4 ARL1 KNOCKDOWN VALIDATION

To better characterize the functional role of ARL1 in CM aggressiveness, we performed *in vitro* assays with A375 and WM115 CM cell lines. *ARL1* was silenced by viral transduction with different shRNAs. Knockdown validation was done to mRNA level by RT-qPCR and to protein level by Western Blot (Figure 4.4). We found that shRNA3 and shRNA5 were the most successful vectors in ARL1 silencing, with a relative expression of 0.29 and 0.15, respectively, in A375 cells (Figure 4.4 A). Regarding ARL1 protein expression, a decrease was visualized in A375 cells transduced with shRNA3 and shRNA5, relative to A375 Empty (Figure 4.4 B). In regards to WM115 cells, only shRNA5 vector significantly decreased *ARL1* mRNA expression (relative expression of 0.36) (Figure 4.4 C), translating to a decrease in protein levels as well (Figure 4.4 D).

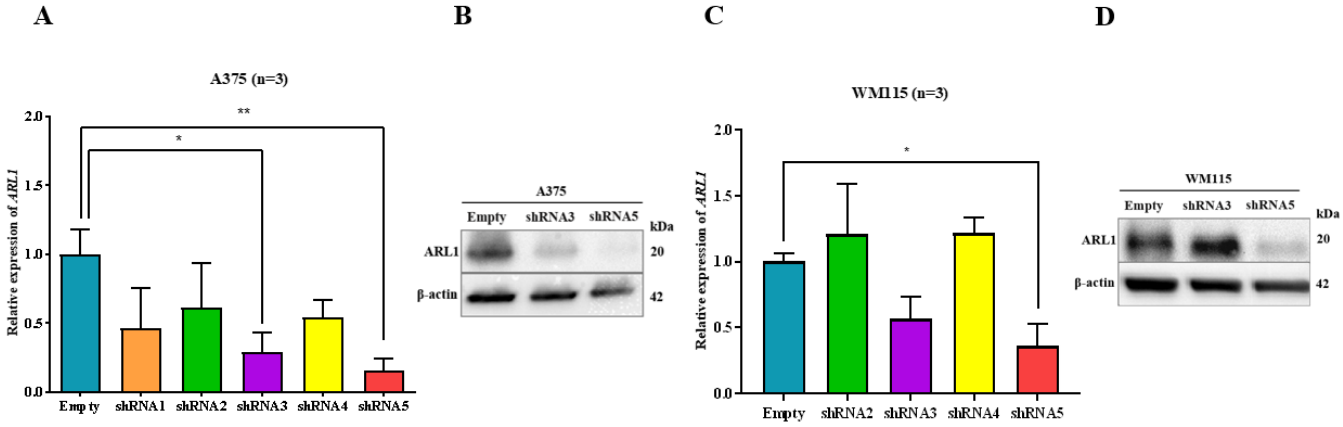


Figure 4.4 Validation of *ARL1* knockdown to mRNA and protein level. Confirmation of *ARL1* silencing by **(A)** RT-qCR (mean ± SD, n=3) and by **(B)** Western Blot in A375 CM cell line. *ARL1* silencing in WM115 CM cell line was also confirmed by **(C)** RT-qPCR (mean ± SD, n=3) and **(D)** by Western Blot. Ordinary one-way ANOVA was used to compare the differences between the control condition (empty) and each vector used for *ARL1* knockdown (shRNA1, shRNA2, shRNA3, shRNA4 and shRNA5). *p-value< 0.05; **p-value< 0.01. By Western Blot we detected the expression of ARL1 protein and β-actin (endogenous control) in each condition, A375 Empty, A375 transduced with shRNA3 and shRNA5, WM115 Empty, WM115 shRNA3 and WM115 shRNA5.

4.5 IMPACT OF ARL1 KNOCKDOWN ON CELL VIABILITY

To evaluate the effect of *ARL1* knockdown in CM cell lines, cell viability assays were performed using trypan blue and CCK8 assays (Figure 4.5). The trypan blue assay on days 3, 6 and 10 showed no statistically significant differences between silenced A375 cell line and A375 Empty cells (Figure 4.5 A). Through the CCK8 assay, after 3 and 6 days, we saw that the metabolic viability of the silenced A375 cell line was similar to the control cells (Figure 4.5 B). No statistically significant differences were also verified in the viability of WM115 cell line to both, the trypan blue (Figure 4.5 C) and CCK8 assays (Figure 4.5 D).

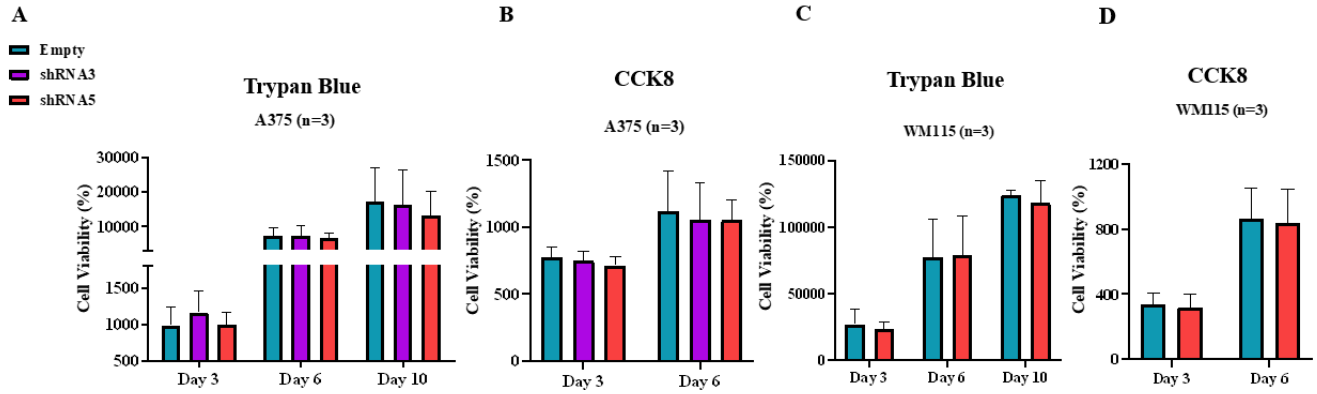


Figure 4.5 Impact of *ARL1* silencing on cell viability. (A) Trypan blue and **(B)** CCK8 were performed to understand whether *ARL1* knockdown could affect viability of A375 cell line (mean ± SD, n=3). **(C)** Using trypan blue and **(D)** CCK8 assays we also evaluated viability of WM115 silenced to *ARL1* (mean ± SD, n=3). Two-way ANOVA was used to assess differences on cell viability.

4.6 IMPACT OF ARL1 KNOCKDOWN ON CELL MIGRATION AND INVASION

Cell migration and invasion are processes responsible for the aggressiveness and progression of cancer. The role of ARLs in migration and invasion has been described[106-121,137,138]. Mainly, ARL1 regulates vesicular trafficking and is associated with increased secretion of MMPs[139]. It appears that deregulation of ARLs can decrease or increase the occurrence of these processes[106-121,137,138]. Therefore, through transwell assays, we decided to explore whether *ARL1* knockdown impacts melanoma aggressiveness, that is, whether it affects the ability of cells to migrate and invade. Our results show that *ARL1* silencing can decrease cell migration in the A375 cell line (Figure 4.6 A). These results are interesting because they are not in line with the favorable prognosis observed in patients with high levels of *ARL1* in the previous bioinformatics study[98]. Additionally, no statistically significant differences were found in the ability of these cells to invade when *ARL1* is silenced (Figure 4.6 B). Regarding WM115 line, we found that *ARL1* knockdown has no impact on the migration and invasion (Figure 4.6 C and D). The differences observed in these cell lines may be related to the different genetic background and metabolism, as well as the different knockdown efficiencies.

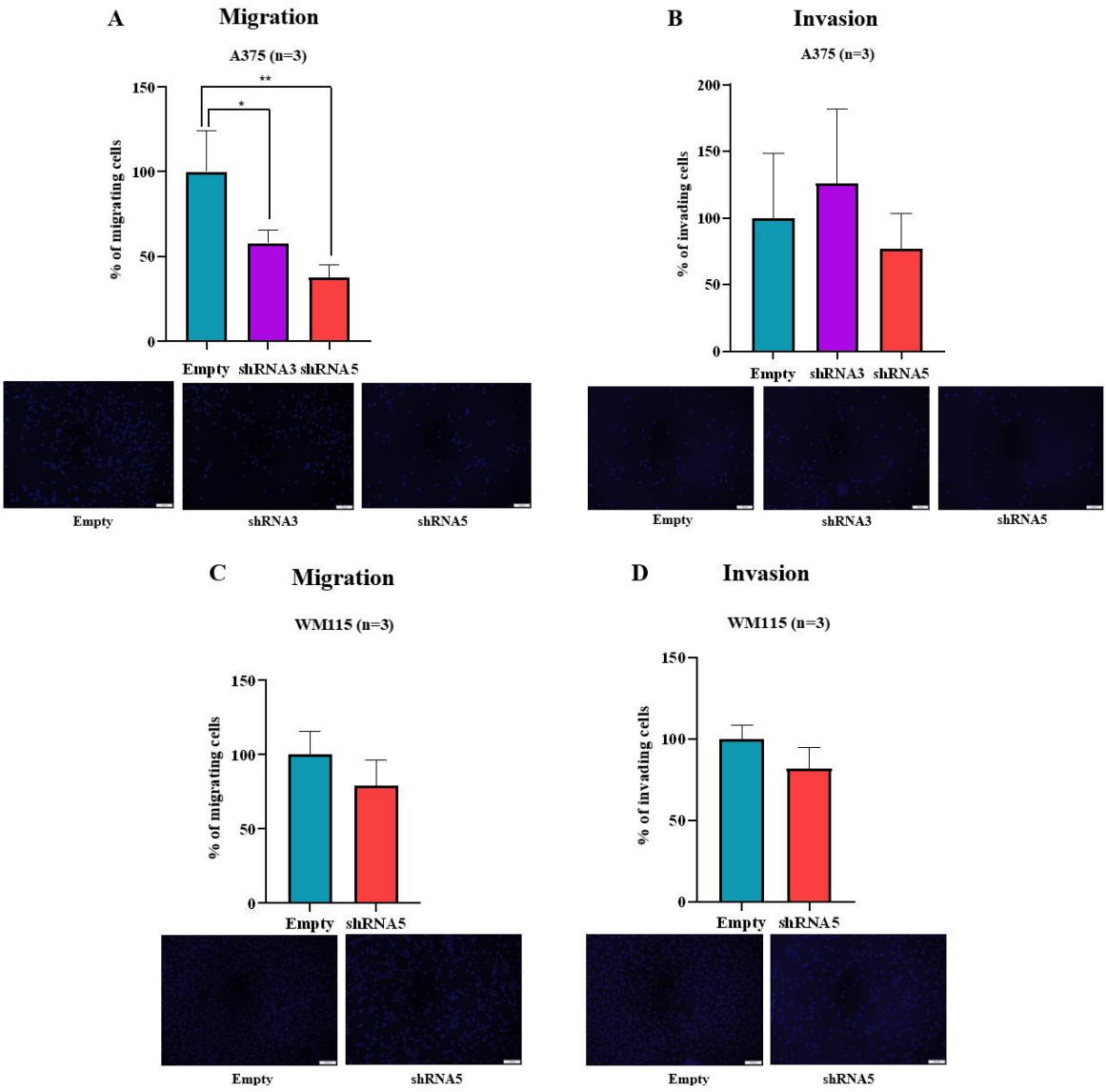


Figure 4.6 Impact of *ARL1* silencing on migration and invasion. Transwell assays were used to assessed **(A)** migration and **(B)** invasion ability after *ARL1* knockdown on A375 cells (mean ± SD, n=3). Representative images of **(C)** cell migration and **(D)** cell invasion after *ARL1* knockdown in WM115 cells (mean ± SD, n=3). One-way ANOVA was used to compare the control and mutated A375 cells at migration and invasion levels. Unpaired t-test was used to analyze cell migration and invasion of WM115. *p-value< 0.05; **p-value< 0.01. Representative images of cell migration and invasion are shown. Inverted fluorescence microscope allowed us to count cells with migratory and invasive capacity. Scale bars = 100 μm.

4.7 ROLE OF ARL1 ON MAPK AND PI3K PATHWAYS

Cells can decode and transmit signals through signaling cascades, which require the involvement of various proteins to ensure cellular homeostasis. Any mistake during this process results in disease, such as cancer. In sporadic CM, the MAPK signaling pathway is the most altered, followed by the PI3K pathway. Alterations in components of these pathways, such as mutations in NRAS and BRAF (MAPK pathway), and mutations in PTEN (PI3K pathway) result in the activation of downstream targets, such as MEK and ERK in the MAPK pathway, and AKT and mTOR in the PI3K pathway, enhancing cell proliferation, migration, invasion and evasion of apoptosis[38,41]. In addition, components of these pathways have been explored as therapeutic targets for treating CM, such as BRAF and MEK inhibitors[28]. NRAS is a well-known GTPase in CM, regulating cell migration and invasion, such as ARL proteins[83,84,87,89,98]. Here, we sought to determine the interference of *ARL1* knockdown in the MAPK pathway, through ERK, and in the PI3K pathway, through AKT. Despite no

differences in total ERK1/2 levels, phosphorylated ERK1/2 levels seem to decrease upon *ARL1* knockdown with shRNA5 for A375 cells, contrary to what happens in WM115 cells, where there is an increase in phosphorylated ERK1/2 upon *ARL1* knockdown (Figure 4.7 A). Additionally, there seem to be a decrease in AKT levels upon *ARL1* knockdown with shRNA5 for A375 cells. For WM115 cells, there seem to be no differences. There appear to be no differences in phosphorylated AKT levels for all conditions. (Figure 4.7 B). Thus, ARL1 silencing may be involved in modulating of MAPK pathway, but with a different effect on both melanoma cell lines: in A375 cells, *ARL1* knockdown decreases ERK1/2 activation; in WM115 cells, *ARL1* knockdown increases ERK1/2 activation. Regarding the PI3K pathway, *ARL1* knockdown seems to be able to modulate AKT levels, particularly decreasing protein levels for A375 shRNA5 cells.

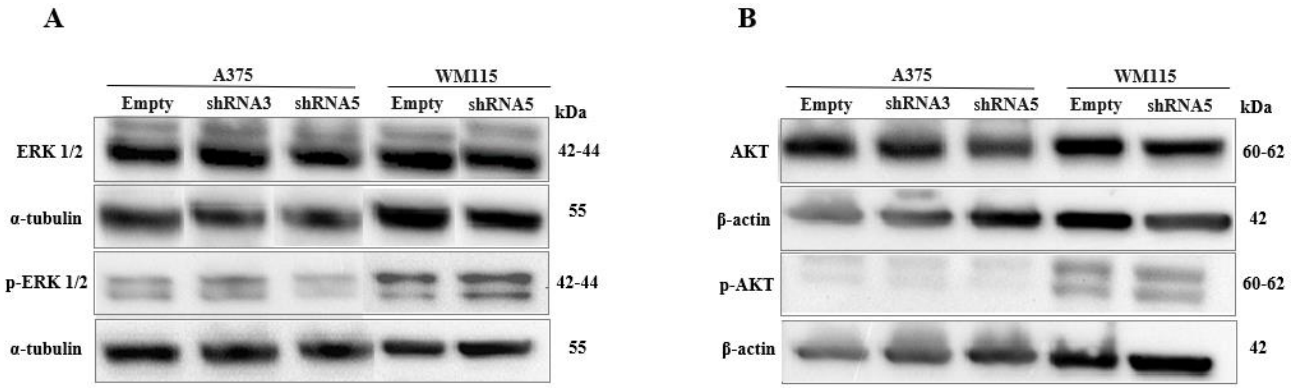


Figure 4.7 *ARL1* interference in the MAPK and PI3K pathways. Western Blot analysis was performed in A375 Empty, WM115 Empty, A375 and WM115 CM cells with low levels of ARL1. **(A)** Detection of ERK1/2 and p-ERK1/2 protein expression level. α-tubulin was used as an endogenous control. **(B)** Detection of AKT and p-AKT protein expression level. β-actin was used as an endogenous control.

4.8 ARL1 KNOCKDOWN AND CELLULAR METABOLISM

Cancer cells, including melanoma cells, need nutrients to sustain their survival and proliferation, so they have the ability to reprogram their metabolism to attend nutrient and oxygen shortages. Cells achieve this by increasing glucose uptake and lactate production and synthesizing amino acids, such as glutamine and cysteine, used a carbon source[76-79]. Therefore, we decided to investigate whether ARL1 can modulate cellular metabolism. For this, we studied a panel of genes involved in lactate production and transport (*LDHA*, *LDHC*, *MCT1* and *MCT4*), phosphate pentose pathway (*G6PD*), glutamine synthesis (*GLS1*, *SNAT1* and *SNAT2*) and cysteine synthesis (*XCT* and *EAAT3*)[76-79]. We found no significant differences for both CM lines between the control condition and *ARL1* knockdown, which suggests that ARL1 might not significantly influence these metabolic pathways (Figure 4.8 A and B).

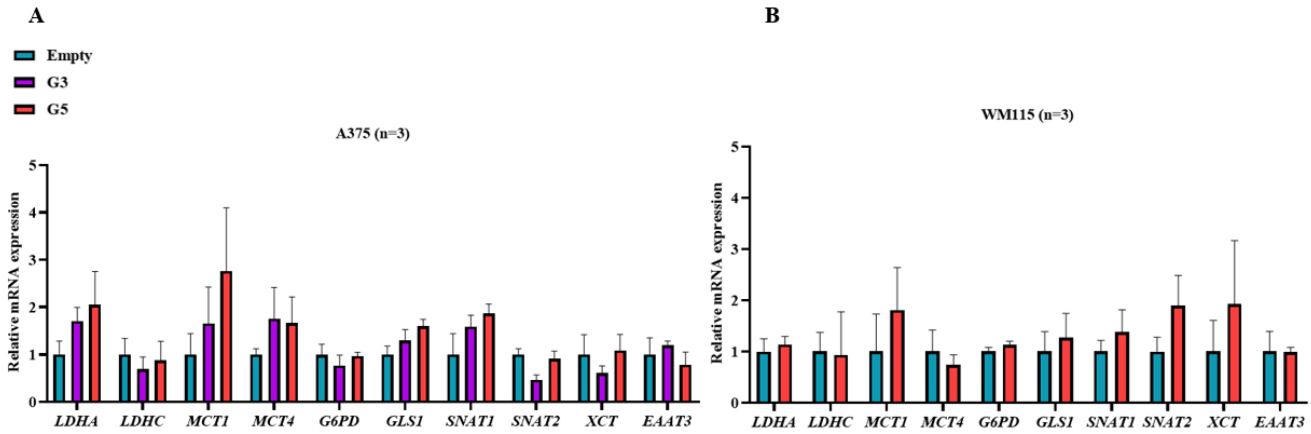


Figura 4.8 Impact of *ARL1* knockdown on cellular metabolism. Representative graphs of expression of several genes involved in metabolic pathways (*LDHA*, *LDHC*, *MCT1*, *MCT4*, *G6PD*, *GLS1*, *SNAT1*, *SNAT2*, *XCT* and *EAAT3*) according to *ARL1* expression for CM cell lines, **(A)** A375 (mean ± SD, n=3) and **(B)** WM115 (mean ± SD, n=3). Kruskal-Wallis and Mann-Whitney tests were used to assess the effect of *ARL1* knockdown on metabolism.

5. DISCUSSION

The invasive and metastatic potential of CM is a significant obstacle in patient outcome and response to therapy, so investigating new players that may be related to the aggressiveness of this tumor and could be prognostic and predictive biomarkers is crucial[98]. The interest in studying GTPases in cancer is understandable due to their role in activation or deactivation of various cellular processes related to tumor development, like proliferation, cell cycle progression and vesicle trafficking[83].

Our group was interested in investigating a particular group of GTPase proteins, the ARL proteins. Considering that there are about 21 known ARLs[89], we started by performing a bioinformatics study, which had as its primary objective to investigate the expression of ARLs in CM. Genotype tissue expression (GTEx) was used to evaluate ARLs expression in NS, and TCGA data was used to assess ARL transcriptional levels in primary and metastatic CM samples, and to perform OS curves. *ARL1*, *ARL11* and *ARL15* showed to be differentially expressed. Furthermore, these genes were the only considered independent prognostic factors in CM[98]. Taking into account the previous results, here we focused on studying ARL1 protein in CM. As far as we know, in addition to the bioinformatics study, this work is the second study focusing on the role of ARL1 in CM.

In this study, we evaluated *ARL1* expression in the IPOLFG cohort (n=21). Thus far, no significant differences were found in *ARL1* mRNA expression between all conditions under study (NS vs CM, stage, sample type and *BRAF* status (Figure 4.1). In the bioinformatics study performed by our group, we found high levels of *ARL1* in CM samples. Since the size of our cohort is significantly smaller than *in silico* cohorts, alterations in *ARL1* expression in our cohort of patients could not be observed, and therefore more samples need to be included to corroborate the results of *ARL1* expression in CM samples. Furthermore, there were no significant differences in PFS and OS between patients with high and low *ARL1* expression, but there seems to be a trend towards better PFS for patients with low *ARL1* expression (Figure 4.2). This trend is not in line with what was observed in the bioinformatics study, high *ARL1* expression was correlated with prolonged OS[98]. However, the bioinformatics study might indicate different potentials for ARL1 in CM; higher levels of *ARL1* in CM samples could be an indication of its importance for CM cells to thrive, but within CM samples, higher *ARL1* expression could hinder CM aggressiveness. Once again, it is important to highlight that our cohort data are only preliminary and limited. Furthermore, considering the pros and cons that an *in silico* study may have compared to cohort analysis, the observation of different results is possible. So

far, there are no studies that show opposing roles of a specific ARL in the same cancer, including ARL1. In fact, the real impact of ARL1 in cancer is poorly explored and only two studies have explored the role of ARL1 in cancer. Ong et al. also found high *ARL1* expression in breast cancer through TCGA, but high *ARL1* expression was associated with lower OS[128]. Zhang et al. using also TCGA data found *ARL1* downregulated in endometrial cancer samples compared to normal tissue, and high levels of *ARL1* were associated with better prognosis, as we observed *in silico* for CM[130]. All this evidence, including the present study, indicates that the role of ARL1 in cancer is not straight-forward; it is highly context-dependent, being able to behave as both an oncogene and a tumor suppressor. More samples are also needed to clarify the role of ARL1 on CM patients prognosis. Alternatively, samples from CM patient can also be evaluated by immunohistochemistry.

After the observation in the bioinformatics study of ARL1, ARL11 and ARL15 value on CM patients prognosis, immune cell infiltrate levels, important components of the tumor microenvironment were investigated in CM. These ARLs were correlated with the infiltration of several immune populations. Thus, in this work we also evaluated the impact of ARL1 on mechanisms associated with tumor aggressiveness: 1) we investigated the correlation between *ARL1* expression in the tumor and different tumor-infiltrated and circulating immune populations, such as CD4+ T cells, CD8+ T cells, Tregs, monocytes and macrophages; 2) we performed *in vitro* assays in two CM cell lines *BRAF* mutated, A375 and WM115, in order to evaluated migration and invasion; 3) we studied the role of ARL1 in cellular metabolism from a panel of genes.

Regarding the impact of ARL1 on the modulation of the immune system in CM, our data showed a positive correlation between *ARL1* expression and tumor-infiltrated CD4+ T cells, CD8+T cells and Tregs expressing CCR4 (Figure 4.3 B). So far, only our previous bioinformatics study investigated the correlation between ARL expression and the infiltration of immune cells in CM; however, in glioma, Wang et al. showed through Tumor immune estimation resource (TIMER) data, that low levels of ARL3 were correlated with an increase of dendritic cells, macrophages, CD4+ T cells and CD8+ T cells[111].

The role of CD4+ T cells in cancer is controversial mainly due to the diversity of CD4+ cells, as well as the secretion of different factors into the tumor microenvironment. CD4+ T cells are a heterogeneous group of cells (Th1, Th2, Th9, Th17 and Tregs) that can have an antitumor or immunosuppressive role by cytokine secretion[133]. In lung cancer, CD4+ Th1 and CD4+ Th17 stimulate the function of CD8+ T cells by reversing their exhausted phenotype[140]. In colorectal cancer, high levels of CD4+ Th17 were associated with liver metastasis and consequently a poor prognosis, while high CD4+ Th1 expression was associated with prolonged PFS[141,142]. However, in ovarian cancer high levels of CD4+ Th17 it was associated with a good prognosis. The presence of CD4+ Th2 *in vivo* was shown to be associated with immune evasion in colon cancer[142]. Overexpression of signal transducers related to CD4+ Th2 differentiation has been correlated with stimulation of M2-like macrophages in melanoma, lymphoma, breast and lung cancer, which is indicative of the immunosuppressive role of CD4+ Th2. In addition, some interleukins secreted by CD4+ Th2 were related to cancer metastasis[143]. Focusing on melanoma, clinically responsive patients showed the presence of neoantigens recognized by CD4+ T cells after therapy[144]. Kreiter et al. and Zander et al. demonstrated the induction of CD4+ T cells responses in murine melanoma models[145,146]. Additionally, neoantigen-based vaccines have led to CD4+ T cell-based anti-tumor responses[147]. Melanoma patients with favorable outcome had more tumor-infiltrated CD4+ T cells than patients with unfavorable outcome[148]. In a previous study, most melanoma patients with regional lymph node metastases had low levels of interleukin-2 (produced by CD4+ Th1), but high levels of interleukin-4, interleukin-6 and interleukin-10 (produced by CD4+ Th2). Furthermore, patients with relapse had

significantly low levels of interleukin-2 and interleukin-12 (produced by CD4+ Th1), suggesting the antitumor and immunosuppressive role of CD4+ Th1 and CD4+ Th2, respectively[149]. On the contrary, Mattes et al. found a decrease in lung CM metastases *in vivo* through CD4+ Th2 cells, whereas CD4+ Th1 cells did not affect tumor growth. This effect caused by CD4+ Th2 cells appears to be dependent on cellular toxins released from eosinophil granules, that can destroy the tumor, whereas the inability of CD4+ Th1 cells to inhibit tumor growth may be related to the recruitment of pro-angiogenic macrophages incapable of inhibiting tumor growth[150]. In the bioinformatic analysis carried out by our group through the TIMER tool, high *ARL1* expression was also negatively correlated with CD4+ Th1 cells and positively with infiltration of CD4+ Th2 cells in CM[98]. Several studies have reported both the antitumor and immunosuppressive role of CD4+ Th17 in melanoma. High levels of CD4+ Th17 can be found in melanoma, for example due to secretion of tumor-associated fibroblast ligands, which bind to CD4+ Th17 receptors, or due to the expression of high levels of interleukins by melanoma cells, resulting in the expansion of CD4+ Th17 cells. The antitumor role may be related to the differentiation of Th17 cells into Th1 cells or due to the ability of Th17 cells to recruit leukocytes stimulating CD8+ activation. On the other hand, the presence of CD4+ Th17 cells may be associated with a protumoral effect in melanoma due to the expression of interleukin-17. A study showed that interleukin-17 potentiated the growth of melanoma by activating genes responsible for tumor progression and pro-survival genes[151]. Finally, CD4+ Th9 cells are described for their antitumor effect in cancer due to favoring the activation of CD8+ T cells and dendritic cells. CD4+ Th9 was shown to reduce melanoma tumors in mice[143]. In this study, high *ARL1* expression is related to increased infiltration of CD4+T cells. The different biological role of each subset of CD4+T cells in the tumor microenvironment may or may not favor the tumor. Thus, it is not possible to assess the biological significance between the interaction of ARL1 and CD4+T cells without studying CD4+T cells individually.

CD8+ T cells are able to recognize and kill cancer cells by releasing of several cytokines[152]. Increasing the infiltration and activity of CD8+ T cells has been proposed as a therapeutic strategy in the treatment of cancer, for example through checkpoint inhibitor antibodies, T cell co-stimulatory molecules, agonistic antibodies, chimeric antigen receptor-T cells, T-cell receptor-transduced T cells, and tumor-infiltrating lymphocytes-based cancer therapy[153,154]. The high expression of CD8+ T cells is indicative of a better prognosis in several cancers[155]. However, the high infiltration of these cells is not always positively associated with good outcomes[156,157]. Some studies related with lung and breast cancer, hepatocellular and basal cell carcinoma mention that the high levels of CD8+ T cells observed are cells with an exhausted phenotype[152]. Exhausted CD8+ T cells have a decreased ability to produce cytokines, high levels of checkpoint molecules, immune surveillance affected by secretion of immunosuppressive molecules, lack of adequate T-cell-costimulation or downregulation of cell-surface MHC Class II protein expression[156,157]. In CM, Piras et al. confirmed what had already been evidenced in other studies about the antitumor role of CD8+ T cells. In this study, CD8+ T cell infiltration was beneficial in survival of CM patients with stage I and II[158]. In another study, the increase of CD8+ T cell infiltrates was associated with positive survival outcomes in primary CM and metastatic melanoma lesions[159]. Kakavand et al. investigated the populations of lymphocytes that interact with metastatic melanoma cells and found a positive correlation between CD8+ T cells and recurrence-free survival[160]. A recent study identified high levels of CD8+ T cells in the sentinel lymph nodes of patients with CM, but these cells had exhaustion markers, capable of reducing their antitumor activity[161]. The positive correlation between CD8+ T cells and *ARL1* expression can be suggestive of the impact of ARL1 on antitumor immunity; however, these cells may also be associated with an exhausted phenotype. Therefore, they must be further characterized with activation/exhaustion markers and functional assays.

Tregs are cells that can develop by stimulation of autoantigens or through naive T cells. They are critical cells in immune evasion during tumor progression, as these cells suppress the functions of CD8+ T cells. High levels of Tregs have been evidenced in metastatic melanoma, gastrointestinal and ovarian cancer, and are related to poor clinical outcome in cancer patients. Furthermore, activation of Tregs may be related to low efficacy of current therapies[143]. In a study carried out to estimate the level of infiltration of immune cells in melanoma, the authors found, using bioinformatic tools, a greater infiltration of immune cells in metastatic melanoma than in primary melanoma, including CD4+ T cells, CD8+ T cells and Tregs. These data suggest a possible activation of immune responses against the tumor along with immunosuppression[162]. In 2018, Di Gennaro et al. found increased levels of Treg cells particularly involved in tumor tolerance in advanced-stage melanoma patients[163]. In the tumor microenvironment, several chemokines are released interacting with specific receptors on Tregs. HLA-DR is a surface receptor expressed in Tregs and CD8+ T cells. Tregs with high HLA-DR expression have greater immunosuppressive ability[164]. However, CD8+ T cells expressing HLA-DR appear to have an antitumor effect[165]. The CCR4 chemokine receptor is expressed at high levels on Tregs during metastasis, being involved in migration and infiltration of Tregs in the tumor[166,167]. Expression of this receptor is associated with a poor prognosis in some cancers (e.g. prostate and lung)[168,169]. Klein et al. showed that CCR4 can be implied in melanoma brain metastasis, since melanoma cells from brain metastasis express more CCR4 than local cutaneous cells from the same melanoma. In addition, CCR4 is overexpressed in samples of melanoma brain metastasis compared with samples of primary melanoma[170]. ARL1 may be associated with an immunosuppressive effect due to the infiltration of Treg cells expressing CCR4.

Finally, we found no significant correlations between *ARL1* expression and circulating immune cells (Figure 4.3 A). Thus, ARL1 appears to have an important role in recruiting immune cells to the tumor, but that does not translate to immune cells in circulation. In fact, considering that ARL1 is a protein that modulates functions at the level of the Golgi complex, described in vesicular traffic, being involved in cell polarity (e.g. E-cadherin transport), innate immunity (e.g. HLA class I, TNFα and interleukin-10 transport)[125], it is more likely to observe the recruitment of immune cells to the tumor, because to see changes in circulating immune cells, *ARL1* expression would have to be very high in tumor. Circulating immune cells can give us indications of the response to therapy, as well as predict patient survival and prognosis in some contexts. For example, a study evaluated the presence of PBMCs in melanoma patients (responders and nonresponders) before initiation of immune checkpoint inhibitor therapy. The authors found that nonresponders had lower percentages of proliferating CD4+ and CD8+ T cells, but higher percentages of checkpoint molecules than responders, which is indicative of more signs of T-cell exhaustion having an influence on OS[164]. Additionally, other studies have focused on the investigation of immune populations through PBMCs in melanoma patients[171,172].

Overall, the immune system in cancer is a difficult and complex field of investigation because of the interaction between different components in tumor microenvironment. The study of the immune system has clearly been crucial in the study of cancer with the development of immunotherapies. Our findings regarding the impact of ARL1 on the immune system allowed us to verify that ARL1 enhances the recruitment of immune cells to the tumor; however, it is still unclear whether the role of ARL1 involves stimulating the infiltration of immune cells (e.g. CD8+ T cell and CD4+ Th1) capable of eliminating the tumor, or whether it confers beneficial advantages to the tumor achieved by increasing Tregs or CD4+ Th2 cells capable of releasing immunosuppressive cytokines, or whether the possible increase in CD8+ T cell infiltration is associated with cells with an exhausted phenotype. It would be interesting to evaluate activation and exhaustion markers or for example, perform functional

proliferation assays in order to distinguish cells with an active from exhausted phenotype, and also use markers of differentiation of CD4+ Th1 and CD4+ Th2 cells.

It is important to highlight that the role of ARL1 in cell immunity has been described before[125]. ARL1 is known to mediate the recruitment of golgin-97 and golgin-245 proteins to the trans-Golgi network. In fact, ARL1 together with golgin-97 or golgin-245 influences the transport of several types of cargo, such as Human leukocyte antigen (HLA) class I, Tumor necrosis factor alpha (TNFα) and interleukin-10[125,173,174]. The overexpression of p230/golgin-245 in Jurkat cells results in downregulation of HLA class I expression, molecules expressed by almost all nucleated cells that allows them to present antigens from inside the cell (e.g. presentation of antigens from cancer cells to CD8+ T cells)[175,176]. Indeed, alterations in HLA expression can result in immune evasion and cancer metastasis[176]. Loss of HLA class I was associated with low survival and poor response during treatment of melanoma patients[177,178]. In addition, loss of HLA class I was also found in melanoma cell lines[179,180]. Therefore, lower expression of *ARL1* may promote HLA class I transport, in other words, an antitumor effect. Depletion of p230/golgin-245 in macrophages impairs post-Golgi TNFα transport *in vitro* and *in vivo*[181]. TNFα is a cytokine that can promote apoptosis, proliferation, angiogenesis and metastasis in cancer[182]. In fact, it is described to play an immunosuppressive role through increasing levels of Tregs or by inhibition of CD8+ T cells. Bertrand et al. showed that inhibition of TNFα signaling blocks the expression of PD-L1 and TIM-3 in mouse melanoma model. Additionally, TNFα is able to potentiate activity of PD-L1 in melanoma cells[183]. We can hypothesize that high levels of ARL1 potentiate TNFα transport contributing to immune evasion. Interleukin-10 transport by p230/golgin-245 was proved through miRNA silencing of p230/golgin-245 in mice, which consequently reduced the secretion of interleukin-10[184]. The role of interleukin-10 is evidenced in cancer and seems to play an immunosuppressive and antitumoral role[185]. Dummer et al. showed that interleukin-10 was more present in metastatic melanoma cell lines and in metastatic lesions, suggesting their role in tumor progression[186]. In fact, interleukin-10 may adversely affect CD4+ T cell function and MHC class II antigen presentation. However, Huang et al. reported that interleukin-10 can regulate macrophage migration into the tumor, resulting in inhibition of angiogenesis and tumor growth promoters. Interleukin-10 expression *in vitro* and *in vivo* decreases metastatic capacity and tumorigenesis of melanoma, respectively[185,187]. These findings may reveal that high levels of ARL1 can promote the secretion of interleukin-10, which has an unclear impact in CM (antitumor or immunosuppressive effect).

To better characterize the role of ARL1 in CM aggressiveness, we performed *in vitro* assays in A375 and WM115 cell lines, in order to evaluated cell viability, migration and invasion. To achieve our goals, we started by reducing *ARL1* expression levels in both CM lines (Figure 4.4). Then, we assessed whether *ARL1* knockdown affected cell viability. No significative differences were found for both CM cell lines (Figure 4.5).

Since *ARL1*, does not play a preponderant role in melanoma cell viability and proliferation, we wanted to see the impact of *ARL1* knockdown on cell migration, an important indicator of tumor aggressiveness. *ARL1* knockdown decreased cell migration of A375 cells, indicating that high levels of *ARL1* may be associated with an increase of CM aggressiveness. However, these results do not, seem to justify the positive correlation observed in our bioinformatics study between high levels of *ARL1* and a favorable prognosis, but it may justify the high levels of *ARL1* observed in CM. Despite the differences observed in the A375 line, no differences were found on cell migration in WM115 cells between the control condition and *ARL1* knockdown (Figure 4.6). As both cell lines are BRAF mutated, it could be expected to obtain similar results, but these CM cells lines have different mutated genes, and therefore, the genetic background may have influence in the different results obtained.

Additionally, the cell metabolism and the percentage of silencing achieved for each line can also justify these results. We also evaluated the role of *ARL1* on cell invasion. *ARL1* knockdown does not appear to affect cell invasion for both lines (Figure 4.6).

The known role of ARLs in processes that regulate cell migration, such as membrane traffic, actin remodelling and tubulin assembly, could justify the impact of ARL1 on A375 cell migration[89,98]. For example, alterations in ARL1 can influence Golgi structure and function. The occurrence of modifications in Golgi can result in aberrant glycosylation and defects in membrane traffic. In cancer, Golgi glycosylation is associated with cancer cell invasion, cell-matrix adhesion, angiogenesis and metastasis[188]. Alteration in membrane traffic pathways may favor cell migration, invasion and metastasis[189,190]. Although there are no differences in cell invasion in this study, Eiseler et al. showed that knockdown of ARL1 and Arfaptin2 impaired the secretion of MMP7 in Panc1 cells[139]. It is known that MMPs are responsible for the degradation of the extracellular matrix resulting in invasion and metastasis[191]. MMPs (MMP2, MMP7, MMP9, MMP14, MMP19, MMP21 and MMP26) have already been documented as contributors to melanoma progression[38,191].

Growing evidence reveals that abnormal expression of ARL genes contributes to the modulation of the malignant phenotype to potentiate proliferation, migration, invasion, and metastasis. When upregulated, ARLs can contribute to tumorigenesis in several cancers. Pang et al. reported *ARL2* knockdown increased proliferation, migration and invasion of colorectal cells[137]. In *in vitro* studies using colon and cervical cancer cells, *ARL2* knockdown by microRNA led to a negative impact on cell growth and inhibition of cell proliferation, migration and invasion, respectively[108,109]. In bladder cancer, *ARL2* upregulation *in vitro* significantly increased mitochondrial activity, promoting the proliferation of cancer cells[110]. In a recent study, the silencing of *ARL4C* by siRNA decreased cell proliferation of lung cancer cells and *ARL4C* expression showed to be involved in the AKT pathway[138]. In colorectal and lung cancer cells showed high levels of *ARL4C* mRNA, and *ARL4C* knockdown by siRNA repressed the migration and invasion of these tumor cells[113,114]. In another study using gastric tumor cells, a similar result was obtained, with *ARL4C* knockdown decreasing migration and invasion[115]. *ARL5A* knockdown showed decreased proliferation in colorectal cancer[116]. *ARL6* is upregulated in rhabdomyosarcoma human cells and mouse tissues. Additionally, *ARL6* knockdown promoted cell apoptosis resulting in defects in ciliogenesis and decrease activation of the Hedgehog pathway[117]. In prostate cancer, *ARL8b* depletion prevented lysosome trafficking in cell lines and the presence of *ARL8b* in a 3D culture model proved to be necessary in protease secretion, matrix degradation and tumor cell invasion. Additionally, *ARL8b* knockdown prevents the growth of prostate tumors in mice[118]. Shao et al. identified that *ARL13b* is able to promote Hedgehog signaling, which in turn results in the proliferation, migration and invasion of gastric cancer cells, as well as in the growth of xenograft gastric tumors[119]. Recently, Casalou et al. found that, in breast cancer, *ARL13b* knockdown inhibits migration and invasion, as well as impairs cancer progression, *in vitro* and *in vivo*, respectively[120]. Finally, in lung adenocarcinoma cells, elimination of *ARL14* by siRNA resulted in a negative impact on proliferation, migration and invasion[121]. Conversely, ARL proteins can also contribute to tumorigenesis when downregulated. In breast cancer, low levels of *ARL2* contribute to the aggressiveness and growth of cancer cells *in vitro* and to tumor enlargement in mice[107]. *ARL2* overexpression is able to suppress proliferation, migration and invasion of glioma cells[106]. Another study evidenced low *ARL4C* expression favors cell migration in ovarian cancer[112]. These findings show that *ARL2* and *ARL4C* can function as both oncogenes and tumor suppressors. Thus, the role of ARLs probably depending on the interaction with different effectors or even of the type of cancer.

To confirm the results already obtained *in vitro*, we decided to evaluate the interaction of ARL1 with the most frequently altered pathways in CM, MAPK and PI3K (Figure 4.7). MAPK and

PI3K are involved in cell cycle control, proliferation, cell death and metastasis[38,41]. In CM, MAPK and PI3K signaling are constitutively active due to mutations in key oncogenes, such as *BRAF* and *NRAS*, in the MAPK pathway, and mutations in tumor suppressor genes like *PTEN*, in the PI3K/AKT pathway. Mutations in these genes result in aberrant activation of the aforementioned signaling pathways, enhancing cell proliferation, survival, invasion and reduction of apoptosis, contributing to the development of CM[38,41]. Our results show a decrease in the activation of the ERK pathway and a decrease in the AKT levels in cell line A375 upon *ARL1* knockdown. Therefore, these data also suggests a decrease in cellular processes such as cell migration, as seen for the A375 cell line by transwell migration assay. Despite *in vitro* assays do not indicate that *ARL1* knockdown is able to potentiate or inhibit migration and invasion for WM115, there seems to be an increase in the activation of the ERK pathway, but we did not see any difference in the AKT pathway. These data suggest that the role of ARL1 is ambiguous and may depend on the context, and could have an oncogene or tumor suppressor role.

Finally, considering the increasing importance of metabolism in cancer, we investigated several genes involved in metabolic pathways. *ARL1* had no significant impact on the expression of the studied genes (Figure 4.8). The reprogramming of metabolism is one of the biggest hallmarks of cancer, as cancer cells, including CM cells, are able to keep up with their energy demands in order to ensure their growth[76]. In CM, dysregulation of the MAPK and PI3K pathways can promote the activation of genes that induce increased glucose intake, lactate secretion and decreasing mitochondrial respiration and oxidative phosphorylation, or downregulation of genes that decrease mitochondrial oxidative metabolism[77]. So far, few studies show the role of ARLs in cellular metabolism. *ARL2* overexpression increases bladder cancer cell proliferation as a result of modulation of mitochondrial metabolism. Cancer cells are known to utilize glycolysis from dysfunctional mitochondria. In addition, mitochondrial alterations can increase levels of glutamine, one of the amino acids preferred by cancer cells as a carbon source[110]. Zhang et al. showed through databases that ARLs were related to metabolic processes, including glycolysis and oxidative phosphorylation in endometrial cancer[130]. ARL8b controls lipid metabolism for maintenance of prostate cancer cell proliferation in nutrient-poor environment[118]. ARL1 is involved in lipid droplet formation[125]. Lipid droplets are organelles responsible for the storage and exchange of lipids in the cell, controlling signaling pathways and interfering with cell growth, proliferation and metabolism. Cancer cells employ lipid droplets in order to ensure redox balance, control autophagy and as a source of energy; therefore, changes in lipid droplet metabolism contribute to the growth of cancer cells[192]. Although our results show that *ARL1* knockdown does not have a significant impact on the expression of genes involved in metabolic pathways, it does not mean that ARL1 does not interfere with the metabolism of melanoma cells. Thus, studying genes involved in lipid droplets may help us to better understand the role of ARL1 in CM. We can assess genes involved in lipid droplet formation (e.g. AUP1 Lipid Droplet Regulating VLDL Assembly Factor and BSCL2 Lipid Droplet Biogenesis Associated, Seipin), genes related to lipogenesis (e.g. Patatin Like Phospholipase Domain Containing 2, Fatty Acid Synthase and Diacylglycerol O-Acyltransferase) and genes regulating lipid homeostasis (e.g. Sterol Regulatory Element Binding Transcription Factor)[193-196].

6. CONCLUSION AND FUTURE PERSPECTIVES

CM is a public health problem. Early detection, immediate treatment, warning about risk behaviors, forms of prevention, identification of new diagnostic and prognostic biomarkers can in fact save many lives. Since the therapies available so far are not completely efficient, the use of molecular biomarkers in the clinical practice is very promising, not only as prognostic and predictive tools, but also in the design of new therapeutic approaches, in order to increase patient's survival and quality of

life. In this sense, understanding the relevance of candidate genes for therapeutic targets is fundamental, especially in a context of metastasis, which is the major problem of mortality in cancer, including CM. Thus, we propose to understand the impact of ARL1 GTPases on CM aggressiveness.

So far, we have not found significant differences in *ARL1* expression between patients with CM from our cohort and NS, as well as between different stages, sample type and *BRAF* mutational status. Additionally, we found that ARL1 might potentiates the recruitment of immune cells to the tumor, suggesting a role for ARL1 in the immune modulation of CM. Our *in vitro* data showed that *ARL1* knockdown had no impact on cell viability and invasion. Furthermore, we found a controversial role of *ARL1* in cell migration. Our *in vitro* results for the A375 cell line seem to show that high *ARL1* expression may be associated with increased aggressiveness in CM. These data are supported by the decrease in the expression level of p-ERK when ARL1 is silenced. However, the results observed *in vitro* do not agree with those observed *in silico*, where we found that the high *ARL1* expression was associated with a good prognosis in CM. Lastly, ARL1 does not appear to influence key metabolism factors: lactate production, pentose phosphate pathway and amino acid synthesis.

As future perspectives, we intend to add more samples to our patient cohort in order to have a more accurate assessment of *ARL1* expression in CM patients. Considering the possible observed role of ARL1 in immune modulation, a better characterization of immune populations, such as CD4+T cell subsets and exhausted CD8+T cells would be important to truly understand the functional role of the recruited populations in tumor aggressiveness. It would also be important to evaluate *ARL1* expression by flow cytometry as we are analysing immune populations to understand whether the cells that are expressing *ARL1* are tumor or immune infiltrates. We are currently silencing ARL1 in a cell line (WM3211) that does not harbor a *BRAF* mutation, in order to see whether ARL1 behaves differently according with distinct genetic backgrounds in CM. Additionally, considering the contradictory results obtained *in vitro*, namely in terms of cell migration, which do not justify the results of the bioinformatics analysis, it would be important to clarify the prognostic value of ARL1 at the protein level by immunohistochemistry. Finally, as ARL1 is involved in lipid droplets we could evaluate genes related to this organelle.

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8. APPENDICES

Table A.1. Antibody panels used for flow cytometry

Panels	Antibodies
Panel 1	anti-CD3-PerCP (HIT3a), anti-CD4-FITC (OKT4), anti-CD25-PE (BC96), anti-CD127-PE/Cy7 (A019D5), anti-HLA-DR-APC (L243) and anti-CCR4-BV421 (L291H4)
Panel 2	anti-CD3-PerCP (HIT3a), anti-CD8a-PE (HIT8a) and anti-HLA-DR-APC (L243)
Panel 3	CD45-PerCP (HI30) and anti-CD14-FITC (63D3)
Panel 4	CD45-PerCP (HI30), anti-CD68-APC (Y1/82A), anti-CD80-FITC (2D10), anti-CD163-PE (GHI/61) and anti-CD206-APC/Cyanine7 (15-2)

Table A.2. Different shRNA sequences (shRNA1, shRNA2, shRNA3, shRNA4 and shRNA5) targeting *ARL1* for transduction with lentivirus.

***ARL1* shRNA**	**Hairpin sequence**
shRNA1	5'-CCGG-GCCATACTGGAGATGTTACTA-CTCGAG-TAGTAACATCTCCAGTATGGC-TTTTTG-3'
shRNA2	5'-CCGG-GTTACTACTATACCTACCATT-CTCGAG-AATGGTAGGTATAGTAGTAAC-TTTTTG-3'
shRNA3	5'-CCGG-GCCTTGATGAGGCAATGGAAT-CTCGAG-ATTCCATTGCCTCATCAAGGC-TTTTTG-3'
shRNA4	5'-CCGG-CAAACACAGATGCAGTCATTT-CTCGAG-AAATGACTGCATCTGTGTTTG-TTTTTG-3'
shRNA5	5'-CCGG-GAACTCGGGAAATGAGAATTT-CTCGAG-AAATTCTCATTTCCCGAGTTC-TTTTTG-3'

Table A.3. Forward and reverse primer sequences for *ARL1*, *HPRT1*, *LDHA*, *LDHC*, *MCT1*, *MCT4*, *G6PD*, *GLS1*, *SNAT1*, *SNAT2*, *XCT* and *EAAT3*.

Genes	**Primer sequences**
ARL1	forward: 5'AGTCTGTTTGGAACTCGGGAA 3' reverse: 5'AGGTTTTTGTACGTCACCGTCT 3'
HPRT1	forward: 5'TGAGGATTTGGAAAGGGTGT 3' reverse: 5'GAGCACACAGAGGGCTACAA 3'
LDHA	forward: 5'CTTGCTCTTGTTGATGTCATC 3' reverse: 5'CAGCCGTGATAATGACCAGC 3'
LDHC	forward: 5'GGATCTTCAGCATGGCAGTC 3' reverse: 5'CTATTCTGGAGTTTGCAGATA 3'
MCT1	forward: 5'GCTGGGCAGTGGTAATTGGA 3' reverse: 5'CAGTAATTGATTTGGGAAATGCAT 3'
MCT4	forward: 5'CACAAGTTCTCCAGTGC 3' reverse: 5'CGCATCCAGGAGTTTGC 3'
G6PD	forward: 5'GGCAACAGATACAAGAACGTGAAG 3' reverse: 5'GCAGAAGACGTCCAGGATGAG 3'
GLS1	forward: 5'CTTCTACTTCCAGCTGTGCTC 3' reverse: 5'CACCAGTAATTGGGCAGAAACC 3'
SNAT1	forward: 5'CATTCTATGACAACGTGCAGTCC 3' reverse: 5'CAGCAACAATGACAGCCAGC 3'
SNAT2	forward: 5'CTGAGCAATGCGATTGTGGG 3' reverse: 5'CTCCTTCATTGGCAGTCTTC 3'
XCT	forward: 5'GGTCCTGTCACTATTTGGAGC 3' reverse: 5'GAGGAGTTCCACCCAGACTC 3'
EAAT3	forward: 5'GTATCACGGCCACATCTGCC 3' reverse: 5'GCAATGATCAGGGTGACATCC 3'