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Ruolo degli endocannabinoidi nel controllo della proliferazione delle cellule tumorali xenotrapiantate in larve di zebrafish (Danio rerio)

Role of endocannabinoids in controlling the proliferation of xenograft tumor cells in zebrafish (Danio rerio)

Tesi di laurea Magistrale di:

Fiorenza Sella

Relatore:

Chiar.ma Prof.ssa Oliana Carnevali

Correlatore:

Prof.ssa Francesca Maradonna

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Ché, rotti i lacci a l'elmo suo, d'un salto
(mirabil colpo!) ei le balzò di testa;
e le chiome dorate al vento sparse,
giovane donna in mezzo 'l campo apparse.

TORQUATO TASSO, Gerusalemme librata, canto III, vv.172-175

Abstract

Il cancro al colon è una delle cause più frequenti di morte riscontrate nella popolazione mondiale, anche dovuta al fatto che i trattamenti addottati attualmente non riescono a bloccare la progressione del tumore e la sua capacità a metastatizzare. In questo contesto, i cannabinoidi hanno suscitato interesse nella ricerca scientifica per la scoperta delle loro proprietà antitumorali. Tra questi, si evidenzia l'anandamide (AEA), un endocannabinoide endogeno studiato per la sua capacità di bloccare la crescita cellulare ed indurre morte nelle cellule tumorali.

Questo lavoro di tesi è incentrato sullo studio dell'effetto dell'esposizione all'Anandamide sul controllo della proliferazione di cellule tumorali della linea umana HCT116 in larve di zebrafish xenotrapiantate. Eseguito lo xenotrapianto a 48 hpf, le larve sono state trattate con AEA fino a 120 hpf. Tramite microscopia confocale è stata valutata l'efficacia dell'esposizione all'endocannabinoide sulla crescita delle cellule tumorali e sulla neo-vascolarizzazione del tumore. Dai risultati ottenuti, nel gruppo trattato con l'anandamide si è osservato un volume del tumore minore rispetto a quello osservato negli altri gruppi sperimentali. Inoltre, in questo gruppo sperimentale i dati hanno mostrato una minore vascolarizzazione intorno al tumore rispetto agli altri gruppi.

Per convalidare i risultati ottenuti dalle osservazioni al microscopio, sono state condotte delle analisi molecolari. L'impiego di tecniche quali RNAseq, qPCR e

Western blot hanno permesso di studiare i livelli di alcuni biomarkers coinvolti nella regolazione di pathways responsabili della proliferazione tumorale. I risultati ottenuti analizzando i livelli di *vegf-c*, VEGF-C e *vegf-d* supportano l'ipotesi dell'effetto antitumorale dell'AEA, mentre quelli relativi a LC3A/B e CASP3 suggeriscono un ruolo marginale dell'autofagia e dell'apoptosi nel processo di crescita tumorale. I risultati dell'RNAseq hanno riportato un differente numero di DEGs tra i vari gruppi sperimentali e nello specifico ci si è concentrati sul confronto dei geni differenzialmente espressi tra il gruppo trattato con AEA e quello di controllo in modo da poter associare delle evidenze molecolari a quelle ottenute tramite microscopia confocale. Dai risultati è stato possibile risalire alla regolazione messa in atto dall'AEA su determinati pathways. Nello specifico, l'endocannabinoide agisce sull'infiammazione riducendo il rilascio di citochine e sul controllo delle cellule del sistema immunitario. Pertanto, i risultati ottenuti mostrano un'azione diretta dell'AEA sullo zebrafish esercitando, conseguenzialmente, un'azione antiproliferativa sulla cellule tumorali iniettate, come suggerito dai livelli di mRNA di *socs3 e pcnp,* e antinfiammatoria come evidenziato dai livelli di *Il-11a, mhc1uba e csf3b*.

Per quanto riguarda le prospettive future, nonostante sia necessario compiere ulteriori studi sulla capacità antiproliferativa e antinfiammatoria dell'AEA, è evidente il suo potenziale effetto sul controllo della crescita tumorale e su come la sua somministrazione potrebbe essere presa in considerazione per un trattamento clinico nella terapia contro il cancro.

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ABBREVIATIONS

AEA= anandamide

BAX = BCL2-Associated X Protein

BCL2 = B-cell lymphoma 2

BCL-XL = B-cell lymphoma-extra larg

CACO2 = human colorectal adenocarcinoma cells

CASP3 = caspase 3

CB = cannabinoid receptor

CDs = dendritic cells

CRC = colorectal cancer

DEGs = different expression genes

ECM = extracellular matrix

ECS = endocannabinoid system

EFM19 = human prolactin-sensitive breast cell line

EMT = epithelial–mesenchymal transition

G-CSF = granulocyte colony-stimulating factor

GI = gastrointestinal

HCT116 = human colorectal carcinoma cell line

IL-1B = interleukin 1b

IL-6 = interleukin 6

IL-8 = interleukin 8

IL-11 = interleukin 11

JAK = janus kinase

LD = light dark

MDA-MB-231 = human breast cancer cell line

Mep1b = meprin 1 b

MHC = Major histocompatibility complex

MMPs = metalloproteases

mTOR = mammalian target of rapamycin

NF-Kb = nuclear factor

NK = natural killer

PCNP = PEST-containing nuclear protein

SOCS3 = suppressor of cytokine signaling 3

STAT1 = Signal Transducer And Activator Of Transcription 1

STAT3 = Signal Transducer And Activator Of Transcription 3

STAT5 = Signal Transducer And Activator Of Transcription 5

SW480 = human colorectal adenocarcinoma cells

TAMs = tumor-associated macrophages

TME = tumor microenvironment

TNF = tumor necrosis factor

VEGF = vascular endothelial growth factor

WT = wild type

Chapter one

INTRODUCTION

Over the years, several studies focused on ECS role in the control of tumorigenesis and suppression of tumors (Moreno et al. 2019) by controlling inflammation and immunomodulation (Ahmed et al. 2021; Guindon and Hohmann 2011; Izzo and Camilleri 2009). Depending on tumor type, the action of the ECS could be antiproliferative, pro-apoptotic, antiangiogenic, anti-metastatic or anti-inflammatory (Ahmed et al. 2021). On the other hand, the dysregulation of ECS, characterized by changes in expression and function of receptors, enzymes activities and in turn concentration of endocannabinoids, in addition to cancer has been associated with several diseases, including neurodegenerative disorders, multiple sclerosis, inflammation, epilepsy, schizophrenia, glaucoma, cardiovascular diseases, and obesity (Laezza et al. 2020).

1.1 The endocannabinoid system (ECS)

The endocannabinoid system (ECS) is composed by a complex network of cannabinoid receptors and endocannabinoid ligands whose levels are regulated by a complex enzymatic machinery that drives their biosynthesis, degradation, and transport (Moreno et al. 2019). The two most important receptors in ECS are the endocannabinoid receptors known as CB1 and CB2. They are distributed in different tissues and have a different expression level: for example, the CB1 can be expressed

in the brain, uterus, testes, ovaries, prostate and colon (Pagano et al. 2021; Wright et al. 2005), while CB2 is primarily expressed only when there is active inflammation.

Furthermore, many non-cannabinoid receptors that are modulated by cannabinoids have been recently discovered. Some of these are: PPARs (peroxisome proliferator-activated receptors), GRP55 (G-protein-coupled receptor 55) and TRPV1 (transient receptor potential cation channel subfamily V member) (Contino and McCormick 2020).

The eCBs are lipidic messengers acting as paracrine and autocrine factors. In addition, probably, due to their nature that allows them to diffuse into membrane, they can act through an endocrine mode of action (Contino and McCormick 2020). The two main eCBs are derivatives of polyunsaturated fatty acids: N-arachidonoylethanolamine (anandamide, AEA), and 2-arachidonoylglycerol (Navarrete et al. 2020; Piomelli 2003). The two major synthetic enzymes are diacylglycerol lipase (DAGL) and N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD). The 2-Arachidonoylglycerol (2AG) hydrolysis may be mediated via monoacylglycerol lipase (MAGL), αβ hydrolase 12 (ABHD12) or αβ hydrolase 6 (ABHD6). On the other side, AEA can be hydrolyzed by fatty acid amide hydrolase (FAAH), FAAH2 or N-acylethanolamine acid amidase (NAAA). Both 2AG and AEA can be transformed into apparently independently bioactive metabolites through cyclooxygenase-2 (COX-2), lipoxygenase (LOX) or epoxygenase/cytochrome P450 (EPOX/CYP) (Hourani and Alexander 2018).

1.2 Role of Anandamide on cancer cells

N-arachidonoyl ethanolamide (anandamide, AEA) was the first endocannabinoid discovered (**Figure 1**) (Maccarrone and Finazzi-Agró 2003; Marzo, et al. 2001).

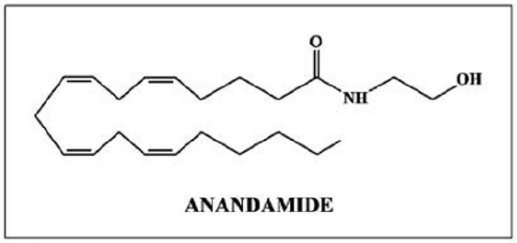

Figure1. *Structure of Anandamide*

AEA is the main ligand of the endocannabinoid system receptors, CB1 and CB2. This interaction has an important role in the activation of several pathways involved in the control of human pathophysiology, being strictly related to cancer disease (Laezza et al. 2020).

Testing AEA on different tumor cell lines, it resulted that it exerts a different anti-tumoral effect, regulating both tumor microenvironment and its metastatic process. Anandamide, indeed, can have a pivotal role in the control of angiogenic process, a process that allows cancer cell proliferation generating new vascular supply (Harmey and Bouchier-Hayes 2002). The most important pathway of angiogenesis is regulated by the vascular endothelial growth factor (VEGF) proteins (Hicklin and Ellis 2005). Specifically, it was reported that AEA (10 μM) downregulated the expression of angiogenic factors including vascular endothelial growth factor-C (VEGF-C), vascular endothelial growth factor–receptor 2 (VEGF-R2), and vascular endothelial growth

factor-receptor 3 (VEGF-R3) in in vivo xenograft model of cholangiocarcinoma (DeMorrow et al. 2008).

Moreover, AEA can influence tumor cell migration process, responsible of metastasis formation. On this regard, studies reported that AEA represses the migration of $CD8^+$ T lymphocytes and SW48 colon carcinoma cells by activating the CB1. (Joseph et al. 2004).

In addition, AEA also affects autophagy, apoptosis, and cell cycle (Yang et al. 2018). Cellular autophagy or auto-phagocytosis is a cellular selective mechanism leading to removal of damaged cytoplasmic components (K. Wang et al. 2019). Autophagy allows the degradation and recycling of cellular components (Hout et al. 2020). In this case, it was reported that AEA binds CB1 and induces autophagy in the mature intestinal epithelium, Caco-2 cell line, causing a reduction of the regulator protein suppressor of cytokine signaling 3 (SOCS3) levels (Pagano et al. 2021). Concerning cell cycle, typical of cancer cell is the loss of cell cycle control, thus its targeting is one of the strategies to fight its proliferation. Among cannabinoids, AEA can influence cell cycle regulation: in the EFM-19 cells (human prolactin-sensitive breast cell line) it was shown that AEA can inhibits cell cycle progression by arresting the G1-S transition (Kisková et al. 2019). It was also reported that it can induce cancer cell death through apoptosis. Typically, in the prostate it was seen an increase of cleaved-caspase-3 levels and a reduction of Bcl-2 levels (Pagano et al. 2021).

1.3 Colon Cancer (CRC)

The colorectal cancer (CRC) is the third most common cancer worldwide behind prostate and lung in male and breast and lung in female (Orrego-González et al. 2020). The development of the cancer is also linked to strong environmental associations and genetic risk factors. The development of the precancerous lesion required an accumulation of genetic mutations by chromosomal instability, mismatch repair, and CpG hypermethylation (Dominic et al. 2020).

Concerning to ECS, the presence of CB was reported in the gastrointestinal tissue. Specifically, the CB1 and CB2 are part of the enteric nervous system and epithelial cells (Pesce et al. 2018). In the case of CRC, it has been found that an upregulation of CB transcripts represents a poor prognosis and advanced stage of disease. Interesting, AEA and the expression of AEA-synthetizing enzymes are more expressed in CRC than in normal mucosa (Laezza et al. 2020). Moreover, it has been demonstrated that endogenous cannabinoid agonists, such as AEA (0.5–5 μM) and its metabolic-stable analogous, Meth-AEA (0.5–5 μM), diminished the volume and the density of gastric carcinomas cells, inducing apoptosis and necrosis, respectively (Ortega et al. 2016).

1.4 Tumor microenvironment

The emerging concept of "tumor microenvironment (TME)" is a complex and continuously evolving entity (Anderson and Simon 2020). The composition of the environment depends on the tumor types, but in general, it includes the immune cells (Labani-Motlagh, et al. 2020; Li et al. 2020), stromal cells (Denton, et al. 2018), blood vessels and extracellular matrix (ECM). It is largely analyzed owing to its anti-tumor or immune suppressive actions. In fact, immune cells in the microenvironment can support tumor formation and promote an anti-tumor microenvironment or can induce tumorigenesis through an immune suppressive microenvironment (**Figure 2**).

The component of the TME are T-cells, B-cells, Natural killer cells, Macrophages, Neutrophils, Dendritic cells (DCs) and Stromal cells. All of them play a role in the development of the tumor (Elinav et al. 2013). In addition, tumor cells have some of the signaling proteins of the inherent immune system such as chemokines and cytokines which promote the migration and metastasis (Lisa M Coussens 2002).

Within the TME it is possible to find distinct cell populations that influence the tumorigenesis, as well as, M1 macrophages involved in tumor cell suppression, while M2 ones, promotes the secretion of IL and support tumor progression (Sumitomo et al. 2019).

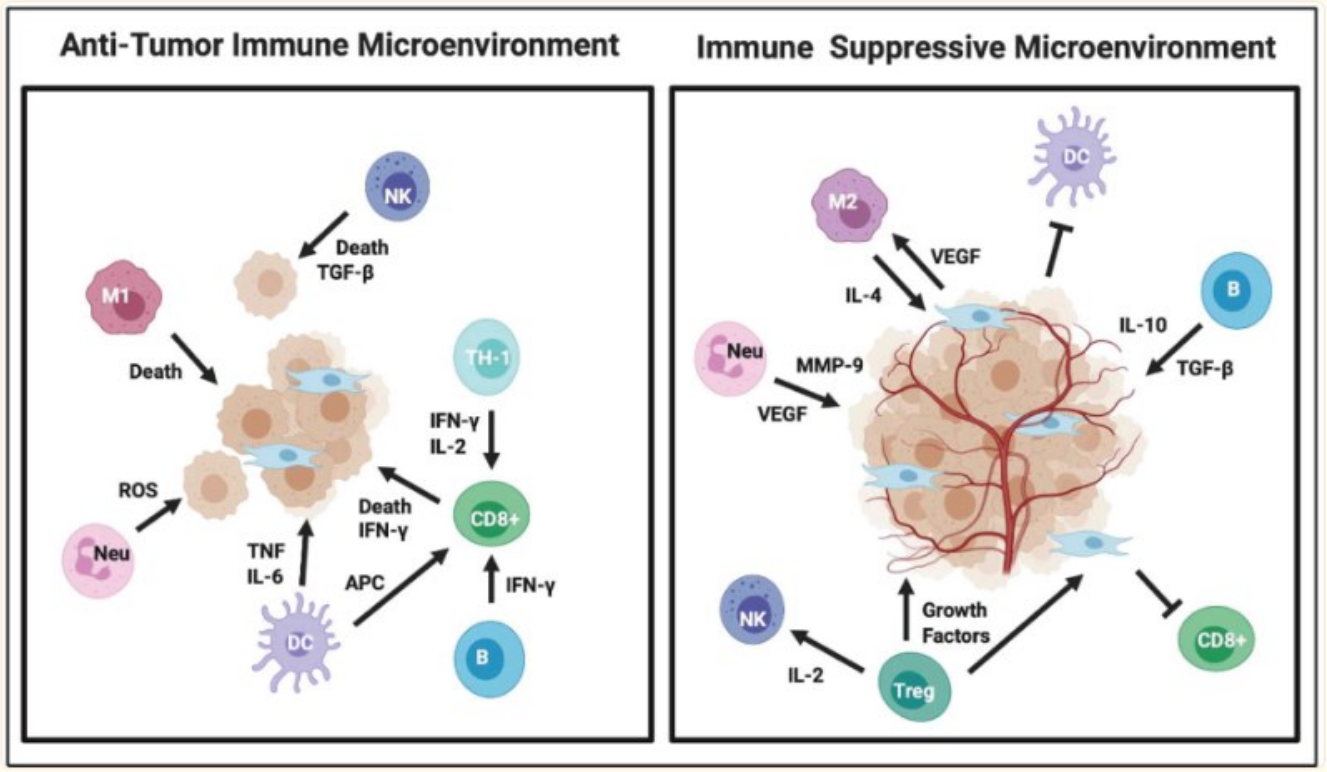

Figure 2. *Impact of immune cells within the TME*

Other hallmark for the tumorigenesis in TME is represented by Stromal cells. They are vascular endothelial cells, fibroblasts, adipocytes, and stellate cells. Their characteristic is represented by the secretion of many factors, once recruited into the TME, involved in angiogenesis, proliferation, invasion, and metastasis (**Figure 3**) (Anderson and Simon 2020; Guo and Deng 2018; Sumitomo et al. 2019).

Concerning to TME regulation, immune cells express on their membrane the CBs and produce endocannabinoids forming an “Immune endocannabinoid system” (Kienzl, et al. 2020).

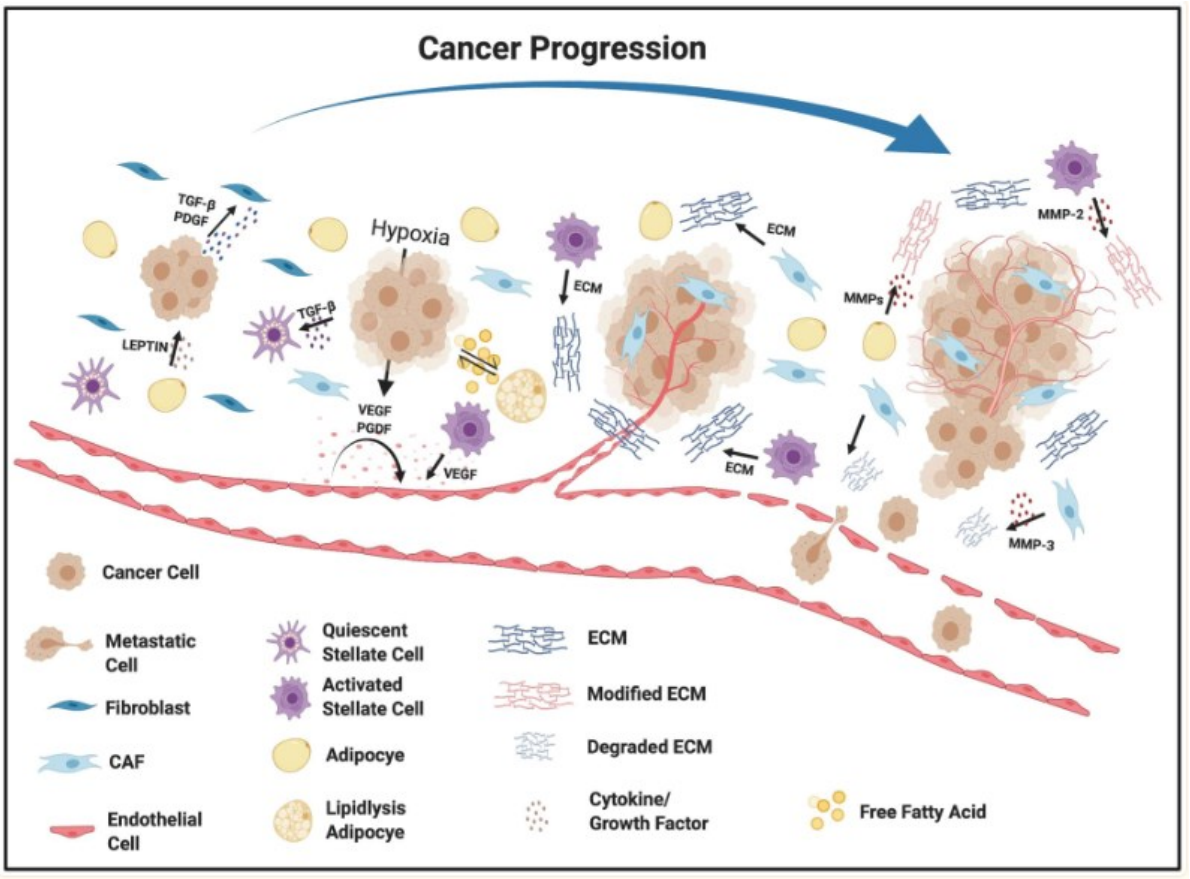

Figure 3. *Defining the role o stromal cells in promoting cancer progression*

The effects of endocannabinoids are mostly associated with the anti-inflammatory functions. In fact, it was reported that the activation of CB1 induces polarization of M1 phenotype (Tian et al. 2017), while CB2 promotes the M2 phenotype conversion (Braun et al. 2017). Furthermore, macrophages produce AEA (Marzo et al. 1996), and it was demonstrated that in the gut AEA contributes to promote the presence of immunosuppressive CX3CR1^{hi} macrophages for the maintenance of homeostasis tissue (Acharya et al. 2017). Also, DCs produce AEA and express CB1 and CB2 levels, especially CB2 is implicated with the DC maturation (Gaffal et al. 2020; Turcotte et al. 2015). This supports the idea that endocannabinoids may shape TME, influencing the immune cell functions (Kienzl, Kargl, and Schicho 2020).

1.5 Zebrafish xenograft model and utility

The zebrafish xenograft model is a useful and low-cost animal model for predicting the efficacy of cancer treatment and exploring mechanisms that lead to tumor growth, metastasis, and responses to therapy (X. Chen et al. 2021). Two of the most important characteristics of zebrafish are that can lay 150-300 eggs every week, thus allowing the generation of biological and technical replicates in a short time, and presents a high synteny, around 82% of ortholog genes, with human genome. Furthermore, it has an optical clarity that allows the visualization of either early embryo development as well as tumor cell growth and dynamics at early stages of cancer development *in vivo* (Hason and Bartůněk 2019).

The Xenograft practice consists in the implantation of different host cell line into zebrafish. The practice has become an emerging model platform in cancer studies. With this practice it is possible to implant various types of cancer cell lines. The model represents an alternative to rodents, especially for the tumor study. On the other hand, there are some limitations using the zebrafish model and one of these is the high mortality after injection. In addition, Zebrafish is a poikilothermic fish living with a preferred optimal temperature set at 28°C, which could represent a bias if considering studies where the mammalian homeostatic temperature would be important (Hason and Bartůněk 2019).

To create a xenograft tumor model, both the use of the embryonic or adult zebrafish could be feasible, and in case of adult fish, the choice of an immune-deficient adult zebrafish strain that lacks T, B and natural killer (NK) cells (Stoletov et al. 2007; Yan

et al. 2019) is recommended, representing similarly to mammals, cell rejection a major issue. Indeed, the advantage of using embryos transplanted at 48 hpf, is represented by the lack of an adaptative immune system, not yet matured thus avoiding the immune suppression (Lam et al. 2004; Nicoli et al. 2007; R. White, et al. 2013). Furthermore, the tumor cell line labelled with fluorescent marker to follow their localization and development utilizing the fluorescent microscopy, confocal microscopy, pathological biopsy, and other techniques (de Sousa Pontes et al. 2017) represents a great advantage in research. In addition, the use of GFP-labeled zebrafish (X. Chen et al. 2021) (**Figure 4**), allow the angiogenesis visualization, providing important insights on cancer growth.

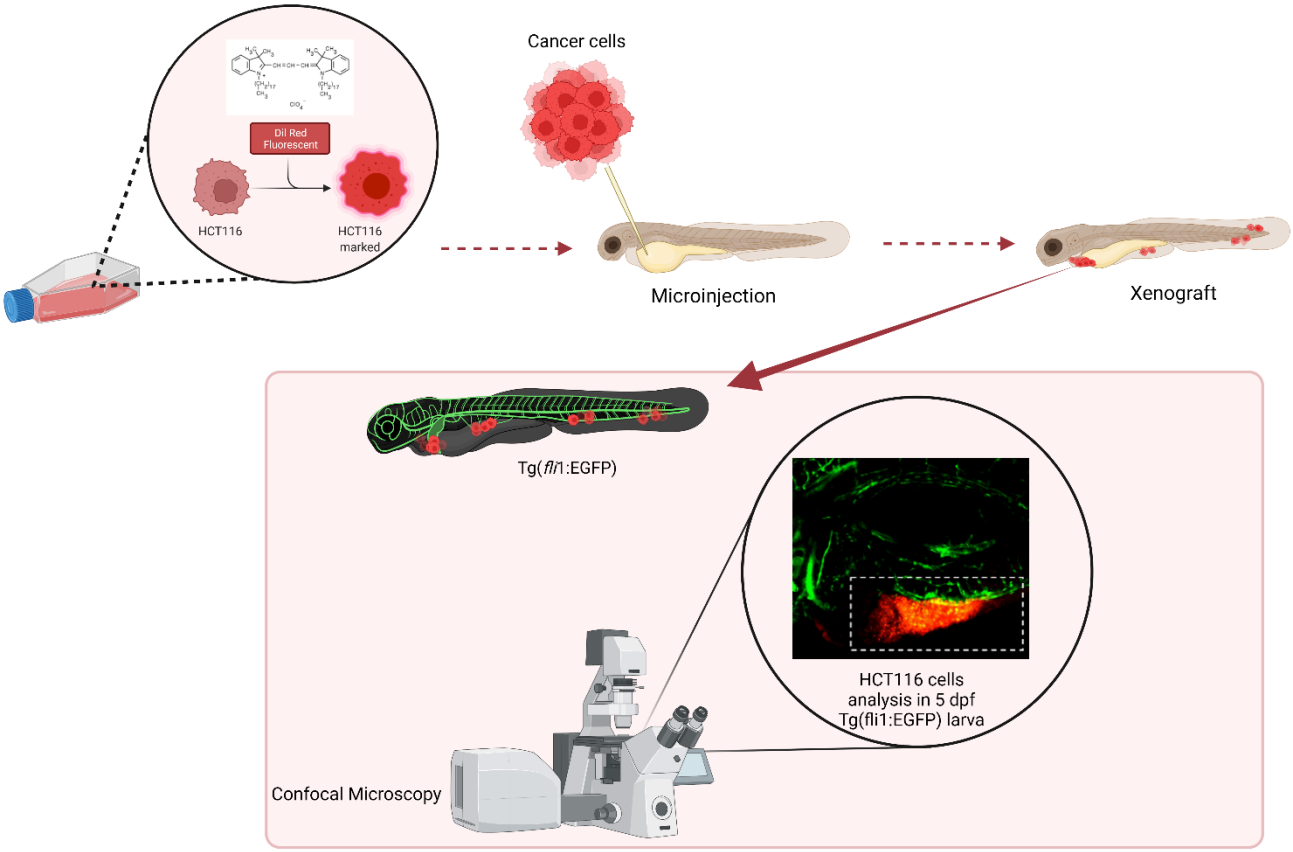

***Figure 4**. Process of zebrafish xenograft model*

Moreover, this model permits to study the tumor microenvironment, when the cancer cells are injected in the yolk, it is possible to stimulate the formation of TME for the presence of hypoxic environment and the lack of vascularization (X. Chen et al. 2021). Trough the transplantation into the yolk, many different cancer cells characteristics can be assessed with the most predominant endpoints being growth, survival, invasion, and metastasis formation (Gamble et al. 2021) and the phenotypic responses are used to provide information on the aggressiveness of the cancer studied (**Figure 5**).

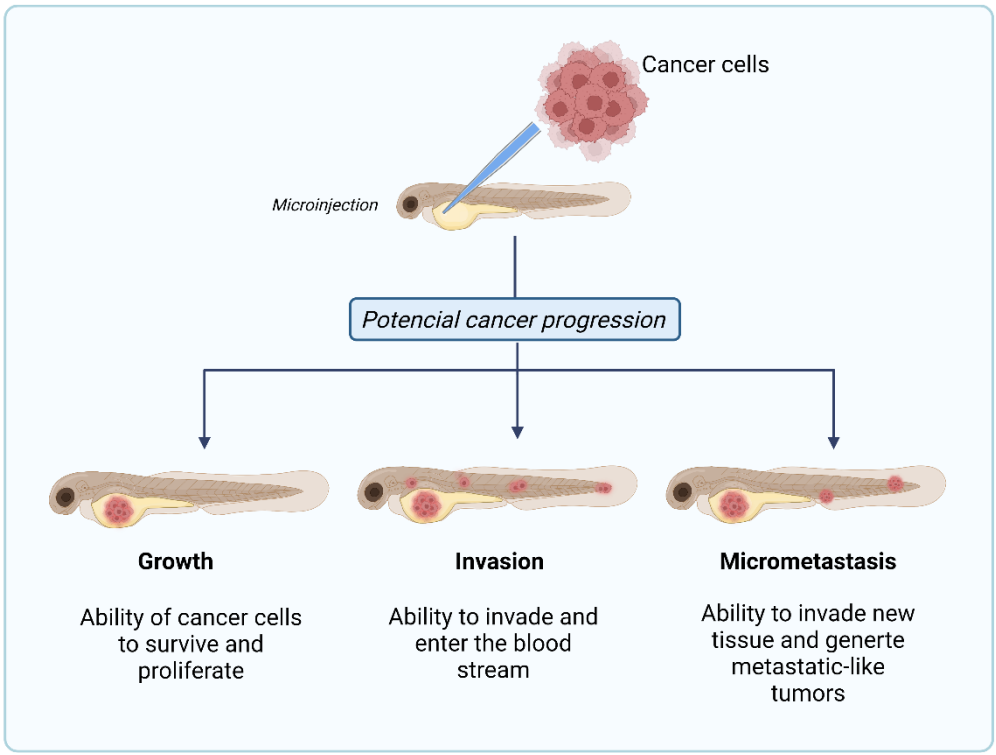

Figure 5. *Cancer progression in zebrafish xeografts*

It represents also a good animal model to test the effect of drug administration: zebrafish has a high permeability to small molecules dissolved in the water, such as drugs used for chemotherapy making zebrafish a good experimental model for clinical trials (Usai et al. 2020; Veinotte et al. 2014). In case of drug screening, the employment

of drugs with low teratogenic effects it should be recommended. In fact, in the long term the drug action can be lethal for the model, or the quantity of the compound added in water can be higher respect the total quantity tolerated in culture by cancer cells (Haney et al. 2021; Konantz et al. 2012). At this regard, the administration of accurate dose to avoid collateral effects on the model is recommended.

1.5.1 Zebrafish and HCT116 cell line xenograft

Zebrafish xenografts have been also recently used as a model for study of colorectal carcinoma implantation and proliferation. In many case the researchers used the HCT116 cell line isolated from patients (Fior et al. 2017) and injected in zebrafish embryos at 48 hpf, resulting in the realization of a rapid model with high sensitivity to unravel human tumor functional heterogeneity.

Concerning the xenograft, the HCT116 cell line is a very aggressive human cell line of colorectal cancer. It is used in many studies to prove the beneficial effect of some treatments, including those with endocannabinoids, either on cell line culture alone in vitro (Sawhney et al. 2002; J. Wang et al. 1995) or with mice xenografts (Rajput et al. 2008). While, *in vitro*, the studies are limited to cell motility and invasion focusing on the premise of single cell locomotion and penetration of an extracellular matrix coating (Rajput et al. 2008), *in vivo*, the orthotopic model of colon cancer provides a tool to dissect the molecular mechanism involved in the metastatic cascade. In zebrafish xenografts, the tumor cell line shows a well-vascularized periphery composed of large vessels that generally do not infiltrate the tumor (Fior et al. 2017).

Chapter two

AIM OF THE STUDY

Cancer is the second leading cause of death in the world. Given that cancer is a highly individualized disease, predicting the best chemotherapeutic treatment for individual patients can be difficult. Ex vivo models such as mouse patient-derived xenografts (PDX) and organoids are being developed to predict patient-specific chemosensitivity profiles before treatment in the clinic. Although promising, these models have significant disadvantages including long growth times that introduce genetic and epigenetic changes to the tumor. On the contrary, the zebrafish xenograft assay is ideal for personalized medicine. Imaging of the small, transparent fry is unparalleled among vertebrate organisms. In addition, the speed (5–7 days) and small patient tissue requirements (100–200 cells per animal) are unique features of the zebrafish xenograft model that enable patient-specific chemosensitivity analyses.

The general aim of this experimental study is to evaluate the effect of Anandamide in colon cancer disease, and in particular its role as antitumor agent. Concerning to this, the use of Zebrafish (*Danio rerio*) xenograft, WT larvae injected with the HCT116 colon cancer cell line, can represent a novel and appropriate model to analyze the beneficial effects of AEA administration on antiangiogenic and anti-inflammatory pathways and in turn on the tumor suppression.

The specific objective of my thesis is to elucidate if AEA has a direct action on the HCT116 colon cancer cell or on the zebrafish immune system.

To answer this question, transcriptomic analysis in zebrafish total body and in transplanted HCT 116 human cancer cells have been performed concomitant with the analysis of the direct effects of AEA on HCT116 *in vitro*.

Chapter Three

MATERIALS AND METHODS

3.1 Zebrafish husbandry

Zebrafish embryos, larvae and adult were maintained according to standard procedures (Westerfield 1995). Embryos were obtained from natural spawning and raised at 28.5°C in a 12:12 light:dark (LD) cycle in fish water (50X: 25 g Instant Ocean, 39.25 g CaSO4 and 5 g NaHCO3 for 1 l). All husbandry and experimental procedures complied with the Italian and European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). For the experiments described in this paper we used wild type (WT), and Tg(*fli1*:EGFP) zebrafish strains. Tg(*fli*1:EGFP) expresses the EGFP under the control of the promotor of the Fli1 gene, a well described endothelial cell marker (Delov et al. 2014; Let et al. 1996; Thompson et al. 1998).

3.2 HCT116 Human cell line maintenance

Human colorectal cancer cells HCT116 were tested for mycoplasma (VenorGeM Classic, Minerva Biolabs GmbH) and cultured in RPMI Medium 1640 with GlutaMAX-I (Life Technologies, Gibco, NY, USA) supplemented with 10 % FBS (Fetal Bovine Serum) (Life Technologies, Gibco, NY, USA) in a humidified atmosphere containing 5% of CO2 at 37°C.

3.3 In vitro exposure of HCT116 cell to AEA: viability assay

Human colorectal cancer cells HCT116 were tested for mycoplasma (VenorGeM Classic, Minerva Biolabs GmbH). Cell viability was determined using the cell counting Kit-8 colorimetric assay (CCK-8, Bimake, USA). HCT 116 cells were seeded in 96-well plates with RPMI 1640 (Gibco, Cat 61,870-010) and 10 % fetal bovine serum (FBS) at a density of 5 × 103 cells/well. After 24 hours incubation at 37°C, the medium was removed and new culture medium containing 0.5 % FBS and Anandamide (AEA) were added. AEA dissolved in ethanol was diluted directly in cell culture medium to 50 µM solution and then serially diluted to give a final test concentration range of 0.005-5 µM. Cells were treated at 37°C for 72 h changing the medium every day after which cell viability was measured. Briefly, 10 µl of CCK-8 reagent (Bimake, USA) were added to each well and incubated for 30 minutes at 37°C, then absorbance at 450 nm was measured using a microplate reader (Infinite F200 PRO Tecan). The absorbance in the control group was regarded as 100 % cell viability. The percentage of viability was calculated using the formula: "[OD (optical density) of treated cells background absorbance/ OD of untreated cells (control)—background absorbance] × 100". All controls and samples were measured by four independent experiments with five replicates for each concentration. Values are expressed as the mean ± SD.

3.4 Xenotransplantation

For zebrafish embryo microinjection, HCT116 cells were washed, trypsinized and re-suspended in RPMI medium containing Dil Vibrant Red Fluorescent dye (Invitrogen) for 20 minutes at 37°C. Successively, cells were centrifugated at 1200 rpm for 5 minutes, the supernatant was discarded, and cells were washed two times with PBS. After this, cells were re-suspended in PBS for injection in the zebrafish yolk.

Concerning to the xenotransplantation, zebrafish embryos at 48 hpf were immobilized with 0.04% tricaine (Sigma-Aldrich, E10521) and positioned on thick film of 2% agarose in fish water. To evaluate the tumor volume and interaction with the host approximately 200-400 Dil-labelled HCT116 cells were injected into the inferior section of the yolk sac. After injection, xenografts were transfected to 35°C until the end of the experiment. At 3 dpi unsuccessfully injected xenografts were discarded.

3.5 Zebrafish xenograft drug administration

AEA and its inhibitor, the AM251 were bought from Merck ((Merck #94421-68-8 and #183232-66-8 respectively) and were dissolved in Ethanol 100%. Working concentration were chosen on the bases of preliminary experiments and was 10 nM for both AEA and AEA inhibitor AM251. Randomly, 6 hpi zebrafish xenografts were distributed among the four treatment groups (**Table 1**).

Group	*Treatment*
Ctrl	EtOH as chemical vehicle
AEA	10 nM AEA
AM251	10 nM AM251
AEA+AM251	10 nM AEA + 10 nM AM251

Table 1*. Treatment for each group of experimentation. Ctrl = control, AEA = Anandamide, AM251 = inhibitor*

The compounds were dissolved in fish water (NaCl, 13.7 mM; KCl, 0.54 mM; MgSO4, 1.0 mM; CaCl2, 1.3 mM; Na2HPO4, 0.025 mM; KH2PO4, 0.044 mM; NaHCO3, 4.2 mM) for three consecutive days, with the fish water completely replaced daily.

3.6 Live imaging

For confocal imaging, xenograft 3 dpi WT and Tg(*fli1*:EGFP) larvae, animals were anaesthetized with 0.04% tricaine, embedded in 1% low-melting agarose and placed on a depression slide. The Nikon C2 confocal system using the software NIS ELEMENTS was used to record images.

3.7 Tumor volume and angiogenesis quantification

All images of WT and Tg(*fli1*:EGFP) xenografts obtained with the Nikon C2 confocal system were analyzed with the imaging software Fiji. The tumor size was obtained by calculating the tumor area (red fluorescent structure) of each Z stack. Concerning to angiogenesis quantification, the results was obtained by calculating the blood vessel

area (green-fluorescent structure) of each Z stack in the area of the tumor, normalized for the tumor volume.

3.8 RNA extraction and cDNA synthesis

Total RNA was extracted from 5 pools of 20 larvae for each experimental group, using RNAzol RT (Sigma-Aldrich, St.Louis, MI, USA). The extracted RNA was diluted in at least 10 µL of RNAse-free water. The RNA integrity and concentration were determined by nanophotometer P330 (Implen, München, Germany) to check concentrations and 260/230 and 260/280 ratios. The extracted RNA was then stored at -80°C. Genomic DNA was removed by DNase I digestion (Sigma-Aldrich, St. Louis, MI, USA) according to the manufacturer's instructions. Part of the total RNA was used for cDNA synthesis, part to create a library for RNA-seq. The reverse transcription was conducted from 1 µg mRNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, USA) following the manufacturer guidelines. The cDNA obtained with the retrotranscription (MyCycler Thermal Cycler System, Bio-Rad, Milan, Italy) was considered as the stock (1:1), this stock was diluted with milli Q water (1:10) to obtain the working concentrations. All cDNAs were stored at – 20 °C.

3.9 RNA-seq

Libraries were created starting from 3 different RNA samples/experimental groups, with the Illumina TruSeq Stranded mRNA Library Prep Kit and then sequenced with an HiSeq2500.

On average, 0.74% of the total reads could be mapped uniquely on the human genome and 81.76 % of the reads could be mapped uniquely on the zebrafish genome. In order to perform disambiguation between the two species, the software disambiguate was used. FeatureCounts (version 2.0.0) was used to calculate gene expression values as raw fragment counts. In addition, a normalization was applied to the raw fragment counts by using the Trimmed Mean of M -values (TMM) and the Fragments Per Kilobase Million (FPKM) normalization.

For the analysis of human transcripts, for each comparison the following procedure was performed: removing all the genes showing 0 read counts for all the samples; checking the quality of the replicates with a Principal Component Analysis and removing outlier samples.

For zebrafish, the following procedure was performed: filtering of lowly expressed genes using the HTSFilter tool; checking the quality of the replicates with a Principal Component Analysis and removing outlier samples.

3.10 Real time PCR

The qRT-PCRs were performed with SYBR green method in a CFX thermal cycler (Bio-rad, Milan, Italy) as previously described (Giommi et al. 2021). For each experimental group, replicates (n = 6) were run in duplicate. Final concentration of primers was 10 pmol/µL. The thermal cycling was as follow: 95 °C for 3 min; 45 cycles of denaturation (10 s at 95 °C) followed by 20 s for annealing at 60 °C for *casp3, vegf-aa,vegf-ab, vegf-c, vegf-d*. Ribosomal protein 13 (*rpl13*) and ribosomal protein 0 (*rplp0*) mRNAs were used as internal standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. the sequences of primers forward and reverse are reported in the table (**Table 2**). mRNA levels of each target gene analyzed were calculated using the Pfaffl method relative to the geometric mean of the two reference genes once demonstrated they were stably expressed by the geNorm algorithm, both implemented in the Bio-Rad CFX Manager 3.1. software. Modification of gene expression among the experimental groups is reported as relative mRNA abundance (arbitrary units). Primers were used at a final concentration of 10 pmol/mL.

GENE	ABB	FORWARD(5'-3')	REVERSE(5'-3')
Caspase-3	*casp3*	GTGCCAGTCAACAAACAAAG	CATCTCCAACCGCTTAACG
Vascular endothelial growth factor-a	*vegfaa*	GACGTTTCGTGTCTCTGTCG	AAAAGAGTGCGTGCAAGACC
Vascular endothelial growth factor-ab	*vegfab*	GGACCTGCAGATGTGACAAA	ATCAAATCCTGTGCTCCGAG
Vascular endothelial growth factor-c	*vegfc*	GGCCTCAACAGAGCTTCAAC	TCTCTTGGGGTCCACGTTAC
Vascular endothelial growth factor-d	*vegfd*	GCTGGACTTCACATGTTGCT	CTCAGTTCCTGCTCCCACTT
Ribosomal protein large P0	*rplp0*	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC
Ribosomal protein L13a	*rpl13*	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG

Table 2. Table of Primers F and Primers R used

3.11 Protein extraction and Western Blotting

For LC3, Caspase3, IL-6 and VEGF-C assays, 5 pools consisting of 20 xenograft larvae from each experimental group were electrophoresed and transferred onto polyvinylidene difluoride membranes. Briefly, 7 mg of each protein sample were separated using 4% stacking and 10% separating sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and electroblotted onto a Bio-Rad filter using a Bio-Rad mini trans-blot electrophoretic transfer cell. The transfer was performed for 2 h at 250 mA and 4°C using a 25mM Tris base, 192 mM glycine, and 20% methanol as the electrode solution. Membranes were soaked in 5% Nonidet-P40 for 30 minutes to remove SDS and incubated with 2% bovine serum albumin (BSA; Sigma) in phosphate-buffered saline (PBS) buffer overnight. Blots were incubated with primary

antibody 2 hour at RT. The LC3A/B primary antibody (BK4108S, Cell Signaling, Beverly, MA, USA), the Caspase 3 polyclonal antibody (BK9661S, Cell Signaling, Beverly, MA, USA), the IL6 (ab208113, abcam, Cambridge, UK) and VEGF-C Antibody (BK2445S, Cell Signaling, Beverly, MA, USA) were diluted 1:1000 in a solution containing 2% BSA and 0.1% TWEEN 20 in PBS1X. Anti-b-actin antibody (BK4967S) was used to normalize sample loading. The reaction was visualized with ECL-PLUS (GE Healthcare, Milano, Italy) chemiluminescent reagent for Western blotting. Densitometric analysis was performed using Fiji software for Windows.

3.12 Statistical analysis

RNAseq statistical analyses were performed with R with the package edgeR. The edgeR package determines differential expression using empirical Bayes estimation and exact tests based on a negative binomial model.

Statistical analysis was performed with Graph Pad Prism V9.0.1. (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the means ± SEM. All the data was analyzed by One-Way ANOVA followed by Dunnett's multiple comparison test. When the collected data was expressed in percentage, arcsine transformation was conducted before ANOVA. Letters and asterisks (*) indicate statistically significant changes among groups. The p-values are indicated with the following symbols: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Chapter Four

RESULTS

4.1 Microinjection experiment optimization

Preliminary experiments were performed to optimize the number of HCT116 cells to use for Zebrafish embryo transplantation. Based on previous studies two different quantities of cells per embryo were injected: 200-400 or 500-1000 cells/embryo (Cornet et al. 2019; Fior et al. 2017; Ye et al. 2020). The inoculation has been done in the yolk, according to standard protocols.

Xenografts were initially exposed to two different AEA concentrations (5 and 10 nM), on the bases of Migliarini and coauthor (Migliarini and Carnevali 2009) and HA (HA et al. 2010) to gain first evidence on changes in cell proliferation. A reduced tumor cell proliferation was observed in xenograft transplanted with 200-400 cells and further treated with both AEA concentrations (**Figure 6A**), while in xenograft injected with 500-1000 cells, exposure to 10 nM of AEA did not affect cell proliferation (**Figure 6B**). Accordingly with these results, for the future trials, 200-400 cells/embryos were used to transplant Zebrafish larvae and set up the main experiment.

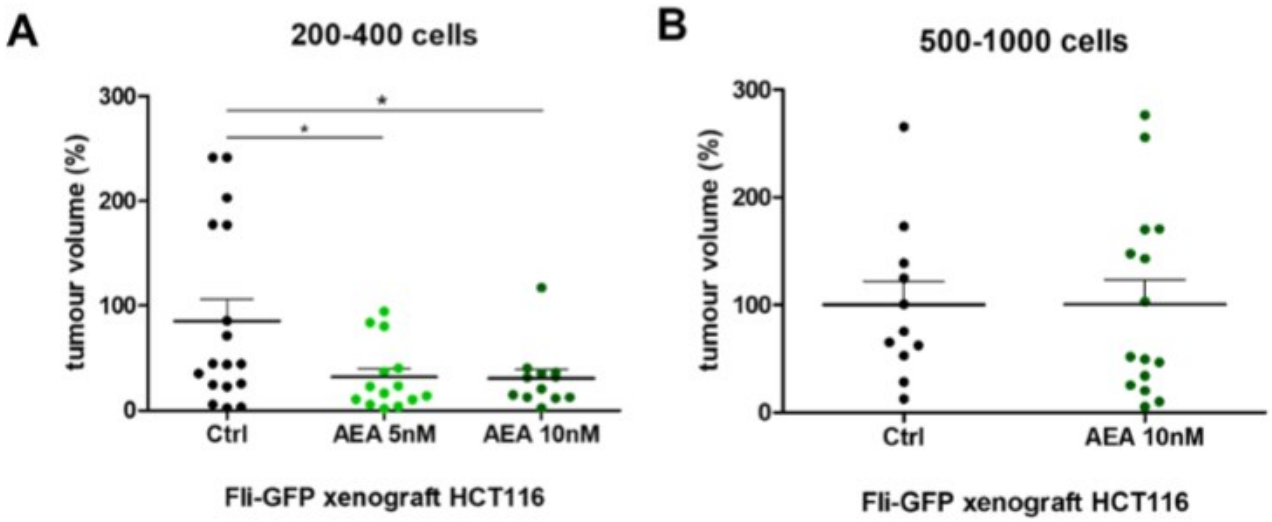

***Figure 6**. A) HCT116 tumour size analysis in 5 dpf (3 dpi) Tg(fli1:EGFP) larvae injected with 200-400 cells. Two EAE concentrations were used for this experiment (AEA 5 nM and 10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 17; AEA 5 nM n = 14; AEA 10 nM n = 12). Statistical analysis was performed using Student's t-test. *, P < 0.05. B) HCT116 tumour size analysis in 5 dpf (3 dpi) Fli-GFP larvae injected with 500-1000 cells. Only one EAE concentration was used for this experiment (10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 11; AEA 10nM n = 15).*

4.1.1 Confocal microscopy analysis to identify AEA effect on tumor growth

To evaluate the consequence of AEA treatment in tumor development, confocal microscopy analysis using Tg(*fli*1-EGFP) transgenic line xenograft at 5 dpf, was performed. Larvae have been exposed to AEA 10 nM, AM251 10nM and AEA+AM251 (10 nM + 10 nM) starting at 6 hpi. Fascinating, AEA exposed xenograft presented a reduced tumor volume compared to Ctrl group (52%), to AM251 group and AEA+AM251 group (**Figure 7B**). No differences of tumor size were seen among Ctrl, AEA+AM251 and AM251 exposed xenograft. The results observed in the AEA treated group suggests that AEA can affect -tumor growth, possibly by binding CB1 and that AM251, specifically bindings the endocannabinoid receptor, thus allowing tumor cell proliferation. In fact, as expected, groups treated only with AM251 and with

the combination of AEA and its inhibitor show a similar tumor size and similar to control volume (**Figure 7A**).

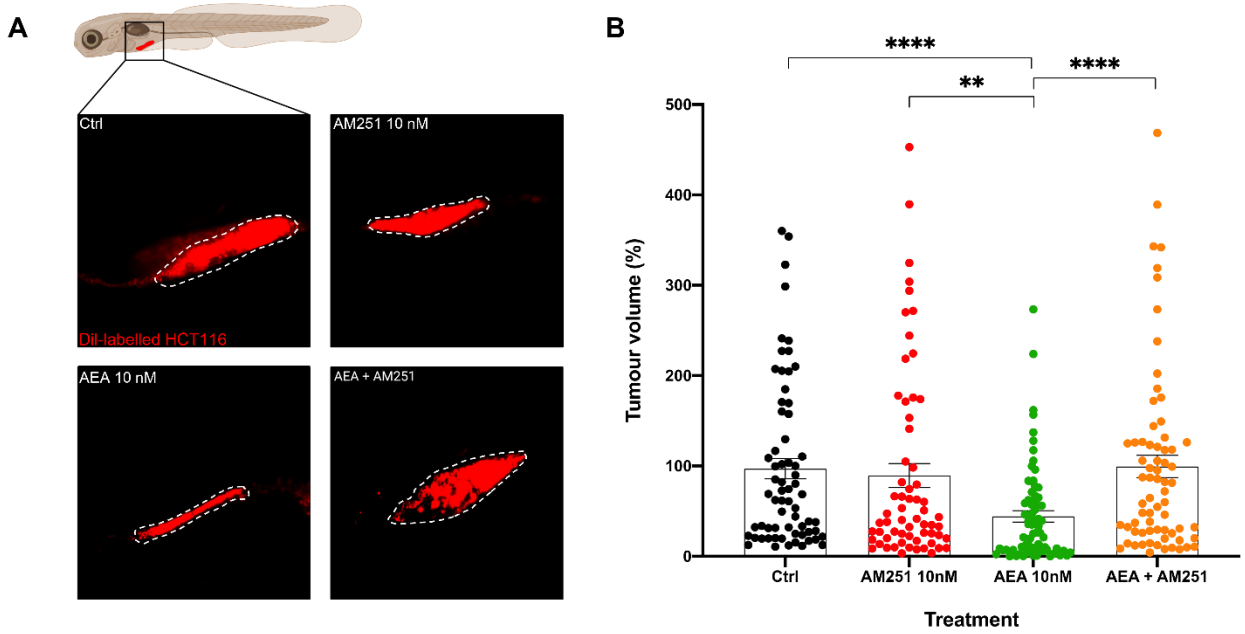

***Figure** 7. A) HCT116 tumour size analysis in 5 dpf (3 dpi) Tg(fli1:EGFP) larvae injected with 200-400 cells. Two EAE concentrations were used for this experiment (AEA 5 nM and 10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 17; AEA 5 nM n = 14; AEA 10 nM n = 12). Statistical analysis was performed using Student's t-test. *, P < 0.05. B) HCT116 tumour size analysis in 5 dpf (3 dpi) Fli-GFP larvae injected with 500-1000 cells. Only one EAE concentration was used for this experiment (10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 11; AEA 10nM n = 15).*

4.1.2 Confocal microscopy analysis to identify AEA effect on vascularization

To evaluate the ability of AEA to modulate tumor development the Tg(*fli1*:EGFP) transgenic line, where the blood vessels are marked with a green fluorescent protein allowing the visualization of larvae vascularization *in vivo, was* used (**Figure 8A**). Specifically, the focus was on the blood vessels closed to the tumor area directly involved in its growth. The results showed that AEA leads to significant reduction of

the tumor vascularization respect to Ctrl group, AM251 and AEA+AM251 groups (**Figure 8B**).

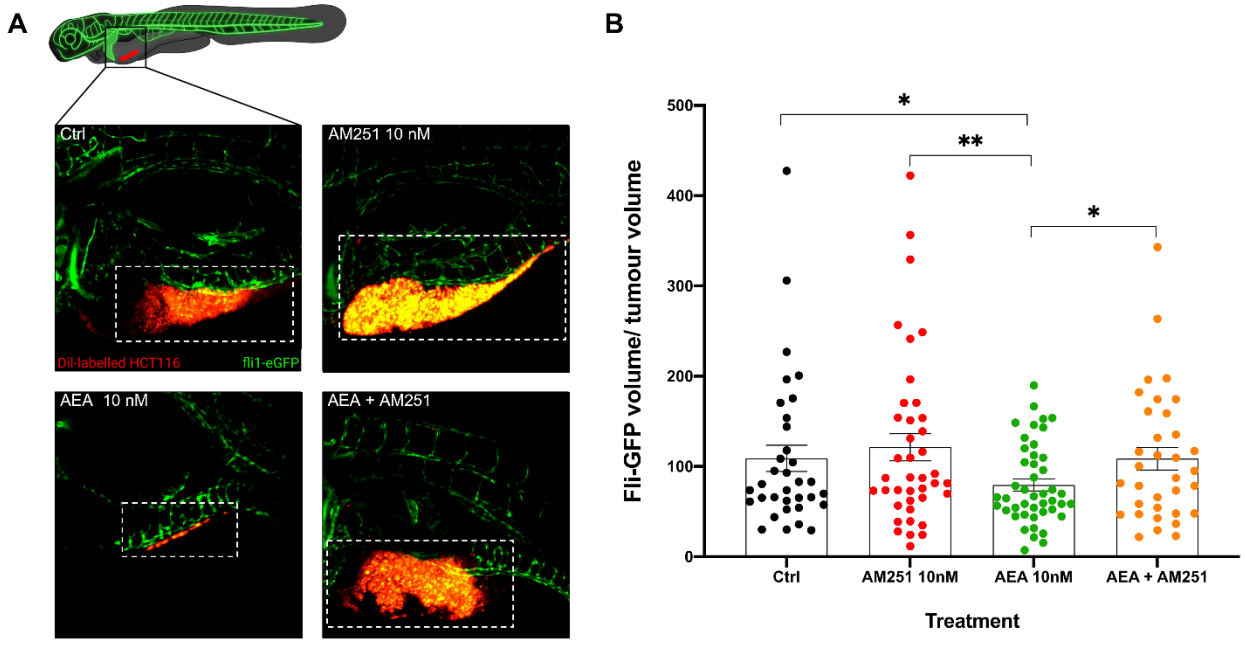

Figure 8. *A) HCT116 tumour size analysis in 5 dpf (3 dpi) Tg(fli1:EGFP) larvae injected with 200-400 cells. Two EAE concentrations were used for this experiment (AEA 5 nM and 10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 17; AEA 5 nM n = 14; AEA 10 nM n = 12). Statistical analysis was performed using Student's t-test. *, P < 0.05. B) HCT116 tumour size analysis in 5 dpf (3 dpi) Fli-GFP larvae injected with 500-1000 cells. Only one EAE concentration was used for this experiment (10 nM). The mean value of the tumour size in the controls was settled as 100%. Error bar indicates SEM (Controls n = 11; AEA 10nM n = 15).*

4.2 In vitro AEA effect on HCT116 cell proliferation

To investigate the effects on cell viability and proliferation HCT 116 cells were reared with 5 different AEA concentrations in the range between 0.005 and 5 µM. The in vitro tests were carried out in low serum concentrations (i.e.0.5% serum) to limit cellular growth and to increase the response of cells to the presence of the compound. Indeed, the effect of cannabinoids is cell context-dependent being modulated by the presence of growth factors (Sainz-Cort et al. 2020). Cell viability was evaluated using the CCK-

8 assay after 72 hours of treatment but in contrast to what previously observed in the animal model all AEA concentrations were found to have no anti proliferative activity (**Figure 9**).

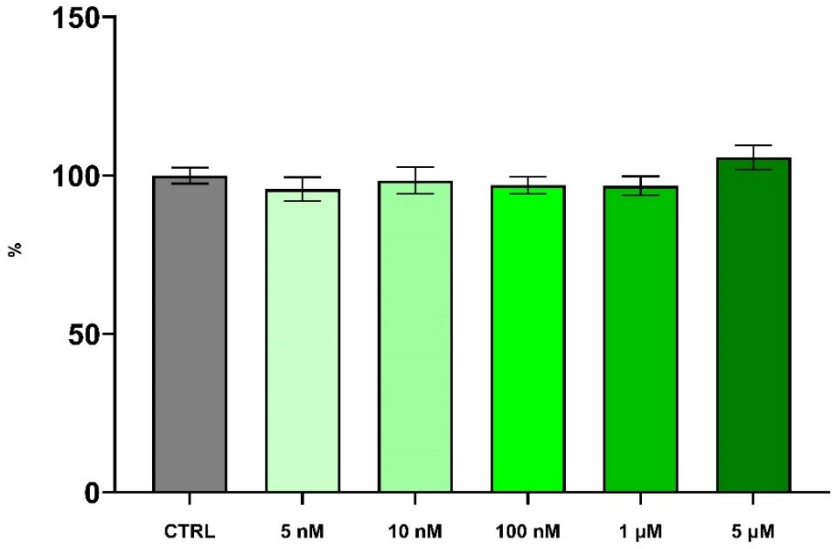

***Figure 9**. Bar plot showing cell proliferation in HCT116 cells exposed to different AEA concentration*

4.3 Identification of DEGs among different groups

By RNAseq analysis of WT xenografts, a series of DEGs were evidenced following comparisons between experimental groups as follows: AEA vs Ctrl, AEA+AM251 vs Ctrl, AM251 vs Ctrl, AEA+AM251 vs AEA, AM251 vs AEA, AEA+AM251 vs AM251. From these comparisons, some genes showed a statistically significant difference with FDR <=0.05. DEGs between experimental groups, the results are reported in the heatmaps (**Figure 10A-B-C-D-E**).

Among transcripts functionally annotated, 39 DEGs were found to be differentially expressed between Ctrl and AEA, 8 upregulated and 31 downregulated, 30 DEGs

were found to be differentially expressed between Ctrl and AEA+AM251, 16 upregulated and 14 downregulated, 94 DEGs were found to be differentially expressed between AEA+AM251 vs AEA, 30 upregulated and 64 downregulated, 30 DEGs were found to be differentially expressed between AM251 vs AEA, 12 upregulated and 19 downregulated and 16 DEGs were found to be differentially expressed between AEA+AM251 vs AM251, 10 upregulated and 6 downregulated. No DEGs were found after comparing AM251 and Ctrl (**Figure 11F**).

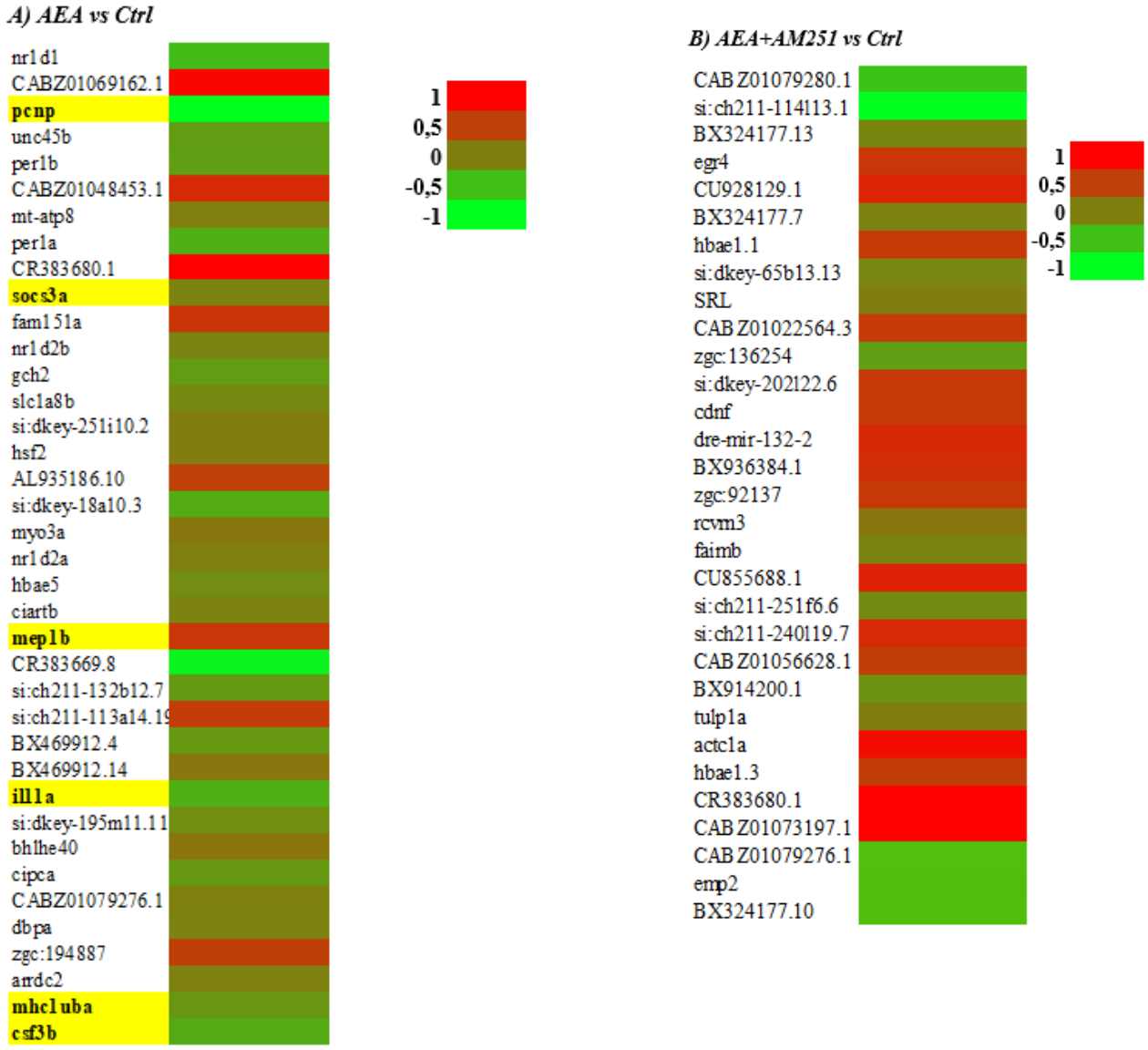

Figure 10A-B. *Heatmaps showing the list of DEG in zebrafish larvae.* ***A)*** *AEA vs Ctrl.* ***B)*** *AEA+AM251 vs Ctrl. Scales of the colors represent fold-change between two groups as described (green = down-regulation, red = up-regulation). DEG with FDR <= 0.05. The DEGs studied in the thesis have been reported in yellow.*

Figure 10C. *Heatmaps showing the list of DEG in zebrafish larvae.* ***C)*** *AEA+AM251 vs AEA. Scales of the colors represent fold-change between two groups as described (green = down-regulation, red = up-regulation). DEG with FDR <= 0.05*

Figure 10D-E. *Heatmaps showing the list of DEG in zebrafish larvae.* ***D)*** *AM251 vs AEA.* ***E)*** *AEA+AM251 vs AM251. Scales of the colors represent fold-change between two groups as described (green = down-regulation, red = up-regulation). DEG with FDR <= 0.05*

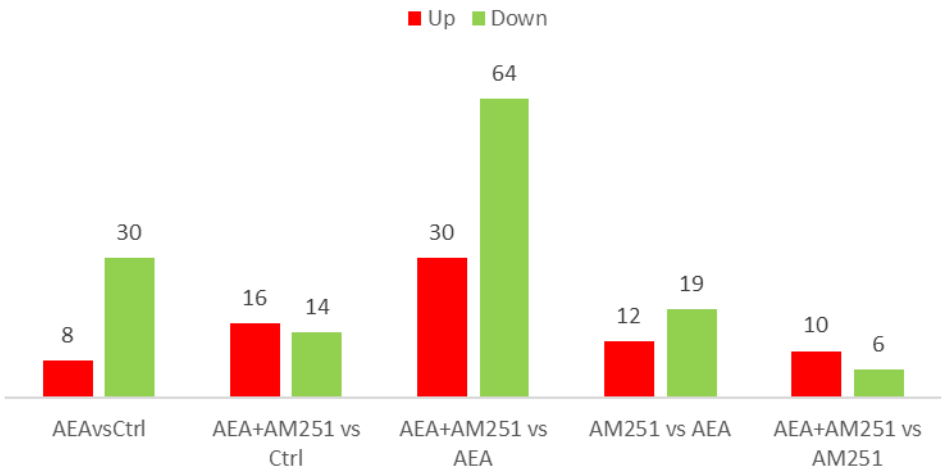

Figure 11F. *Hierarchical cluster analysis of DEG expression profiles in three treatment group compared to Ctrl group.*

Heatmaps represents DEGs between experimental groups. Starting from the evidence obtained by confocal microscopy and in order to gain knowledge on the possible mechanisms activated by AEA to counteract tumor cell proliferation, in this study, attention was mainly focus on DEGs between Ctrl and AEA groups. Among not differential expressed mRNA, Figure 12 reports some gene which have a key role in some of the pathways regulated by AEA (**Figure 12**).

Figure 12. *Heatmaps showing the list of DEG in zebrafish larvae. AEA vs Ctrl. Scales of the colors represent fold-change between two groups as described (green = down-regulation, red = up-regulation). DEG with FDR => 0.05*

Considering DEGs between AEA and Ctrl, and focusing on the principal aim of this study, attention was made on all signals involved in cell proliferation, angiogenesis, and immune system.

4.3.1 Identification of DEGs between Ctrl group and AEA, AM251 AND AEA+AM251 groups in Human

Regarding DEG analysis on human HCT116 cell transcripts, no genes showed an FDR <=0.05. Nevertheless, some gene expression, despite not statistically significant, were considered to support the study aim (**Figure 13**).

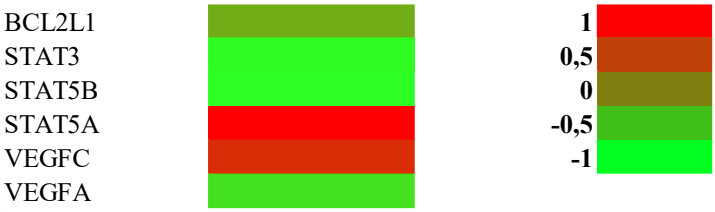

Figure 13. *Heatmaps showing the list of DEG in zebrafish larvae. AEA vs Ctrl. Scales of the colors represent fold-change between two groups as described (green = down-regulation, red = up-regulation). DEG with FDR => 0.05*

4.4 Expressions of the genes controlling angiogenesis in xenografts larvae, by qRT-PCR

To test the effects of AEA on angiogenesis, transcript levels for VEGF family members were measured. In WT xenograft larvae, while no significant changes were observed for the *vegf-aa* and *vegf-ab* transcript levels (**Figure 14a,b**) among experimental groups, the AEA treatment induced a significant *vegfc* and *vegfd* mRNA reduction respect to Ctrl. A similar, although not significant reduction was observed also in group AEA+AM251. AM251 treatment did not induce changes respect to Ctrl group (**Figure 14c,d**).

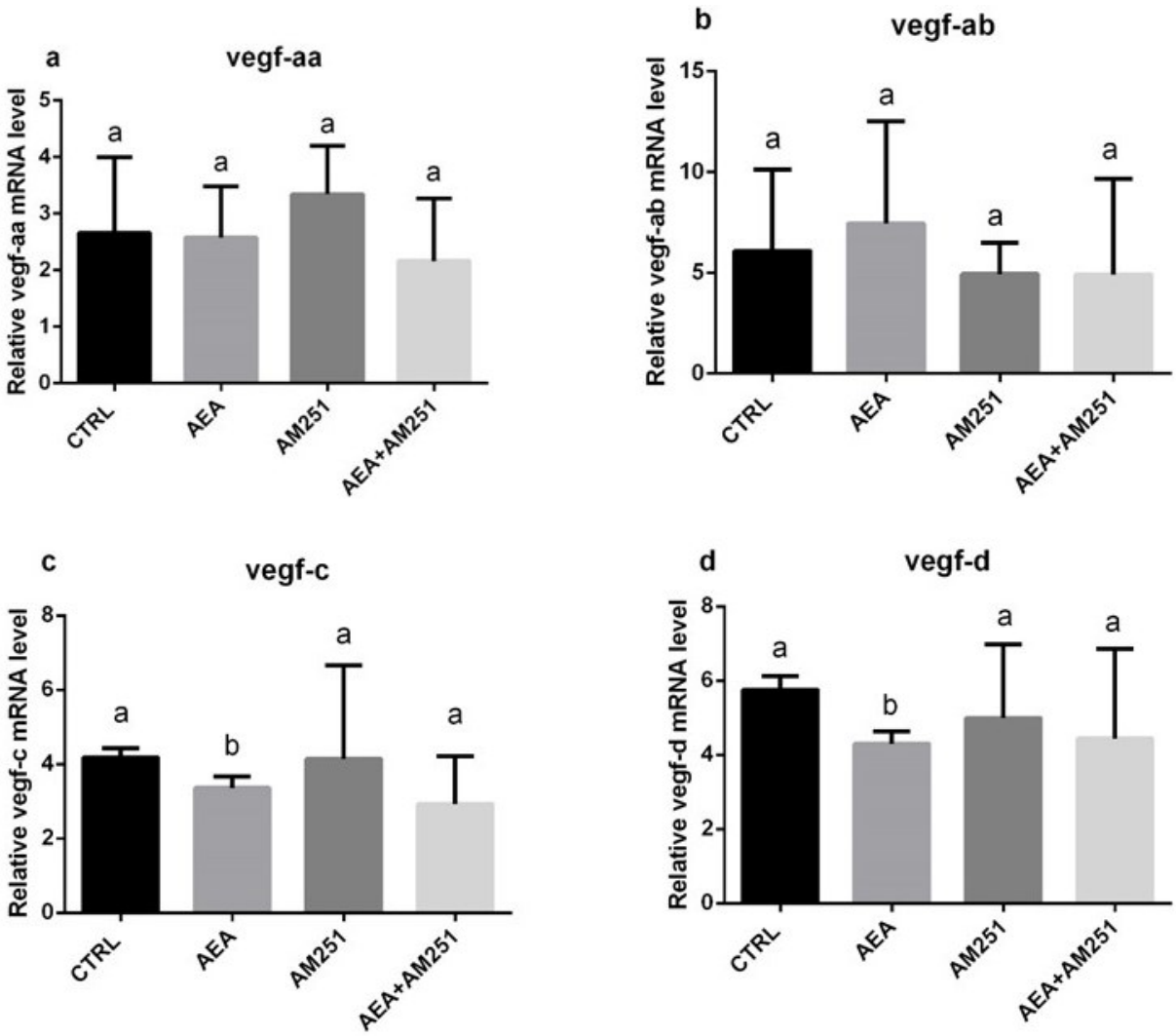

Figure 14. *Transcriptional profiles of genes coding for angiogenic process: vegf-aa (a), vegf-ab (b);vegf-c (c), vegf- (d) expressed as mean of the relative mRNA abundance of 4 replicates ± SEM and determined by Bio-Rad Laboratories iQ5 manager software. Ctrl: control, AEA: Anandamide, AM251: inhibitor. Different letters above each column indicate statistical differences among groups, while identical letters indicate no statistical differences (one-way ANOVA, $p < 0.05$).*

Regarding the gene coding for protein involved in the apoptotic pathway, the levels of casp3 in treated WT xenograft, did not significantly change respect to Ctrl. A significantly decreased was found in the group treated with AEA respect to the group treated with AM251 (**Figure 15**).

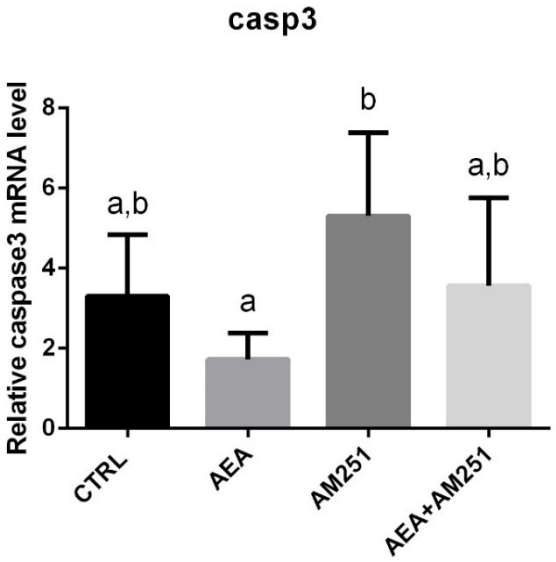

Figure 15. *Transcriptional profile of gene casp3 coding for apoptotic process. Expressed as mean of the relative mRNA abundance of 4 replicates ± SEM and determined by Bio-Rad Laboratories iQ5 manager software. Ctrl: control, AEA: Anandamide, AM251: inhibitor. Different letters above each column indicate statistical differences among groups, while identical letters indicate no statistical differences (one-way ANOVA, $p < 0.05$).*

5.5 Molecular analysis of biomarkers involved in tumor cell survival/ growth

Western blot analysis on WT xenografts of LC3A/B (**Figure 16a**) and CASP3 (**Figure 16b**) did not show any significant changes among experimental groups. On the contrary, VEGF-C (**Figure 16c**) and IL6 (**Figure 16d**) western blot revealed a significant reduction of protein level in xenograft exposed to AEA. The other experimental treatment did not induce statistical changes respect to Ctrl.

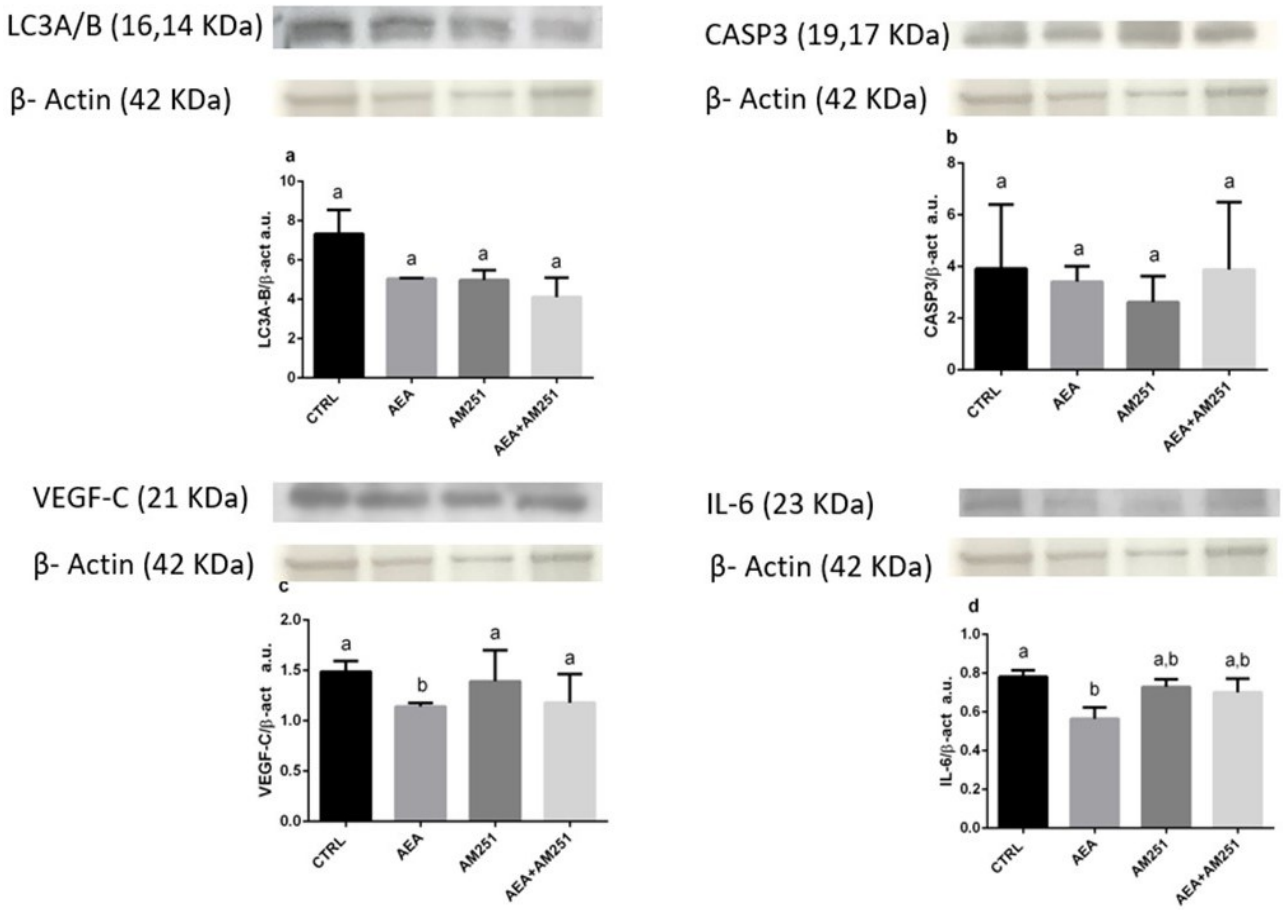

Figure 16. *Insert shows a representative LC3A/B, CASP3, VEGF-C, IL6 and B-ACT Western Blot in different experimental groups. Densitometric analysis of 3 independent experiments a.u. (arbitrary units) a) LC3A/B b) CASP3, c)VEGF-C, d) IL-6. Different letters above each column indicate statistical differences among groups, (one-way ANOVA, $p < 0.05$).*

Chapter Five

DISCUSSION

In this thesis, a variety of approaches were used to closely investigate and profile the effect of AEA on the proliferation of cancer cells in zebrafish larval xenograft. Combining the results regarding the *in vitro* HCT116 cell proliferation and the evidence coming from confocal microscopy, it emerged that AEA treatment probably mainly affects the tumor microenvironment than the tumor cells, thus acting on the organism factor responsible for tumor development. Indeed, observing the results obtained through the confocal microscopy, it emerged that AEA inhibits angiogenesis and thus allowing a reduced tumor growth.

To validate the data, the images were coupled with RNA-seq analysis, qPCR and Western blot on different xenograft larvae groups. A series of DEGs emerged from RNAseq analysis in AEA group, involved in angiogenesis, proliferation, and the immune system.

Among DEGs, previous studies demonstrated that the "suppressor of cytokine signaling 3 (Socs3)" regulates the IL-6/JAK/STAT3 pathway by inhibiting Janus kinase (JAK)-signal transduce, which in turn, affects STAT transcription signaling in a negative auto-regulatory loop (Jin et al. 2007). Thus, the activation of this pathway regulates the expression of key proteins involved in inflammation, angiogenesis, and cell survival, including VEGF, IL-6 and BCL-2 (Kaplan 2013; Sepúlveda et al. 2006; Wei et al. 2003).

Studies reported that the downregulation of *socs3* alone, is implicated in the development of colorectal-, breast- and ovarian cancer (Dai et al. 2021; Ghafouri-Fard et al. 2018; Huang et al. 2016).

Results obtained in this study, unveiled AEA ability to downregulate socs3 mRNA, and IL6 and VEGF protein levels, thus suggesting the inhibition of IL-6/JAK/STAT3 pathway. These results are also supported by the RNA seq analysis, showing a downregulation, although not significant, also of *jak1* and *stat3* mRNA, and are in line with studies reporting the antiproliferative effect of AEA in controlling the expression of genes regulating cell survival and proliferation (Huang et al. 2011; Laezza et al. 2020; Petrocellis et al. 1998).

Regarding these last aspects, VEGF family members have a pivotal role in angiogenesis and in the activation of tumorigenesis (Harmey and Bouchier-Hayes 2002). Thus, it can be assumed that a lower expression of *Vegf* family factors concurs to the reduction of tumor volume affecting angiogenesis (Chen et al., 2017; Falcon et al., 2011; Loges et al., 2009). In fact, as observed in hepatocellular carcinoma (Zou, and Guo, 2015), osteosarcoma (N. Peng et al. 2016) and in gastric cancer (Chen et al. 2013), the silencing of VEGF caused the inhibition of tumor angiogenesis. Therefore, as seen in this study, AEA regulates tumor size possibly by reducing VEGF levels. It can be speculated that this can occur by blocking the VEGF/VEGFR2-mediated microenvironmental crosstalk between endothelial cells and HCT116 cells as previously observed *in vitro* (Liu et al. 2017) and in a mouse xenograft model (Liu et al. 2017). In addition, in a parallel study using our same fish, the inhibition of

VEGF/VEGFR2 pathway could explain the lack of micrometastis observed in our xenograft. Futhermore, it was reported that AEA inhibits breast tumor-induced angiogenesis in human breast cancer cell line MDA-MB-231, where the downregulation of VEGF and IL-8 promoted a reduction of inflammatory condition (Ciaglia et al. 2014). In addition, VEGF can be produced also by other cell types present in the tumor microenvironment. Among these cells, noteworthy are the tumor-associated macrophages (TAMs) which (Inagaki et al. 2021), under hypoxic conditions, secrete VEGF (Galdiero et al. 2013; Goel and Mercurio 2013). The secretion of this factor contributes to the development of blood vessels and tumor (Ferrao et al. 2018). On this regard, although a parallel study performed in our laboratory, did not show changes of macrophage number in the microenvironment, it could be supposed that AEA may not affect TAMs migration but only VEGF secretion (Kumari and Choi 2021; J. Peng et al. 2012). Concerning to this, it was reported that the activation of CB1 and CB2 in human lung-resident macrophages inhibits the release of angiogenic and lymphangiogenic factors (Staiano et al. 2016).

Focusing on HCT116 cells injected, it is well known that this cell strain expresses high levels of VEGF receptors (Liu et al. 2017; Samuel et al. 2011), thus promoting their growth with a paracrine and autocrine vascular endothelial signaling (Lichtenberger et al. 2010). Indeed, in CRC, the high expression of Vegf is correlated with poor prognosis (Tokunaga et al. 1998), and the downregulation of VEGF-C leads to the reduction of tumor-initiating cells and inhibition of metastasis (Khromova et al. 2011). In this contest, it can be assumed that the decrease of VEGF secretions in the tumor

microenvironment by AEA, could reduce tumor cell proliferation decreasing its binding to VEGFR. This assumption can be supported by RNA-seq data on human transcripts, which lacked significant changes among experimental groups and is also supported by our *in vitro* results showing that the different treatments do not affect proliferation and further supports the role of the larval response in tumor progression.

Also, the expression of the granulocyte colony-stimulating factor (G-CSF), a pleiotropic cytokine that promotes the activation of monocytes and macrophages, wound healing and can synthesize itself (Bussolino et al. 1989), resulted downregulated. The interesting aspect of this factor relies in its involvement in the promotion of angiogenesis, by synthesizing VEGF, starting from the early stage of primitive endothelial tubule formation (Okazaki et al. 2005). Thus the statistically significant downregulation of G-csf suggests that AEA can influence the angiogenesis of blood also by affecting the levels of this factor.

Noteworthy, among DEGs, STAT3 is the main mediator of the cytokine family functions and directly regulates IL6 levels (Cheng et al. 2020; Xu et al. 2021). The property of IL6 is controversial: it may have anti-inflammatory or pro-inflammatory functions. In xenografts and tumor conditions, inflammation is an important mediator for the progression of cancer. IL-6 high production causes a rich inflammatory environment and can promote the malignant transformation of cancer cells supporting their proliferation, survival, and metastatic dissemination (Fisher et al. 2011; Hartman et al. 2013; Rose-John 2012).

Several studies reported that AEA treatments can reduce inflammation via the regulation of inflammatory target genes and activation of inflammasome components (Cris et al. 2011; Pflüger-Müller et al. 2020; Sedeighzadeh et al. 2021; Shi et al. 2012). In this contest, both RNA-seq analysis and Western blot data revealed the negative regulation of the transcription and level of this cytokine in the group treated with AEA, supporting its beneficial role.

It is in fact known that IL-6 leads to STAT3 phosphorylation and its dimerization by acting on transcription of antiapoptotic-, angiogenic-, proliferating- and immune response factors including BCL-2, BCL-xL, VEGF, and MMP2/9 (Xu et al. 2021). Concerning the antiapoptotic Bcl-2 family proteins, they are frequently overexpressed in cancer suggesting that tumor cell turnover in not regulated by apoptotic process (Sasi et al. 2009; Walensky 2006; Zhou et al. 2010).

On this regard, in this study, both human and zebrafish RNA-seq results showed a *bcl-2* downregulation, although not statistically significant, which can be ascribed to the reduction of IL-6 levels, as previously observed in IL-6-/- mouse, where IL6 KO causes a decrease of Bcl-2 and Bcl-xL mRNA expression (Kovalovich et al. 2001). The lack of significant changes of Bcl2 levels herein observed, suggests a marginal role of apoptosis in either tumor physiology and during this stage of larval growth and is endorsed by caspase3 analysis, which mRNA and protein levels, performed on the xenograft, showed the lack of differences among experimental groups. According to this, AEA has been tested by several research groups to observe its role on apoptosis

and it was evaluated that it promotes the apoptotic events through activation of CASP3 (Dross 2009; Gómez et al. 2014; Nazıroğlu et al. 2019).

Moreover, IL-6 in colorectal cancer positively regulates the EMT program (Rokavec et al. 2014), an important process involved in tissue response to injury (Nieto et al. 2016) and in tumor spread (Al-Ismaeel et al. 2019). It was demonstrated that AEA can have an anticancer effect by inhibiting EMT in MDA-MB-231 human breast cancer (Laezza et al. 2012). These results lead as imagine that a similar situation can also act on HCT116 cells does inhibiting cell proliferation/invasion, as herein observed by confocal analysis. In addition,

IL-6 is highly expressed by macrophages and intestinal epithelial cells (Hosokawa et al. 1999) and modulate monocyte differentiation towards type M2 macrophages (Chomarat et al. 2000; Heusinkveld et al. 2011) by using their autocrine M-CSF. Furthermore, it was demonstrated in wild-type mice that IL-6 signaling promotes M2 polarization of macrophages (Mauer et al. 2014)).

With the downregulation of IL-6 and the inhibition of the IL-6/JAK/STAT3 pathway mediated by AEA in our model, it could be speculated about a possible differentiation of macrophageM2 in macrophage of type M1, as observed in a study during the development of hepatocellular carcinoma" (Yin et al. 2018). Furthermore, M1 macrophage contributes to the suppression of tumor growth and angiogenesis (Yuan et al. 2015), thus enforcing our result about the conditioning of the tumor microenvironment by AEA. Thus, the possibility of indirect TAMs polarization to M1-

subtype macrophage or eliminating M2-subtype macrophage mediate by AEA might represent useful treatment for cancer.

Thereby, the endocannabinoid may regulate/reduce the production of IL-6 (Kunath et al. 2013; Lowin et al. 2011), mitigating the pro-inflammatory cytokine effect in the tumor microenvironment with, consequentially, not excessive stimulation of macrophages M2 differentiation as previously observed in NIH3T3/Src cell line (Chen et al. 2018).

Recently, a novel nuclear factor involved in cell cycle regulation, transcription, and apoptosis was identified. The PEST-containing nuclear protein (PCNP) role consists in the degrading of residual proteins linked with cell growth and differentiation and acting as a tumor promoter or suppressor in a tissue-specific manner (Afzal et al. 2019; Wu et al. 2018). The study on lung adenocarcinoma cancer reported a significant higher level of PCNP in tumor tissue, promoting proliferation, invasion, and migration of cancer cells, than in adjacent non-tumor tissue (Wang et al., 2019). In this light, in this study, AEA treatment leads to a significant downregulation of zebrafishf *pcnp*, associated with a decrease (although not significant), of *stat3* and *stat5*. This might imply that lower levels of *PCNP* suggest a reduction of activated STAT3/5 and a lower transcription of downstream genes including *vegf, bcl-2* and *il6*. Our hypothesis is supported by previous data obtained in CRC cells showing that STAT5 is involved in tumor growth by upregulating genes involved in angiogenesis and cell survival (Xiong et al. 2009). Furthermore, inhibition of the tumor proliferative ability was demonstrated in HCT116 and SW480, CACO2 following PCNP silencing (Xu et al. 2022). In

addition, PCNP is involved in the regulation of STAT3 which is influenced by AEA and plays a key role in IL6/JAK/STAT3 signaling, above described.

In addition, looking at our data, we can assume that AEA can influence not only IL-6 levels but also *il11* transcript. Interleukin-11 is a cytokine related to IL-6 cytokine family controlling the release of inhibitory nuclear factor (NF)-kB which serves as a transcriptional activator for proinflammatory cytokines. It has been detected in many organs including gastrointestinal tracts and liver (Calon et al. 2012; Fung et al. 2022) and it has been shown to promote the progression of CRC (Putoczki et al. 2013) and prostate cancer (Campbell et al. 2001). Similar to IL6, it is involved in STAT3 and STAT1 phosphorylation, that promotes chronic gastric inflammation, as observed in a mouse model of gastric cancer. Indeed, the silencing with antisense oligonucleotides of Stats corresponds to a reduction of gastric tumorigenesis, gastric inflammation, and IL-11 expression (Ernst et al. 2008). For the first time, in our model, it was shown that AEA administration induces a significant downregulation of IL-11 affecting IL-11/STAT3-1 pathway. This result is coherent with the data collected before, where it is reported the action of endocannabinoids on the regulation of JAKs and STAT family members and interleukins. Since IL-11 is a functionally dominant inducer of neoplastic STAT activity in the GI epithelium, in this contest, AEA plays a role as a molecular entity with strong anti-tumorigenesis potentiality (Putoczki et al. 2013).

Autophagy is considered a double-edged sword because although it is a tumor-suppression mechanism, it also enables tumor cell survival (White and Dipaola, 2009). The high expression of LC3, a key marker of autophagy, is correlated with a bad

prognosis in pancreatic and gastrointestinal cancer tissue (Fujii et al. 2008; Yoshioka et al. 2008). Therefore, the biological significance and clinical impact of LC3 levels in cancer seems to be related to tumor type and tissue context (Chen and Karantza-Wadsworth, 2009).

In this study, LC3 protein levels does not change significantly among experimental groups, although two possible scenarios regulated by autophagy can be assumed in our xenografts: in control ones, autophagy regulates metabolite uptake favoring HCT116 proliferation, as previously observed in different cancer cell lines (Hout et al. 2020; Lozy and Karantza 2012) in AEA exposed xenograft, it can be assumed that autophagy exerts a cytoprotective, tissue-protective and anti-inflammatory action (Chandrika et al. 2015; K. Wang et al. 2019; Yoshizaki et al. 2012). This last hypothesis is strongly supported by IL downregulation in the treated group. In this contest, some researchers have reported that AEA suppresses proinflammatory T-cell responses through a CB1 mediated mTOR inhibition, the suppressor of autophagy, in human keratinocyte cells thus limiting the expression of proinflammatory chemokines (Breton et al. 2016).

The Major histocompatibility complex (MHC) is one of the protagonists in the immune response and in the recognition of non-self, expressed on the membrane of all nucleated cells. The principal function of MHC-I is the presentation of peptide antigens to CD8+ T cells triggering their differentiation (Daniels et al. 2001) and thus aiding the immune response. RNAseq analysis on zebrafish transcriptome revealed a downregulation of the U lineage gene, which shares a high synteny with mammalian MHC (Dirscherl et

al. 2014) and suggests that AEA treatment activates the Natural Killer (NK) mediated immune response, thus promoting tumor cell death (Cornel et al. 2020). However, several studies on this aspect are still required since the control of AEA on recognition of non-self by MHC-I should be deepened. Based on our transcript results, we can speculate that its reduction could be correlated to the decrease of *il11* mRNA levels, as recently demonstrated in HD11 cell line (Truong et al. 2020) . It could be interesting to investigate how the endocannabinoids can mitigate the antigen-immune response not only focusing on the control of inflammation, but also on the regulation of self from non-self. Considering HCT116 cells, in our results Mhc-1 transcript resulted, despite not significantly, upregulated, suggesting the tentative of the tumor cell to evade from NK cytotoxic action, as previously observed (Jonges et al. 2000; Miguel et al. 2014).

The metalloproteases (ADAMs) are extracellular proteases involved in connective tissue homeostasis, intestinal barrier function and immunological processes. Among them, meprins have a particular structure and functional features: meprin a is a pro-angiogenic enzyme and promotes tumor progression, while meprin b is mainly related to some diseases (Tredup and Becker-Pauly 2016).

The substrates for the metalloproteases are many transmembrane proteins such as pro-inflammatory cytokines including TNF-a and IL-6R (Althoff et al. 2000; Cosman et al. 1995; Matthews et al. 2003). Moreover, meprins balance the immune environment modulating the activity of IL-1B, IL-18 and IL-6 which are the major products released in response to tissue injury and inflammation (Banerjee and Bond 2008; Herzog, et al. 2019). They are also involved in ECM remodeling, when imbalance of cytokines and

mononuclear cells occurs in response to mechanical stress or ROS production (Arnold et al. 2017). Surprisingly, in our study the RNAseq analysis, revealed an increase of *mep1b* gene expression, which, as reported in several studies and it should be associated to an increase of cytokines through the leukocyte influx promotion (Herzog et al. 2019) and tumor cell migration (Breig et al. 2017). Conversely among DEG, ILs resulted downregulated, suggesting that in our xenograft model, mep1b could be only involved in larval development, possibly controlling the active cellular progression typical of an early-stage larva.

Chapter six

CONCLUSION

Collectively, our data suggest a pivotal role of AEA on the anti-angiogenic, anti-proliferative, and anti-inflammatory process in intercellular tumor-endothelial cell communication resulting in the containment of tumor. According to these results, AEA could be proposed among novel therapeutic strategies with endocannabinoids for the treatment of cancer. All the genes regulated by AEA have been reported in the figure 17 within the tumor microenvironment (**Figure 17**).

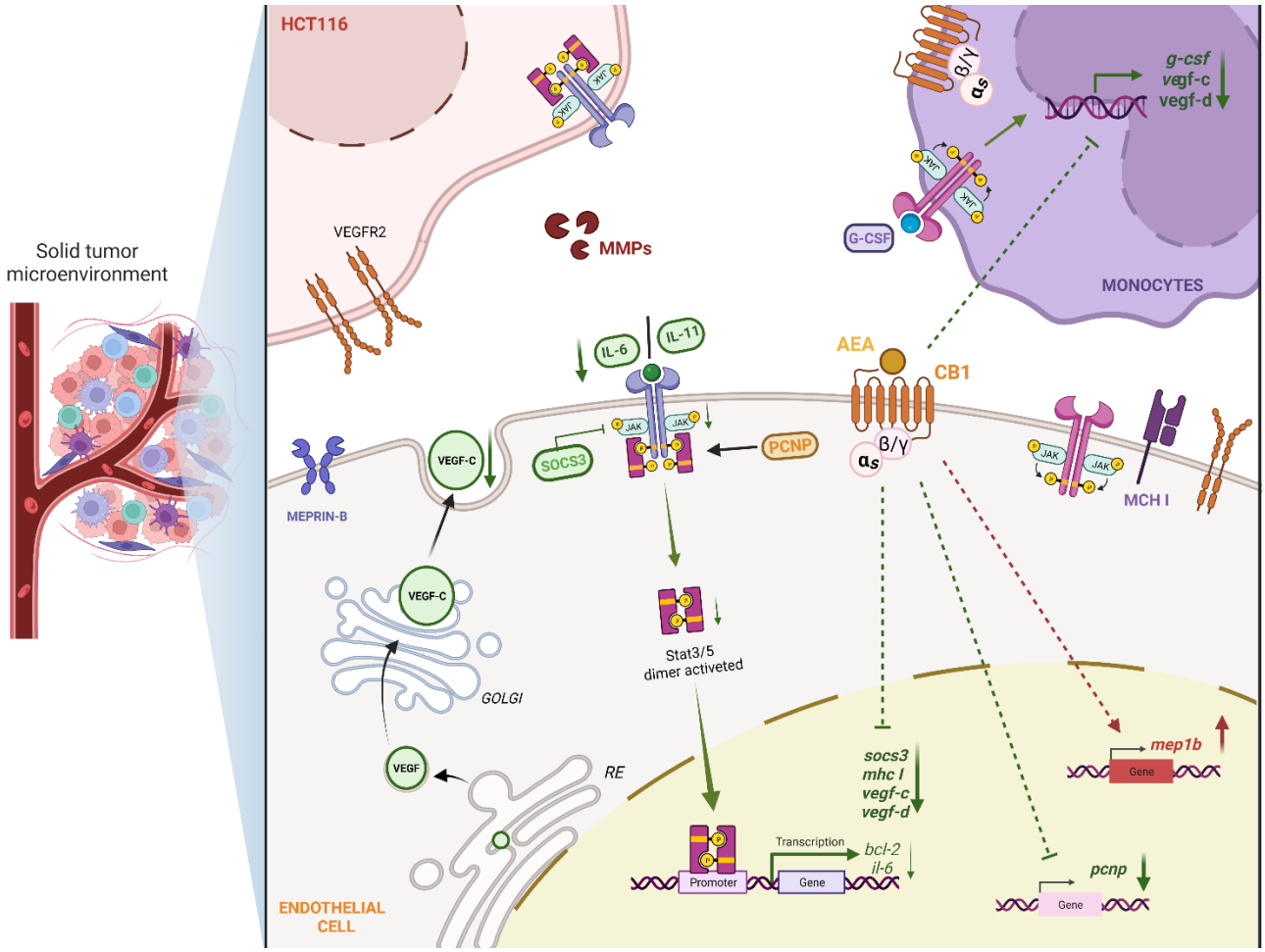

Figure 17*. Schematic figure representing pathways regulated by AEA in xenograft tumor microenvironment. IL-6, VEGF-C, socs3, mhc I, pcnp and g-csf are significantly downregulated, while mep1b is significantly upregulated. The significantly downregulated genes are marked in dark green and a downregulated not significant genes in light green. The significantly upregulated gene is marked in red. RE = endoplasmic reticulum, MMPs = metalloproteases. The image has done with Biorender.*

Chapter Seven

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