PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: FIORENZA SELLA

Title of PhD research: Exploring the main potential roles of Endocannabinoid system in different biological models

Name of PhD supervisor: Prof.ssa OLIANA CARNEVALI
Research lab name: LABORATORIO DI BIOLOGIA DELLO SVILUPPO E DELLA RIPRODUZIONE

Cycle:
[ ] XXXVI
[X] XXXVII

PhD Curriculum::
[ ] Marine biology and ecology
[X] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[X] Advanced Instrumentation lab
[X] Aquarium
[ ] MassSpec lab
[X] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate:

ABSTRACT (1000 characters, including spaces):

Over the last 25 years, the endocannabinoid system (ECS) has been discovered to play a proven role in the management of several physiological conditions. Endocannabinoids or cannabinoids are lipid molecules which bind with cannabinoid receptors and impact the physiological process of the body. Different studies have investigated the biological role of ECS in health and disease "conditions". It is well known the role of ECS on bone homeostasis, bone metabolism and bone disease such as osteoporosis (OP). Similarly, different studies reported the ability of endocrine disruptors (EDcs) to impair bone metabolism causing osteoporosis and rheumatoid arthritis. Thus, one of the main aims of this research is to highlight if PFOA, among EDCs, could affect bone homeostasis by targeting the ECS.
More specifically, exposing human fetal osteoblast (hFOB1.19) to physiological PFOA concentrations, evidence regarding the ability of PFOA to affect the regulation of oxidative stress response and the

endocannabinoid receptors levels. Furthermore, PFOA does not appear to stimulate or inhibit OB differentiation, but it could affect the collagenous composition of the bone ECM.
In addition, in recent years, several studies focused on the role played by the Endocannabinoid System (ECS) in tumorigenesis and tumour suppression by controlling inflammation and immunomodulation. So, the second aim of the research was to investigate the cannabinoid ability to reduce inflammation in zebrafish apc mutants exposed to THC. After a histopathological analysis, it has been reported that there is any neoplasia (adenoma or adenocarcinoma) or preneoplastic lesions in any lesions of the subjects of the various groups. Among phytocannabinoid, the efficacy of THC dietary uptake by molecular analysis is under evaluation and the characterization of the ECS through confocal microscopy analysis demonstrate the presence of CB1 and CB2 receptor in the gut folds.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

The endocannabinoid system (ECS) system was discovered less than 30 years ago, and its role and actions have been found to be associate with the control of physiological functions. ECS is composed by a complex network of cannabinoid receptors (CB1 and CB2), endocannabinoid ligands (AEA and 2-AG) and a series of enzymes responsible for their biosynthesis (DAGL and NAPE-PLD) and degradation (FAAH and MAGL) (1). The cannabinoid receptors belong to the super family of GPCR: the primary receptor is CB1, found in the peripheral and central nervous systems, microcirculation, in reproductive system and in gastrointestinal system (2). The second one, the CB2, is found in multiple lymphoid organs and it is highly expressed in the inflammatory system and in bone (3). In combination with CB2 and CB1 there are other molecular receptors like peroxisome proliferators activator receptor (PPARs) and transient receptor potential channels (TRPV) that regulate the functioning of endocannabinoids in the human body (3).

A lot of studies have investigated the biological role of ECS in health and disease “conditions” and among others, it plays a proven role in the management of bone, gastrointestinal, immunological, cardiovascular disease, and cancer (4).

it is known that in skeletal remodeling, determinant is the role of the endocannabinoids in bone formation, resorption, and growth, on age, gender, and specie-specific manner. Specifically, the endocannabinoid receptors CB1, CB2 and TRPV1, expressed by bone cells, can regulate cell survival, differentiation, and activity, contributing to bone maintenance and remodeling. In this regard, the osteoblasts and osteoclasts, the main component of bone, express the CB receptors suggesting that they might act together to balance the bone mineralization and resorption. The bone diseases are very common worldwide, and osteoporosis is the principal cause of bone loss, and it can be caused by several factors. Considering the involvement of ECS receptors in bone remodeling, is likely to hypothesize that they could influence the balance in all pathological conditions where an altered osteoblast/osteoclast activity is observed (5).

Moreover, it is known that CB1 receptors is implicated in the gastrointestinal tract as a potent gastroprotective agent (6). At the same time, the CB2 receptors were mostly found in the whole segment region of the intestine indicating the presence of the immune cells (7). Over the years, several studies focused on ECS role in the control of tumorigenesis and suppression of tumors (1) by controlling inflammation and immunomodulation (8; 9; 10). Depending on tumor type, the action of the ECS could be antiproliferative, proapoptotic, antiangiogenic, anti-metastatic or anti-inflammatory (11). In the case of colorectal cancer (CRC), the third most common cancer in the world, it has been found that an upregulation of CB transcripts represents a poor prognosis and advanced stage of disease. Recently, some studies have investigated the biological role of cannabinoid treatment in health and disease, such us cardiovascular disease, inflammation and cancer for the ability of cannabinoids to inhibit cell proliferation and induce tumor cell death (12; 13,14). Otherwise, it is known that the anticancer effects are elicited by a wide range of biological pathways involved

in cell survival, proliferation, and apoptosis such as p38 MAPK, cyclic AMP, ERK and ceramide pathways (15). Currently, it has been studied that cannabinoids could affect proliferation, viability and invasiveness of cancer cells acting on the proliferation, cell-cycle arrest, and apoptosis process. It has been studied that one of the most important phytocannabinoids, the Δ9-tetrahydrocannabinol (THC), could cause the inhibition of cancer growth in vivo due to its cannabinoid receptor-activating properties in the tumor microenvironment (16).

- SCIENTIFIC AIMS

The scientific aims of my PhD researchare to investigate and evaluate the activity and the roles of ECS in two different biological models. In this regard, I'm focusing on bone homeostasis, investigating, *in vitro*, the role and the response of ECS on human osteoblast cell culture treated with PFOA, an endocrine disruptor chemical (EDC) which leads the breaking of bone homeostasis and compromise the bone metabolism. Moreover, I'm working on the ability of THC to modulate the inflammation *in vivo* using zebrafish APCmcr mutants. On this, regard, the mutation on adenomatous polyposis coli (APC) could determine the insurgence of inflammation and the treatment with THC could maintain immune homeostasis.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. - Objective.

The objective of the study is to evaluate the effects of PFOA treatment on hFOB 1.19 (human fetal osteoblast) homeostasis, by studying its impact on the ability of these cells to deregulate the proliferation and the differentiation processes into mature OBs and alter the formation and initiation of ECM mineralization. Furthermore, effect of PFOA on the endocannabinoid system and antioxidant response, responsible of bone metabolism was also evaluated.

Methods.

a)

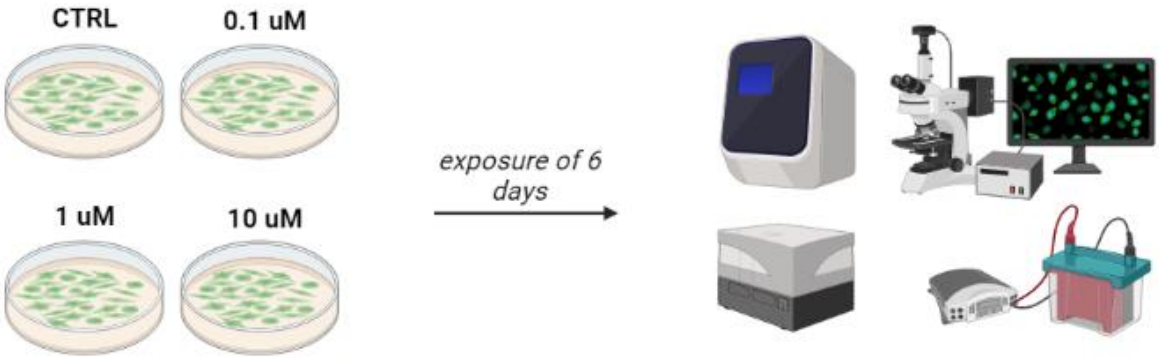

Figure 1. a) experimental design of the treatment

- **Cell culture of hFOB1.19** (**Figure 1**)
- **Viability assay** - **MTT**
- **Alizarin Red staining**
- **RNA extraction, cDNA synthesis and Real Time PCR**
- **Protein extraction and Western Blotting**
- **Immunocytochemistry**
- **Statistical analysis**

Expected/Obtained Results.

- To select PFOA concentrations that could affect hFOB 1.19 proliferation phase, MTT assay, as viability test, was performed. Below, is reported the result of MTT test using five different concentrations. (Figure 1). DMSO, at the concentration of 0.02%, was used as vehicle. The osteoblasts were treated with a concentration of 0.1, 1, 10, 50 and 100 uM of PFOA for 144h. After the MTT results, only the first three concentration (0.1, 1, and 10 uM) were selected for subsequent analysis based on the viability obtained. Normality of Cell morphology was daily checked under the microscope.

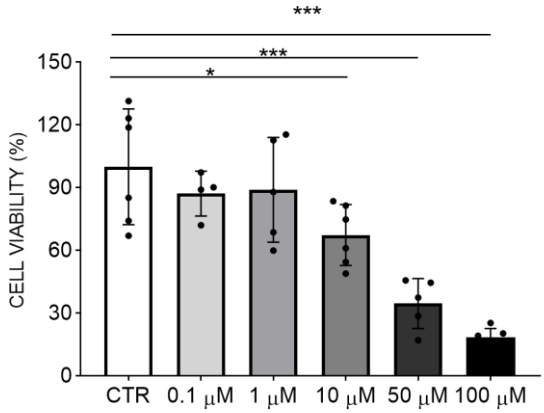

Figure 1. Cell viability of hFOB 1.19 cells exposed to different concentrations of PFOA tested by MTT assay. The graph shows the mean ± standard deviation (n = 5). Asterisks indicate statistically significant changes compared with the control group (*, P <0.05; ****, P< 0.0001).

- To assess the impact of PFOA on mineralization, cells were stained with alizarin red and observed at the Lionheart. To quantify the calcium deposits, the absorbance at 570 nm of isopropanol were quantified using a spectrophotometer (**Figure 2**). In this case, the PFOA does not promote any significant differences in the presence of the calcified deposits compared to control group.

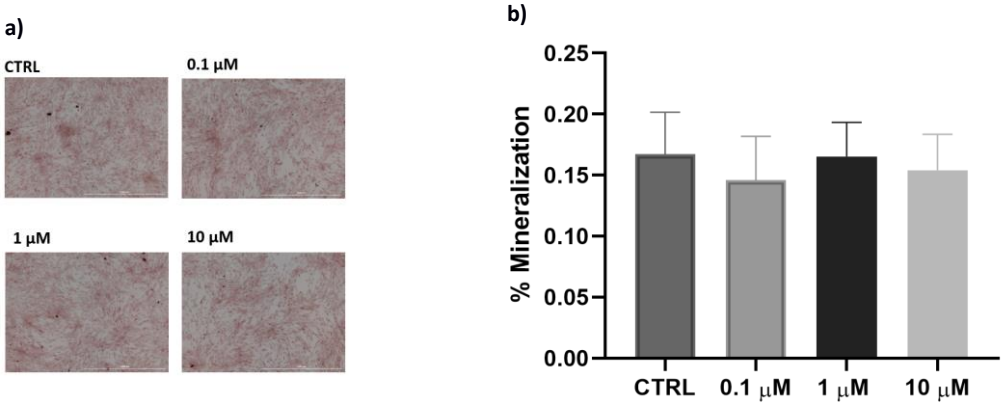

Figure 2. Alizarin Red staining of the hFOB 1.19 cells cultured with different PFOA treatments. **a)** Representative images of control group and experimental groups treated with 0.1 mM, 1 mM and 10 mM of PFOA; Scale bar: 1000 mM. **b)** Quantitative analysis of the ECM mineralization of all groups by absorbance measurement with spectroscopy. The graph shows the mean ± standard deviation (n = 9).

- To study the effect of PFOA on the bone homeostasis molecular analysis by Western Blot. The analysis was performed to quantify the levels of markers responsible for the early stage of OB differentiation and matrix maturations and mineralization. In this case, the functional ALP 200 kDa protein decrease in the group treated with 1 uM of PFOA compared with the control group.

Regarding the bone extracellular matrix, it is possible to see a reduction in the COL1A2 in the group treated with 10 uM (**Figure 3**).

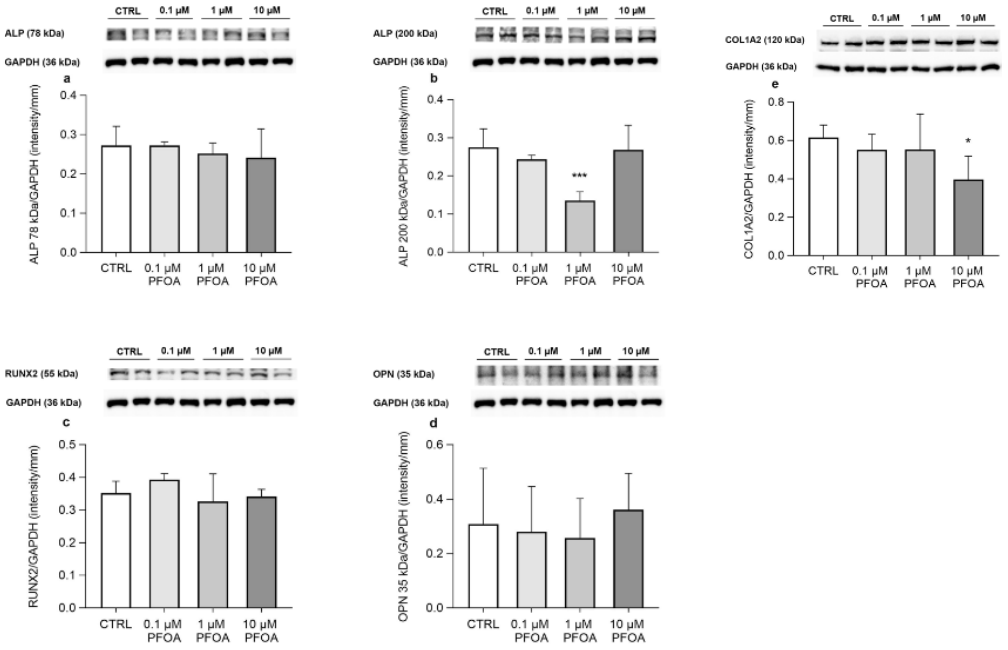

Figure 3. Western blot analysis of osteogenic marker expression, a) ALP 78 kDa, b) ALP 200 kDa, c) RUNX2, d) OPN and e) COL1A2 in hFOB 1.19 cells exposed to different PFOA concentrations. The graph shows the mean ± standard deviation (n = 5). Statistical analysis was performed with the one-way ANOVA test. Asterisks indicate the statistically significant data of the experimental groups compared with the control group (*, P< 0.05; ***,P< 0.001).

- To study the effect of PFOA on the oxidative stress defence, the levels of CAT, SOD-1 and TRX proteins have been evaluated. In the case of CAT levels, there is a significantly decrease of the proteins for all groups treated with PFOA compared to the CTRL. For the SOD-1 levels there were a significantly decrease after the exposure to 1 and 10 uM of PFOA when compared with the control, and also it is possible to observe a low levels of protein in the cells treated with the lowest dose of pollutant (**Figure 4**).

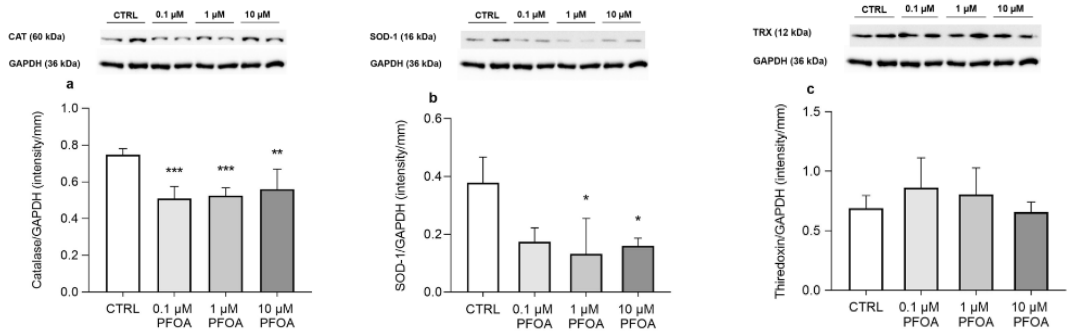

Figure 4. Western blot analysis of the expression of oxidative stress defence related proteins, a) CAT, b) SOD-1 and c) TRX in hFOB 1.19 cells exposed to different PFOA concentrations. The graph shows the mean ± standard deviation (n = 5). Statistical analysis was performed with the one-way ANOVA test. Asterisks indicate the statistically significant changes compared with the control group (*, P< 0,05 **, P< 0.01 ***, P< 0.001).

- To assess the effect of PFOA on the endocannabinoid system, the levels of CB1 protein were significantly reduced in the cells treated with 10 uM of PFOA when compared with control, while CB2 and TRPV1 levels did not change respect the CTRL group (**Figure 5**).

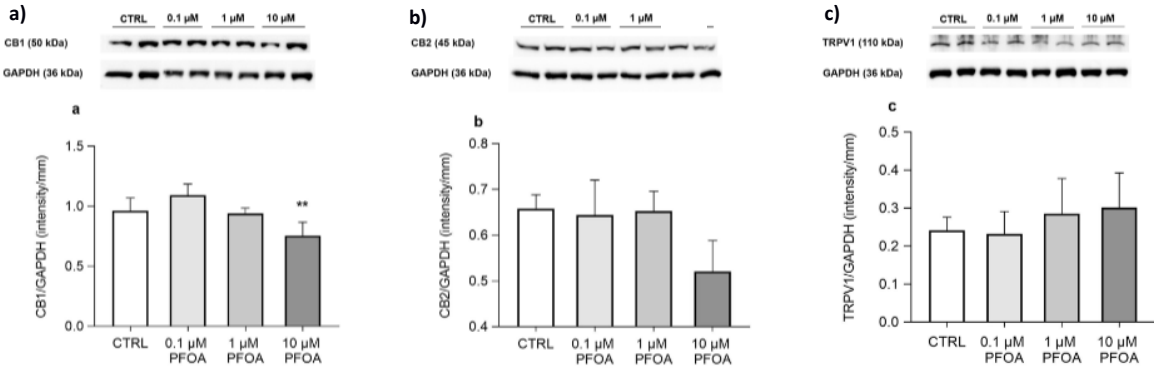

Figure 5. Western blotting analysis of endocannabinoid receptors, a) CB1, b) CB2 and c) TRPV1, in hFOB 1.19 cells exposed to different PFOA concentrations. The graph shows the mean ± standard deviation (n = 5). Statistical analysis was performed with the one-way ANOVA test. Asterisks indicate the statistically significant changes compared with the control group (** P<0.01).

- To assess the possible effects of PFOA on epigenentic changes it has been evaluated the H3K9ac and H4K12ac levels by immunocytochemistry analyses. In this case, the acetylation levels of lysine 9 and 12, in the H3 and H4 histones respectively analyzed did not evidence possible pollutant-induced epigenotoxicity (**Figure 6**).

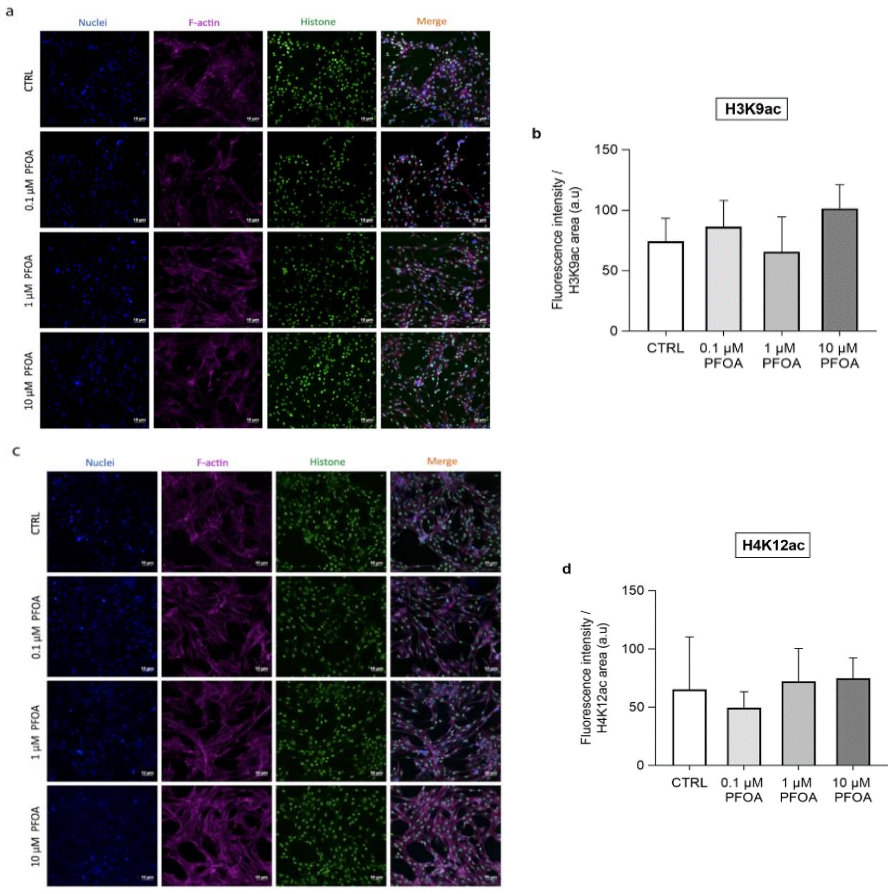

Figure 6. Analysis of histone H3K9 (a-b) and H4K12 (c-d) acetylation levels in hFOB 1.19 cells exposed to different PFOA concentrations by immunocytochemistry. a,c) Representative confocal images of nuclei (blue), F-actin cytoskeleton (purple), histone acetylation (green) and merged are shown. Scale bar: 10 µm. b,d) Quantification of histone acetylation levels are represented by the graphs. The graph shows the mean and the standard deviation (n = 5).

WP 2.

Objective.

The aim of this study is to investigate the role of ECS in inflammatory cases using zebrafish APCmcr mutant uptake dietary with THC focusing on the characterization of endocannabinoid receptors in the gut and in the contribution of the treatment to modulate the inflammation and regulate the immune systems of the experimental model.

Methods.

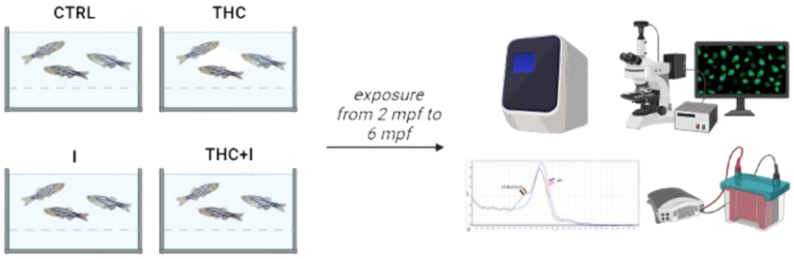

Figure 7. a) experimental design of the treatment

- Genotypization to detect apc mutation
- RNA extraction, cDNA synthesis and Real Time PCR
- H&E staining
- Immunocytochemistry
- Statistical analysis

Expected/Obtained Results.

- To study the biological effect of THC on gut intestine the histological analysis of gut has been conducted. The results demonstrate that the THC uptake dietary could influence the presence of globlet respect the CTRL group. In this case there a significantly increase in the number of goblet cells in the THC group respect the control groups (**Figure 8**).

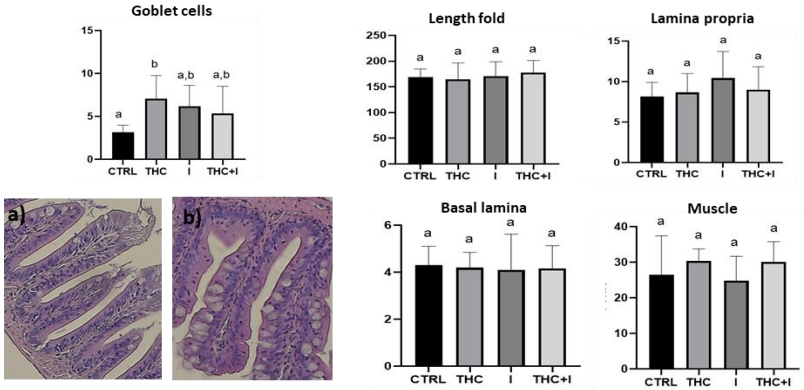

Figure 8. Histological analysis of gut from CTRL, THC, I and THC+I. Eosin and Mayer's haematoxylin staining in gut. A-b) Representative images of the goblet cells results (a) CTRL, (b) THC group

- To assess the possible occurrences of polyps, a histopathological analysis was performed, observing that there is any neoplasia (adenoma or adenocarcinoma) or preneoplastic lesions in any lesions of the subjects of the various groups (**Figure 9**)**.** The graphic represents %Score of pathological changes summarizing tree parameters studied: neoplasia (adenoma/adenocarcinoma), blunting/fusion of intestinal folds, hyperplasia of goblet cells**.**

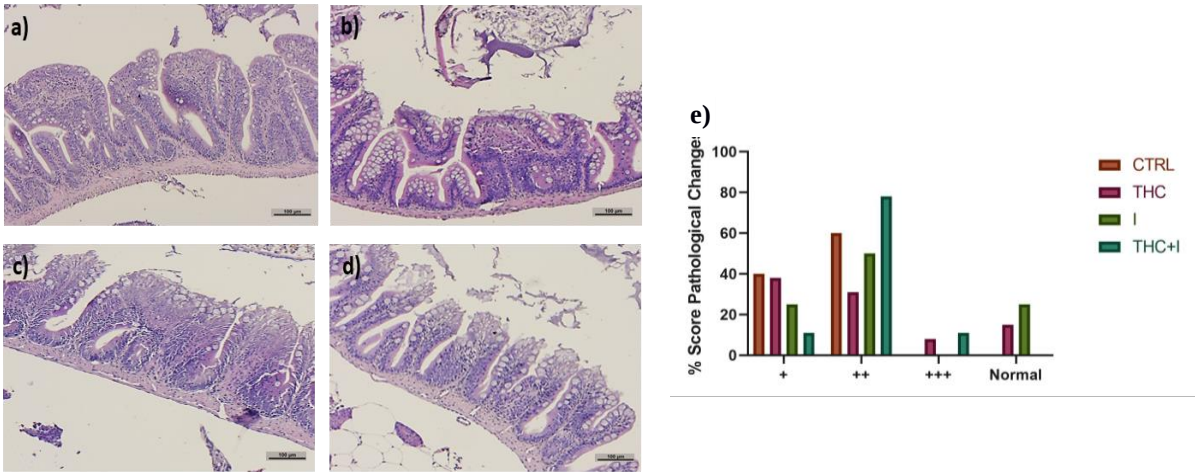

Figure 9. Intestinal section in apc -/+ fish. Section of blunting intestinal folds from 6-month-old apc-/+ fish-stained Eosin and Mayer's haematoxylin. In some disorganized intestinal area, there are some cells with high levels of cytoplasmic accumulation. A) CTRL, b) THC 0,4 ug/kg, c) I AM251+AM360 (1mg/kg bw), d) THC+I. Scale bar: 100 uM. e) Graphic representing the %Score of pathological changes in CTRL, THC, I, THC+I. The scoring is mild: +, moderate: ++, severe:+++

- To characterize the presence of CB1, CB2, the B-catenin and the VEGF-C proteins, an immunohistochemistry has been done (**Figure 11**).

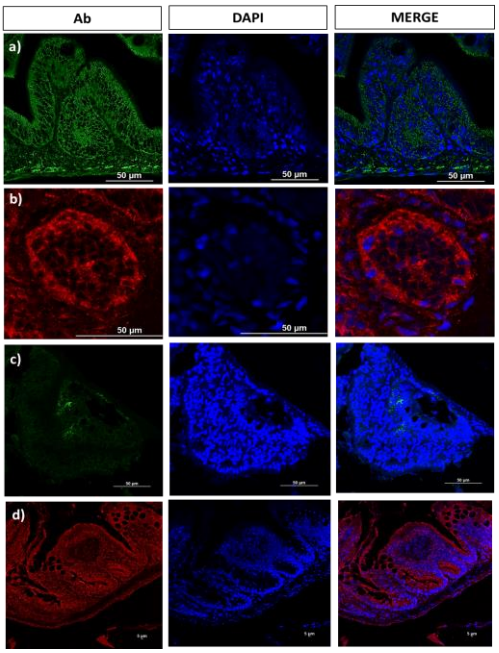

Figure 11. Representative immunofluorescence detection images of CB1 1:100 (a), CB2 1:100 (b), VEGF-C 1:100 (c), beta-catenin 1:100 (d), and nuclei (DAPI) in 6 mpf zebrafish gut section (5 µm) stained without any treatment. Scale bar: 50 and 5 µm. e) Representative of zebrafish gut

Histological analysis show that the anatomy and architecture of adult zebrafish intestinal tract did not present any neoplasia or preneoplastic regions in all groups. Despite the absence of adenocarcinoma, the

goblet cells could represent a possible marker for the presence of local inflammatory phenomena. In this regard, the inflammatory markers, such as IL1 and IL6, are under investigation to verify the possible role of THC in the occurrence of inflammatory events.

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Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Seminar: "Il futuro della fertilità umana sarà davvero in provetta?" – Dr. Nina Montik, 29/11/2022

2. SHARPER – European Researchers' Night 2023

3. Structural and functional aspects of RNA and RNA-proteins interactions – Professor Anna La Teana

List of periods spent abroad

1.

2.

...

List of conferences/workshops attended and of contributions eventually presented

1. INBB – poster presentation

2. BaCELL 3D – BUILDING ADVANCED MULTICELLULR SYSTEMS IN 3D

3. GEI – poster presentation

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

J1. Maradonna F, Fontana CM, Sella F, Giommi C, Facchinello N, Rampazzo C, Caichiolo M, Hoseinifar SH, Dalla Valle L, Van Doan H, Carnevali O. A zebrafish HCT116 xenograft model to predict anandamide outcomes on colorectal cancer. Cell Death Dis. 2022 Dec 23;13(12):1069. doi: 10.1038/s41419-022-05523-z. PMID: 36564370; PMCID: PMC9789132.

J2. Damiani E, Sella F, Astolfi P, Galeazzi R, Carnevali O, Maradonna F. First In Vivo Insights on the Effects of Tempol-Methoxycinnamate, a New UV Filter, as Alternative to Octyl Methoxycinnamate, on Zebrafish Early Development. Int J Mol Sci. 2023 Apr 5;24(7):6767. doi: 10.3390/ijms24076767. PMID: 37047738; PMCID: PMC10094805.

List of publications on conference proceedings

C1. ...

C2. ...

List of other publications (books, book chapters, patents)

B1. ...

B2. ...

12/10/2023

Student signature

Supervisor signature