UNIVERSITÀ DI SIENA 1240

Dipartimento di Medicina Molecolare e dello Sviluppo

Dottorato in Medicina Molecolare

XXXVII° Ciclo

Coordinatore: Prof. Vincenzo Sorrentino

Unveiling the role of Carbonic Anhydrase XII in human melanoma and dendritic cells in a hypoxic microenvironment

Settore scientifico disciplinare: BIOS-06/A

Candidato
Dott. Alessandro Falsini
Dipartimento di Medicina Molecolare
E dello Sviluppo, Università di Siena

Firma digitale del candidato

Supervisore
Prof.ssa Antonella Naldini
Dipartimento di Medicina Molecolare
E dello Sviluppo, Università di Siena

Co-supervisore
Dott.ssa Irene Filippi
Dipartimento di Medicina Molecolare
E dello Sviluppo, Università di Siena

Anno accademico 2024/2025

Università degli Studi di Siena
Dottorato in Medicina Molecolare e dello Sviluppo
XXXVII° Ciclo

Data dell'esame finale
12 maggio 2025

Commissione giudicatrice
Prof.ssa Daniela Rossi
Dr.ssa Elena Sticchi
Prof. Giovanni Casini

Supplenti
Prof.ssa Cecilia Paolini

Insieme
Il passato nei ricordi, il futuro solo nei sogni

A mio babbo

INDEX

ABSTRACT 7
1. INTRODUCTION 8
1.1 Hypoxia 8
1.1.1 Hypoxia inducible factor 1 α (HIF-1α) 9
1.2 Carbonic Anhydrases 13
1.2.1 Carbonic anhydrases and pathology 14
1.3 The tumor microenvironment 15
2. AIMS 18
3. INTRODUCTION (PART I): Melanoma, the Hedgehog pathway and Carbonic Anhydrases 19
3.1 Melanoma 19
3.1.1 Hypoxia and melanoma 21
3.2 The Hedgehog pathway 21
3.2.1 Hypoxia and the Hedgehog pathway 26
3.2.2 Melanoma and the Hedgehog pathway 26
3.3 Carbonic Anhydrases and melanoma 27
4. RESULTS (PART I) 28
4.1 HIF-1α and SMO expression in SK-MEL-28 and A375 cell lines 28
4.2 Chemical compounds GlaB and C22 inhibit SMO and GLI1 expression in SK-MEL-28 and A375 31
4.3 Hh inhibition resulted in melanoma cell migration impairment 33
4.4 Hh inhibition resulted in melanoma cell invasion impairment 34
4.5 CAXII expression can be modulated by Hh interfering compounds 35
5. DISCUSSION (PART I) 38
6. INTRODUCTION (PART II): Dendritic Cells and Carbonic Anhydrases 40
6.1 The tumor microenvironment and infiltrating immune cells 40
6.2 The Immune system 40
6.2.1 Innate immunity 41
6.3 Dendritic Cells 43
6.3.1 Immature Dendritic Cells 44
6.3.2 Mature Dendritic Cells 44
6.4 Dendritic Cells and Hypoxia 45
6.5 Dendritic Cells and Carbonic Anhydrases 46
7. RESULTS (PART II) 47
7.1 CAXII expression is enhanced by LPS in normoxic DCs 47
7.2 CAXII expression is enhanced by LPS in hypoxic DCs 48
7.3 DC migration is reduced by AAZ treatment 50
7.4 CAXII downregulation by siRNA in normoxic and hypoxic DCs 52
7.5 CAXII siRNA resulted in DC migration impairment 54
8. DISCUSSION (PART II) 55
9. FINAL DISCUSSION AND CONCLUSION 56
10. MATERIALS AND METHODS 58
10.1 Cell lines cultures 58
10.2 Chemical compounds 58
10.2.1 Chemical compounds I 58
10.2.2 Chemical compound II 58
10.3 Primary cells cultures 59
10.3.1 Primary Cell culture Reagents. 59

10.3.2 Human monocyte isolation and monocyte-derived DC preparation. ... 59
10.3.3 DC culture conditions... 60
10.4 CAXII inhibition by RNA interference. ... 60
10.5 GENE AND PROTEIN EXPRESSION ANALYSIS. ... 60
10.5.1 Western Blotting and antibodies. ... 60
10.5.2 RNA extraction and qRT-PCR. ... 61
10.6 Immunofluorescence ... 62
10.7 Wound healing assay ... 62
10.8 Modified Boyden chamber ... 63
10.9 Statistical Analysis ... 63
11. ADDENDUM ... 64
12. REFERENCES ... 65
GRATIARUM ACTIO ... 77

ABSTRACT

The tumor microenvironment (TME) is characterized by low oxygen levels, a condition known as hypoxia which plays a critical role in cancer progression, immune evasion and resistance to therapy. Along with cancer cells, the TME is populated by stromal cells, which include infiltrating immune cells. The aim of the thesis was to study the relevance of Carbonic Anhydrase XII (CAXII) in the migration of Dendritic Cells (DCs) and the interplay between CAXII and Hedgehog (Hh) pathway in the migration of melanoma cells, through new small molecules, especially under hypoxia.

At first, we decided to study the relationship between the Hh pathway and CAXII, underlining their involvement in melanoma cell migration under hypoxia. Indeed, such signaling is well-known for its role in various physiological processes but aberrantly re-activated in cancer cells and carbonic anhydrases (CAs), key enzymes in pH regulation, have been linked to cell migration. In this study, we targeted components of the Hh pathway using Glabrescione B and C22, as well as Cyclopamine and SAG, to assess their impact on CAXII expression, particularly under hypoxic conditions. Migration and invasion assays were conducted on two melanoma cell lines, SK-MEL-28 and A375, where Smoothened, a pivotal upstream regulator of the Hh pathway, and GLI1, its primary transcription factor, were chemically inhibited. The findings revealed a connection between CAXII and the Hh pathway, demonstrating that their inhibition significantly impaired melanoma migration and invasion, especially under hypoxia.

Then, due to the relevance of DCs in the TME and DCs-based immunotherapy in melanoma, we focused our attention on DCs with the aim to study CAXII expression and role. For the first time, we observed an increased expression of CAXII in mature DCs (stimulated with lipopolysaccharide, LPS) either under normoxia or hypoxia. To understand the involvement of CAXII in DC migration we first inhibited CAs with the chemical compound acetazolamide (AAZ), observing a downregulation of CAXII expression. We performed a migration assay in the presence of AAZ, observing a reduction in DC migration. In addition, to define if CAXII itself was crucial in DC migration, we knocked down the protein with a siRNA approach under normoxia and hypoxia, either in the presence or not of LPS. Finally, we performed a migration assay, demonstrating that CAXII siRNA significantly reduced DC migration under all experimental conditions.

Overall, these data suggest a similar role of CAXII in two different cellular models highlighting its involvement in cell migration, specifically related to Hh pathway in melanoma cells, especially under hypoxic conditions. These findings acquire a major impact on the development of new therapeutical strategies targeting CAXII in a hypoxic microenvironment, a typical feature of physiological and pathological conditions.

1. INTRODUCTION

1.1 Hypoxia

Accumulation of oxygen (O_2) in Earth's atmosphere starting approximately 2.5 billion years ago led to the evolution of the system of oxidative phosphorylation that transfers chemical energy stored in carbon bonds of organic molecules to the high-energy phosphate bond in ATP, which is the main source of energy used to power chemical reactions in living cells [Lane, N., & Martin, W., 2010]. From the little primitive metazoan species, where O_2 could diffuse from the atmosphere to organism's thousand cells (e.g. *Caenorhabditis elegans*), we arrive at a more complex organism (e.g. Drosophila melanogaster), where a system designed to conduct air to cells evolved to guarantee sufficient O_2 delivery. The final step in even more complex organisms (e.g. Vertebrates) is represented by the evolution of complex respiratory and circulatory systems designed to efficiently capture and distribute O_2 to hundreds of millions of millions of cells [Semenza, 2012].

O_2 is crucial for normal physiological function and aerobic metabolism in most living organisms, especially eukaryotes [MacIntyre, 2014]. Indeed, the maintenance of tissue and cell homeostasis in response to O_2 fluctuations is a result of a complex evolution of mechanisms, which sense changes in O_2 levels in the atmosphere and generate a response to adapt to these changes [Nakazawa et al., 2016]. In normal circumstances, O_2 acts as the main character in mitochondria, during oxidative phosphorylation to produce ATP [Cummins et al., 2016]. In addition, the presence of non-mitochondrial O_2 is necessary for its reserve and for acting, as a sensor signal, to understand if specific tissues are receiving a sufficient O_2 supply or not, based on their ongoing metabolic process [Taylor, 2008].

However, when the state of O_2 homeostasis is disrupted, it occurs hypoxia conditions [Cummins et al., 2016], in which O_2 availability is reduced [Semenza, 2020] as O_2 demand exceeds supply [Samanta and Semenza, 2018]. As a response to hypoxia some cells can activate the transcription of specific genes to better adapt to the new microenvironment [Semenza, 2020], while other cells can go under apoptosis [Martin et al., 2010]. However, hypoxia is a characteristic of both physiological and pathological conditions [Monaci etal., 2022]. We find physiological hypoxia within fetal development and hematopoiesis [Taylor and Colgan, 2017]. Tissue hypoxia can be associated with some pathological conditions such as hypoxemia, given by high altitude, where oxygen tension (pO_2) is decreased [Taylor and Scholz, 2022], impaired oxygen delivery to tissues, heart attack, stroke, ischemic diseases, tumors and inflammation disease [MacIntyre, 2014; Wu et al., 2022]. In fact, inflammatory conditions are marked by an increased production of inflammatory enzymes, cytokines, and activated neutrophils, which are drawn to the inflammation site, resulting in a higher oxygen demand [Cummins et al., 2016]. Additionally, hypoxia plays a role in tumor immune evasion, as the release of immunosuppressive molecules by cancer cells can inhibit the maturation of DCs [Vaupel and Multhoff, 2018; Wu et al., 2022].

1.1.1 Hypoxia inducible factor 1 α (HIF-1α)

Hypoxia is a condition that occurs frequently in both physiological and pathological conditions, the reason why organisms have developed adaptive cellular responses to maintain homeostasis avoiding bioenergetics crisis and tissue damage. HIFs (Hypoxia Inducible Factors) are the main transcription factors that play a crucial role in cellular responses to hypoxia. Indeed, a hypoxic inducible factor was first discovered by Gregg Semenza while he was studying the upregulation of erythropoietin (EPO) levels in low O_2 environments [Semenza et al., 1991; Semenza and Wang, 1992]; some years later he identified this factor as HIF [Wang and Semenza, 1995].

HIF is and heterodimeric transcription factor not only associated with the induction of adaptive responses to O_2 reduction, through several pathways including metabolic and functional ones [Taylor and Scholz, 2022], but also is involved in angiogenesis, cell survival and metastasis of tumors [Semenza, 2002].

Three HIF isoforms have been identified in mammals: HIF-1, HIF-2 and HIF-3. HIF consisted of two subunits: HIF-1α, which expression is O_2 dependent, and HIF-1β constitutively expressed by all types of cells [Wang and Semenza, 1995; Semenza, 2012].

As described in Figure 1, in normoxic conditions, O_2 and different cellular pathways such as ERK/AKT/mTOR, IKK/NF-κB and RAS/RAF/MEK/ERK [McGettrick and O'Neill, 2020] increased HIF1-α expression, which is immediately degraded through the ubiquitin-proteasome system. In particular, O_2-dependent hydroxylation of HIF-1α is done by three prolyl hydroxylases (PHDs), PHD1, PHD2 and PHD3 directly on two specified proline residues, Pro-402 and Pro-564 [Giaccia et al., 2004]. These two hydroxylated prolines are recognized by the Von Hippel-Lindau protein (VHLp), which is a component of ubiquitin E3 ligase complex that led to degradation by proteasomes [McGettrick and O'Neill, 2020; Taylor and Scholz, 2022]. Moreover, in normoxia, Factor Inhibiting HIF (FIH), by the modification of an asparagine residue on HIF-1α, inhibits the binding of HIF with its coactivator p300/CRB [Lando et al., 2002].

However, in hypoxic conditions, both PHDs and FIH are inhibited due to the lack of their necessary substrate, which is O_2. As a result, HIF-1α accumulates and translocates into the nucleus where, after its interaction with HIF-1β and p300/CRB coactivators [Nizet and Johnson, 2009], bind to Hypoxia Response Elements (HREs) with the consequent activation of the transcription of its target genes [Taylor and Scholz, 2022]. Furthermore, STAT3 impairs VHLp binding to HIF-1α and along with mTOR promotes the binding of hypoxia factor to its coactivators p300/CRB [McGettrick and O'Neill, 2020].

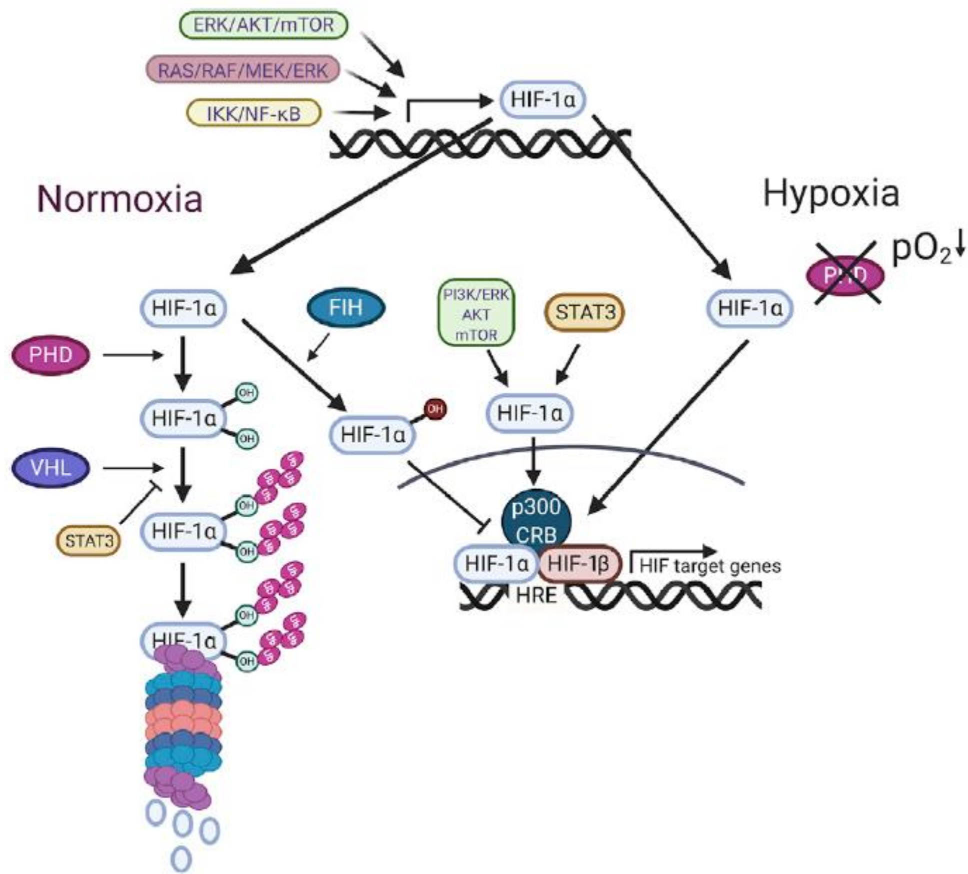

Figure 1. HIF-1α regulation under normoxia and hypoxia. Several pathways, including PI3K/AKT/mTOR, RAS/RAF/MEK/ERK and IKK/NF-κB can promote HIF-1α expression, which is constitutively expressed. In normoxic conditions, HIF-1α is degraded by ubiquitin proteasome complex, but under hypoxia, the degradation is inhibited, HIF-1α is accumulated in cytoplasm and translocates to the nucleus, where, after its dimerization with HIF-1β, it binds to HRE leading to the transcription of HIF target genes [From McGettrick and O'Neill, 2020].

As said before, HIF-1α is expressed in all cell types, whereas HIF-2α and HIF-3α have tissue-specific expression patterns. Notably, HIF-3α, the most recently discovered member of the HIF family, has multiple splicing variants and frequently functions as an antagonist to the other two isoforms, which share a similar structural composition [Bao et al., 2021].

The expression of more than one hundred genes can be regulated by HIF, as they are involved in a wide variety of functions, as schematized in figure 2, to regulate low O_2 stress: metabolism, angiogenesis (e.g. VEGF), cell differentiation and apoptosis either in physiological or pathological conditions. In addition, HIF plays a pivotal role in modulating the immune response in both innate and adaptive immunity. Also the apoptotic process is influenced by HIF: BNIP-3 and BNIP-3-L, members of the BH3-only protein family of cell death factors, are associated with Bcl-2 and Beclin-1 complex, but under hypoxic conditions, this complex is disrupted, and due to their upregulation by HIF-1α, they can activate autophagy in hypoxic cells [Nakazawa et al., 2016].

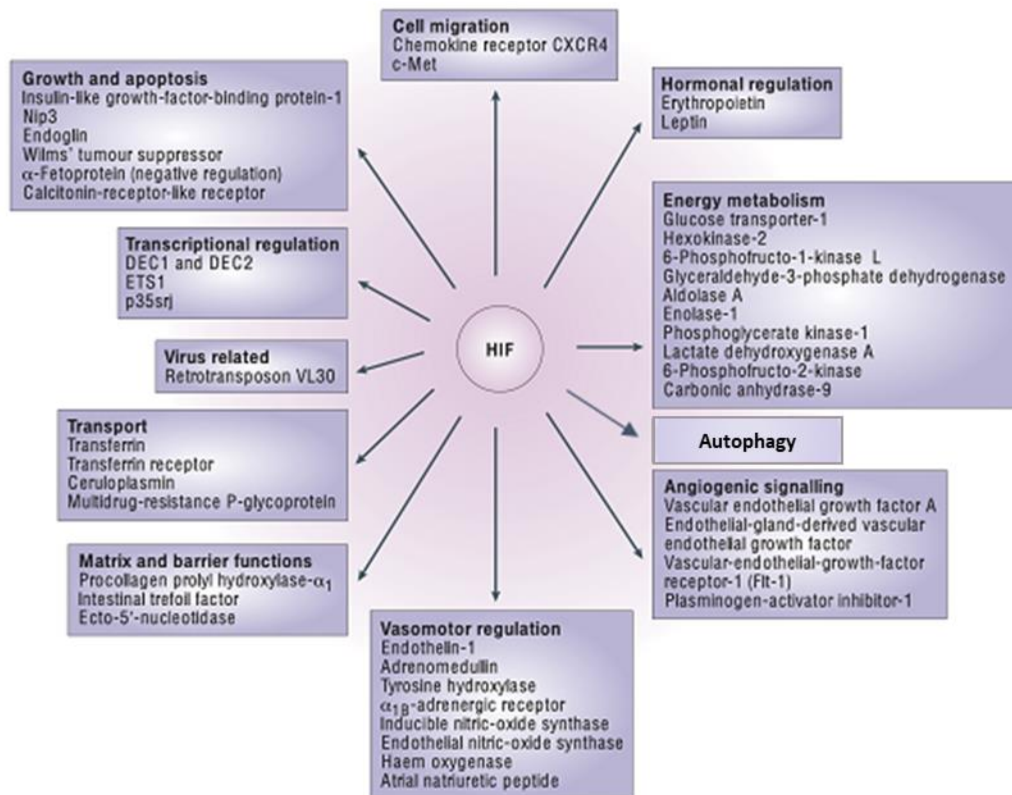

Figure 2. HIF involvement in cellular processes. HIFs modulates diverse cellular and systemic adaptive responses through inducing the activation of its multiple and different target genes. [Adapted from Schofield and Ratcliffe, 2004].

Mitophagy, a process of autophagy-mediated mitochondrial degradation, can also be activated to protect cells exposed to low oxygen levels, from excessive reactive oxygen species (ROS) production and DNA damage, thereby preventing apoptosis [Rubinsztein et al., 2012; Zhang and Ney, 2009; Mazure and Pouysségur, 2010]. The expression of the pro-apoptotic protein BNIP3 is upregulated under moderate hypoxia, but its ability to induce cell death depends on acidosis, a condition caused by metabolic alterations in response to HIF activation. Notably, in severe hypoxia, as described in figure 3 the activation of Carboxy-terminal Transcription Activation Domain (C-TAD) genes, such as CAIX, becomes crucial, but due to the activation of N-TAD genes (under moderate hypoxia), we have the expression of the pro-apoptotic protein BNIP-3, which require acidosis to promote cell death. Indeed, in the tissue microenvironment, oxygen levels vary depending on a cell's proximity to blood capillaries, following a decreasing gradient, while lactate and CO_2 concentrations increase with a consequent decrease extracellular pH. In response to hypoxia, CAIX expression is increased in order to maintain intracellular pH balance. Meanwhile, the proapoptotic protein BNIP-3 is activated under moderate hypoxia but requires acidosis to induce cell death effectively.

The oxygen gradient from the blood vessel to the tumor core also regulates the activity of PHD and FIH proteins. The Michaelis constant of these enzymes indicates that PHD proteins have a lower oxygen affinity than FIH, making g them more susceptible to inhibition. At

moderate hypoxia, HIF-1α remains stable due to PHD inactivation, yet genes requiring C-TAD activity are still repressed by FIH. However, only N-TAD genes are expressed. As oxygen levels drop further, FIH inhibition is lifted, allowing HIF-1α to reach full transcriptional activity. This dual regulatory mechanism of HIF-1α enables distinct gene expression patterns depending on FIH activity. [Pouysségur, 2006].

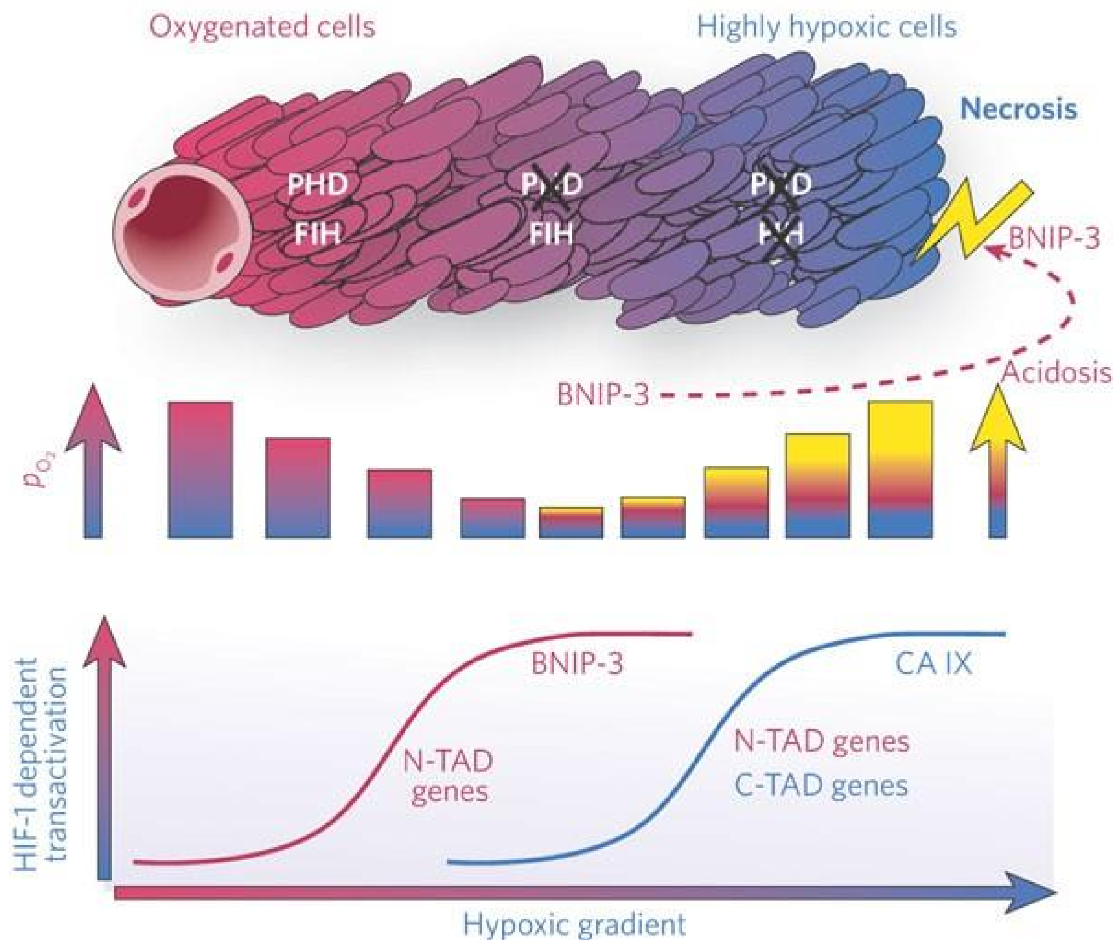

Figure 3. HIF-1 dependent genes activation and cellular adaptation through increment of hypoxic gradient. Severe hypoxia C-TAD genes induced the expression of carbonic anhydrase IX (CAIX), which is involved in balancing intracellular pH, but, due to the activation of N-TAD genes, which occurs under moderate hypoxia, it is induced the expression of the pro-apoptotic protein BNIP-3, which require acidosis to enhance cell death. [From Pouysségur et al., 2006]

1.2 Carbonic Anhydrases

Carbonic Anhydrases (CAs) are a group of evolutionary enzymes conserved in all living organisms. CAs catalyze the reversible hydration of carbon dioxide (CO_2) to yield bicarbonate (HCO_3^-) and protons (H^+) and are involved in physiological and pathological processes. CAs are involved in lots of physiological processes in all organisms in which they are present, such as respiration, photosynthesis, pH and CO_2 homeostasis control, CO_2 and bicarbonate transport [Supuran, 2008].

For instance, mammalian cells produce carbon dioxide because of sugar and fat breakdown, and they need to be removed. The CO_2 produced in peripheral tissues by cellular aerobic metabolism leaves the cells and enters the bloodstream. CAs convert to HCO_3^- the CO_2, which flows into red blood cells. The bicarbonate exits the red blood cells via anion exchanger protein and arrives to the lungs via the bloodstream. At this level, through the action of the reverse reaction catalyzed by CAs, bicarbonate is transformed into water and CO_2. The CO_2 produced is then released into the bloodstream and, passing through the alveolus walls, is exhaled [Boron, 2010].

In plants, CO_2 is stored as bicarbonate ions: CAs convert HCO_3^- ions to CO_2, which is concentrated in the proximity of the enzyme Ribulose Bisphosphate Carboxylase/Oxygenase (RuBisCO) present in the stroma of the chloroplasts. As a result, the performance of RuBisCO carboxylation reaction is increased [Monti et al., 2013].

Furthermore, CAs play a pivotal role in coral outer surface calcification [Del Prete et al., 2017] and in bacteria, the CAs-catalyzed reaction is the only known process used to balance endogenous levels of CO_2, H_2CO_3, and HCO_3^-, rapidly [Supuran andCapasso, 2016].

Eight classes of CAs (α, β, γ, δ, ζ, η, θ and ι) are known at present days and while lots of organisms express two or more CAs classes, only α-CAs are present in mammals and 16 isoforms have been identified, as represented in figure 4. They play a pivotal role in physiological processing and maintaining homeostasis, but CAs dysregulation is often associated with pathological conditions [Nocentini et al., 2021].

The physiological roles of CAs span multiple systems in humans. For instance, in the lungs and peripheral tissues, carbonic anhydrases play a vital role in the transport of CO_2, a byproduct of cellular metabolism. Within red blood cells, CAII facilitates the rapid hydration of CO_2 to bicarbonate. This bicarbonate is transported out of the cells in exchange for chloride ions, allowing CO_2 to be carried in the blood plasma to the lungs [Supuran, 2008]. In the kidneys, CAs are essential for maintaining acid-base balance by modulating urinary acidification and bicarbonate reabsorption. Isoforms such as CAII and CAIV are expressed in renal tubular cells, where they are involved in the regulation of reabsorption along the nephron [Lee et al., 2023]. Finally, they are involved in bone remodeling, as osteoclasts rely on CAII for bone resorption. The enzyme generates protons that are secreted into the extracellular space, dissolving hydroxyapatite and enabling the breakdown of bone matrix, a process vital for bone remodeling and calcium homeostasis [Sly et al., 1983].

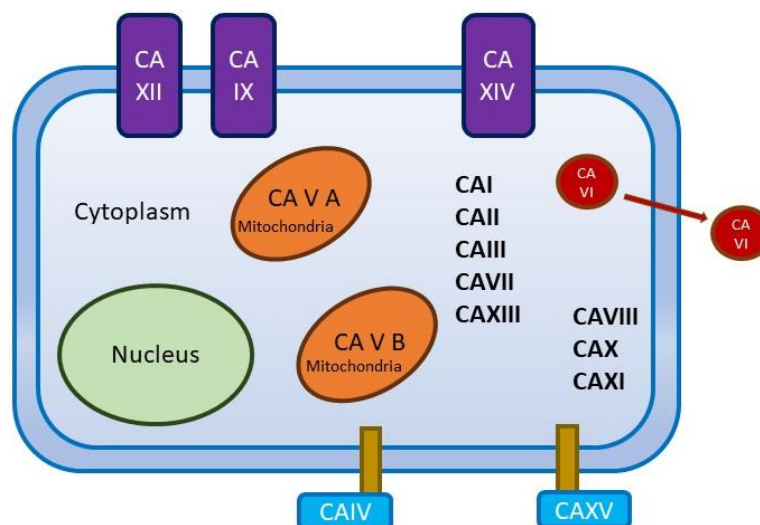

Figure 4. Schematic representation of human α-CAs. Representation of α-CAs along with their different subcellular localizations [realized with Microsoft Power Point].

1.2.1 Carbonic anhydrases and pathology

However, dysregulation of CAs activity or expression is often related to the arise of pathological conditions. Indeed, one of the most studied pathological roles of CAs is in cancer biology. Isoforms such as CAIX and CAXII (figure 5) are frequently overexpressed in solid tumors, particularly under hypoxic conditions driven by HIF-1. High CAIX expression has been correlated with aggressive phenotypes and poor prognosis in cancers such as renal cell carcinoma, breast cancer, and glioblastoma. Due to these roles, CAIX and CAXII have emerged as promising targets for the development of anticancer drugs, with specific inhibitors currently under investigation [Supuran, 2020].

Furthermore, dysregulation of CAs activity is relevant in other diseases. CAII, found in renal tubules, brain, and osteoclasts, is critical in acid-base homeostasis and bone remodeling. Deficiency of CAII gives rise to a syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification with associated developmental delay. It is inherited in an autosomal recessive way. Bone marrow stem cell replacement cures the osteoclast component of CAII deficiency, but it appears to have little or no effect on the renal lesions [McMahon et al., 2021].

CAs activity also plays a critical role in glaucoma, where excessive production of aqueous humor is linked to increased intraocular pressure. Pharmacological inhibition of CAs, particularly CAII and CAIV, with sulfonamide-based inhibitors such as acetazolamide, has been a cornerstone in the management of this condition [Tsikas, 2024]. Moreover, CA

inhibitors have therapeutic applications in treating altitude sickness, epilepsy, and certain diuretic needs [Pastorekova., et al. 2008].

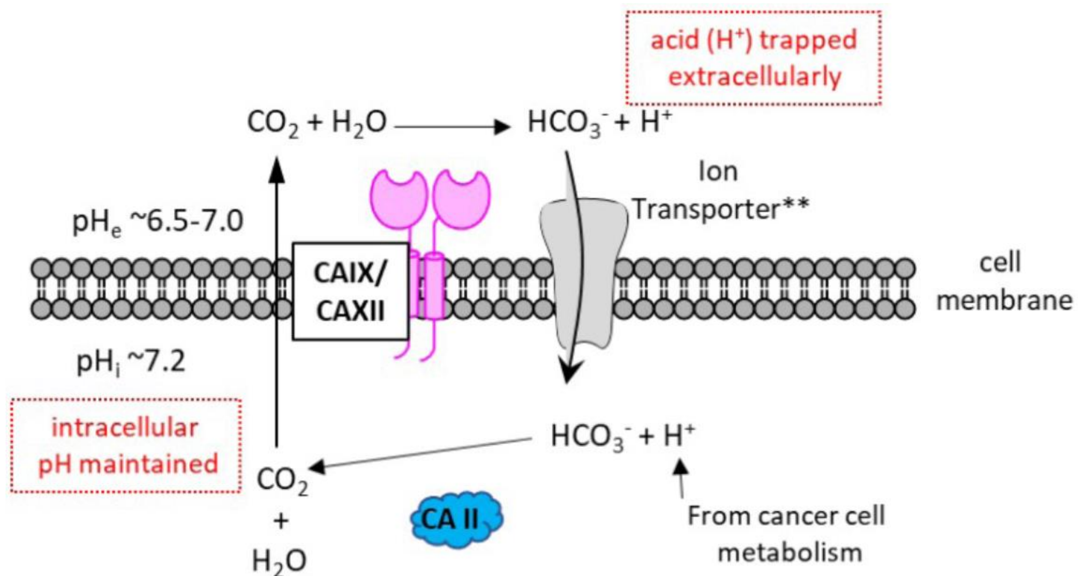

Figure 5. CAIX/CAXII working model. CAs catalyze the reversible hydration of carbon dioxide (CO_2) to yield bicarbonate (HCO_3^-) and protons (H^+). In detail CAIX/CAXII and CAII which, when dysregulated, are involved in pathological conditions [From Tonissen et al., 2021].

1.3 The tumor microenvironment

The tumor microenvironment (TME) is characterized by low O_2 levels and this condition plays a critical role in cancer progression, metastasis, immune evasion and resistance to therapy [King R. et al., 2021]. Tumor hypoxia arises due to the rapid proliferation of cancer cells outpacing the development of an adequate blood supply, leading to regions within the tumor that are deprived of oxygen. HIF-1α regulates the expression of various genes involved in angiogenesis, metabolism, invasion, and survival, thereby promoting tumor adaptation and aggressiveness [Semenza, 2012]. For instance, under hypoxia, HIF-1α induces the expression VEGF, which stimulates the formation of new blood vessels from pre-existing ones to supply the tumor with nutrients and oxygen. However, these newly formed vessels are often abnormal and inefficient, contributing to a chaotic blood flow and perpetuating the hypoxic conditions within the tumor [Monaci et al. 2024]. In addition to this, HIF-1α upregulates the expression of genes involved in epithelial-mesenchymal transition (EMT), a process by which cancer cells acquire migratory and invasive properties. This enables them to detach from the primary tumor, to invade surrounding tissues, and eventually disseminate to distant organs. Moreover, hypoxia-induced changes in cellular metabolism, such as a shift towards glycolysis (known as the Warburg effect), support the

survival of cancer cells in the nutrient-deprived conditions of the tumor microenvironment [Rankin, E. B., & Giaccia, A. J. 2016].

Particularly HIF-1α promotes angiogenesis via VEGF and metabolic reprogramming toward anaerobic glycolysis [Semenza, 2010]. The reliance on glycolysis produces excessive lactate, which, together with protons, is exported by monocarboxylate transporters (MCTs), contributing to extracellular acidification. This acidic environment (pH ~6.5–6.8) is further intensified by the activity of CAIX and CAXII, which are upregulated in hypoxic tumors [Swietach et al., 2010] influencing tumor progression and promoting immune evasion by impairing cytotoxic T cells and natural killer cell functions [You L. et al., 2021]. Moreover, acidification enhances the invasive and metastatic potential of cancer cells by activating cathepsins and matrix metalloproteinases (MMPs), which degrade the extracellular matrix (ECM), and by upregulating epithelial to EMT pathways [Estrella et al., 2013].

Indeed, the presence of hypoxia within tumors is associated with poor prognosis in many cancers, as it not only promotes more aggressive tumor behavior but also contributes to resistance to conventional therapies, including chemotherapy and radiotherapy. Indeed, the altered metabolism and increased drug efflux in hypoxic cancer cells reduce the effectiveness of many chemotherapeutic agents [Vaupel, & Mayer, 2007]. In addition, the hypoxic TME is infiltrated by immune cells, with an effect of hypoxia on them, as hypoxia control mechanisms that suppress anti-tumor immunity and promote tumor progression [Diaz-Montero et al. 2009].

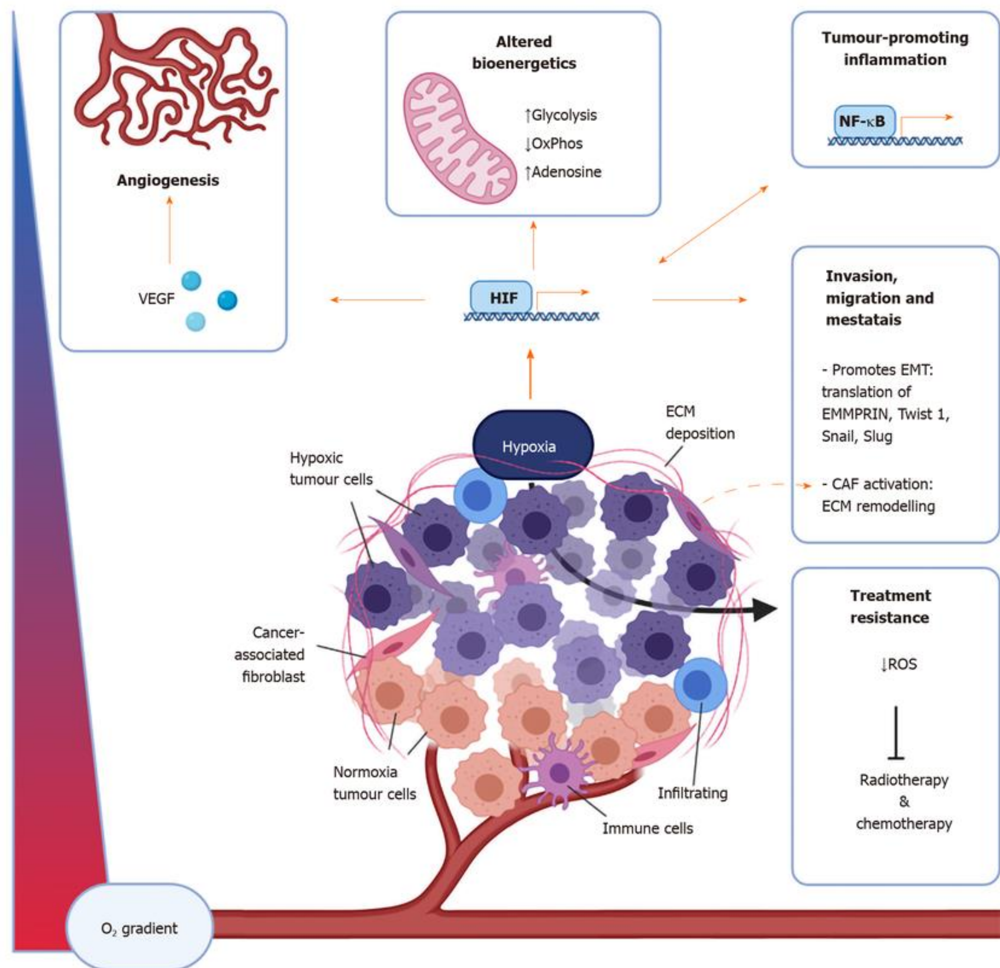

Figure 6. **Schematic representation of the TME.** TME within the cells composition: cancers cells, immune cells, cancer-associated fibroblast exposed to a hypoxic gradient with the associated expressed pathways [King et al., 2021].

2. AIMS

The TME is characterized by a variety of different cells, including cancer cells and immune cells, such as the Dendritic Cells (DCs). In addition, the TME is characterized by hypoxia, which is related to the expression of specific CAs (including CAXII). Indeed, CAs are necessary to maintain intracellular pH, thus their dysregulation is related to pathological conditions.

Thus, the first aim of this thesis was to address the potential impact of CAXII in cancer cells such as melanoma and, in particular the involvement of CAXII in cell migration especially under hypoxia and in relation with the Hedgehog (Hh) pathway

The second aim of the thesis was to analyze CAXII expression in human monocytes derived DCs and its involvement in DC migration abilities, especially under hypoxia.

3. INTRODUCTION (PART I): Melanoma, the Hedgehog pathway and Carbonic Anhydrases

3.1 Melanoma

The first known description of melanoma appeared in Hippocrates' writings in the 5th century B.C., [Rebecca et al., 2012] but the earliest evidence of melanoma was detected in in the skeletons of Pre-Colombian mummies (~2400 years old) found in Peru [Urteaga, Pack, 1966].

At present days, melanoma is the third most common skin cancer after basal cell carcinoma and squamous cell carcinoma [Sundararajan et al., 2022] and it is the most aggressive and lethal form, responsible for over 80% of skin cancer-related deaths [Saginala et al., 2021]. In the U.S., an estimated 100,000 new cases of invasive melanoma were expected in 2020, with around 7,000 deaths attributed to the disease and the trend is still increasing. Women tend to have better prognosis and survival rates than men, likely due to interactions between sex hormones and melanoma cells. Other risk factors include fair skin, numerous moles, UV exposure, age, and a family history of skin cancer [Eddy & Chen, 2020].

However, UV light represents a major contributor to cutaneous melanoma genesis due to direct DNA damage, generating thymidine-dimers, which, if unrepaired by nucleotide excision repair (NER), cause errors in DNA replication, with consequent mutations in cell signaling molecules, and ultimately carcinogenesis, as schematized in figure 7 [D'Orazio et al., 2013].

As a matter of fact, RAS is mutated in about 15% of human cancers. The three RAF genes code for cytoplasmic serine/threonine kinases that are regulated by binding RAS [Teixido et al., 2021]. Indeed, approximately 50% of advanced melanomas derive from BRAF somatic missense mutations, primarily at codon 600, with V600E being the most common variant. These mutations drive the activation of the MAPK pathway, which is critical for tumor growth and progression [Cheng et al., 2018].

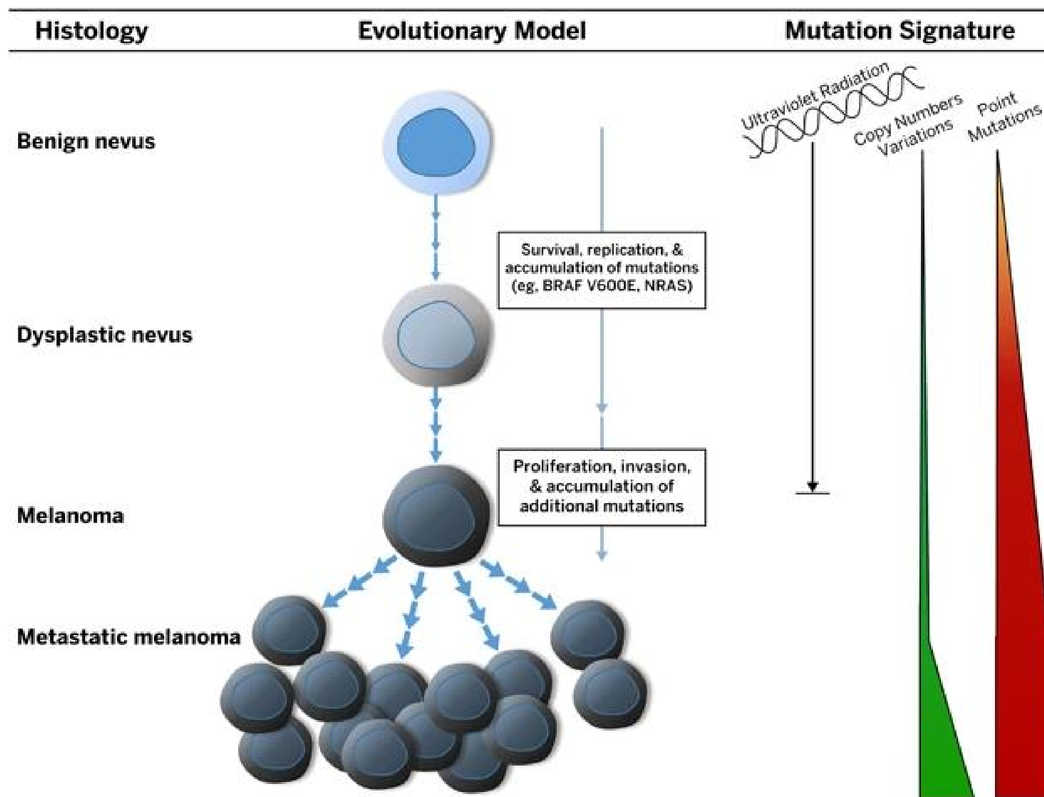

Figure 7. From normal nevus to melanoma. The main source of DNA damage is related to UV exposure, this can generate mutation that leads to melanoma progression. The further accumulation of mutations determines an increased cell proliferation and invasion (metastatic melanoma) [Cheng et al., 2018].

Melanoma can be categorized into three types: cutaneous melanoma, mucosal melanoma, and ocular melanoma, with cutaneous melanoma being the most prevalent. This type of cancer carries a significant risk of metastasis due to its origin from melanocytes, which are derived from neural crest cells in the skin [Rebecca et al., 2020]. Early detection allows for surgical removal of the primary tumor, which is the most effective treatment approach. However, due to melanoma's high metastatic potential, this remains a major concern. In advanced stages, the disease progresses to metastatic melanoma, where tumor cells spread from the primary site to distant locations [Damsky et al., 2011]. The treatment modalities currently used for metastatic melanoma include surgery, immunotherapy [Eggermont et al., 2016; Larkin et al., 2019], target therapy [Jenkins & Fisher, 2021] and chemotherapy.

3.1.1 Hypoxia and melanoma

Hypoxia, a condition characterized by reduced O_2 levels in tissues, plays a critical role in the progression and metastasis of melanoma. TME frequently experiences hypoxia due to inadequate vascularization and the rapid proliferation of cancer cells, which outpaces the oxygen supply [Wilson, 2011]. In melanoma, hypoxia has been shown to upregulate VEGF and other pro-angiogenic factors, fostering the formation of abnormal blood vessels that paradoxically exacerbate hypoxic conditions [Muz et al., 2015]. Moreover, hypoxia enhances the EMT, a process by which melanoma cells gain migratory and invasive properties, mediated in part by signaling pathways such as PI3K/AKT and MAPK, and the transcriptional repression of E-cadherin [Giatromanolaki et al., 2013; Halsey et al., 2013]. In addition, hypoxia induces metabolic reprogramming in melanoma cells, shifting them towards glycolysis via the Warburg effect to adapt to low oxygen conditions [Faubert et al., 2020]. This metabolic shift supports survival while creating an acidic extracellular environment that promotes immune evasion and extracellular matrix degradation [Fais et al., 2014]. Hypoxia also influences the immune microenvironment, reducing the efficacy of antitumor immune responses by upregulating immune checkpoint proteins such as programmed death-ligand 1 (PD-L1) and promoting the recruitment of immunosuppressive cells like regulatory T cells and myeloid-derived suppressor cells [Noman et al., 2015]. Furthermore, hypoxia is implicated in resistance to targeted therapies such as BRAF inhibitors and immune checkpoint inhibitors, partially through the activation of drug-resistance pathways and the selection of resistant cell clones [Vito et al., 2020; Eales et al., 2016].

3.2 The Hedgehog pathway

The Hh pathway was first discovered in *Drosophila melanogaster* and Hh was identified as a gene involved in segmental embryonic development including the development of Drosophila imaginal discs [Nusslein-Volhard and Eric Wieschaus, 1980].

In vertebrates three members of Hh were found: Sonic Hedgehog (SHh), Desert Hedgehog (DHh) and Indian Hedgehog (IHh). Even if DHh is the closest homolog of Drosophila Hh, its expression (and the same could be assessed for IHh expression) is related to restricted areas, SHh has the highest activity and is the most studied. The Hh pathway plays a pivotal role during embryonic development, but it is expressed also in the adult organism. It is involved in cancer progression, as overexpression of Hh signaling components has been associated with tumor spread out [Robbins et al., 2012].

In *Drosophila*, in the absence of Hh, Patched (PTCH) prevents Smoothened (SMO) membrane localization and activation so that SMO is retained in endosomal vesicles. In this situation, full-length Cubitus interruptus (Ci) is detained in a microtubule-associated complex containing the kinesin-like protein Costal2 (Cos2), the kinase Fused (Fu), and Suppressor of Fused (Sufu), which promotes phosphorylation of Ci and its partial proteasomal processing into a transcriptional repressor. Binding of Hh to the receptor PTCH

(and its co-receptor Ihog) results in the internalization of the ligand-receptor complex and phosphorylation and translocation of SMO to the plasma membrane. SMO interacts with Cos2 leading to the release of Ci and activation of the heterotrimeric Gi protein [Robbins et al., 2012].

As seen in figure 8, Hh signaling in vertebrates is similar to Hh signaling in *Drosophila*, with the important distinction that signaling takes place on primary cilia (PC).
The canonical Hh pathway activation is related to Hh ligand interaction with his receptor PTCH: this interaction determines the activation of a seven-pass transmembrane protein, SMO. SMO, when inactive, is internalized in endosomal vesicles, but when Hh interacts with Patched, SMO inhibition is no longer effective and translocates into the cell membrane. As said before, in vertebrates, the Hh pathway is associated with the PC, a sensory organelle that extends from the surface. This microtubule-based organelle acts as a sensor of the extracellular environment and oxygen levels, it is involved in the cell cycle and mediates several signaling pathways [Schneider et al., 2025]. SMO localizes to the PC when activated: PTCH inhibits Hh signaling by blocking the entry of SMO into the PC. The interaction between SHh to PTCH removes PTCH from the PC, allowing SMO to enter and, its activation, propagate the Hh signal. The precise mechanism by which PTCH inhibits SMO is still unclear. Indeed, the activation of Hh pathway, through the binding of Hh ligand, leads to the release of GLI from Sufu and the formation of activated GLI, which enters into the cell nucleus. Activated GLI exits the PC, translocates to the cell nucleus, and binds to its binding motif on DNA (5'-GACCACCC-3'), activating the transcription of target genes. On the other hand, in the absence of Hh ligand, GLI is phosphorylated by PKA, GSK3 and CK1 and retained in PC, promoting GLI3 activation, which inhibits the pathway [Teperino et al., 2014]. [Teperino et al., 2014]. GLI1 is the main transcription factor which through a ZZ domain binds to the DNA and activates the transcription of its target gene (Hh responsive gene) such as GLI1 itself, PTCH, SNAIL1, EMT genes, cyclins. On the other hand, GLI3 is a repressor of Hh responsive genes transcription, while GLI2 can act as both activator and repressor.

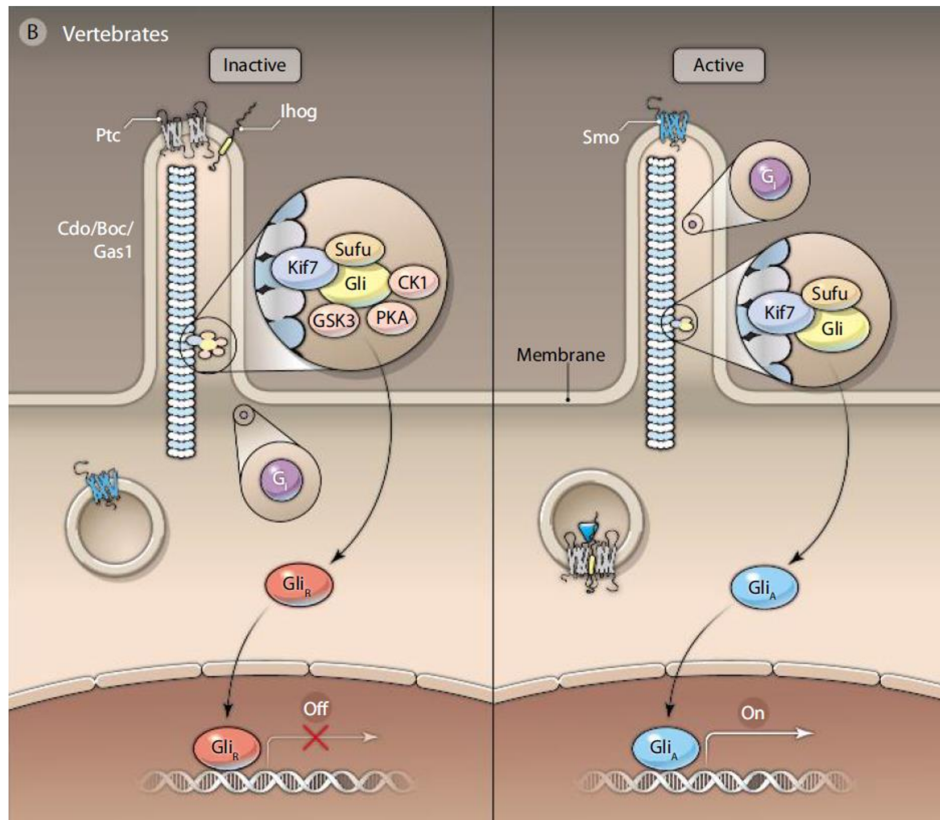

Figure 8. The Canonical Hh pathway in vertebrates. When the pathway is inactive SMO is retained in endosomal vesicles and GLI1 is not allowed to enter the cell nucleus. On the other hand, the activation of the pathway results in SMO activation, allowing GLI1 activation, translocation into the cell nucleus and transcription of its target genes [from Robbins et al., 2012].

In addition, the expression of PC in cancer cells is variable. Besides the “classical Hh pathway” (canonical pathway) there is evidence of a non-canonical Hh pathway, which can be SMO-dependent and SMO-independent. In cancer cells generally it is find a reduction in PC presence. Indeed, as represented in figure 9, canonical activation of Hh pathway relies on the activation of GLI, but GLIs can be activated by other signaling such as PI3K/AKT, RAS, ERK (non-canonical type-I SMO independent) and AMPF, Ca^{2+}; Rho, Rac (non-canonical type-II SMO dependent); in both cases, type-I or -II non-canonical Hh do not require GLI transcription factors [Robbins et al., 2012]. This highlights the possibility of having an activation of the Hh pathway, with the final result of GLI expression, which does not require the canonical pathway or the presence of SMO itself, as other signaling (e.g. PI3K/AKT) can directly affect GLI expression.

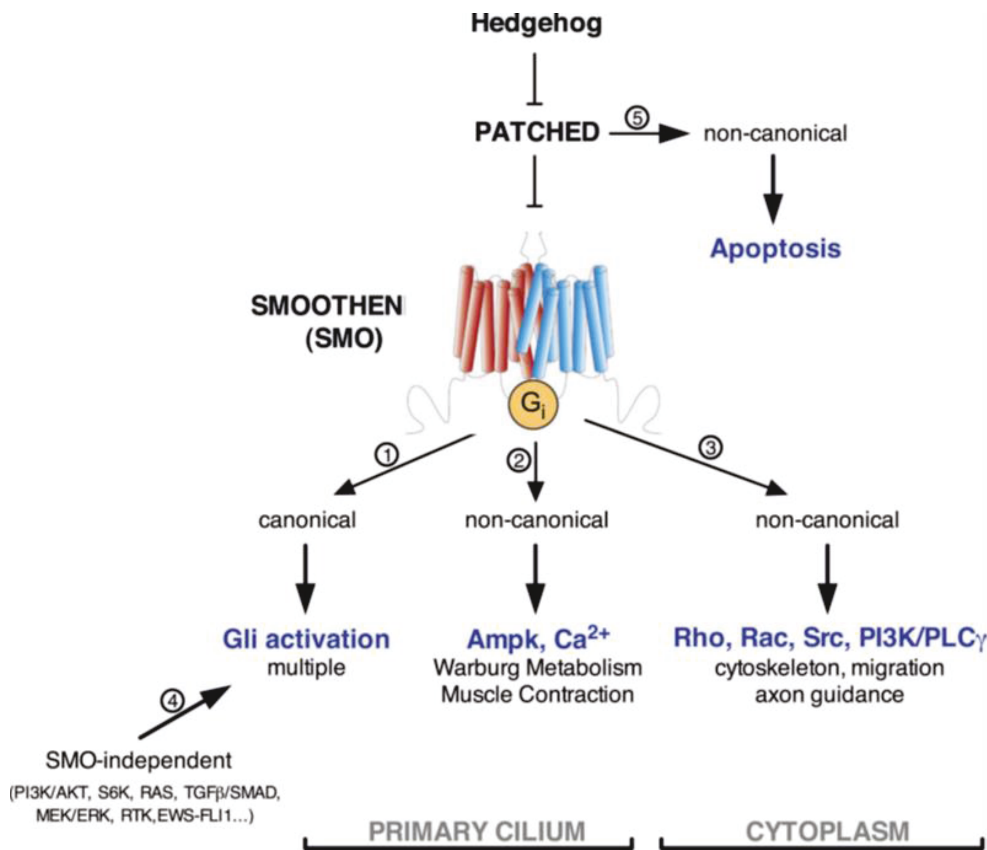

Figure 9. Different Hh pathway activation. Canonical Hh SMO dependent or SMO independent results in GLIs activation; Hh non-canonical pathway can be related to PC or cytoplasm only generating different responses [From Teperino et al., 2014].

In addition, aberrant activation of the Hh signaling pathway is associated with pathological conditions, such as developmental deformities and cancers [Taipal, Beachy, 2021]. According to the latest studies, the Hh signaling contributes to the development of one-third of all malignant tumors [Murone et al., 1999]. As a matter of fact, the dysregulation of any component within the Hh pathway leads to its aberrant activation, resulting in the malignant transformation of cells. There are three proposed mechanisms of aberrant Hh activation in different cancer types [Rubin, 2006].

Firstly, as described in figure 10, we find the ligand-independent activation of Hh signaling (type I) determined either by the activating mutations of SMO or inactivating mutations of PTCH or SUFU (Hh signaling negative regulators) causing a constitutive activation of Hh signaling in the absence of the ligand (a). This Hh aberrant activation was first discovered in patients with a rare autosomal dominant disorder, i.e., BCNS (Gorlin syndrome), where a PTCH mutation on chromosome 9 was found [Johnson et al., 1996]. Type- II Ligand-dependent autocrine/juxtacrine Hedgehog signaling (b) activates the pathway in a cell-autonomous manner, as Hh ligand is produced by and taken up by the same or surrounding tumor cells. The overexpression of this Hh signaling has been found in various tumors including stomach, esophageal, pancreatic [Berman al., 2003], breast [Kubo et al., 2004],

melanomas [O'Reilly et al., 2013], and other extracutaneous tumors. Finally, Hh signaling can be activated in paracrine way (type III ligand-dependent Hh signaling), which resulted in playing an important role during embryonic development, as Hh acts as a morphogen molecule (c). However, paracrine activation of the Hh pathway in stromal cells has also been associated with various cancers. In this contest, Hh ligands, secreted by tumor cells, bind to PTCH receptor on tumor stromal cells, which then activate the Hh pathway, generating a feedback loop, in which the stromal cells transmit the growth signals (e.g. VEGF, WNT) to tumor cells, supporting and promoting their proliferation and differentiation [Jiang et al., 2008; Skoda et al., 2018].

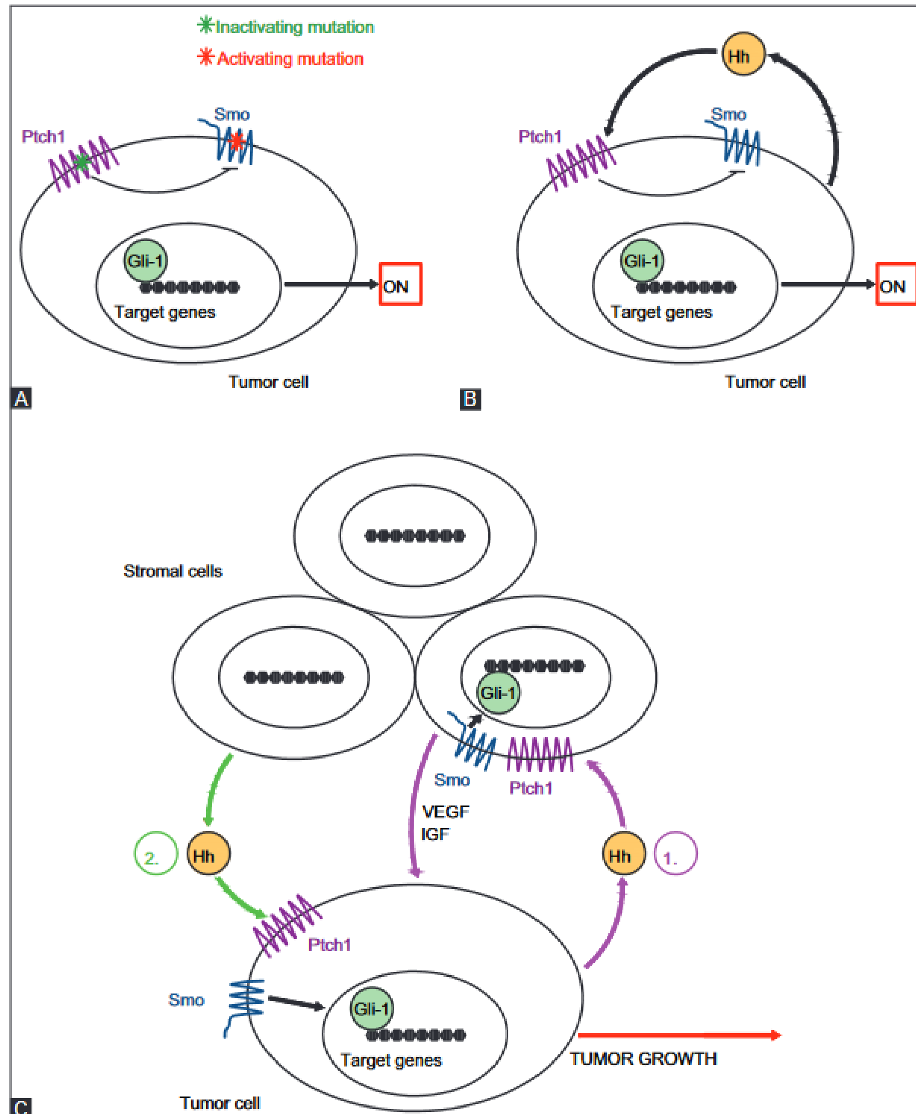

Figure 10. Three aberrant activations of the Hh pathway. Type-I ligand-independent activation of Hh signaling; type- II ligand-dependent autocrine/juxtacrine signaling; type-III ligand-dependent paracrine and reverse paracrine signaling [From Rubin, 2006].

3.2.1 Hypoxia and the Hedgehog pathway

Hypoxia plays a pivotal role in various biological processes, from evolutionary adaptations to disease pathogenesis and there is evidence of the cross-talk between the Hh pathway (in which a correct activation is crucial for embryonic development) and hypoxia condition. In the surface fish and cave fish *Astyanax mexicanus* (different morphs of the same species, which diverged from a common ancestor), the most molecular difference is related to the different expression of SHh during the embryonic development [Menuet, et al., 2007]. Indeed, in the cavefish *Astyanax mexicanus*, the interplay between hypoxia and the Hh signaling pathway has been identified as a driver of primitive hematopoiesis, crucial for survival in hypoxic subterranean environments. Elevated SHh signaling, stimulated by hypoxia, expands hematopoietic domains in the lateral plate mesoderm, increasing erythrocyte production. This hypoxia-driven SHh plasticity underscores a key evolutionary mechanism enabling cavefish to prosper in oxygen-limited habitats [van der Weele et al., 2024]. In addition, hypoxia and Hh pathway are involved in the development of odontogenic cysts, which are like autosomal dominant polycystic kidney disease: dysfunction of primary cilia and HIF-1α cause cystic transformations, with Sonic the Hh signaling driving epithelial proliferation and cavity formation [Szaraz, et al., 2023].

Furthermore, hypoxia-induced non-canonical Hh signaling in hepatocellular carcinoma accelerates tumor aggressiveness by promoting epithelial-mesenchymal transition through reactive oxygen species and GLI1 activation. Hypoxia and Hh pathway are linked in controlling embryonic development (and evolution), and, when dysregulated, cancer progression [Liu et al., 2017]. Of note, there is evidence of a close relationship between hypoxia and SMO, as hypoxia promotes the transcription SMO gene in pancreatic ductal adenocarcinoma (PDAC), in correlation with over-expression of the transcription factor Mastermind-like 3 (MAML3). Briefly, the study suggests that the MAML3 could regulate SMO transcription with a crosstalk with Notch signaling under hypoxia, driving the arise of the malignant phenotypes observed in hypoxic PDAC [Onishi et al., 2016].

3.2.2 Melanoma and the Hedgehog pathway

The Hh pathway contributes to melanoma tumor progression by modulating the microenvironment, promoting angiogenesis, and inducing drug resistance mechanisms [Kasper et al., 2006]. It has been shown that the overexpression of SMO and the upregulation of GLIs are associated with a more aggressive melanoma phenotype and poorer prognosis [Yang et al2010]. Moreover, the Hh signaling appears to interact with other oncogenic pathways, such as the MAPK/ERK pathway, which is frequently mutated in melanoma, creating a complex network that promotes tumor growth and metastasis [Atwood et al., 2012]. Pharmacological inhibition of the pathway has shown promising results in some types of tumors. In the last years, chemical compounds, such as SMO inhibitors (Vismodegib), GLI inhibitors (Gant61), or Hh ligand inhibitors (Ronbotinikinin) [Wang, X., et al., 2024] demonstrated to be effective therapeutic approaches for targeting the Hh pathway in contrast

to the traditional chemotherapy and radiotherapy. However, their application in melanoma is still limited by factors such as genetic variability and the development of resistance [Sekulic et al., 2012].

Nevertheless, targeted therapeutic combinations that include Hh pathway inhibitors could represent an innovative strategy to overcome resistance to BRAF and MEK inhibitors commonly used in advanced melanoma, improving clinical outcomes for patients [Liu et al., 2020]. In addition, it has been seen that ERK5 is necessary for Hh-GLI-dependent melanoma cell proliferation, as Hh signaling regulates proliferation and survival, while ERK5 supports cell cycle progression and proliferation and that it is often upregulated in a variety of tumors, including melanoma [Tusa et al., 2021]. Development of new small molecules capable of inhibiting Hh pathway could be crucial to manage melanoma cells spread out.

3.3 Carbonic Anhydrases and melanoma

In the context of melanoma, certain isoforms of carbonic anhydrase, particularly CAIX and CAXII, have been implicated in promoting tumor progression and metastasis. CAIX and CAXII are often overexpressed in hypoxic regions of tumors, where they play a critical role in regulating the acidic extracellular environment characteristic of cancer, which is necessary for invasive behavior [Supuran, 2020]. Recent studies suggest that CAIX expression correlates with poor prognosis in melanoma patients, underscoring its potential as a biomarker for disease aggressiveness. Indeed, melanoma cells release hypoxia-induced small extracellular vesicles expressing CAIX mRNA, that can be a potential liquid biopsy biomarker [Venturella et. al, 2023]. Additionally, pharmacological inhibition of CAs has emerged as a promising therapeutic strategy as they are capable of impairing cell proliferation and invasion [Pastorekova et al., 2007].

4. RESULTS (PART I)

4.1 HIF-1α and SMO expression in SK-MEL-28 and A375 cell lines

Firstly, as hypoxia can modulate melanoma cell migration and invasion, we analyzed HIF-1α accumulation in melanoma cell lines SK-MEL-28 (a) and A375 (b), after exposition to hypoxia. As seen in figure 11, a significant increase in HIF-1α accumulation was described under hypoxic conditions at all-time points, compared to the relative normoxic controls, in both cell lines. Concerning the same kinetics end-time, we analyzed SMO protein levels by western blot analysis. We highlighted an increased trend of SMO protein expression under hypoxic conditions, after 24h and 48h of exposure in both cell lines.
This result indicates an activation of the Hh pathway.

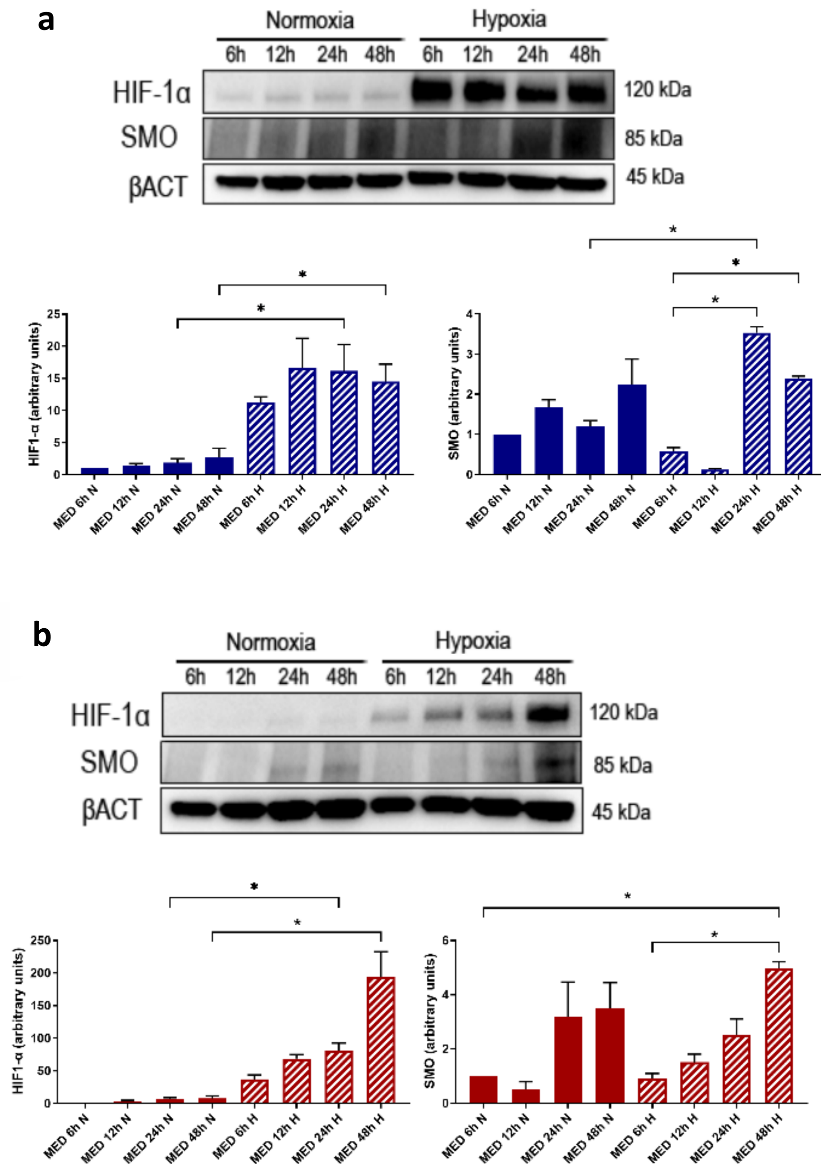

Figure 11. HIF-1α and SMO accumulation in melanoma cell lines. HIF-1α and SMO Western blot in SK-MEL-28 (a) and A375 (b) cell lines after exposure to normoxia and hypoxia. β-actin was used as loading control for Western Blot. Blots are representative images and data are presented as the mean and ± SEM of three independent experiments. * $p \leq 0.05$ indicates statistically significant differences.

In addition, as HIF-1α is a transcription factor, which translocates in the cell nucleus and dimerizes with HIF-1β to bind target sequences on DNA, we performed immunofluorescence analysis to confirm its nuclear translocation. We exposed SK-MEL-28 and A375 to hypoxia for 24h and looked for HIF-1α accumulation. As expected, and represented in figure 12, HIF-1α signal under hypoxia was significantly enhanced with respect to normoxic control. Furthermore, we performed a co-localization analysis between HIF-1α and the cell nucleus, which has been labeled with Hoechst, highlighting a present co-localization.

We concluded that HIF-1 was activated in our melanoma cell lines under hypoxia.

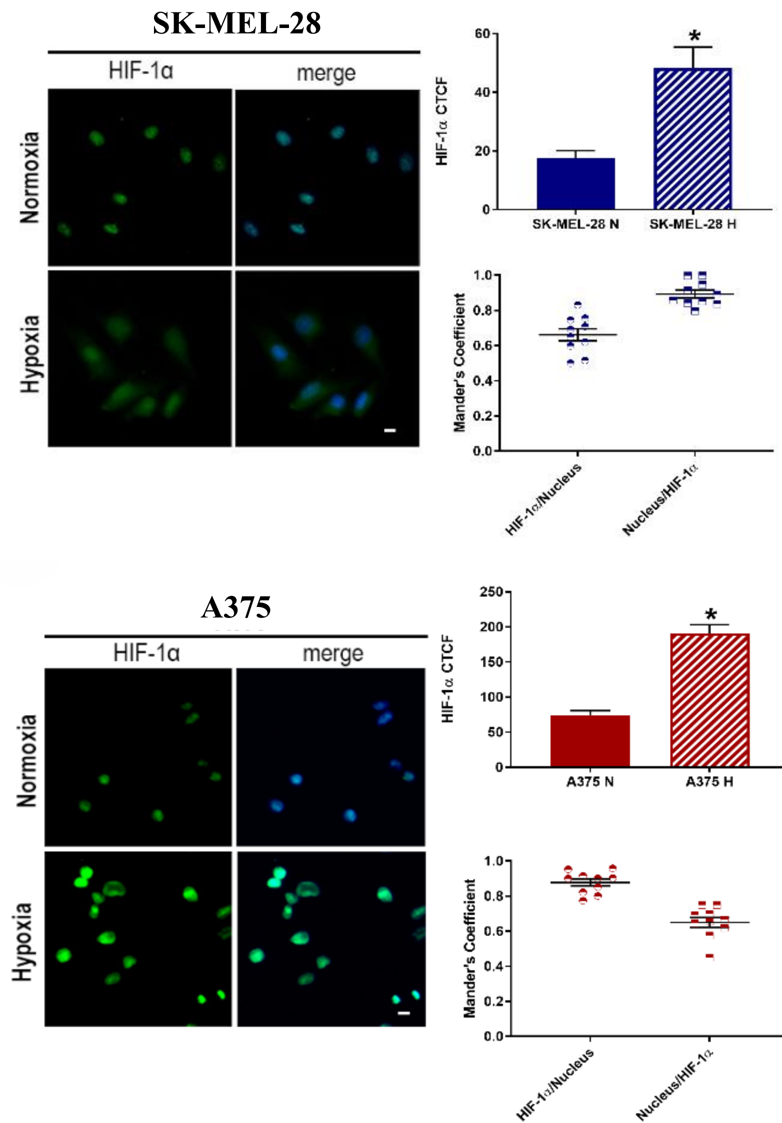

Figure 12. HIF-1α immunofluorescence. HIF-1α levels detected by immunofluorescence (20× magnification, scale bar 10 μm) in SK-MEL-28 and A375 under normoxia and hypoxia. Co-localization analysis of HIF-1α and cell nucleus indicating an effective co-localization expressed by Mander's coefficient as mean ± SEM (10 cells/sample) 0 (absence of co-localization) and 1 (maximum of co-localization); means ± SEM are presented. (n = 3; * p ≤ 0.05 indicates statistically significant difference).

4.2 Chemical compounds GlaB and C22 inhibit SMO and GLI1 expression in SK-MEL-28 and A375

We then decided to inhibit the Hh pathway by using new small molecules, GlaB and C22, which inhibit GLI1 and GLI1/SMO respectively. C22 is a derivate of GlaB isoflavone, which is present in a legumonosae. Thus, we treated SK-MEL-28, with 1 μM of our compounds and exposed cells to normoxia and hypoxia. The impact of the treatment was monitored by immunofluorescence. As shown in Figure 13, treatment with C22 reduced SMO expression under normoxia and significantly under hypoxia. Then, we analyzed GLI1 expression after chemical treatment: GLI1 protein level was reduced in normoxia and hypoxia by the two chemical compounds.

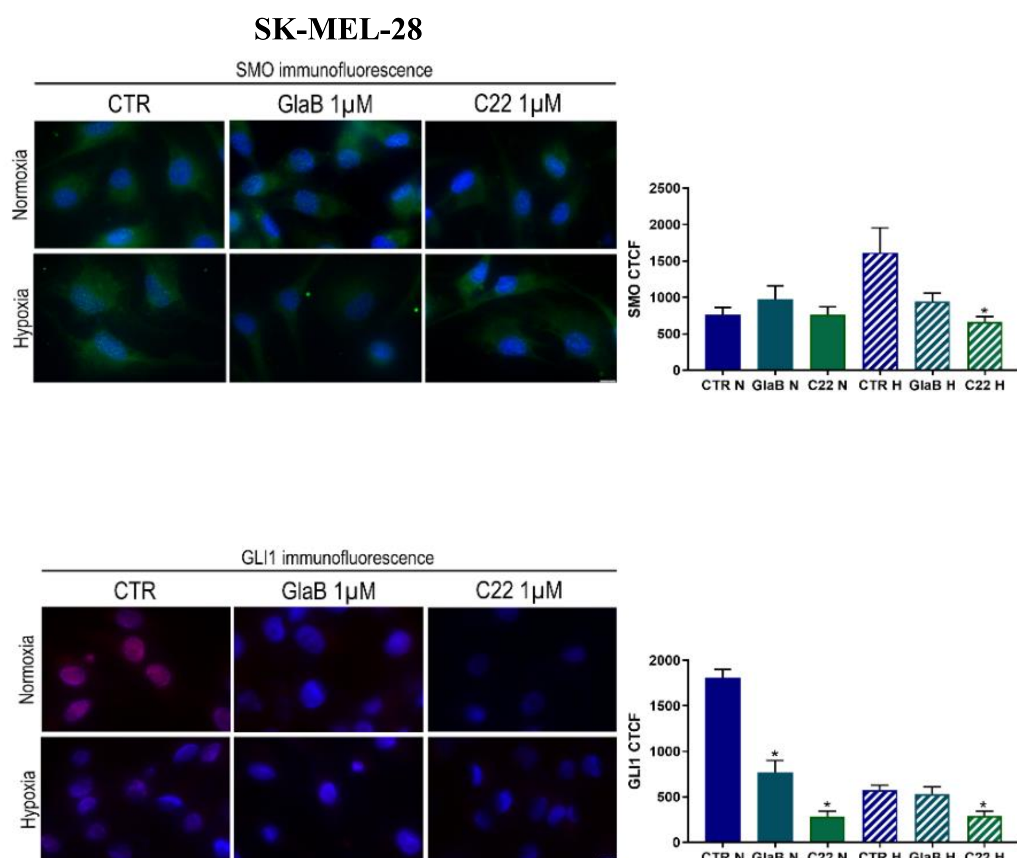

Figure 13. Chemical compounds inhibit SMO and GLI1 expression in SK-MEL-28. SMO and GLI1 immunofluorescence (60X magnification, scale bar 10 μm) after treatment with GlaB or C22 in SK-MEL-28 under normoxia and hypoxia. Images are representative of three independent experiments. Means ± SEM are presented. ($n = 3$; * $p \leq 0.05$ indicates statistically significant difference)

Then we replicated the chemical treatment in A375 cell line to see if they were effective also in this highly invasive cell line. We treated A375 with 1 μM of our compounds and exposed cells to normoxia and hypoxia for 24h. As shown in Figure 14, treatment with C22 reduced SMO expression under normoxia and more evidently under hypoxia. Then, we observed that GLI1 protein level was reduced in normoxia and hypoxia after chemical treatments. Of note, the reduction effect promoted by C22 was more evident and always significant under hypoxia in both cell lines. Interestingly, we detected an effect of GlaB, which is a specific GLI1 inhibitor, on SMO even if it was not significant. This phenomenon can be explained by the fact that in some cellular models, SMO could be a GLI1 target gene: indeed, inhibiting GLI1 activity can result in the downregulation of SMO itself [Doheny et al., 2020]. As a result, we demonstrated that new small molecules GlaB and C22 were able to impair GLI1 and SMO expression.

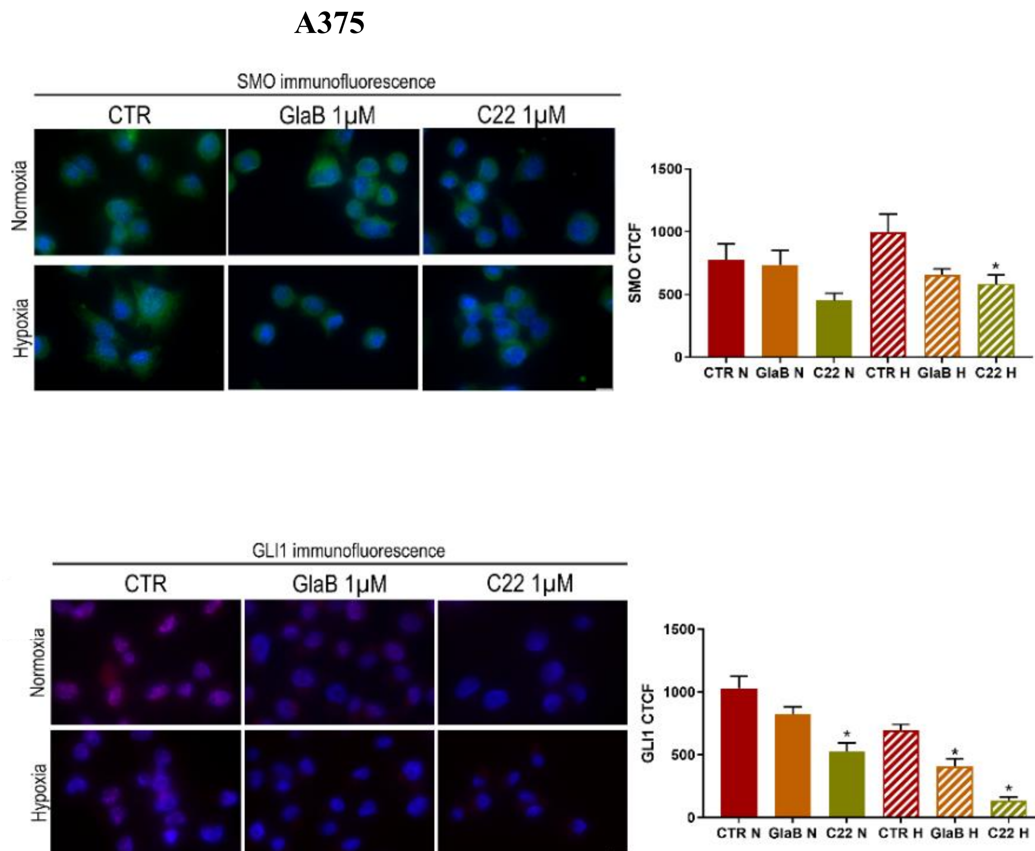

Figure 14. Chemical compounds inhibit SMO and GLI1 expression in A375. SMO and GLI1 immunofluorescence (60X magnification, scale bar 10 μm) after treatment with GlaB or C22 in A375 under normoxia and hypoxia. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference)

4.3 Hh inhibition resulted in melanoma cell migration impairment

To assess if Hh inhibition was able to impair melanoma cell migration we performed a migration assay in both cell lines after treatments with the two small molecules, GlaB and C22. We added AraC 2.5 µg/mL to culture media to inhibit cell proliferation. As shown in figure 15, images were acquired at 24h and 48h of treatment under normoxia and hypoxia. SK-MEL-28 migration (fig. 15a) was significantly impaired by the two compounds both under normoxia and hypoxia. About A375 cells (fig 15b) we showed a reduced migration ability in the presence of the two chemical compounds under normoxia and hypoxia. In the latter experimental condition, the reduced ability to repair the wound is statistically significant for C22. Of interest, the two small molecules were effective in impairing migration in both cell lines, either under normoxic and hypoxic conditions.

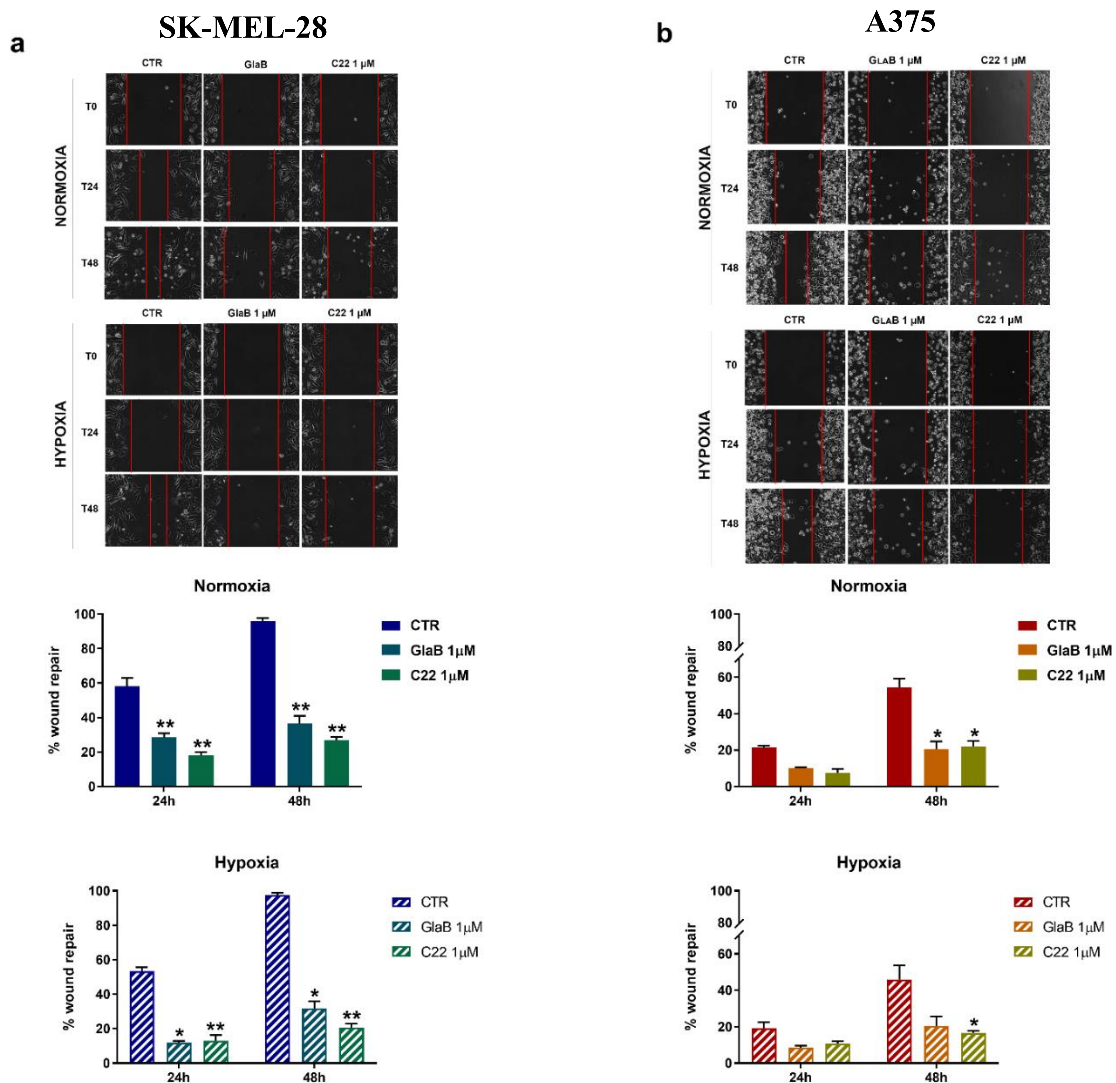

Figure 15. Melanoma cells migration after Hh inhibition. Cell migration measured by wound-healing assays under normoxic or hypoxic conditions in SK-MEL-28 (A) and A375 (B) treated with GlaB and C22 chemical compounds. Pictures are representative of three independent experiments. Statistical significance markers compare GlaB or C22 condition

to control (CTR). Means ± SEM are presented. (n = 3; * $p \leq 0.05$ and ** $p \leq 0.01$ indicate statistically significant differences).

4.4 Hh inhibition resulted in melanoma cell invasion impairment

As a next step, we tested the invasion ability of both melanoma cell lines performing a modified Boyden chamber assay and acquiring images of invaded cells at 24 h in order to observe the effect of GlaB and C22. In figure 16, we demonstrate that SK-MEL-28 invasion (fig 16a) was significantly reduced by GlaB under hypoxia, while C22 significantly reduced the number of invaded cells both in normoxia and hypoxia. Then we evaluated A375 invasion ability (fig 16b). Under normoxia and hypoxia, the two small molecules significantly reduced the invasion of A375 cells which present a more invasive phenotype with respect to SK-MEL-28.

As a result, we demonstrated that melanoma cell invasion can be inhibited by chemically targeting the Hh pathway under normoxia and hypoxia.

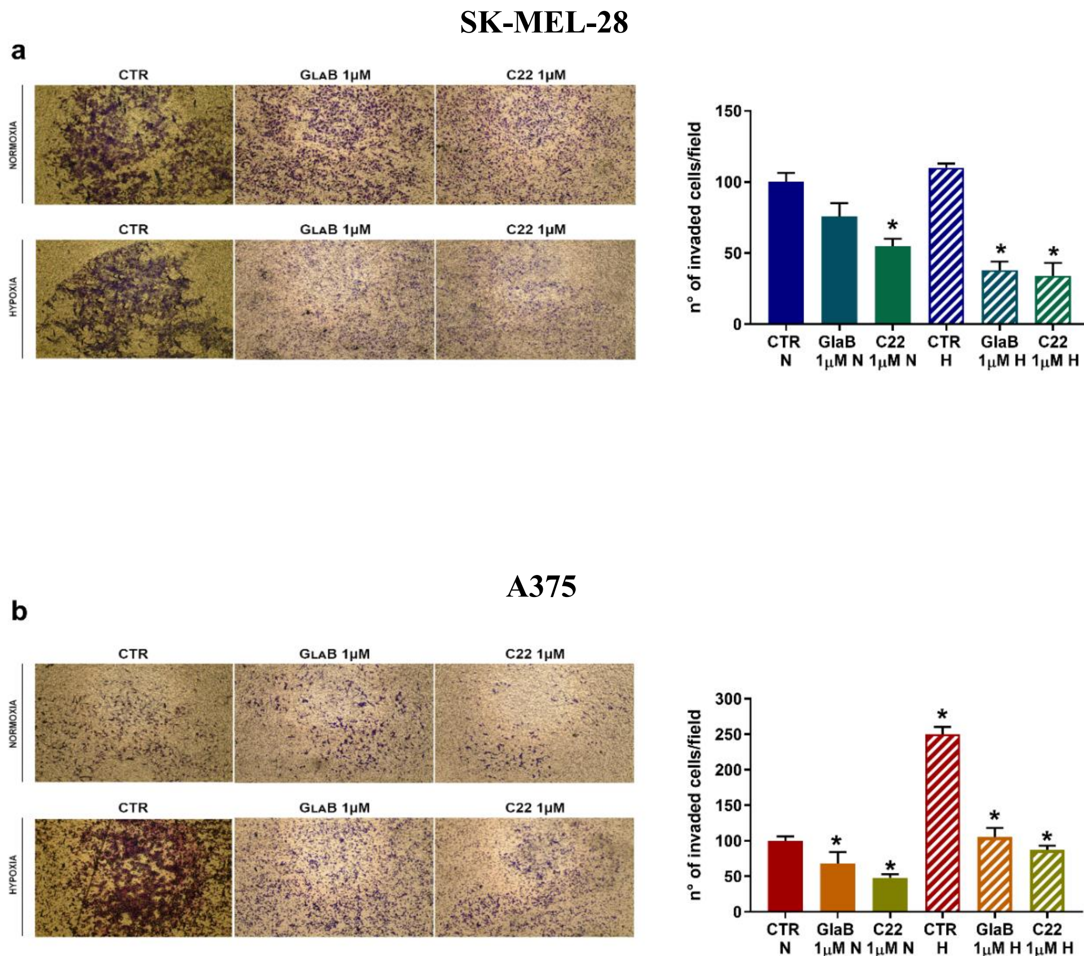

Figure 16. Melanoma invasion impairment after Hh inhibition. Cell invasion measured by modified Boyden chamber assay in SK-MEL-28 (A) and A375 (B) cells treated with GlaB and C22 under normoxic and hypoxic conditions. Pictures are representative of three independent experiments. Statistical significance markers compare GlaB or C22 condition to control (CTR), while CTR H is compared to CTR N. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference).

4.5 CAXII expression can be modulated by Hh interfering compounds

Then we studied CAXII role in melanoma cells with the aim to see if our novel small molecules could affect its expression. By immunofluorescence, we evaluated CAXII expression in SK-MEL-28 treated with GlaB and C22. As seen in figure 17, inhibition of GLI1 and SMO resulted in a downregulation of CAXII, which was significant under hypoxia in the presence of C22. Of interest, we detected a dotted appearance of CAXII in these cell lines, due to possible membrane accumulation. We managed to count CAXII dots demonstrating a significant accumulation in hypoxic conditions and a reduction when cells were treated with C22 chemical compound.

SK-MEL-28

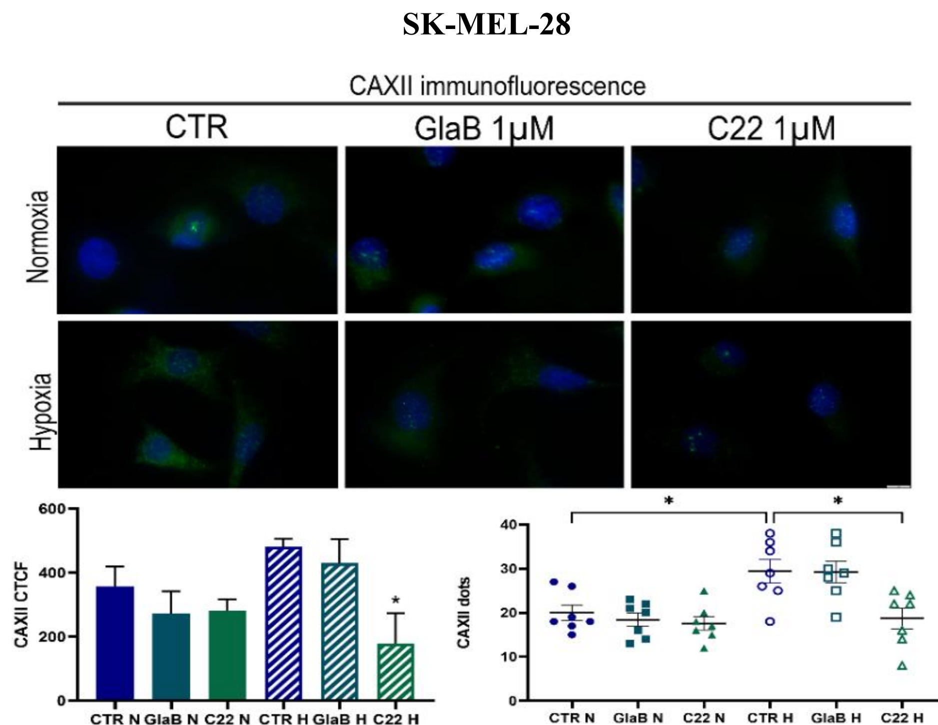

Figure 17. CAXII expression in SK-MEL-28. CAXII western blot and immunofluorescence (60X magnification, scale bar 10 μm) after treatment with GlaB or C22 in SK-MEL-28 under normoxia and hypoxia. CAXII dots were counted in at least 10 cell per condition. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference

Then we moved on A375 and repeated the treatment with GlaB and C22, evaluating CAXII expression. As seen in figure 18, the inhibition of GLI1 and SMO resulted in a significant downregulation of CAXII under hypoxic conditions. Even in this cells line, we detected a dotted appearance of CAXII, and we managed to count dots demonstrating a significant accumulation in hypoxic conditions and a significant reduction in GlaB- and C22-treated cells under hypoxia. In the A375 cell line, which is more aggressive, both compounds, GlaB and C22, determined a significant reduction in dots presence.

A375

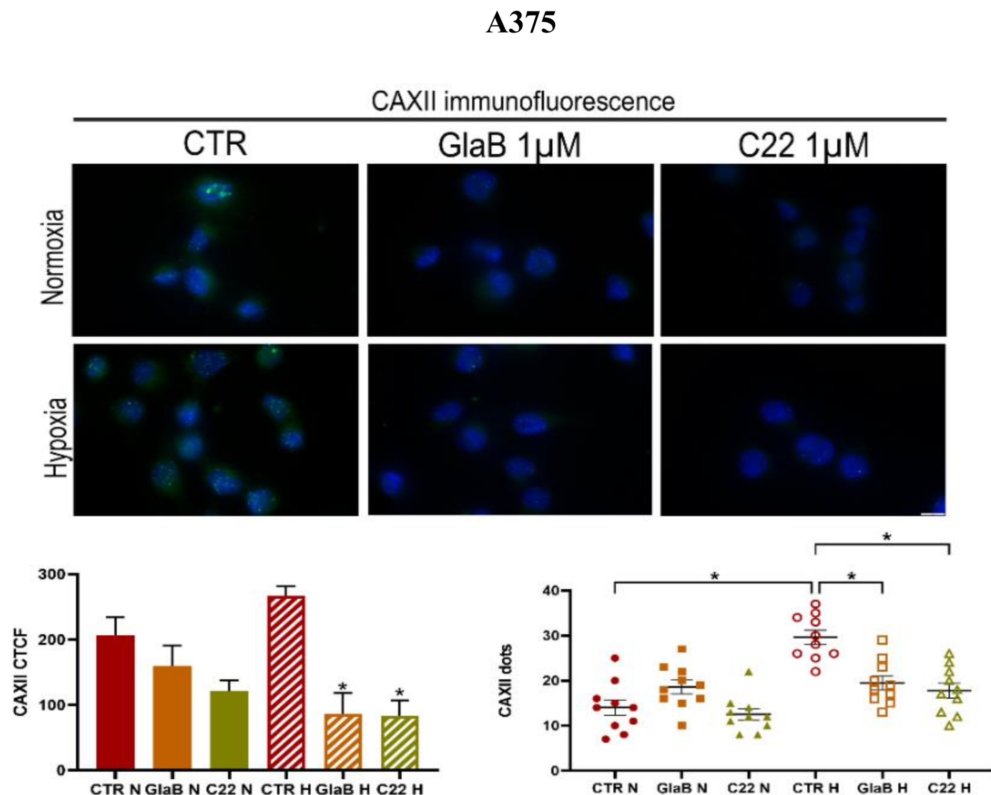

Figure 18. CAXII expression in A375 cell line. CAXII immunofluorescence (60X magnification, scale bar 10 μm) after treatment with GlaB or C22 in A375 under normoxia and hypoxia. CAXII dots were counted in at least 10 cell per condition. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference)

To further confirm the relationship between the Hh pathway and CAXII we treated SK-mel-28 and A375 with Cyclopamine and SAG, which are canonical Hh inhibitor and activator, respectively, under normoxia and hypoxia. As represented in Figure 19, we observed a significantly increased CAXII expression in hypoxia with respect to the normoxic control in

both cell lines. Treatment with Cyclopamine reduced CAXII protein level, while SAG increased CAXII expression under normoxia and hypoxia in both cell lines. Of note, data was significant in hypoxic conditions. Notably, despite the increased CAXII determined by hypoxia, treatment with SAG resulted in an even higher expression of CAXII, confirming the involvement of Hh signaling.
The overall results highlight that inhibition or activation of Hh pathway resulted in consequent CAXII modulation, underlining the crosstalk with the metalloenzyme.

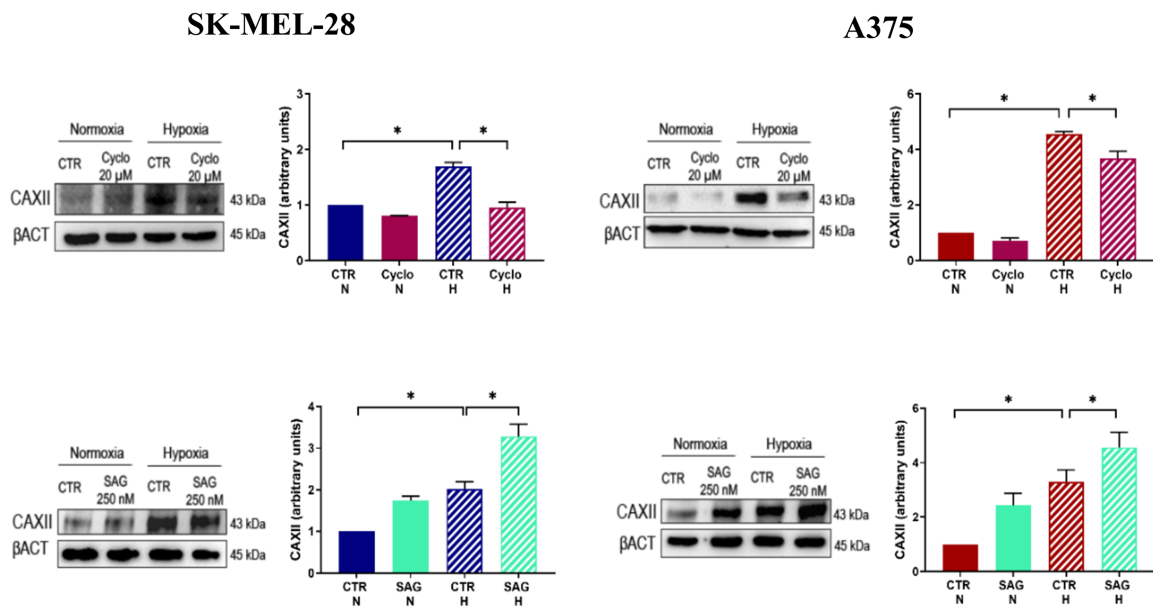

Figure 19. CAXII expression after Cyclopamine or SAG treatment. CAXII Western blot after treatment with Cyclopamine or SAG under normoxic or hypoxic conditions for 24 h. β-actin was used as loading control. Blots are representative of three independent experiments. Means ± SEM are presented. ($n = 3$; * $p \leq 0.05$ indicates statistically significant difference).

5. DISCUSSION (PART I)

Hypoxia, a condition marked by low oxygen levels in tissues, is a key factor in the progression and metastasis of melanoma. The TME often experiences hypoxia due to insufficient vascularization and the rapid growth of cancer cells, which exceeds the available oxygen supply [Wilson, 2011]. Additionally, hypoxia promotes EMT, a process through which melanoma cells acquire migratory and invasive characteristics [Giatromanolaki et al., 2013; Halsey et al., 2013].
The first step in our study regarding melanoma cells was validating the hypoxic microenvironment by detecting HIF-1α protein accumulation and its translocation into the cell nucleus under hypoxia. According to previous studies [Zbytek, et al., 2013], also in our experimental model, HIF-1α was accumulated and activated by low pO_2.

Then, as the Hh pathway is aberrantly re-activated in melanoma [Tusa et al., 2021], we looked for SMO expression, confirming its expression, particularly in hypoxic conditions. In order to better understand Hh involvement in cell migration and invasion, we decided to inhibit the Hh pathway using new small molecules GlaB and C22.
For the first time, we demonstrated the efficacy of GlaB and C22 to inhibit GLI1 and SMO in melanoma cells, either under normoxia or hypoxia. Thus, we tested SK-MEL-28 and A375 cell lines for migration and invasion abilities, adding GlaB and C22 to culture media. We showed that Hh-interfering small molecules were able to impair melanoma cell migration and invasion, especially under hypoxia with respect to control. The application of these compounds has been investigated in other cell lines [Infante et al., 2021], but their use has not been documented in melanoma models yet, particularly in hypoxic conditions. In recent years, the growing resistance to conventional therapies [Wang, X., et al., 2024] has increased the necessity of exploring novel chemical compounds, especially small molecules. GlaB is an isoflavone naturally found in the seeds of Derris glabrescens (Leguminosae) and a direct GLI1 inhibitor, as inhibit of GLI1/DNA interaction. The versatility of the isoflavone scaffold has been further exploited for targeting the Hh signaling pathway at multiple levels, and the GlaB derivative C22 was identified as the first and most efficient multitarget Hh inhibitor that simultaneously targets both SMO and GLI1 [Lospinoso et al., 2019].

In melanoma, specific isoforms of CAs have been associated with tumor progression and metastasis. Within the hypoxic TME, the overexpression of CAIX and CAXII is commonly observed, where they are crucial in modulating the acidic extracellular milieu, a condition essential for promoting invasive behavior [Supuran, 2020]. Moreover, in our previous papers [Guerrini et al., 2018; Guerrini et al., 2019], it has been demonstrated that CAXII expression is related to the Hh pathway, as its impairment resulted in CAXII, cell migration and invasion downregulation in breast cancer. Thus, we decided to focus our attention on the interplay between the Hh signaling and CAXII in melanoma model. Since we already demonstrated this interplay using a siRNA approach targeting Hh signaling proteins [Giuntini et al., 2022], we decided to confirm it by using novel chemical compounds. Indeed, by using Hh interfering small molecules we detected a reduction in CAXII expression. In addition, the immunofluorescence analysis revealed the presence of CAXII dots in both cell lines, especially under hypoxia, which are attributable to membrane accumulation sites. A similar condition has been detected by Horikawa et al, in breast cancer where intracellular trafficking of CAIX is regulated by AMAP1 protein [Horikawa et al., 2022].

In our study, we investigated the inhibition of Hh pathway only in two melanoma cell lines, characterized by different migration and invasion abilities. The cell lines allowed us to perform preliminary studies on the effects of new small molecules on melanoma, as C22 was before tested only on Hh-dependent medulloblastoma in vitro and in vivo model [Lospinoso et al., 2019].

However further experiments to clarify the efficacy of these new Hh inhibitors may be performed in primary melanoma. Indeed, it would be relevant to study the behavior of primary cells treated with new small molecules, along with patient-specific gene mutation. Of note, Das et al. have demonstrated in cell lines established from two subcutaneous metastatic nodules from the same patient that cyclopamine is able to inhibit the Hh pathway, along with osteopontin expression reduction (as osteopontin gene is regulated by GLI1). In addition they demonstrated that the knockdown of GLI1 resulted in a reduction of the malignant behavior of primay tumor cells [Das et al., 2009]. However, few studies has been performed on primary melanoma cells, as sample are often difficult to obtain from patients.

In conclusion, in this work, we highlight the opportunity to target CAXII indirectly with chemical compounds, which could be further developed as a new pharmacological strategy to manage melanoma spread out.

6. INTRODUCTION (PART II): Dendritic Cells and Carbonic Anhydrases

6.1 The tumor microenvironment and infiltrating immune cells

The hypoxic TME is infiltrated by immune cells, with an effect of hypoxia on them, as hypoxia control mechanisms that suppress anti-tumor immunity and promote tumor progression. Myeloid-Derived Suppressor Cells (MDSCs), a diverse group of immature myeloid cells, are recruited to the TME under hypoxic conditions. Hypoxia, via HIF1-α, promotes MDSC accumulation and their differentiation into tumor-associated macrophages (TAMs). MDSCs suppress anti-tumor immunity and are linked to higher IL-6 levels, which further amplifies their immunosuppressive effects and correlates with tumor progression [Diaz-Montero et al., 2009]. TAMs are a major TME component associated with worse outcomes. Hypoxia enhances TAM-mediated pro-tumorigenic activities, including angiogenesis and immune evasion. TAM density correlates with increased PD-L1 expression, which further suppresses immune responses [Henze, Mazzone. 2016]. Of note, DCs, essential for initiating adaptive immune responses, exhibit impaired antigen uptake and cytokine production in hypoxic TMEs, as they can acquire a tolerogenic phenotype, characterized by reduced antigen uptake and reduced migration abilities. In addition, high expression of markers like LAMP3 in DCs correlates with metastasis and poor outcomes [Elia et al., 2008]. Overall, this environment diminishes the efficacy of anti-tumor immune responses and contributes to treatment resistance.

6.2 The Immune system

The immune system functions as a complex defense mechanism composed of organs, cells, and molecules that work together to protect the host from a wide range of pathogenic microbes and other infectious agents [Chaplin, 2010]. Beyond its primary role in host defense, the immune system also plays key roles in maintaining tissue homeostasis and regulating physiological processes such as embryonic development [Reemst et al., 2016], angiogenesis [Sunderkötter et al., 1994], as well as tissue regeneration and repair [Sattler, 2017]. In terms of host defense, the immune system is divided into innate and adaptive components [Arneth, 2021]. The innate immune system serves as the body's initial line of defense, composed of physical and chemical barriers such as the skin and mucous membranes, which include epithelial layers with tight junctions [Chaplin, 2010]. These barriers, along with substances like acids, enzymes, and mucus, provide the first level of resistance against infections. When these barriers are breached, additional innate components, including cytokines and chemokines that recruit inflammatory leukocytes, as well as complement system proteins, defensins, and other enzymes that promote tissue inflammation, quickly respond [Weaver and Murphy, 2017]. This nonspecific response employs various pathogen-elimination strategies such as phagocytosis [Uribe-Querol et al., 2020], autophagy [Tian et al., 2024], or the activation of the complement Membrane Attack

Complex (MAC) and inflammasomes [Diaz-Del-Olmo et al., 2021]. Furthermore, innate effectors collaborate with the adaptive immune system to eradicate foreign invaders [Yatim et al., 2015]. The adaptive immune system, on the other hand, is highly specific, targeting pathogens, toxins, or allergens. Upon encountering an antigen, adaptive immune cells must proliferate to initiate an effective response [Chaplin, 2010]. The primary cells involved in adaptive immunity are B and T lymphocytes, and a defining feature of this system is its capacity for memory, which allows for a faster and more efficient immune response upon subsequent antigen exposure [Marshall et al., 2018]. While innate immunity is traditionally thought to lack immunological memory, recent research has shown that certain cellular and molecular mechanisms can mediate a form of innate immune memory [Sherwood et al., 2022].

6.2.1 Innate immunity

The first line of defense of the human is represented by the skin, which protects the living organism when exposed to a pathogen. In case of infection, the innate immune system is activated [Mccomb et al., 2019]. When the pathogen invades the host cell, neutrophils and natural killer cells start digesting the pathogen. However, the macrophages and DCs phagocytose the pathogen and act as APC, producing a cytokine storm for attracting other immune cells to amplify the response, as described in figure 20 [Wang et al., 2024].

More precisely, innate immune memory is initiated by the activation of germline-encoded pattern recognition receptors (PRRs), which rapidly identify and respond to a wide spectrum of pathogens by recognizing conserved microbial molecules, such as bacterial cell wall components (e.g., lipopolysaccharides) (LPS) and viral double-stranded RNA. These molecules, termed pathogen-associated molecular patterns (PAMPs) [Marshall et al., 2018], as well as endogenous molecules released from damaged cells, known as damage-associated molecular patterns (DAMPs) [Takeuchi and Akira, 2010], bind to PRRs to trigger specific signaling pathways. This leads to the expression of immunomodulatory molecules like cytokines, type I interferons, and other antiviral proteins, all coordinating to mount an appropriate immune response against external or internal threats [Jentho et al., 2021; Taguchi et al., 2019]. Key PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-leucine-rich repeat (LRRs)-containing receptors (NLRs), retinoic acid-inducible gene 1-like (RIG-1) receptors (RLRs), C-type lectin receptors (CLRs) [Takeuchi and Akira, 2010], and the stimulator of interferon genes (STING) [Ishikawa and Barber, 2008]. These receptors are expressed not only in immune cells like macrophages and dendritic cells but also in various non-professional immune cells [Takeuchi and Akira, 2010]. PRR activation induces inflammation and can also stimulate the complement system, a biochemical cascade that can be activated via three pathways: classical, alternative, and mannose-binding lectin [Turvey et al., 2010]. The complement system primarily protects the host from infections and inflammation by recruiting innate immune cells and enhancing phagocytosis. The formation of the MAC results in target cell lysis by creating functional pores in the cell membrane [Nesargikar et al., 2012; Morgan, 2016]. Additionally, the adaptive immune response may be activated through the recruitment and activation of APCs.

Various cells contribute to the innate immune response, including phagocytes (sub-divided into monocytes and macrophages), mast cells, granulocytes (basophils, eosinophils, and neutrophils), innate lymphoid cells (ILCs), natural killer (NK) cells, and DCs [Marshall et al., 2018]. Among them, eosinophils are a minor population of granulocytes that are mostly explored in asthma and allergic disorders. Their influence on primary and metastatic tumors, however, has recently come to light, as they participate in innate and adaptive immunity, and shape TME and tumor outcomes [Ghaffari & Rezaei 2023]. Indeed, eosinophils are accessory cells that can affect the response to various forms of T cell-mediated immunotherapies and might be therapeutically targeted to improve cancer immunotherapy [Grisaru-Tal et al., 2022].

In addition, in the bone marrow, pluripotent hematopoietic stem cells differentiate into common lymphoid progenitor cells, which give rise to ILCs and NK cell lineages, while myeloid stem cells differentiate into almost all other innate cells, including monocytes, macrophages, mast cells, and granulocytes. Dendritic cells can originate from either lymphoid or myeloid precursors, giving rise to classical and plasmacytoid DCs [Weaver and Murphy, 2017; Huston, 1997].

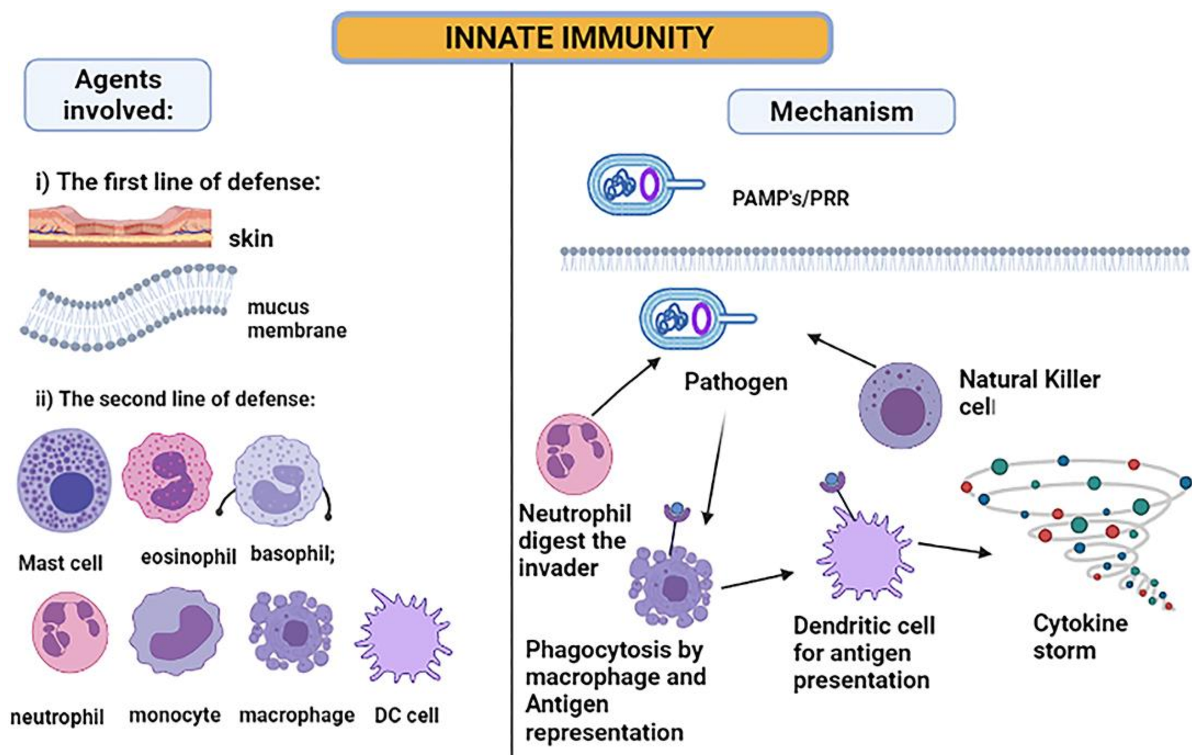

Figure 20. Schematic representation of innate immunity. When a pathogen manages to cross the first line of defense (skin) and invades a host cell, neutrophils and natural killer (NK) cells initiate its digestion. Meanwhile, macrophages and dendritic cells (DCs) engulf the pathogen through phagocytosis, functioning as antigen-presenting cells. These cells release a surge of cytokines, known as a cytokine storm, to recruit additional immune cells and amplify the immune response [From Wang et al., 2024].

6.3 Dendritic Cells

Ralph Steinman and Zanvil Chon first discovered DCs in 1973 during their experiments on mice spleen, when they identified a novel type of immune cells with a specific morphology comparable to neuronal dendrites, characterized by membrane processes that blowout from the cell body [Steinman and Chon, 1973]. DCs are a complex and diverse group of APCs, distinguished by notable phenotypic variability and functional adaptability [Liu et al., 2021]. DCs are characterized by their expression of MHC class II molecules [Steinman and Witmer, 1978] and the surface integrin CD11c [Crowley et al., 1990]. They also express a variety of PRRs, including TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and C-type lectins. These receptors enable them to recognize molecular patterns associated with pathogens or cellular damage [Pearce and Everts, 2015].

DCs develop bone marrow from CD34+ hematopoietic stem cell progenitors, which give rise to both myeloid and lymphoid precursors. DCs specifically arise from common myeloid progenitors (CMPs). CMPs that express the transcription factor Nur77 first differentiate into monocytes and subsequently into monocyte-derived DCs (moDCs). In the absence of Nur77, CMPs differentiate into common dendritic cell progenitors (CDPs), which give rise to both plasmacytoid DCs (pDCs) and conventional DCs (cDCs) [Geissmann et al., 2010]. DCs serve as a critical bridge between the innate and adaptive immune systems [Liu et al., 2021], as they are highly specialized cells that, upon recognizing pathogens via PRRs, initiate the innate immune response. Furthermore, they present antigens to naïve T cells, thereby activating the adaptive immune response [He et al., 2019]. To facilitate this function, activated DCs upregulate MHC-peptide complexes and costimulatory molecules necessary for T-cell activation [Pearce and Everts, 2015]. Beyond their role in antigen-specific immunity, DCs also contribute to self-tolerance by capturing, processing, and presenting both self and non-self antigens, enabling the host to distinguish between them [Marciscano and Anandasabapathy, 2021]. DCs are classified into four main types: plasmacytoid DCs (pDCs), conventional DCs (cDCs) — further subdivided into cDC1 and cDC2 subsets — monocyte-derived DCs (moDCs), and Langerhans cells (LCs) [He et al., 2019]. Each DC subtype is distinguished by its specific production of interferons, cytokines, and chemokines [Liu et al., 2021]. For example, moDCs represent an inflammatory DC subset that differentiates in response to various inflammatory stimuli via CCR2-CCL2 chemokine signaling [Marciscano and Anandasabapathy, 2022]

6.3.1 Immature Dendritic Cells

DCs are found in two distinct functional and phenotypic states: immature dendritic cells (iDCs) and mature dendritic cells (maDCs). iDCs (figure 21) are terminally differentiated cells distributed across various body tissues, especially in peripheral regions, where they contribute to immune surveillance and tolerance mechanisms [Mellman and Steinman, 2001; Mellman, 2013]. In their immature phase, they are highly efficient in capturing antigens through mechanisms such as macropinocytosis, phagocytosis, and clathrin-mediated endocytosis. However, they lack the capacity to present these antigens to T cells effectively, as their surface expression of MHC class I and II molecules is minimal, and most of these molecules accumulate within late endosomes and lysosomes. Similarly, they produce low levels of costimulatory molecules and immunostimulatory cytokines [Tibúrcio et al., 2019]. iDCs can develop to a mature phenotype in response to environmental signals, particularly through the recognition of microbial components [Hammer and Ma, 2013]. This maturation involves reduced endocytic activity [Mellman and Steinman, 2001] and can result in either tolerogenic or immunogenic phenotypes, influencing T-cell responses accordingly [Mellman, 2013]. Pro-inflammatory chemokine receptors such as CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2 are expressed on iDCs, allowing them to migrate to inflammatory sites [Tiberio et al., 2018]. Moreover, under steady-state conditions, iDCs migrating from peripheral tissues to primary and secondary lymphoid organs can process and present self-antigens to T cells, thus supporting the maintenance of self-tolerance [Bonifaz et al., 2002].

6.3.2 Mature Dendritic Cells

Mature DCs (maDCs), as described in figure 21, are characterized by an upregulated expression of MHC class II molecules, which occurs upon sensing PAMP or DAMPs through PRRs. This process is accompanied by the increased expression of costimulatory molecules (CD83, CD80, CD86, and CD40), a reduction in phagocytic and endocytic receptor activity, and a switch in chemokine receptor expression. maDCs downregulate inflammatory chemokine receptors (CCR1, CCR2, CCR5, CCR6, and CXCR1) while upregulating homeostatic ones (CCR7 and CXCR4), which are essential for homing to secondary lymphoid organs [Bosco and Varesio, 2012; Sozzani, 2005; Liu et al., 2021; Turley et al., 2000]. Among these, CCR7 plays a crucial role in directing maDC migration to lymph nodes via afferent lymphatics through interactions with its ligands, CCL19 and CCL21 [Förster et al., 1999]. The maturation process involves extensive gene reorganization, culminating in significant morphological and functional changes. Once in the lymph nodes, maDCs present antigens to activate naïve T cells. Intracellular pathogen-derived antigens presented on MHC-I molecules activate CD8+ T cells, while exogenous antigens presented on MHC-II molecules activate CD4+ helper T cells [Braun et al., 2011; Granucci et al., 2005; Joffre et al., 2012]. The expression of TLRs, such as TLR4, which specializes in bacterial antigen recognition, is crucial for activation of inflammatory

pathways [Marciscano and Anandasabapathy, 2022]. TLRs also detect viral components and nucleic acids [Dudek et al., 2013]. For example, the bacterial LPS, crucial in hypoxic conditions for increasing proinflammatory cytokines and interferons [Kiani et al., 2021], is recognized by TLR4. LPS also robustly activates the MAPK/NF-κB pathway, critical for DC migration and maturation [Halligan et al., 2016]. The coordination of DC migration and maturation is governed by complex signaling pathways, including PI3K/AKT and MAPK/NF-κB. These pathways are essential for TLR-induced maturation and activation of DCs via inflammatory cytokines like IL-1, IL-6, and TNF [Liu et al., 2021]. Notably, iDCs can spontaneously mature into maDCs, even in the absence of inflammatory stimuli, upregulating CCR7 during this process [Liu et al., 2021.

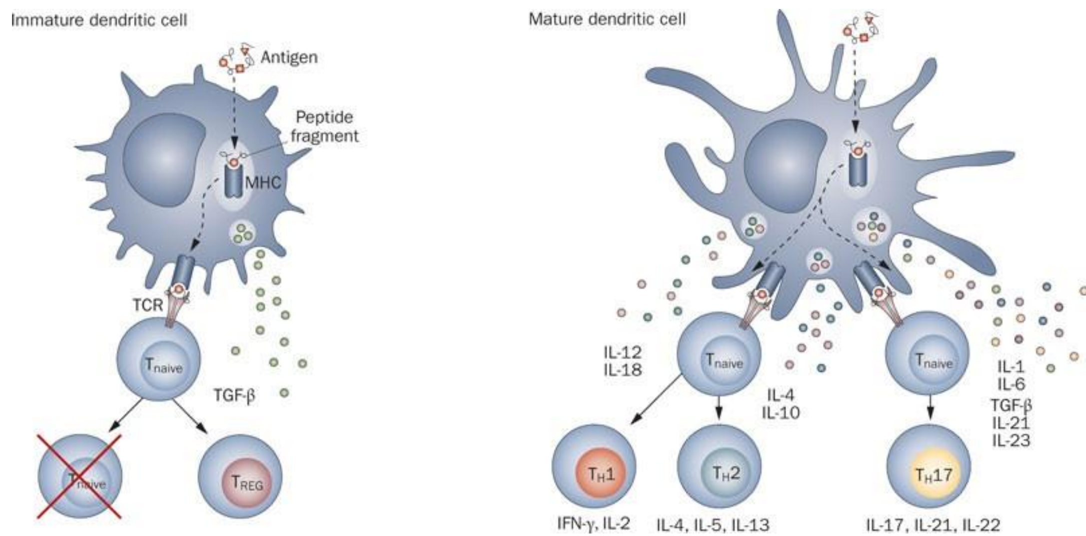

Figure 21. iDCs and maDCs. Schematical representation of the difference between iDCs and maDCS, with the latter expressing a pattern of inflammatory cytokines necessary to induce an inflammatory response [Comabella et al., 2012].

6.4 Dendritic Cells and Hypoxia

During their development, DCs often experience changes in pO_2 tensions, due to their migration from peripheral tissues, which are characterized by variable O_2 levels, to secondary lymphoid organs, physiologically hypoxic, or to inflammatory sites and TME, where the pO_2 is even lower. As a matter of fact, hypoxia affects DC behavior and physiology, with a high impact on their maturation. The result is that the ability of DCs to adapt to this condition is crucial for them to survive and efficiently exert their functions [Bosco & Varesio, 2012; Naldini et al., 2012].

Different findings have been reported regarding the impact of hypoxia on DC maturation and inflammatory functions. It has been observed that hypoxia alone does not significantly influence the expression of pro-inflammatory cytokines such as IL-6, TNF, IL-10, and IL-

12p40 in murine iDCs. However, in maDCs, hypoxia enhances the levels of TNF and IL-6. Additionally, when hypoxia is combined with LPS stimulation, murine DCs exhibit substantial increases in the expression of costimulatory molecules and the capacity to induce allogeneic lymphocyte proliferation compared to LPS stimulation alone [Jantsch et al., 2008; McGettrick & O'Neill, 2020]. In contrast, others demonstrated that hypoxia impairs the differentiation and full maturation of human monocyte-derived DCs: specifically, they found that oxygen deprivation reduces the expression of key maturation markers, including CD40, CD80, CD83 and MHC-II. At the same time, hypoxia enhances the inflammatory capabilities of iDCs by increasing cytokine secretion and promoting chemotaxis toward inflammatory sites [Mancino et al., 2008].

However, different times of exposure to hypoxia have diversely affected DCs: short-term hypoxic exposure primarily enhances iDC migration through the activity of HIF-1α. In contrast, in maDCs, HIF-1α works in conjunction with the Akt pathway to enhance migratory behavior under hypoxic conditions [Filippi et al., 2014]. However, prolonged hypoxic exposure induces iDC death through HIF-1α-mediated upregulation of pro-apoptotic factors such as BINP3 and BAX, as well as PARP cleavage, caspase 3 activation, and downregulation of BCL-2. However, LPS-induced maturation of DCs mitigates this effect, as maDC survival under hypoxia is supported by TLR4-mediated activation of the PI3K/Akt pathway [Naldini et al., 2012]. Notably, cell survival under hypoxic conditions can also involve autophagy as a key metabolic adaptation strategy [Mazure & Pouyssegur, 2010]. Hypoxia has a different effect on DC behavior depending on their functional state and time exposure to hypoxia.

6.5 Dendritic Cells and Carbonic Anhydrases

DC-based vaccines and immunotherapy have been developed recently and they represent a novel approach to manage tumor spread out. After isolation of precursor cells from patients, specific antigens are loaded into differentiated DCs which then exercise their role of APC more proficiently [Santos et al., 2012]. In addition, it has been demonstrated that DCs pulsed with a recombinant CAIX-Acinetobacter baumannii outer membrane protein A (CAIX-AbOmpA) fusion proteins generate a specific anti-tumor immune response against renal cell carcinoma and that they can be employed in immunotherapy for this kind of tumor.

Indeed, in this study, Kim et al. investigated the ability of DCs pulsed with this fusion protein in a murine renal cell carcinoma model demonstrating that DCs CAIX-AbOmpA-treated were able to produce interleukin 12 and going through maturation. Furthermore, the encounter of pulsed DCs with naïve T cells was able to induce the secretion of IL-2, interferon γ and tumor necrosis factor (TNF)-α in T cells [Kim et al., 2012]. However, the role of CAs in DCs remains unclear, highlighting the necessity for further studies to better understand their function in this cellular model.

7. RESULTS (PART II)

7.1 CAXII expression is enhanced by LPS in normoxic DCs

After having displayed a link between CAXII and melanoma cell migration, we asked if CAXII may play a similar role in immune cells. Focusing on DCs, we investigated CAXII expression and its role in cell migration, a critical aspect of DC functionality, particularly after maturation. Firstly, we exposed DCs to normoxia for 24h and treated them with LPS and detects HIF-1α accumulation (fig. 22a) in this condition. Then, to evaluate CAXII expression, we performed immunofluorescence (fig. 22b) and western blot analysis (fig. 22c). For the first time, we observed an increased CAXII protein level under LPS stimulation with respect to control.

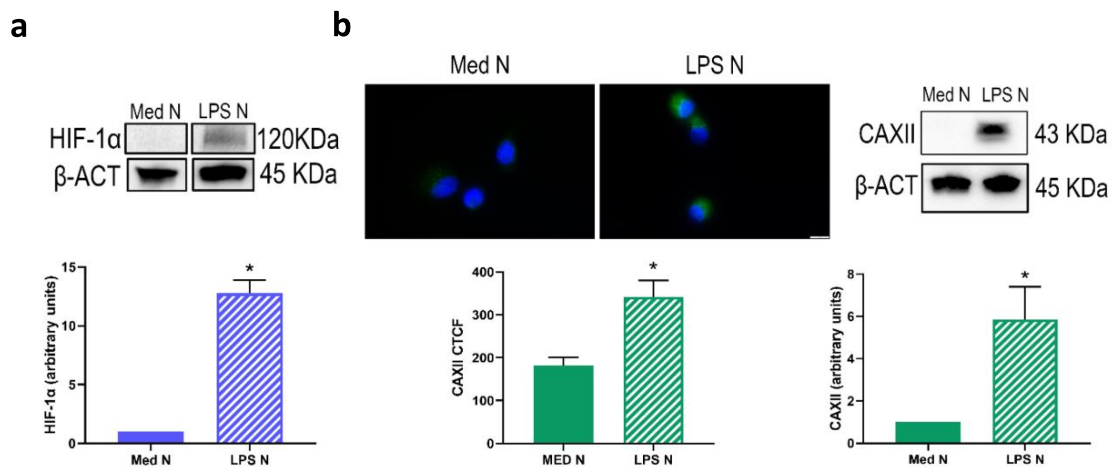

Figure 22. HIF-1α and CAXII expression in normoxic DCs. HIF-1α western blot (a) and CAXII western blot and immunofluorescence (b) (60X magnification, scale bar 10 μm) after treatment with LPS after 24h normoxic exposure. β-actin was used as loading control. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference)

We then decided to treat DCs with the chemical compound AAZ, a CAs inhibitor, at the concentration of 10 nM. As seen in immunofluorescence (fig 23a) and western blot analysis (fig 23b), CAXII expression was significantly reduced by AAZ in LPS-treated cells, with respect to control, along with a CAXII mRNA downregulation (fig 23c).

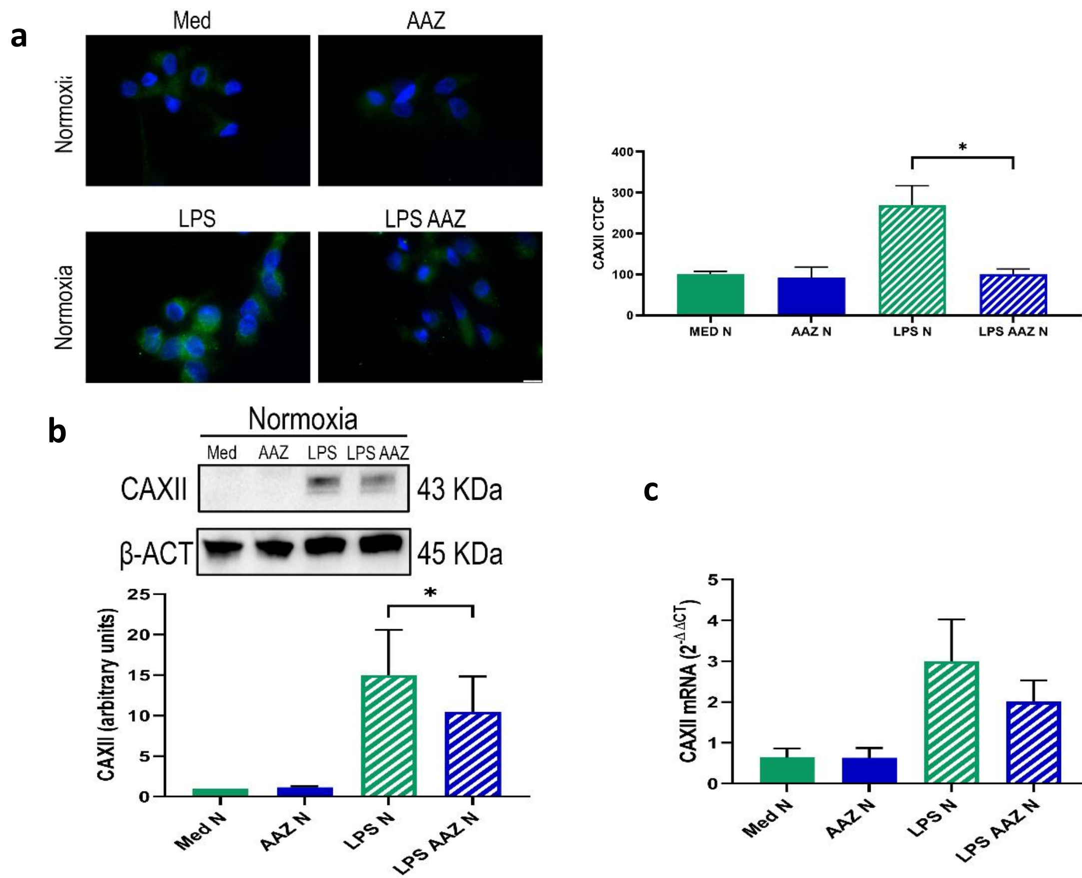

Figure 23. AAZ reduced CAXII expression in normoxic DCs. CAXII immunofluorescence (a) (60X magnification, scale bar 10 μm), western blot (b) and RT-qPCR (c) after treatment with LPS and AAZ under normoxic condition. β-actin was used as loading control. β-actin and L-32 were used as housekeeping genes for RT- qPCR Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference)

7.2 CAXII expression is enhanced by LPS in hypoxic DCs

As DCs encounter different hypoxic microenvironments, we exposed them to hypoxia and treated them with LPS. Firstly, after 24h exposure and treatment, we detected HIF-1α protein level accumulation (fig 24a). Then, we investigated CAXII expression under hypoxic conditions and, for the first time, demonstrated its upregulation following LPS treatment, as shown by immunofluorescence (Fig. 24b) and western blot analysis (Fig. 24c).
This suggests that LPS is probably the main responsible for CAXII increase in normoxic and hypoxic conditions.

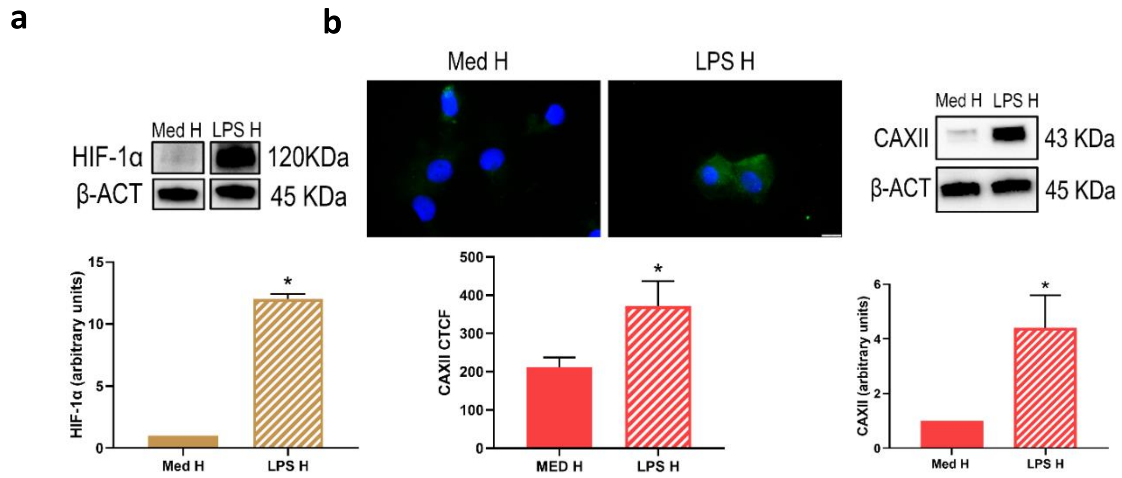

Figure 24. HIF-1α and CAXII expression in hypoxic DCs. HIF-1α western blot (a) and CAXII immunofluorescence (60X magnification, scale bar 10 μm) and western blot (b) after treatment with LPS after 24h Hypoxic exposure. β-actin was used as loading control. Images are representative of three independent experiments. Means ± SEM are presented. ($n = 3$; * $p \leq 0.05$ indicates statistically significant difference)

Thereafter, we inhibited CAXII also in hypoxic DCs with 10 nM AAZ. As shown in figure 25, AAZ significantly reduced CAXII protein (fig.25a and b) and mRNA (fig.25c) levels in LPS-treated cells.

These results demonstrate that AAZ chemical inhibition of CAXII is effective in both DC aerobic and hypoxic culture conditions.

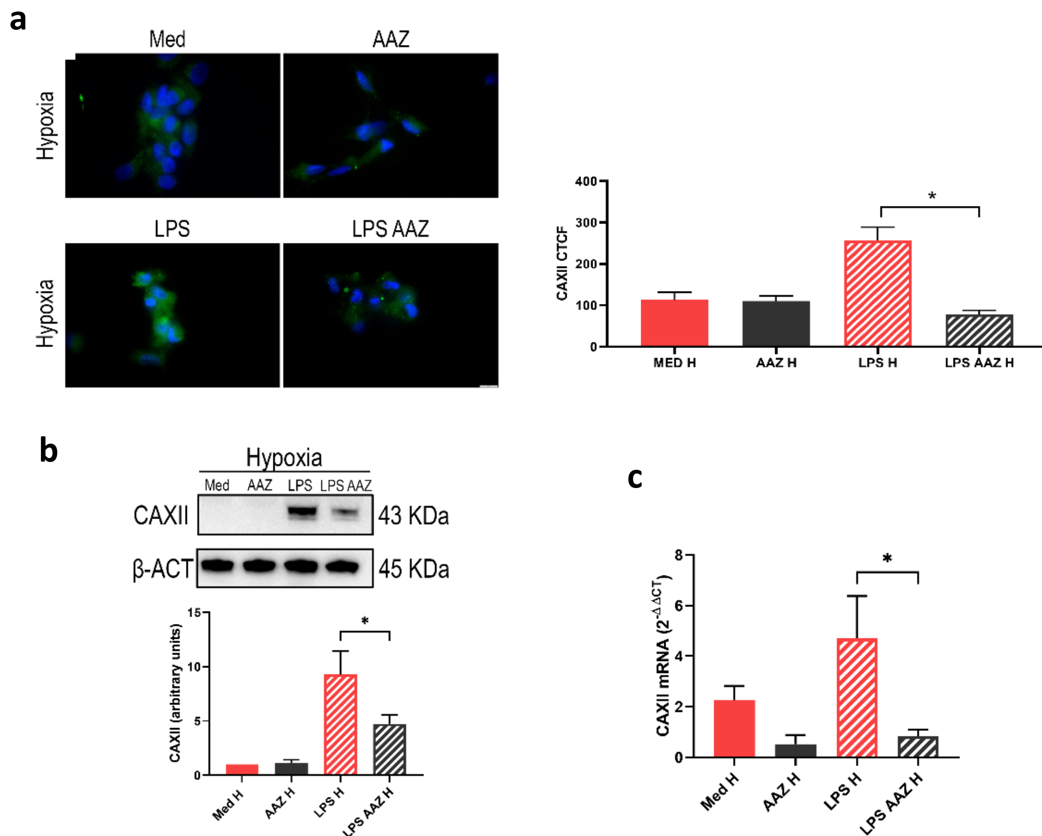

Figure 25. AAZ reduced CAXII expression in hypoxic DCs. CAXII immunofluorescence (60X magnification, scale bar 10 µm) western blot and RT- qPCR (after treatment with LPS and AAZ under hypoxic condition. β-actin was used as loading control. β-actin and L-32 were used as housekeeping genes for RT- qPCR. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference)

7.3 DC migration is reduced by AAZ treatment

To test the effect of CAXII downregulation on DC migration, we performed a modified Boyden chamber assay. As demonstrated in figure 26a, LPS-induced DC migration was significantly reduced by AAZ under normoxia. Of interest, LPS was able to significantly enhance DCs migration under hypoxic condition and, again, AAZ treatment resulted in the reduction of that increase (fig. 26b).

These results suggest that AAZ effectively impaired DC migration after their final maturation with LPS either in normoxia or hypoxia.

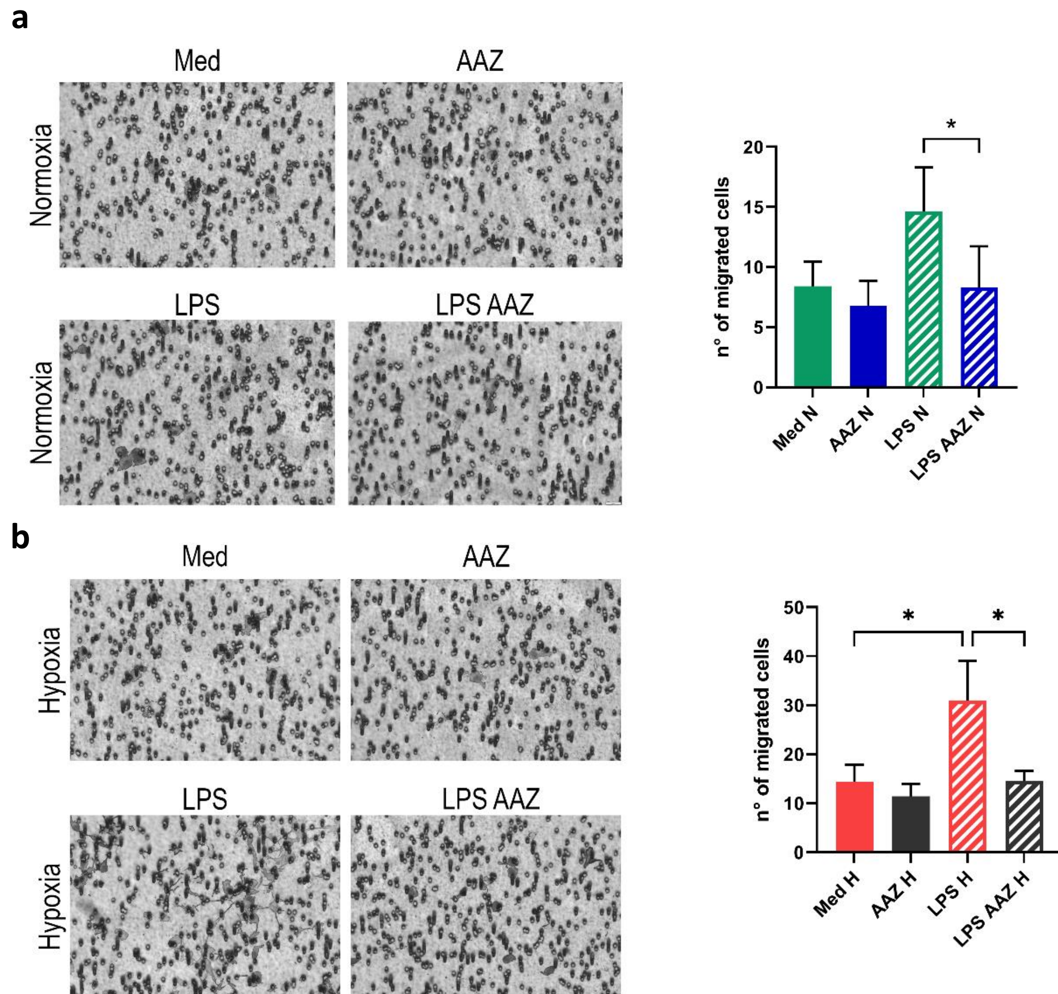

Figure 26. DCs migration after AAZ treatment. Cell migration measured by modified Boyden chamber under normoxic or hypoxic conditions in DCs treated with AAZ. Pictures are representative of three independent experiments (20X magnification, scale bar 20 µm). Means ± SEM are presented. ($n = 3$; * $p \leq 0.05$ and indicate statistically significant differences).

7.4 CAXII downregulation by siRNA in normoxic and hypoxic DCs

To assess if CAXII may play a role in DC migration, we then decided to silence CAXII with a siRNA approach under normoxia. As shown in immunofluorescence (fig. 27a) we observed a downregulation of CAXII protein, which was significant in LPS-siRNA treated cells with respect to control. These results were confirmed by western blot (fig. 27b) and mRNA data (fig. 27c), as we detected a significant reduction of CAXII expression in all experimental condition.

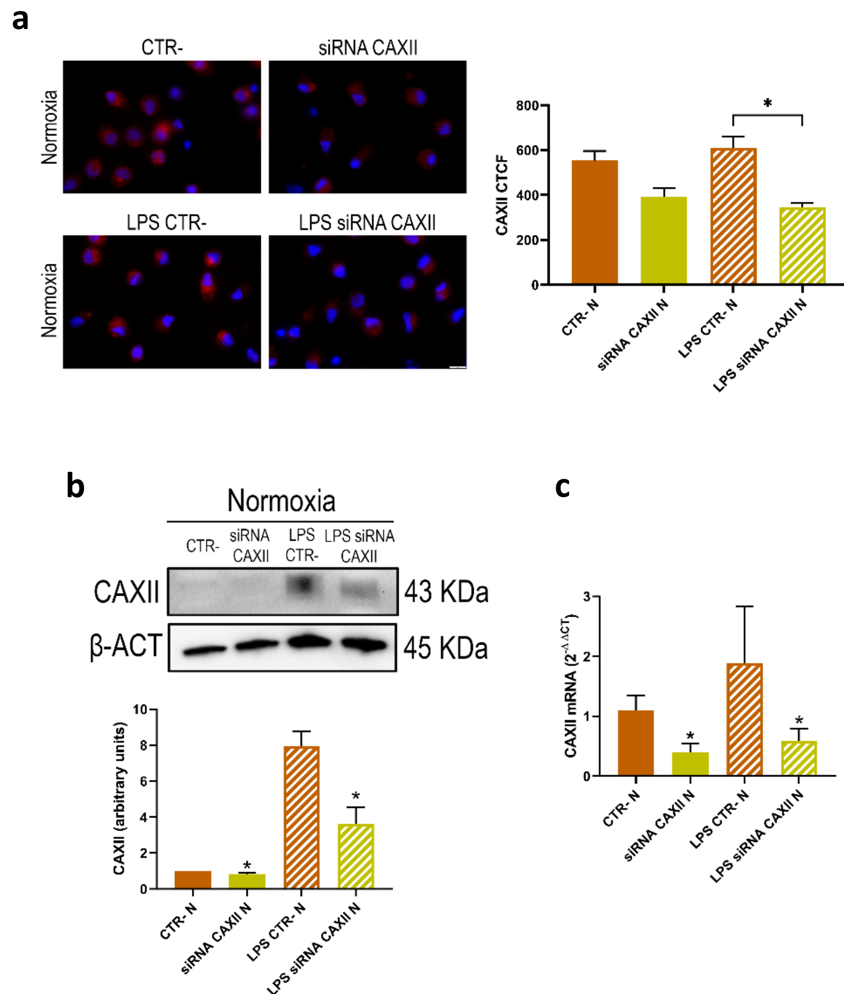

Figure 27. CAXII siRNA in DCs exposed to normoxia. CAXII western blot, immunofluorescence (60X magnification, scale bar 10 μm) and RT-qPCR after siRNA treatment in presence or not of LPS after 24h normoxic exposure. β-actin was used as loading control for western blot. β-actin and L-32 were used as housekeeping genes for RT- qPCR. Images are representative of three independent experiments. Means ± SEM are presented. ($n = 3$; * $p \leq 0.05$ indicates statistically significant difference)

Of interest, DCs silencing by CAXII siRNA was observed also under hypoxic conditions. As demonstrated in figure 28, immunofluorescence (fig. 28a), western blot (fig. 28b) and mRNA (fig. 28c) analysis showed a significant downregulation of CAXII also in the presence of LPS.

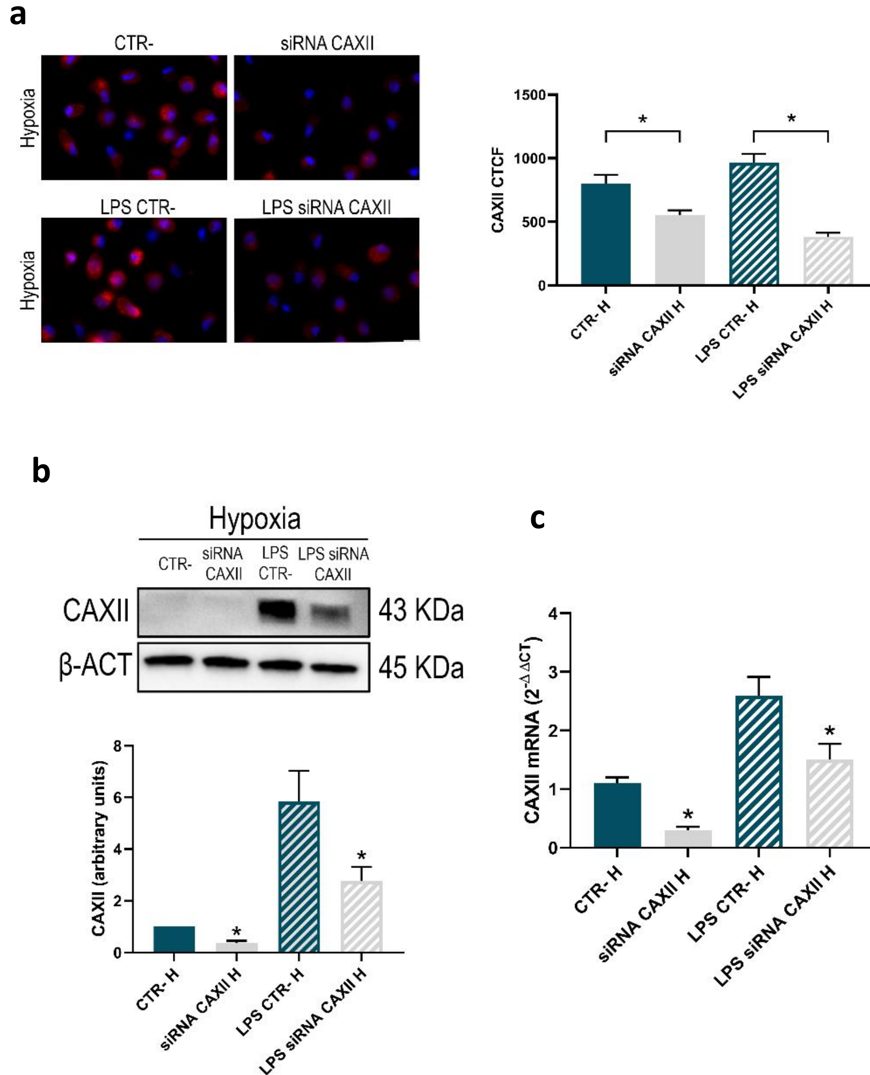

Figure 28. CAXII siRNA in DCs exposed to hypoxia. CAXII western blot, immunofluorescence (60X magnification, scale bar 10 µm) and RT-qPCR after siRNA treatment in presence or not of LPS after 24h hypoxic exposure. β-actin was used as loading control for western blot. β-actin and L-32 were used as housekeeping genes for RT- qPCR. Images are representative of three independent experiments. Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicates statistically significant difference

7.5 CAXII siRNA resulted in DC migration impairment

Finally, we asked if CAXII silencing was able to reduce DC migration. To this end, we performed modified Boyden Chamber assay under normoxia and hypoxia. As shown in figure 29a, we detected a significant reduction of migrated DCs cells when CAXII was silenced by siRNA. Notably, the downregulation was also effective and significant following LPS stimulation. In addition, when the siRNA treatment was performed under hypoxia, we obtained the same results (fig. 29b).
These data highlight that CAXII is directly involved in DC migration in all experimental conditions.

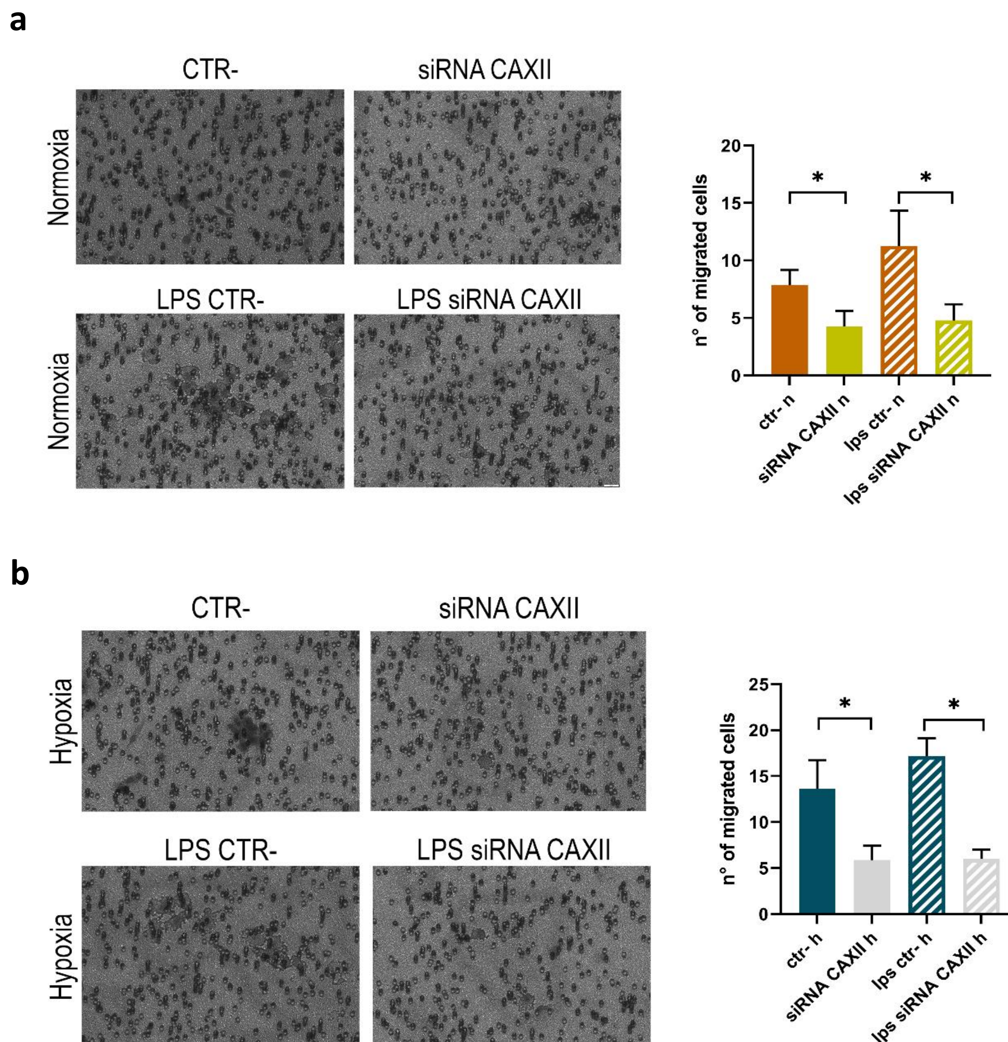

Figure 29. CAXII siRNA impaired DCs migration abilities. Cell migration measured by modified Boyden chamber under normoxic or hypoxic conditions in DCs after CAXII siRNA treatment. Pictures are representative of three independent experiments (20X magnification, scale bar 20 μm). Means ± SEM are presented. (n = 3; * $p \leq 0.05$ indicate statistically significant differences).

8. DISCUSSION (PART II)

DCs undergo significant physiological adaptation due to varying pO_2 during their development. They migrate from peripheral tissues, which exhibit fluctuating O_2 levels, to secondary lymphoid organs, which are naturally hypoxic, or to sites of inflammation and TME, where O_2 levels are even more reduced. Hypoxia significantly influences DC behavior and physiology, particularly impacting their maturation process [Bosco & Varesio, 2012; Naldini et al., 2012]. The acquirement of a migratory phenotype is necessary for DC role as APC. With this in mind, we exposed DCs to normoxia and hypoxia and first detected HIF-1α accumulation, confirming the presence of a hypoxic microenvironment, as previously reported [Filippi et al., 2014].

Studies have shown that hypoxia, through the activation of HIFs, regulates the expression of specific CA isoforms, such as CAIX [Swayampakula et al., 2017]. These isoforms are closely associated with hypoxic tumor microenvironments and contribute to maintaining intracellular pH homeostasis [Pastorekova et al., 2008]. Furthermore, this acidification creates favorable conditions for cell survival in hypoxic and otherwise adverse environments, enhancing the migratory and invasive potential of tumor cells [Supuran, 2018]. In this context, since DCs are present in TME, we wondered whether CAs, particularly CAXII, have an important role also in DC migration. In this study, for the first time, we have identified a link between CAXII and migration in DCs exposed to hypoxia.

Firstly, we showed a significant increment of CAXII expression after LPS stimulation in DCs, under both normoxia and hypoxia. Then, we demonstrated that CAXII plays a pivotal role in DC cell migration, as its inhibition resulted in a reduced cell migratory ability. All these data have never been reported previously in the literature. Of note, we use a chemical inhibitor of CAs, AZZ, which could be the base for a potential development of novel molecules capable of regulating CAXII expression. However, the link between CAXII and LPS, which seems to be the main responsible for CAXII upregulation, is still not well understood. Further studies are necessary to establish whether CAXII is a novel player in DC final maturation, particularly regarding the migratory ability. In this regard, and as reported by Kim et al, it is possible to pulse DCs with CAIX protein and specifically with CAIX-AbOmpA fusion proteins, thus generating a specific anti-tumor response against renal cell carcinoma [Kim et al., 2012]. Further studies could be also conducted to overexpress CAXII in DCs in order to generate better efficient DC-cell based vaccines.

9. FINAL DISCUSSION AND CONCLUSION

The overall results, underline the involvement of CAXII in cell migration in two distinct players in the TME: cancer cells (melanoma cells) and immune cells, DCs.
As represented in figure 30, CAXII indirect downregulation (through the inhibition of the Hh pathway in melanoma) or direct inhibition (either by AAZ or specific targeting siRNA in DCs), resulted in impairment of cell migration. These data may have important implications for novel pharmacological approaches targeting CAXII also with small molecules.

In this context, it is important to point out that a reduced cancer cell spread out results in the impairment of cancer progression. Thus, CAXII inhibition seems to be a promising strategy to control melanoma cell migration. On the other hand, as CAXII reduction is associated with DC migration impairment, it would be reasonable to overexpress this enzyme to improve DC functions, especially at the lymph node levels. Here, they encounter T-cells to present the antigen and such event occurs in a hypoxic microenvironment. In the TME, the ability of DCs to respond to danger signals, to elaborate the antigen, to migrate and to present it to T-cells is crucial for generating specific T-cell immunity. Anti-cancer treatments can directly and indirectly modulate DC function [Marciscano et al., 2021]. As a matter of fact, overexpressing CAs or pulsing DCs with CAs fusion protein can generate a specific anti-tumor immune response [Kim et al., 2012]. Considering this evidence, we can hypothesize a pharmacological strategy based on overexpressing CAXII protein in DCs with the aim to increase their migration abilities and to promote a stronger immune response.

In this context, novel approaches to deliver CAXII protein or CAXII inhibitors are under investigation. Among them studies based on nanocarriers, as new systems to deliver drugs, are being developed. Indeed, liposomes and nanoparticles can improve drug stability, bioavailability, and targeting of specific cells. Nanocarriers can enable controlled drug release and precision targeting of pathological sites recognizing inflammatory markers. They can be released triggered by stimuli like pH changes, reactive oxygen species, or temperature. With this new approach skin disorders, as psoriasis (targeting CD44 proteins in inflamed skin) or melanoma (targeting specific receptors with ligands like epidermal growth factor) could be treated [Qu et al., 2022]. In addition, cell membrane-coated nanoparticles are being developed to directly target specific cells, such as cancer cells, in order to directly insert a specific drug into the cells cytoplasm [Fang et al., 2023] and this application could be also expanded to immune cells, in order to potentiate their activity. Indeed, in our contest, we could think to generate nanoparticles specifically directed against DCs, loaded with CAXII protein to increase their activity.
Specific DC- or melanoma- targeting nanocarriers could be developed to a better performance in conditions where different pH or hypoxia may activate the drug release and the cargo delivery precisely into target cells (avoiding a premature release).

In addition, DCs-based immunotherapy has emerged as a promising approach in the treatment of melanoma, which is often resistant to conventional therapies. In melanoma immunotherapy, autologous or allogeneic DCs are typically loaded with tumor-associated antigens and subsequently reinfused into patients to stimulate cytotoxic T lymphocyte responses capable of targeting melanoma cells. Recent studies have demonstrate a prolonged

progression-free survival and enhanced overall survival in patients receiving DC vaccines, particularly when combined with immune checkpoint inhibitors or cytokine-based adjuvants [Lesterhuis et al., 2011; Palucka & Banchereau, 2013]. Indeed, we could generate autologous or allogeneic DCs from melanoma patients and then overexpress CAXII protein to improve DCs efficiency.

In conclusion, our results may suggest a relevant role of CAXII in the physiology of either melanoma and DCs with important implications for the development of new therapeutical strategies against pathologies characterized by hypoxic microenvironments.

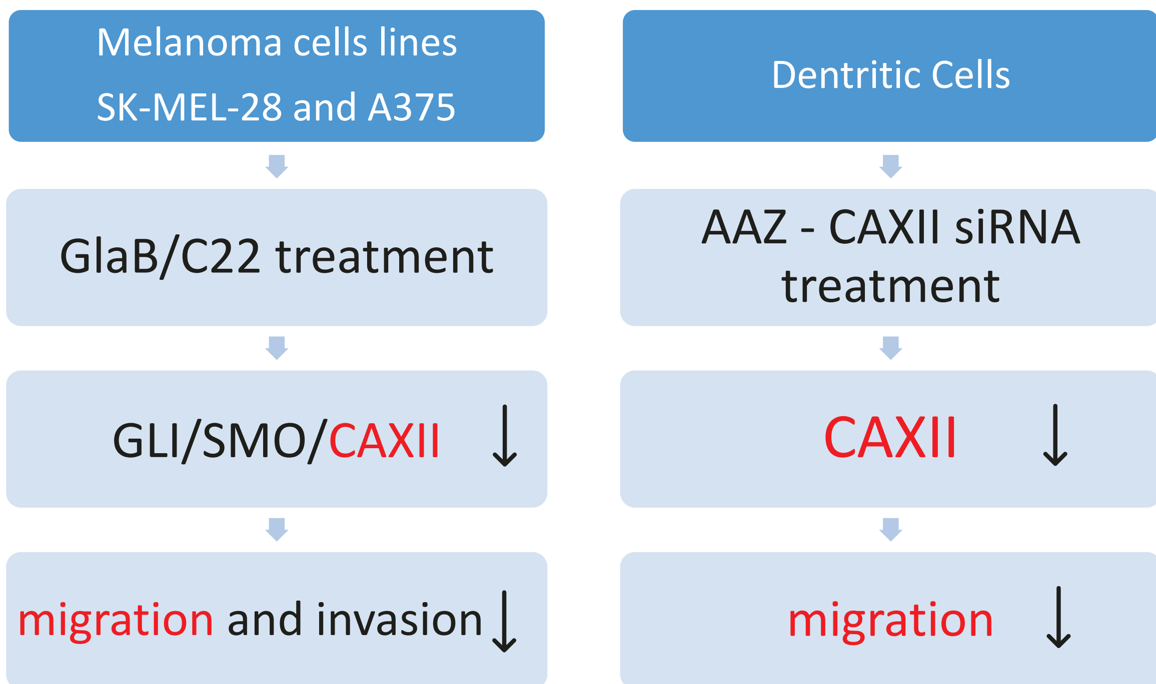

Figure 30. Graphical representation of main results. The migration abilities of melanoma cells and DCs are impaired when CAXII expression is reduced. In addition, in melanoma cells lines CAXII expression reduction was observed after Hh pathway inhibition, highlighting a link between CAXII and Hh pathway.

10. MATERIALS AND METHODS

10.1 Cell lines cultures

SK-MEL-28 and A375 melanoma cell lines were supplied by Dr. Francesca Chiarini (University of Bologna, Bologna, Italy) and Prof. Luisa Bracci (University of Siena, Siena, Italy), respectively. SK-MEL-28 was cultured with RPMI 1640 and A375 with DMEM, both supplemented with penicillin/streptomycin, L-Glutamine and 10% fetal bovine serum (FBS) (all purchased from Euroclone, Devon, UK). The two cell lines were maintained in a humidified atmosphere at 37 °C, 5% CO_2, 20% O_2, or at 37 °C, 5% CO_2, 2% O_2 to mimic a hypoxic microenvironment for experimental procedures by using the O_2 Control InVitro Glove Box (Coy Laboratory Products, USA).

10.2 Chemical compounds

10.2.1 Chemical compounds I

Cyclopamine (AlphaAesar, Haverhill, MA, USA) was resuspended in DMSO and used at a final concentration of 20 μM. SAG dihydrochloride (Sigma Aldrich, Saint Louis, MO, USA) was employed at a final concentration of 250 nM and was diluted in distilled water (ddH_2O). Glabrescione B (GlaB) and C22 were prepared according to the synthetic procedure reported previously [Lospinoso et al., 2019; Berardozzi Set al., 2018]. The structure was unambiguously confirmed through nuclear magnetic resonance (NMR) spectroscopy and by electrospray ionisation–high-resolution mass spectrometry (ESI-HRMS).

10.2.2 Chemical compound II

We used pan-CAs inhibitor Acetazolamide (AAZ) (Sigma-Aldrich, St.Louis, MO, USA): resuspended in dimethyl sulfoxide (DMSO) and diluted in water and used at a final concentration of 10 nM. Specifically, AAZ is a pharmacological sulphonamide-type inhibitor of several CA isoforms and is clinically used in various diseases such as, epilepsy and glaucoma [Tsikas, 2024]

10.3 Primary cells cultures

10.3.1 Primary Cell culture Reagents.

For human monocyte isolation Fycoll/Lympholite and Percoll were purchased from Cederlane Labs, Burlington, ON, Canada and Amersham Bioscience, Pittsburgh, PA, USA, respectively. RPMI 1640, fetal bovine serum (FBS), buffered saline solution (PBS), penicillin/streptomycin, and L-Glutamine (all purchased from Euroclone, Devon, UK), were used. Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-13 (IL-13), both by ProSpec TechnoGene, East Brunswick, NJ, USA, were used. Limulus Amebocyte Lysate assay was purchased from Cambrex, East Rutherford, NJ, USA, and used to assess that all reagents contained <0.125 endotoxin units/ml.

For DC terminal maturation LPS from Escherichia coli strain O26:B6 (Sigma–Aldrich, Milano, Italy) was added to cell culture medium.

10.3.2 Human monocyte isolation and monocyte-derived DC preparation.

Human monocytes were isolated from anonymous healthy donor buffy coats, provided from South-East Tuscany Blood Establishment, AOUS, Siena, Italy.

As a first step, platelets were removed by buffy coats centrifugation for 15 minutes at 900 x g, followed by washing with sterile PBS (pH 7.4 and without Ca^{2+} and Mg^{2+} to avoid coagulation) and centrifugation at 900 x g for 10 minutes. After these steps, blood was diluted with PBS in the ratio 1:1 and stratified on Ficoll (Lympholyte) in the ratio 2:1. Stratified blood was centrifuged at 800 x g for 30 minutes, with the result of erythrocytes and granulocytes sedimentation on the bottom of the tube, along with the formation, at the interface between the sample and the separation medium, of a ring composed of PBMCs (peripheral blood mononuclear cells), which include lymphocytes and monocytes. PBMCs were collected, washed for three times with PBS, stratified on percoll 285 mmOsm, in the ratio 1:1, and centrifuged for 30 minutes at 771 x g. This step allowed the precipitation of lymphocytes in the tube bottom, while monocytes ring appeared between the supernatant and the separation medium. The ring was collected, cells were washed and monocytes expressing CD14 (> 95%) were recovered. Then, monocytes were diluted in with RMPI with 5% FBS, at the concentration of 1x106/mL, seeded in 6 well plates (Corning, New York, NY, USA) and incubated to allow monocytes adhesion.

After 2 hours, cells were washed twice with PBS and then RMPI with 10% FBS and supplemented with 50 ng/ml GM-CSF and 20 ng/ml IL-13, was added and cells were incubated for 6 days under normoxia (atmospheric pO_2 levels: 21% O_2, 5% CO_2, and 74% N_2 corresponding to a pO_2 ~ 140 mmHg).

10.3.3 DC culture conditions.

At the end of the 6 days, iDCs, expressing >90% CD1a and <5% CD14, were obtained. Upon differentiation, cells were collected, by using cold PBS and cell scraper, and seeded at diverse concentration based on the further experimental condition, and treated with established compounds cells were cultured in the absence (iDCs) or in the presence of LPS (maDCs), at a concentration of 100 ng/ml, triggering their terminal maturation, and were exposed either to normoxia, in an incubator (New Brunswick Scientific, Edison, NJ) which was set at 5% CO_2 and 20.9% O_2 (pO2 ~ 140 mmHg; atmospheric pO2), or to hypoxia (5% CO_2 and 2% O_2, corresponding to a p O_2 ~ 14 mmHg) in the workstation O_2 Control InVitro Glove Box (Coy Laboratory Products, USA). The hypoxic pO_2 was choose since it resembles the average O_2 tension experienced by immune cells in the lymphoid organs.

Immature DCs were induced to final differentiation with LPS at a concentration of 100 ng/ml.

10.4 CAXII inhibition by RNA interference.

CAXII inhibition was reached out by an RNA interference approach. A specific CAXII-targeting siRNA were purchased from Sigma-Aldrich, St. Louis, MO, USA. As a control (Ctr-), the MISSION® siRNA Universal Negative Control #1 (SIC001) siRNA (Ctr-), was selected and purchased from Sigma-Aldrich, St. Louis, MO, USA. Each siRNA (CAXII and Ctr-), were used at final concentration of 46 nM. Transient transfection was carried out by using Lipofectamine RNAi MAX (Invitrogen, Paisley, UK).

iDCs were seeded and incubated for 2 h to allow adhesion. Then, siRNAs diluted with OPTI-MEM® (1X) (Gibco, Thermo Fisher Scientific, Cleveland, OH, USA) was added to tubes containing lipofectamine (diluted with OPTI-MEM® too), and the solution was gently mixed. After 20 minutes of incubation, the lypofectamine-siRNA complexes were added to the cells and DCs were incubate d for 24 h under normoxia. After 24 h, the culture medium was replaced and DCs were exposed to normoxic or hypoxic conditions for indicated times.

10.5 GENE AND PROTEIN EXPRESSION ANALYSIS.

10.5.1 Western Blotting and antibodies.

Western Blot was performed in order to detect protein expression. To this end, DCs 5 x 10^5 cells/mL were plated in 35x10 mm Petri's dishes (Corning, New York, NY, USA), treated as experiment design and exposed to normoxia and hypoxia. At indicated times, supernatants were collected and centrifuged to collect suspended cells, while adherent cells were washed with cold PBS and lysed with RIPA buffer (40 µL) supplemented with a protease inhibitor

cocktail (purchased from Sigma-Aldrich, St. Louis, MO, USA). Samples were centrifuged at 14000 x g for 20 minutes.

SK-MEL-28 and A375 were seeded in Petri dishes, treatments with the compounds of interest were performed and experiments were conducted under normoxia and hypoxia. Cells were lysed with Laemmli buffer with proteinase inhibitors added (Sigma Aldrich, Saint Louis, MO, USA). Samples were sonicated.

The total protein concentration of each sample was determined by Micro BCA Protein Assay Reagent kit (Rockford, USA). Then, equal amounts of proteins were loaded onto SDS-PAGE electrophoresis gel and, at the end of the run, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for aspecific binding sites with Bio-Rad EveryBlot Blocking Buffer (Bio-Rad, Hercules, CA, USA) for 5 minutes.

Membranes were incubated overnight, at 4°C, with the following primary antibodies: HIF-1α (BD Biosciences, San Jose, CA, USA, 1:500), SMO and CAXII (Cell Signaling, Denver, CO, USA, 1:1000) and β-actin (Sigma-Aldrich, 1:50000). Anti-mouse Cell Signaling Technologies, Danvers, MA, 1:2000) and anti-rabbit (Cell Signaling Technologies, Danvers, MA, 1:2000) IgG HRP-conjugated were used as secondary antibodies. Protein detection and image acquisition were performed using the ChemiDocXRS (Bio-Rad, Hercules, CA, USA) and the Image Lab software (Bio-Rad, Hercules, CA, USA) was used to quantify band intensity.

10.5.2 RNA extraction and qRT-PCR.

To analyze gene expression, DCs were seeded in 6-well plates (Corning, New York, NY, USA) at the density of 2,5 x 10^5 cells/well and lysed with EuroGOLDTM Trifast reagent (500 μL), purchased from Euroclone, Devon, UK.

Then, chloroform was used to separate the sample in three phases: the upper phase containing total RNA was collected and, following washes with isopropanol and cold ethanol (75%), was centrifuged at 12.000 x g. The obtained pellet was resuspended in nuclease-free water and boiled for 10 minutes at 56°C.

Total RNA was measured with Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, complementary DNA (cDNA) was obtainied by iScriptTMcDNA Synthesis kit (Biorad laboratories, Bio-Rad, Hercules, CA, USA) used to convert 1 µg of RNA to cDNA. RT-qPCR was performed with the iTaqTMSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and primers for CAXII, β-actin and L-32 (the last two used as housekeeping gene). Gene expression was analyzed with the CFX DUET Real Time – PCR System and Maestro Software (Bio-Rad, Hercules, CA, USA) by using the 2-ΔΔCT method (Livak & Schmittgen, 2001).

10.6 Immunofluorescence

SK-MEL-28 or A375 at a density of 25,000 cells for each condition were seeded into 24-well plates with cover slides 10 mm in diameter and exposed to normoxia or hypoxia for 24 h.

DCs were seeded at a concentration of 50.000 cells/well in 24-well plates and treated according to experimental condition; after 24h cells were collected, centrifuged and pellet resuspendend in PBS. Immunofluorescence slides (Epredia, Epredia Netherlands B.V., Essendonk) were pre coated with Poly-D-Lysine (Sigma Aldrich, Saint Louis, MO, USA) in order to facilitate cell adesion for 20 minutes RT and let dry and cells were seeded 30 µl/well containg 50.000 cells. Cells were let attach to te slid for 20 min RT.

Cells were fixed and permeabilised with methanol at −20 °C. Primary antibodies HIF-1α (BD Biosciences, San Jose, CA, USA), CAXII, SMO (Santa Cruz, Dallas, TX, USA) and GLI1 (Cell Signaling, Denver, CO, USA) were diluted 1:300 in PBS 2% BSA and incubated overnight at 4 °C in a humidified chamber. The next day, after 2 washing steps with PBS 0.2% BSA, secondary antibody antimouse 488 conjugated (Thermo Fisher Scientific, Cleveland, OH, USA) or antirabbit 550 conjugated (Thermo Fisher Scientific, Cleveland, OH, USA, 1:300) was added and incubated at RT for 1 h. Nuclei were stained with Hoechst 33342 (Fluka, Sigma Aldrich, Saint Louis, MO, USA) 1 mg/mL (1:1000) for 4 min at RTafyter final washing, cover slides were mounted onto glass microscope slides, or coversildes were mounted into immunofluorescence slides with Mowiol® 4-88 Reagent (EMD Millipore, Burlington MA, USA).

Images with 20X or 60X magnification were acquired with an Olympus IX81 microscope (Olympus,Tokyo, Japan) and analysed with ImageJ software. Fluorescence intensity was expressed by corrected total cell fluorescence (CTCF) = integrated density-(area of selected cell X mean fluorescence of background readings); colocalisation analysis was performed with JACoP plugin, calculating Mander’s coefficient [Manders et al., 1992].

10.7 Wound healing assay

SK-MEL-28 and A375 were seeded in a culture-insert 2 well in a 35 mm µ-Dish (ibidi culture-insert 2 well, ibidi GmbH, Martinsried, Germany). The day after, the culture-insert was removed and non-adherent cells were washed with PBS. New culture medium with 10% FBS, AraC (2.5 µg/mL) and selected chemical compounds were added. Cells were incubated either under normoxia (37 °C, 5% CO2, 20% O2) or hypoxia (37 °C, 5% CO2, 2% O2) for 24 h and 48 h. At the established times of 0 h, 24 h, 48 h, images of the wound gap were acquired with a phase-contrast microscope Olympus IX81 with 10x magnification (Olympus, Tokyo, Japan) and analysed with Cell F© software 11.21 (Olympus, Tokyo, Japan). Migration was calculated as (1 − AxA0) and data expressed in % of wound repair (Ax and A0 represent the empty area at the acquisition times).

10.8 Modified Boyden chamber

Detecting invasion for melanoma cells: Boyden 48-well microchemotaxis chambers (Neuro Probe, Gaithersburg, MD, UK) with 8µm pore size polycarbonate polyvinylpyrrolidone-free nucleopore filters, precoated with 100 µL of 0.2 mg/mL Matrigel (Corning, Life Science, Corning, Tewksbury, MA, USA), were used to test melanoma invasion ability. Cells, with or without compound treatments, were seeded in the upper chamber in 50 µL RPMI or DMEM with 0.1% BSA. NIH3T3 supernatant was employed as chemoattractant in the lower chamber. Then, cells were fixed and stained with Diff Quick (Merz-Dade, Düdingen, Switzerland) and images were acquired at 10× magnification with an Olympus IX81 microscope (Olympus, Tokyo, Japan). Data were expressed as % of invaded cells.

Detecting migration for DCs: Boyden 48-well microchemotaxis chambers (Neuro Probe, Gaithersburg, MD, UK) with 5µm pore size polycarbonate polyvinylpyrrolidone-free nucleopore filters, precoated with 100 µL of 0.2 mg/mL Gelatin from porcine skin (Corning, Life Science, Corning, Tewksbury, MA, USA), were used to test melanoma invasion ability. Cells, with or without compound treatments, were seeded in the upper chamber in 50 µL RPMI with 0.1% BSA. NIH3T3 supernatant was employed as chemoattractant in the lower chamber. Then, cells were fixed and stained with Diff Quick (Merz-Dade, Düdingen, Switzerland) and images were acquired at 20× magnification with an Olympus IX81 microscope (Olympus, Tokyo, Japan). Data were expressed as N. of invaded cells.

10.9 Statistical Analysis

The data are shown as the mean ± SEM of at least three independent experiments. Statistical analyses were performed with Graph-Pad Prism (San Diego, CA, USA). Analysis of variance (ANOVA) and unpaired two-tailed Student's t test were used to test for significant numerical differences among data groups. Difference of $p \leq 0.05$ was statistically significant (*$p \leq 0.05$; **$p \leq 0.01$).

11. ADDENDUM

Part of this thesis work has been published in the following manuscript:

Falsini, A., Giuntini, G., Mori, M., Ghirga, F., Quaglio, D., Cucinotta, A., Coppola, F., Filippi, I., Naldini, A., Botta, B., & Carraro, F. (2024). Hedgehog Pathway Inhibition by Novel Small Molecules Impairs Melanoma Cell Migration and Invasion under Hypoxia. Pharmaceuticals (Basel, Switzerland), 17(2), 227.

12. REFERENCES

Arneth B. (2021). Trained innate immunity. Immunologic research, 69(1), 1–7.

Atwood, S. X., Chang, A. L., & Oro, A. E. (2012). Hedgehog pathway inhibition and the race against tumor evolution. The Journal of cell biology, 199(2), 193–197.

Bao, X., Zhang, J., Huang, G., Yan, J., Xu, C., Dou, Z., . . . Zhang, H. (2021). The crosstalk between HIFs and mitochondrial dysfunctions in cancer development. *Cell Death & Disease*, *12*(2), 215.

Berardozzi S., Bernardi F., Infante P., Ingallina C., Toscano S., De Paolis E., Alfonsi R., Caimano M., Botta B., Mori M., et al. Synergistic inhibition of the Hedgehog pathway by newly designed Smo and Gli antagonists bearing the isoflavone scaffold. Eur. J. Med. Chem. 2018;156:554–562.

Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, et al. Widespread requirement for hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;425(6960):846-51

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., & Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. The Journal of experimental medicine, 196(12), 1627–1638.

Boron WF. Evaluating the role of carbonic anhydrases in the transport of HCO_3^- related species. Biochim Biophys Acta 2010 (1804), 410–21

Bosco, M. C., & Varesio, L. (2012). Dendritic cell reprogramming by the hypoxic environment. Immunobiology, 217(12), 1241–1249.

Braun, A., Worbs, T., Moschovakis, G. L., Halle, S., Hoffmann, K., Bölter, J., Münk, A., & Förster, R. (2011). Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. Nature immunology, 12(9), 879–887.

Chaplin D. D. (2010). Overview of the immune response. The Journal of allergy and clinical immunology, 125(2 Suppl 2), S3–S23.

Cheng, L., Lopez-Beltran, A., Massari, F., MacLennan, G. T., & Montironi, R. (2018). Molecular testing for BRAF mutations to inform melanoma treatment decisions: a move toward precision medicine. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 31(1), 24–38.

Comabella, M., Montalban, X., Münz, C., & Lünemann, J. D. (2010). Targeting dendritic cells to treat multiple sclerosis. Nature reviews. Neurology, 6(9), 499–507

Crowley, M. T., Inaba, K., Witmer-Pack, M. D., Gezelter, S., & Steinman, R. M. (1990). Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. Journal of immunological methods, 133(1), 55–66

Cummins, E. P., Keogh, C. E., Crean, D., & Taylor, C. T. (2016). The role of HIF in immunity and inflammation. Molecular Aspects of Medicine, 47, 24-34.

D'Orazio J., Jarrett S., Amaro-Ortiz A., Scott T. UV radiation and the skin. Int. J. Mol. Sci. 2013;14:12222–12248.

Damsky, W. E., Curley, D. P., Santhanakrishnan, M., Rosenbaum, L. E., Platt, J. T., Gould Rothberg, B. E., Taketo, M. M., Dankort, D., Rimm, D. L., McMahon, M., & Bosenberg, M. (2011). β-catenin signaling controls metastasis in Braf-activated Pten-deficient melanomas. *Cancer cell*, *20*(6), 741–754.

Das, S., Harris, L. G., Metge, B. (J., Liu, S., Riker, A. I., Samant, R. S., & Shevde, L. A. (2009). The hedgehog pathway transcription factor GLI1 promotes malignant behavior of cancer cells by up-regulating osteopontin. The Journal of biological chemistry, 284(34), 22888–22897

Del Prete S, Vullo D, Zoccola D, et al. Kinetic properties and affinities for sulfonamide inhibitors of an a-carbonic anhydrase (CruCA4) involved in coral biomineralization in the Mediterranean red coral Corallium rubrum. Bioorg Med Chem 2017;25:3525–30

Diaz-Del-Olmo, I., Worboys, J., Martin-Sanchez, F., Gritsenko, A., Ambrose, A. R., Tannahill, G. M., Nichols, E. M., Lopez-Castejon, G., & Davis, D. M. (2021). Internalization of the Membrane Attack Complex Triggers NLRP3 Inflammasome Activation and IL-1β Secretion in Human Macrophages. Frontiers in immunology, 12, 720655.

Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 2009; 58: 49-59

Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 2009; 58: 49-59

Doheny D., Manore S.G., Wong G.L., Lo H.W. Hedgehog Signaling and Truncated GLI1 in Cancer. Cells. 2020;9:2114.

Dudek, A. M., Martin, S., Garg, A. D., & Agostinis, P. (2013). Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity. Frontiers in immunology, 4, 438.

Eales, K. L., Hollinshead, K. E., & Tennant, D. A. (2016). Hypoxia and metabolic adaptation of cancer cells. Oncogenesis, 5, e190

Eddy, K., & Chen, S. (2020). Overcoming immune evasion in melanoma. In International Journal of Molecular Sciences 21(23) 1–48).

Eggermont, A. M. M., Chiarion-Sileni, V., Grob, J.-J., Dummer, R., Wolchok, J. D., Schmidt, H., Hamid, O., Robert, C., Ascierto, P. A., Richards, J. M., Lebbé, C., Ferraresi, V., Smylie, M., Weber, J. S., Maio, M., Bastholt, L., Mortier, L., Thomas, L., Tahir, S., …

Testori, A. (2016). Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. New England Journal of Medicine, 375(19), 1845–1855.

Elia AR, Cappello P, Puppo M, Fraone T, Vanni C, Eva A, Musso T, Novelli F, Varesio L, Giovarelli M. Human dendritic cells differentiated in hypoxia down-modulate antigen uptake and change their chemokine expression profile. J Leukoc Biol 2008; 84: 1472-148)

Estrella, V., Chen, T., Lloyd, M., Wojtkowiak, J., Cornnell, H. H., Ibrahim-Hashim, A., Bailey, K., Balagurunathan, Y., Rothberg, J. M., Sloane, B. F., Johnson, J., Gatenby, R. A., & Gillies, R. J. (2013). Acidity generated by the tumor microenvironment drives local invasion. *Cancer research*, *73*(5), 1524–1535.

Fais, S., Venturi, G., & Gatenby, R. A. (2014). Microenvironmental acidosis in carcinogenesis and metastases: New strategies in prevention and therapy. Cancer Metastasis Reviews, 33(4), 1095–1108

Fang, R. H., Gao, W., & Zhang, L. (2023). Targeting drugs to tumours using cell membrane-coated nanoparticles. Nature reviews. Clinical oncology, 20(1), 33–48

Faubert, B., Li, K. Y., Cai, L., et al. (2020). Lactate metabolism in human lung tumors. Cell, 183(6), 1519–1535

Filippi, I., Morena, E., Aldinucci, C., Carraro, F., Sozzani, S., & Naldini, A. (2014). Short-term hypoxia enhances the migratory capability of dendritic cell through HIF-1α and PI3K/Akt pathway. Journal of cellular physiology, 229(12), 2067–2076.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., & Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell, 99(1), 23–33.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. Science (New York, N.Y.), 327(5966), 656–661.

Ghaffari, S., & Rezaei, N. (2023). Eosinophils in the tumor microenvironment: implications for cancer immunotherapy. Journal of translational medicine, 21(1), 551

Giaccia, A. J., Simon, M. C., & Johnson, R. (2004). The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes & development, 18(18), 2183–2194.

Giatromanolaki, A., Sivridis, E., Maltezos, E., et al. (2013). Hypoxia inducible factor-1α and vascular endothelial growth factor in melanoma: A relationship with microvessel density and prognosis. Melanoma Research, 23(3), 210–216.;

Giuntini, G., Coppola, F., Falsini, A., Filippi, I., Monaci, S., Naldini, A., & Carraro, F. (2022). Role of the Hedgehog Pathway and CAXII in Controlling Melanoma Cell Migration and Invasion in Hypoxia. Cancers, 14(19), 4776.

Granucci, F., Foti, M., & Ricciardi-Castagnoli, P. (2005). Dendritic cell biology. Advances in immunology, 88, 193–233.

Grisaru-Tal, S., Rothenberg, M. E., & Munitz, A. (2022). Eosinophil-lymphocyte interactions in the tumor microenvironment and cancer immunotherapy. Nature immunology, 23(9), 1309–1316

Guerrini, G., Criscuoli, M., Filippi, I., Naldini, A., & Carraro, F. (2018). Inhibition of smoothened in breast cancer cells reduces CAXII expression and cell migration. Journal of cellular physiology, 233(12), 9799–9811.

Guerrini, G., Durivault, J., Filippi, I., Criscuoli, M., Monaci, S., Pouyssegur, J., Naldini, A., Carraro, F., & Parks, S. K. (2019). Carbonic anhydrase XII expression is linked to suppression of Sonic hedgehog ligand expression in triple negative breast cancer cells. Biochemical and biophysical research communications, 516(2), 408–413

Halligan, D. N., Murphy, S. J., & Taylor, C. T. (2016). The hypoxia-inducible factor (HIF) couples immunity with metabolism. Seminars in immunology, 28(5), 469–477.

Halsey, S. M., Crowther, L. M., Walker, R., et al. (2013). Hypoxia-inducible factor (HIF)-1α-driven responses in melanoma progression. International Journal of Molecular Sciences, 14(4), 8481–8501.).

Hammer, G. E., & Ma, A. (2013). Molecular control of steady-state dendritic cell maturation and immune homeostasis. Annual review of immunology, 31, 743–791.

Henze AT, Mazzone M. The impact of hypoxia on tumor associated macrophages. J Clin Invest 2016; 126: 3672-3679

Horikawa, M., Sabe, H., & Onodera, Y. (2022). Dual roles of AMAP1 in the transcriptional regulation and intracellular trafficking of carbonic anhydrase IX. Translational oncology, 15(1), 101258

Huston DP. (1997). The biology of the immune system. JAMA.; 278:1804–1814

Infante, P., Malfanti, A., Quaglio, D., Balducci, S., De Martin, S., Bufalieri, F., Mastrotto, F., Basili, I., Garofalo, M., Lospinoso Severini, L., Mori, M., Manni, I., Moretti, M., Nicoletti, C., Piaggio, G., Caliceti, P., Botta, B., Ghirga, F., Salmaso, S., & Di Marcotullio, L. (2021). Glabrescione B delivery by self-assembling micelles efficiently inhibits tumor growth in preclinical models of Hedgehog-dependent medulloblastoma. Cancer letters, 499, 220–231

Ira Mellman; (2013). Dendritic Cells: Master Regulators of the Immune Response. Cancer Immunol Res; 1 (3): 145–149.

Ishikawa, H., Barber, G. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455, 674–678.

Jantsch, J., Chakravortty, D., Turza, N., Prechtel, A. T., Buchholz, B., Gerlach, R. G., Volke, M., Gläsner, J., Warnecke, C., Wiesener, M. S., Eckardt, K. U., Steinkasserer, A., Hensel, M., & Willam, C. (2008). Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. Journal of immunology (Baltimore, Md.: 1950), 180(7), 4697–4705.

Jenkins, R. W., & Fisher, D. E. (2021). Treatment of Advanced Melanoma in 2020 and Beyond. In Journal of Investigative Dermatology (Vol. 141, Issue 1, pp. 23–31). Elsevier B.V.

Jentho, E., & Weis, S. (2021). DAMPs and Innate Immune Training. Frontiers in immunology, 12, 699563.

Jiang J, Hui CC. Hedgehog signaling in development and cancer. Dev Cell 2008;15(6):801-12.]

Joffre, O. P., Segura, E., Savina, A., & Amigorena, S. (2012). Cross-presentation by dendritic cells. Nature reviews. Immunology, 12(8), 557–569.

Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas JM, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science 1996;272(5268):1668-71

Kasper, M., Regl, G., Frischauf, A. M., & Aberger, F. (2006). GLI transcription factors: mediators of oncogenic Hedgehog signalling. *European journal of cancer (Oxford, England : 1990)*, *42*(4), 437–445

Kiani, A. A., Elyasi, H., Ghoreyshi, S., Nouri, N., Safarzadeh, A., & Nafari, A. (2021). Study on hypoxia-inducible factor and its roles in immune system. Immunological medicine, 44(4), 223–236.

Kim, B. R., Yang, E. K., Kim, D. Y., Kim, S. H., Moon, D. C., Lee, J. H., Kim, H. J., & Lee, J. C. (2012). Generation of anti-tumour immune response using dendritic cells pulsed with carbonic anhydrase IX-Acinetobacter baumannii outer membrane protein A fusion proteins against renal cell carcinoma. *Clinical and experimental immunology*, *167*(1), 73–83.

King, R., Hayes, C., Donohoe, C. L., Dunne, M. R., Davern, M., & Donlon, N. E. (2021). Hypoxia and its impact on the tumour microenvironment of gastroesophageal cancers. World journal of gastrointestinal oncology, 13(5), 312–331.

Kubo M, Nakamura M, Tasaki A, Yamanaka N, Nakashima H, Nomura M, et al. Hedgehog signaling pathway is a new ther¬apeutic target for patients with breast cancer. Cancer Res 2004;64(17):6071-4.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., & Bruick, R. K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes & development, 16(12), 1466–1471.

Lane, N., & Martin, W. (2010). The energetics of genome complexity. *Nature*, *467*(7318), 929–934.

Larkin, J., Chiarion-Sileni, V., Gonzalez, R., Grob, J.-J., Rutkowski, P., Lao, C. D., Cowey, C. L., Schadendorf, D., Wagstaff, J., Dummer, R., Ferrucci, P. F., Smylie, M., Hogg, D., Hill, A., Márquez-Rodas, I., Haanen, J., Guidoboni, M., Maio, M., Schöffski, P., … Wolchok, J. D. (2019). Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. New England Journal of Medicine, 381(16), 1535–1546.

Lee, S. K., Boron, W. F., & Occhipinti, R. (2023). Potential Novel Role of Membrane-Associated Carbonic Anhydrases in the Kidney. International journal of molecular sciences, 24(4)

Lesterhuis, W. J., Haanen, J. B., & Punt, C. J. (2011). Cancer immunotherapy--revisited. Nature reviews. Drug discovery, 10(8), 591–600.

Liu, J., Zhang, X., Cheng, Y., & Cao, X. (2021). Dendritic cell migration in inflammation and immunity. Cellular & molecular immunology, 18(11), 2461–2471.

Liu, Z., Tu, K., Wang, Y., Yao, B., Li, Q., Wang, L., Dou, C., Liu, Q., & Zheng, X. (2017). Hypoxia Accelerates Aggressiveness of Hepatocellular Carcinoma Cells Involving Oxidative Stress, Epithelial-Mesenchymal Transition and Non-Canonical Hedgehog Signaling. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology, 44(5), 1856–1868.

Lospinoso Severini L., Quaglio D., Basili I., Ghirga F., Bufalieri F., Caimano M., Balducci S., Moretti M., Romeo I., Loricchio E. A smo/gli multitarget hedgehog pathway inhibitor impairs tumor growth. Cancers. 2019;11:1518.

MacIntyre, N. R. (2014). Tissue hypoxia: implications for the respiratory clinician. Respiratory Care, 59(10), 1590-1596.

Mancino, A., Schioppa, T., Larghi, P., Pasqualini, F., Nebuloni, M., Chen, I. H., Sozzani, S., Austyn, J. M., Mantovani, A., & Sica, A. (2008). Divergent effects of hypoxia on dendritic cell functions. Blood, 112(9), 3723–3734.

Manders E.M., Stap J., Brakenhoff G.J., van Driel R., Aten J.A. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. Pt 3J. Cell Sci. 1992;103:857–862

Marciscano, A. E., & Anandasabapathy, N. (2021). The role of dendritic cells in cancer and anti-tumor immunity. Seminars in immunology, 52, 101481

Marshall, J.S., Warrington, R., Watson, W. et al. (2018). An introduction to immunology and immunopathology. Allergy Asthma Clin Immunol 14 (Suppl 2), 49.

Martin, D. S., Khosravi, M., Grocott, M. P., & Mythen, M. G. (2010). Concepts in hypoxia reborn. Critical care (London, England), 14(4), 315.

Mazure, N. M., & Pouysségur, J. (2010). Hypoxia-induced autophagy: cell death or cell survival? Current opinion in cell biology, 22(2), 177–180.

Mccomb S, Thiriot A, Akache B, Krishnan L, Stark F. Introduction to the immune system. Methods Mol Biol. (2019) 2024:1–24. doi: 10.1007/978-1-4939-9597-4_1).

McGettrick, A. F., & O'Neill, L. A. J. (2020). The Role of HIF in Immunity and Inflammation. Cell metabolism, 32(4), 524–536.

McMahon, C., Will, A., Hu, P., Shah, G. N., Sly, W. S., & Smith, O. P. (2001). Bone marrow transplantation corrects osteopetrosis in the carbonic anhydrase II deficiency syndrome. Blood, 97(7), 1947–1950.

Mellman and Steinman, 2001; Mellman, 2013

Mellman, I., & Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. Cell, 106(3), 255–258.

Menuet, A., Alunni, A., Joly, J. S., Jeffery, W. R., & Rétaux, S. (2007). Expanded expression of Sonic Hedgehog in Astyanax cavefish: multiple consequences on forebrain development and evolution. Development (Cambridge, England), 134(5), 845–855

Monaci, S., Coppola, F., Filippi, I., Falsini, A., Carraro, F., & Naldini, A. (2024). Targeting hypoxia signaling pathways in angiogenesis. Frontiers in physiology, 15, 1408750.

Monti, S. M., De Simone, G., Dathan, N. A., Ludwig, M., Vullo, D., Scozzafava, A., Capasso, C., & Supuran, C. T. (2013). Kinetic and anion inhibition studies of a β-carbonic anhydrase (FbiCA 1) from the C4 plant Flaveria bidentis. Bioorganic & medicinal chemistry letters, 23(6), 1626–1630.

Morgan B. P. (2016). The membrane attack complex as an inflammatory trigger. Immunobiology, 221(6), 747–751.

Murone M, Rosenthal A, de Sauvage FJ. Hedgehog signal transduction: From flies to vertebrates. Exp Cell Res 1999;253(1):25-33.

Murphy K., Weaver C. (2017). Janeway's Immunobiology, Garland Science.

Muz, B., de la Puente, P., Azab, F., & Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia, 3, 83–92

Nakazawa, M. S., Keith, B., & Simon, M. C. (2016). Oxygen availability and metabolic adaptations. Nature reviews. Cancer, 16(10), 663–673.

Naldini, A., Morena, E., Pucci, A., Miglietta, D., Riboldi, E., Sozzani, S., & Carraro, F. (2012). Hypoxia affects dendritic cell survival: role of the hypoxia-inducible factor-1α and lipopolysaccharide. Journal of cellular physiology, 227(2), 587–595.

Nesargikar, P. N., Spiller, B., & Chavez, R. (2012). The complement system: history, pathways, cascade and inhibitors. European journal of microbiology & immunology, 2(2), 103–111.

Nizet, V., & Johnson, R. S. (2009). Interdependence of hypoxic and innate immune responses. Nature reviews. Immunology, 9(9), 609–617.

Nocentini, A., Supuran, C. T., & Capasso, C. (2021). An overview on the recently discovered iota-carbonic anhydrases. *Journal of enzyme inhibition and medicinal chemistry*, *36*(1), 1988–1995.

Noman, M. Z., Desantis, G., Janji, B., et al. (2015). PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. Journal of Experimental Medicine, 211(5), 781–790

Nusslein-Volhard and Eric Wieschaus, 1980

O'Reilly KE, de Miera EV, Segura MF, Friedman E, Poliseno L, Han SW, et al. Hedgehog pathway blockade inhibits mela¬noma cell growth in vitro and in vivo. Pharmaceuticals (Basel) 2013;6(11):1429-50.

Palucka, K., & Banchereau, J. (2013). Dendritic-cell-based therapeutic cancer vaccines. Immunity, 39(1), 38–48

Pastorekova, S., et al. (2008). Carbonic anhydrases: Current state of the art, therapeutic applications, and future prospects. Journal of Medicinal Chemistry, 51(1), 1–18.).

Pastorekova, S., Kopacek, J., & Pastorek, J. (2007). Carbonic anhydrase inhibitors and the management of cancer. Current topics in medicinal chemistry, 7(9), 865–878.

Pearce, E. J., & Everts, B. (2015). Dendritic cell metabolism. Nature reviews. Immunology, 15(1), 18–29.

Pouysségur, J., Dayan, F., & Mazure, N. M. (2006). Hypoxia signaling in cancer and approaches to enforce tumor regression. Nature, 441(7092), 437–443

Qu, F., Geng, R., Liu, Y., & Zhu, J. (2022). Advanced nanocarrier- and microneedle-based transdermal drug delivery strategies for skin diseases treatment. Theranostics, 12(7), 3372–3406

Rankin, E. B., & Giaccia, A. J. (2016). Hypoxic control of metastasis. Science, 352(6282), 175-180.

Rebecca VW, Sondak VK, Smalley KS (2012) A brief history of melanoma: from mummies to mutations. Melanoma Res 22 (2):114-122

Rebecca, V. W., Somasundaram, R., & Herlyn, M. (2020). Pre-clinical modeling of cutaneous melanoma. In Nature Communications (Vol. 11, Issue 1). Nature Research.

Reemst, K., Noctor, S. C., Lucassen, P. J., & Hol, E. M. (2016). The Indispensable Roles of Microglia and Astrocytes during Brain Development. Frontiers in human neuroscience, 10, 566.

Robbins, D. J., Fei, D. L., & Riobo, N. A. (2012). The Hedgehog signal transduction network. Science signaling, 5(246), re6.

Robbins, D. J., Fei, D. L., & Riobo, N. A. (2012). The Hedgehog signal transduction network. *Science signaling*, *5*(246), re6

Rubin LL, de Sauvage FJ. Targeting the hedgehog pathway in cancer. Nat Rev Drug Discov 2006;5(12):1026-33.

Rubinsztein, D. C., Codogno, P., & Levine, B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. Nature reviews. Drug discovery, 11(9), 709–730.

Saginala K, Barsouk A, Aluru JS, Rawla P, Barsouk A (2021) Epidemiology of Melanoma. Med Sci (Basel) 9 (4)

Samanta D, Semenza GL. (2018). Metabolic adaptation of cancer and immune cells mediated by hypoxia inducible factors. Biochem. Biopsy's. Acta Rev. Cancer 1870:15–22

Santos, P. M., & Butterfield, L. H. (2018). Dendritic Cell-Based Cancer Vaccines. Journal of immunology; Sabado, R. L., Balan, S., & Bhardwaj, N. (2017). Dendritic cell-based immunotherapy. Cell research, 27(1), 74–95

Sattler S. (2017). The Role of the Immune System Beyond the Fight Against Infection. Advances in experimental medicine and biology, 1003, 3–14.

Schneider, P., Fandrey, J., & Leu, T. (2025). Primary cilia as antennas for oxygen. American journal of physiology. Cell physiology, 328(2), C381–C386.

Schofield, C. J., & Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. Nature reviews. Molecular cell biology, 5(5), 343–354.

Sekulic, A., Migden, M. R., Oro, A. E., Dirix, L., Lewis, K. D., Hainsworth, J. D., Solomon, J. A., Yoo, S., Arron, S. T., Friedlander, P. A., Marmur, E., Rudin, C. M., Chang, A. L., Low, J. A., Mackey, H. M., Yauch, R. L., Graham, R. A., Reddy, J. C., & Hauschild, A. (2012). Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *The New England journal of medicine*, *366*(23), 2171–2179.

Semenza G. L. (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. Trends in molecular medicine, 8(4 Suppl), S62–S67.

Semenza G. L. (2010). Oxygen homeostasis. Wiley interdisciplinary reviews. Systems biology and medicine, 2(3), 336–361.

Semenza G. L. (2012). Hypoxia-inducible factors in physiology and medicine. Cell, 148(3), 399–408

Semenza G. L. (2020). The Genomics and Genetics of Oxygen Homeostasis. Annual review of genomics and human genetics, 21, 183–204

Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and cellular biology, 12(12), 5447–5454.

Semenza, G. L., Nejfelt, M. K., Chi, S. M., & Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proceedings of the National Academy of Sciences of the United States of America, 88(13), 5680–5684.

Sherwood, E. R., Burelbach, K. R., McBride, M. A., Stothers, C. L., Owen, A. M., Hernandez, A., Patil, N. K., Williams, D. L., & Bohannon, J. K. (2022). Innate Immune Memory and the Host Response to Infection. Journal of immunology (Baltimore, Md.: 1950), 208(4), 785–792.

Skoda, A. M., Simovic, D., Karin, V., Kardum, V., Vranic, S., & Serman, L. (2018). The role of the hedgehog signaling pathway in cancer: A comprehensive review. In *Bosnian Journal of Basic Medical Sciences* (Vol. 18, Issue 1, pp. 8–20). Association of Basic Medical Sciences of FBIH

Sly, W. S., Hewett-Emmett, D., Whyte, M. P., Yu, Y. S., & Tashian, R. E. (1983). Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(9), 2752–2756

Sozzani S. (2005). Dendritic cell trafficking: more than just chemokines. Cytokine & growth factor reviews, 16(6), 581–592.

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. The Journal of experimental medicine, 137(5), 1142–1162.

Steinman, R. M., & Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proceedings of the National Academy of Sciences of the United States of America, 75(10), 5132–5136.

Sundararajan, S., Thida, A. M., Yadlapati, S., Mukkamalla, S. K. R., & Koya, S. (2024). Metastatic Melanoma. In *StatPearls*. StatPearls Publishing

Sunderkötter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., & Sorg, C. (1994). Macrophages and angiogenesis. Journal of leukocyte biology, 55(3), 410–422.

Supuran C. T. (2008). Carbonic anhydrases--an overview. Current pharmaceutical design, 14(7), 603–614).

Supuran C. T. (2018). Carbonic anhydrase activators. Future medicinal chemistry, 10(5), 561–573

Supuran C. T. (2020). Experimental Carbonic Anhydrase Inhibitors for the Treatment of Hypoxic Tumors. Journal of experimental pharmacology, 12, 603–617

Supuran C. T. (2020). Experimental Carbonic Anhydrase Inhibitors for the Treatment of Hypoxic Tumors. *Journal of experimental pharmacology*, *12*, 603–617

Supuran CT, Capasso C. New light on bacterial carbonic anhydrases phylogeny based on the analysis of signal peptide sequences. J Enzyme Inhib Med Chem 2016;31:1254–60)

Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008(7), 168–81.

Swayampakula, M., McDonald, P. C., Vallejo, M., Coyaud, E., Chafe, S. C., Westerback, A., Venkateswaran, G., Shankar, J., Gao, G., Laurent, E. M. N., Lou, Y., Bennewith, K. L., Supuran, C. T., Nabi, I. R., Raught, B., & Dedhar, S. (2017). The interactome of metabolic enzyme carbonic anhydrase IX reveals novel roles in tumor cell migration and invadopodia/MMP14-mediated invasion. Oncogene, 36(45), 6244–6261.

Swietach, P., Hulikova, A., Vaughan-Jones, R. D., & Harris, A. L. (2010). New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. *Oncogene*, *29*(50), 6509–6521

Szaraz, D., Danek, Z., Lipovy, B., Krivanek, J., Buchtova, M., Moldovan Putnova, B., Putnova, I., Stembirek, J., Andrasina, T., Divacka, P., Izakovicova Holla, L., & Borilova Linhartova, P. (2023). Primary cilia and hypoxia-associated signaling in developmental odontogenic cysts in relation to autosomal dominant polycystic kidney disease - A novel insight. Heliyon, 9(6), e17130.

Taguchi, T., & Mukai, K. (2019). Innate immunity signaling and membrane trafficking. Current opinion in cell biology, 59, 1–7.

Taipale J, Beachy PA. The hedgehog and wnt signalling pathways in cancer. Nature 2001;411(6835):349-54

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. Cell, 140(6), 805–820.

Taylor, C. T., & Colgan, S. P. (2017). Regulation of immunity and inflammation by hypoxia in immunological niches. Nature reviews. Immunology, 17(12), 774–785.

Taylor, C. T., & Scholz, C. C. (2022). The effect of HIF on metabolism and immunity. Nature reviews. Nephrology, 18(9), 573–587.

Taylor, C.T., (2008). Mitochondria and cellular oxygen sensing in the HIF pathway. Biochem. J. 409 (1), 19–26

Teixido C, Castillo P, Martinez-Vila C, Arance A, Alos L (2021) Molecular Markers and Targets in Melanoma. Cells 10 (9)

Teperino, R., Aberger, F., Esterbauer, H., Riobo, N., & Pospisilik, J. A. (2014). Canonical and non-canonical Hedgehog signalling and the control of metabolism. *Seminars in cell & developmental biology*, *33*, 81–92.

Tian, J., Liu, H., Che, J., & Song, L. (2024). Editorial: Innate immunity against intracellular bacteria: mechanisms and strategies. Frontiers in immunology, 15, 1396114.

Tiberio, L., Del Prete, A., Schioppa, T., Sozio, F., Bosisio, D., & Sozzani, S. (2018). Chemokine and chemotactic signals in dendritic cell migration. Cellular & molecular immunology, 15(4), 346–352.

Tibúrcio, R., Nunes, S., Nunes, I., Rosa Ampuero, M., Silva, I. B., Lima, R., Machado Tavares, N., & Brodskyn, C. (2019). Molecular Aspects of Dendritic Cell Activation in Leishmaniasis: An Immunobiological View. Frontiers in immunology, 10, 227.

Tsikas D. (2024). Acetazolamide and human carbonic anhydrases: retrospect, review and discussion of an intimate relationship. Journal of enzyme inhibition and medicinal chemistry, 39(1), 2291336.

Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M., & Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. Science (New York, N.Y.), 288(5465), 522–527.

Turvey, S. E., & Broide, D. H. (2010). Innate immunity. The Journal of allergy and clinical immunology, 125(2 Suppl 2), S24–S32.

Tusa, I., Gagliardi, S., Tubita, A., Pandolfi, S., Menconi, A., Lulli, M., Dello Sbarba, P., Stecca, B., & Rovida, E. (2021). The Hedgehog-GLI Pathway Regulates MEK5-ERK5 Expression and Activation in Melanoma Cells. *International journal of molecular sciences*, *22*(20), 11259

Uribe-Querol, E., & Rosales, C. (2020). Phagocytosis: Our Current Understanding of a Universal Biological Process. Frontiers in immunology, 11, 1066.

Urteaga, O., & Pack, G. T. (1966). On the antiquity of melanoma. Cancer, 19(5), 607–610

van der Weele, C. M., Hospes, K. C., Rowe, K. E., & Jeffery, W. R. (2024). Hypoxia-Sonic Hedgehog Axis as a Driver of Primitive Hematopoiesis Development and Evolution in Cavefish. bioRxiv : the preprint server for biology, 2024.06.09.598120

Vaupel, P., & Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. Cancer and Metastasis Reviews, 26(2), 225-239.

Vaupel, P., & Multhoff, G. (2018). Hypoxia-/HIF-1α-Driven Factors of the Tumor Microenvironment Impeding Antitumor Immune Responses and Promoting Malignant Progression. Advances in experimental medicine and biology, 1072, 171–175.

Venturella, M., Falsini, A., Coppola, F., Giuntini, G., Carraro, F., Zocco, D., Chiesi, A., & Naldini, A. (2023). CA-IX-Expressing Small Extracellular Vesicles (sEVs) Are Released by Melanoma Cells under Hypoxia and in the Blood of Advanced Melanoma Patients. *International journal of molecular sciences*, *24*(7), 6122.

Vito, A., El-Sayes, N., Mossman, K., & Akens, M. (2020). The impact of hypoxia on the immune landscape in melanoma and therapy resistance. Frontiers in Oncology, 10, 385.

Wang, G. L., & Semenza, G. L. (1995). Purification and characterization of hypoxia-inducible factor 1. The Journal of biological chemistry, 270(3), 1230–1237

Wang, R., Lan, C., Benlagha, K., Camara, N. O. S., Miller, H., Kubo, M., Heegaard, S., Lee, P., Yang, L., Forsman, H., Li, X., Zhai, Z., & Liu, C. (2024). The interaction of innate immune and adaptive immune system. *MedComm*, *5*(10), e714

Wang, X., Wang, T., Song, X., Gao, J., Xu, G., Ma, Y., & Song, G. (2024). Current Status of Hedgehog Signaling Inhibitors. *Current topics in medicinal chemistry*, *24*(3), 243–258.

Wilson, W. R., & Hay, M. P. (2011). Targeting hypoxia in cancer therapy. Nature Reviews Cancer, 11(6), 393–410

Wu, Q., You, L., Nepovimova, E., Heger, Z., Wu, W., Kuca, K., & Adam, V. (2022). Hypoxia-inducible factors: master regulators of hypoxic tumor immune escape. Journal of hematology & oncology, 15(1), 77

Yang, L., Xie, G., Fan, Q., & Xie, J. (2010). Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. Oncogene, 29(4), 469–481

Yatim, K. M., & Lakkis, F. G. (2015). A brief journey through the immune system. Clinical journal of the American Society of Nephrology: CJASN, 10(7), 1274–1281.

You, L., Wu, W., Wang, X., Fang, L., Adam, V., Nepovimova, E., Wu, Q., & Kuca, K. (2021). The role of hypoxia-inducible factor 1 in tumor immune evasion. Medicinal research reviews, 41(3), 1622–1643

Zbytek, B., Peacock, D. L., Seagroves, T. N., & Slominski, A. (2013). Putative role of HIF transcriptional activity in melanocytes and melanoma biology. Dermato-endocrinology, 5(2), 239–251.

Zhang, J., & Ney, P. A. (2009). Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell death and differentiation, 16(7), 939–946.

GRATIARUM ACTIO

Se siete arrivati a leggere fino a qui, ci possono essere alcune spiegazioni: avete appena girato l'ultima pagina, dopo aver letto attentamente tutta la tesi; avete sfogliato velocemente le pagine, guardando le figure, oppure era vostra intenzione saltare tutto il resto proprio per cercare questa sezione. In ogni caso spero che le poche righe che seguono siano capaci di raccontare qualcosa di me e di coloro che mi sono vicino.

Negli ultimi anni, ultimi tre inclusi, sono successe tante cose che non sono facili da superare, da descrivere, ma che si portano dietro uno strascico di emozioni che, silenziose, come un mantello vesto tutti i giorni. Il mio numero perfetto è sempre stato il cinque, sembrava che fosse infinito, poi in poco tempo la perfezione ha smesso di esistere e il cinque è diventato un due. Ultimamente la trappola sicura della quotidianità caratterizza le mie giornate, che proseguono per la necessità di permettere la sopravvivenza dei ricordi. Di conseguenza il "grazie" per i miei nonni e il mio babbo risuonerà all'infinito. A questo si aggiunge il ringraziamento a mia mamma, che ogni giorno vive come me circondata da fotografie silenziose; e poi ai miei zii e cugini che ci stanno vicino.

Comunque in tutto questo credo anche di essere una persona fortunata perché ho conosciuto tante persone che col tempo si sono ritagliate il loro spazio nella mia vita, arricchendola.

Stefano, un fratello trovato, che mi è stato sempre vicino, soprattutto nei momenti più tristi, col quale ho passato attimi unici, vissuto avventure alla "Rotta x casa di Dio", caratterizzate dall'alternarsi tra discorsi sul perché della vita e sul nostro futuro e dialoghi assurdi su vicende inutili. In casa sua, che per me è diventata quasi una seconda casa, attorno al tavolo di legno della cucina insieme ai suoi coinquilini Andrea, Roberto e Stefano sono nate innumerevoli chiacchierate, che annullavano il significato del tempo. Progetti e fantasie, discorsi sul mondo, sulla scienza, su noi stessi che hanno reso quei momenti e quelle serate appaganti, le più belle di questi anni.

Federica, una persona che non avrei mai immaginato di incontrare giunto a quest'età, un'amica che puoi avere la fortuna di incontrare una sola volta nella vita; la compagna leale di mille avventure, con la quale dirsi tutto. La vita di laboratorio non sarebbe stata la stessa e non sarebbe stata così bella se lei non ci fosse stata. Tramite la sua amicizia ho incontrato Marianna, una delle persone più coraggiose che abbia mai incontrato.

Massimo, mattiniero come me, è il compagno fisso di tutte le colazioni, un amico importante che ha spesso trovato le parole giuste per parlare di vicende complicate, il primo che mi ha abbracciato in un giorno difficile, al quale ho avuto il piacere di insegnare tutto quello che sapevo.

A tutti loro si aggiungono tanti altri con i quali ho condiviso svariati momenti, tra cui spiccano amici come Simone e i nostri dialoghi sulla coltivazione dell'orto, Clelia con la passeggiatina abituale nei corridoi di San Miniato, Luca con i suoi esperimenti; amici di più vecchia data, Matteo ed Antonio, fino ad arrivare agli ultimi incontri come Giulia.

Un ringraziamento alla professoressa Antonella Naldini e al professor Fabio Carraro che mi hanno accolto nei loro laboratori permettendomi di fare esperimenti, studiare i processi cellulari, "fare Scienza", e con la loro guida di vivere un percorso estremamente gratificante.

Il mio amico Carlo che, tra le altre cose, condivide con me la passione per i computer e per Star Trek, una delle poche persone col quale posso dire “Enterprise” ed essere compreso.

E poi gli amici Irene, Sara Ilenia, Paolo, Barbara e Jlenia che rallegrano momenti svariati della giornata portando quella nota di imprevedibile, necessaria per fuggire per qualche secondo dal mondo reale.

Da ultimo è necessario menzionare la Scienza, che giorno dopo giorno mi permette di stare a contatto col mondo dell’infinitamente piccolo per studiare quello che ancora non si conosce.