Evaluating the Role of Endocrine-Disrupting Chemicals in Colon Cancer Aggressiveness: Insights from Cell Cultures, Organoids, and *Danio rerio*

Background: The environment has a substantial biological impact on human health and diseases. On a daily basis, we are exposed to numerous environmental compounds that can adversely affect our health (Caserta et al., 2022). Among these compounds are endocrine-disrupting chemicals (EDCs). EDCs are exogenous chemicals that cause adverse health effects in an organism and its offspring due to their impact on endocrine function (Darbre, 2017). Typically, these are synthetic chemicals produced for various human activities, including pesticides, industrial and household products, plastics, and detergents. The release of these substances into the environment leads to detrimental effects on ecosystems and human health following oral, inhalation, or dermal exposure (Yilmaz et al., 2020; Darbre, 2017). EDCs can interfere with the synthesis, action, and metabolism of sex steroid hormones, leading to developmental problems, infertility, and hormone-sensitive tumors. EDCs act via nuclear hormone receptors, nonnuclear membrane receptors, nonsteroidal receptors, and enzymatic pathways involved in steroid biosynthesis and metabolism (Ahn and Jeung, 2023).
To date, most studies aim to identify the effects and modes of action of individual compounds (Caporale et al., 2022). However, given the ubiquitous distribution of EDCs, we are simultaneously exposed to multiple substances. The combined effects of these substances could be additive, resulting in more pronounced manifestations even at minimal doses (Caserta et al., 2022).
A study conducted by Jun et al. (2021) provided evidence of an association between bisphenol A (BPA) and human colon cancer, demonstrating that this endocrine disruptor promotes the proliferation, migration, and growth of colon cancer cells in both *in-vitro* and *in-vivo* models. BPA increases the phosphorylation of extracellular signal-regulated kinase (ERK); inhibition of the ERK pathway attenuates proliferation and migration induced by BPA. Furthermore, BPA reduces E-cadherin expression, a key factor that prevents epithelial-mesenchymal transition—a critical event in tumor progression—and enhances the expression of 5-HT3 receptors, which are important mitogenic factors. Finally, BPA treatment in xenograft mouse models resulted in a tumor mass volume increase of 4-6 times compared to the untreated control group (Jun et al., 2021).

Aim of the Project and PhD Plan:
Given the direct and continuous contact of the digestive system with EDCs, the primary objective of this project is to investigate the effect of EDCs on colon cancer cells (CCCs), e.g. HT-29 cells, and the main pathways involved in EDC-induced cancer aggressiveness. Specifically, a mixture of EDCs known to accumulate in the human body (Caserta et al., 2022) will be used at variable concentrations and time intervals. The specific aims of my project are as follows:

AIM 1: Determining the effects on CCCs *in-vitro* (1-12 months). Proliferation, migration, and tumor growth will be evaluated in 2D and 3D cell cultures (organoids). Through transcriptomic, metabolomic, and proteomic analyses, I intend to identify, for each condition, the intracellular pathways involved and the different responses upon exposure to EDCs mixtures. CCCs cultures not exposed to the EDCs will serve as controls.

AIM 2: Determining the effects on CCCs *in-vivo* (6-30 months). Based on the results obtained in these in-vitro models, I intend to continue with validation studies on *Danio rerio* (zebrafish), a small freshwater fish commonly used in studies on human diseases and ecotoxicity. Freshwater, brackish, and marine environments represent distinct aquatic ecosystems where EDCs have been detected and impact living organisms (Ismail et al., 2017). Hence, evaluating the impact of these pollutants on zebrafish bearing CCCs is crucial. Through xenograft techniques (cell transfer), it is possible to transplant CCCs into zebrafish larvae or immunocompromised adult fish to analyze and characterize tumor proliferation and survival (Astell and Sieger, 2020; Fazio et al., 2020).

Task 1.1: Writing and approval request of the *in-vivo* protocol by the relevant institutional commissions and Ministry of Health (6-12 months).

Task 1.2: Analysis of tumor growth and validation of the pathways involved (12-24 months). The transplanted tumor cells can be easily traced using fluorescent labeling, allowing direct visualization of tumor growth (Fazio et al., 2020) in fish exposed to EDCs mixtures compared to the untreated control group. This treatment will enable the analysis of tumor progression under continuous administration of the compound mixtures in water, compared to the control group consisting of CCCs transferred to zebrafish not exposed to the mixtures. The most relevant pathways identified in AIM 1 will be examined in the explanted tumor masses using standard molecular biology and biochemical techniques (e.g., qPCR, WB, IHC).

Task 1.3: CCCs gene editing of the relevant gene(s) and analysis of tumor growth *in-vivo* (24-32 months). Understanding the deregulated pathways will allow for the modification of the expression of the relevant gene(s) involved upon exposure to EDCs mixtures. This will be achieved using the CRISPR-Cas9

tool. After proper screening of the effective gene editing and testing on CCCs behavior *in-vitro*, edited CCCs will be transferred into zebrafish specimens, and the effects on tumor mass growth will be analyzed.

AIM 3: Thesis writing and dissemination of the results (30-36 months).

Expected Results:
The results obtained from this project could establish an association between exposure to mixtures of EDCs (which contaminate ecosystems) and tumor progression, and determine the minimum concentrations of EDCs that can lead to this condition in the long term.

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