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Table of Contents

1	INTRODUCTION	3
1.1	Declining Human Fertility: The Role of Environmental Exposures.....	3
1.1.1	Endocrine Disrupting Chemicals (EDCs)	3
1.1.2	Mechanism of action.....	4
1.1.3	Flavonoids	5
1.1.4	Bisphenol A.....	7
1.1.5	Phthalate	12
1.1.6	Perfluoroalkyl substances (PFASs).....	17
1.1.7	Pesticides.....	21
1.2	The Ovary	25
1.2.1	Oogenesis	25
1.2.2	Folliculogenesis	26
1.2.3	Steroidogenesis	28
1.2.4	Follicular Microenvironment.....	31
1.2.5	Taste Receptors.....	32
1.3	The Endometrium	35
1.3.1	Cellular Composition of the Endometrium	35
1.3.2	Human Endometrial Cycle: Phases and Functions	36
1.3.3	Window of Implantation	37
1.3.4	Endometrial Organoids	38
2	MATERIALS AND METHODS: PART I.....	41
2.1	Human Granulosa Cell Line 5 (hGL5).....	41
2.2	Human Primary Granulosa Cells Collection and Isolation.....	41
2.3	Compounds	42
2.4	Cell Proliferation Assay.....	42
2.5	Cytotoxicity Assay.....	43
2.6	Mitotracker	43
2.7	RNA Extraction and Complementary DNA Preparation	43
2.8	Western Blot Analysis	44
2.9	Oil Red O Staining and Morphological Observation.....	45
2.10	Transmission Electron Microscopy.....	46

2.11	Elisa Test.....	46
2.12	Statistical Analysis	46
3	MATERIALS AND METHODS: PART II.....	47
3.1	Endometrial Tissue Collection	47
3.2	Endometrial Glands Isolation and Organoid Assembly.....	47
3.3	EEO Treatment	48
3.4	Viability Assay.....	49
3.5	Sequencing and Differential Gene Expression and Enrichment Analysis.....	50
4	RESULTS AND DISCUSSION: PART I	52
4.1	hGL5 Cell Line Expresses Bitter Taste Receptors	52
4.2	Effect of EDCs Exposure on Viability of hGL5 Cells	53
4.3	EDCs Act as Agonists on TAS2Rs Affecting hGL5 Mitochondrial Footprint.....	54
4.4	BCA and Caffeine Affect TAS2Rs Relative Abundance	57
4.5	BCA and Caffeine Affect Intracellular Lipid Storage and Steroid Secretion	58
4.6	Primary human granulosa cells: BCA affects TAS2R14 and TAS2R43 expression and regulates steroidogenesis	61
4.7	BCA affects Lipid droplet (LD) homeostasis	66
4.8	TAS2R antagonist reverts the effect of BCA on mitochondrial dynamics.....	67
5	Results and Discussion: PART II	72
5.1	Establishment and characterization of EEOs as a suitable model for <i>in vitro</i> analyses	72
5.2	Optimization of <i>In Vitro</i> Parameters for Using EEOs as a Platform to Screen EDC.....	77
5.3	Modulation of Molecular Pathways by Phthalate Mixture.....	80
5.4	Effect of the "Bad Actor" Mixture on the Transcriptome of EEOs: Focus on the Mid-Secretory Phase	86
6	Conclusion.....	92
	References	94

1 INTRODUCTION

1.1 Declining Human Fertility: The Role of Environmental Exposures

1.1.1 Endocrine Disrupting Chemicals (EDCs)

Endocrine Disruption is a concept which was first recognized in 1991, after observation that exposure to several chemicals impact health later in life. In 2002, World health organization and international program on chemical safety reported the definition of EDCs, which continues to be accepted as well nowadays: “Exogenous substance or mixture that alters function of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or subpopulations” (Solecki *et al.*, 2017).

These chemicals may be natural or industrial. The latter are of a bigger concern since there is limited data regarding their effect on human organism as well as they are found on a huge number. Close to 85,000 synthetic chemicals are produced worldwide, with the majority of them coming in contact daily (Mattiske and Pask, 2021). At least 1000 of them display endocrine-interfering properties, a fact which indicates a necessity for more investigations in this field.

Humans are mostly exposed unconsciously to these chemicals, as they are transmitted by different routes including ingestion, inhalation and dermal uptake (Gore *et al.*, 2015). Ingestion of these chemicals amounts to 90% of the total exposure. Products like fish, meat, dairy and poultry can contain endocrine disruptors from previous exposures and serve as a source to human exposure by ingestion (Law *et al.*, 2012). Some of them, due to their lipophilic nature can bioaccumulate on fatty tissue (Lu *et al.*, 2024). This exposure is prolonged in time since these chemicals are stored in adipose tissue. Meanwhile, other chemicals, such as plasticizers, do have a shorter half-life, but they are found at larger quantities and a broad range of dietary products, which makes their continues exposure raise an alarm about the endocrine disruptive effects (Mukherjee *et al.*, 2021). Another source of exposure for humans is inhalation of some of these chemicals due to their volatile nature (Carpenter, 2015).

1.1.2 Mechanism of action

One of the most common effects of EDCs is binding to a hormone receptor and acting as an agonist, therefore causing adverse biological effects. These chemicals, although presenting lower receptor affinity compared to physiological hormones, still compete with the latter due to their abundance in the environment (Montes-Grajales and Olivero-Verbel, 2015).

Data suggests that EDCs can interact with both nuclear receptors, like estrogen and androgen receptors, as well as membrane receptors. Bisphenol A, which will be explained more in detail in the following chapter, can bind nuclear receptor ER α and ER β , as well as G protein coupled estrogen receptor 1. The binding site and duration of exposure to these chemicals influence a wide range of effects on cellular function and the endocrine system.

The most studied effect of EDCs is the interaction with hormone receptors. Such interaction can be agonistic or antagonistic. Structural features similar to estrogen (E2), make such compounds to bind to ER. Differences in the aminoacids lining the ligand binding pockets between ER α and ER β are detrimental for the ligand specificity. Although some chemicals exhibit low affinity for these binding pockets, their specificity for a particular ER subtype indicates potential alterations in specific signaling pathways. On the other hand, some chemicals exhibit high affinity for the receptors, leading to the regulation of both genomic and nongenomic ER signaling pathways, which in turn complicates the understanding of their adverse effects on cells (Shanle and Xu, 2011). Such agonistic effects allow the substances to mimic the action of the natural hormone, triggering a biological response that may be similar or different from the response induced by the binding of the natural hormone (Li *et al.*, 2012).

The expression of hormone receptors is a crucial physiological and temporal event, as they mediate hormonal signaling pathways to initiate specific cellular responses (Zup and Forger, 2017). Expression of such, serves as an indicator of hormone concentration and the intensity of the hormonal response (Charlton, 2009). Some EDCs bind to hormone receptors, such as estrogen receptors, but they do not activate the typical biological response associated with the natural hormone. These chemicals prevent the binding of the endogenous hormone (like estrogen), blocking its effects, acting as an antagonist (Vandenberg *et al.*, 2012b).

1.1.3 Flavonoids

Flavonoids consist of a large group of polyphenolic compounds that are abundant in fruits and vegetables, with humans consuming approximately 203 ± 243 mg per day (Mullie et al., 2007). Beyond being essential nutrients, flavonoids play a role as modulators, contributing to health management and disease prevention, often utilized in medicines and health supplements (Harborne, 2013). Over 4,000 distinct flavonoids have been identified and grouped into six subclasses based on the type of heterocyclic ring they contain: flavonols, flavones, flavanols, flavanonols, flavanones, and isoflavones (Manach *et al.*, 2004). Flavonoids have a dual role in mutagenesis and carcinogenesis, acting as both antimutagens and promutagens, as well as antioxidants and pro-oxidants. Flavonoids are an important category of phytoestrogens that exhibit estrogenic activity, which is associated with diverse physiological effects as well as ecological and social implications. Their estrogenic properties influence numerous biological processes, including programmed cell death, cell cycle regulation, DNA damage repair, epigenetic and chromatin changes, cytoskeletal organization, immune response, inflammation, and neural functions. They are also involved in key signaling pathways such as autophagy, cellular metabolism, and processes of cellular development and differentiation (Kiyama and Zhu, 2014).

Plant flavonoids, particularly isoflavones, mimic vertebrate estrogens due to their molecular structure. Isoflavones, mainly found in soybeans and their derivatives like daidzein and genistein, exhibit estrogenic and antiestrogenic effects. These compounds are considered chemoprotective and may help treat a variety of hormonal disorders, including breast and prostate cancer, cardiovascular issues, osteoporosis, and menopausal symptoms. However, isoflavones may also act as endocrine disruptors, posing potential risks to certain populations or the environment (Křížová *et al.*, 2019).

Flavonoids, including compounds like apigenin, genistein, and kaempferol, can interact with non-estrogen nuclear receptors such as progesterone (PR) and androgen (AR) receptors. Their effects vary, functioning as agonists, antagonists, or a combination of both, depending on factors like receptor type, cellular or tissue context, and compound-specific conditions such as

concentration and metabolic state. Additionally, flavonoids like biochanin A and formononetin act as AhR agonists, leading to anti-estrogenic effects through receptor crosstalk mechanisms (Dean *et al.*, 2017).

In a study using human adrenocortical H295R cells, the isoflavones daidzein and genistein, and the flavone apigenin, along with their mixture, were examined for effects on cortisol, aldosterone, testosterone, and estradiol secretion. The results showed that cortisol and testosterone secretion were the most vulnerable to inhibition, with daidzein and genistein affecting them at concentrations below 1 μ M. An equimolar mixture of the three flavonoids caused additive inhibition of cortisol, aldosterone, and testosterone secretion (Ohlsson *et al.*, 2010). Apigenin, a flavonoid present in various fruits and vegetables, shows promise as an anticancer agent, but its impact on male reproductive health remains unclear. In a study with mouse spermatogonia, apigenin reduced cell proliferation, extended the S and G2/M phases, and increased apoptosis at concentrations of 10-20 μ M. This effect was linked to a decrease in protein arginine methyltransferase 7 expression, a protein crucial for male germ cell development. Transcriptome analysis revealed 287 differentially expressed genes, and further experiments suggested that the Prmt7/Akt3 pathway mediates apigenin's effects. The findings indicate that apigenin influences spermatogonia proliferation (Wang *et al.*, 2021).

Table 1. The table summarizes the literature data and describes the effect of flavonoids.

Authors	Country	Purpose	Summary points
(Kiyama and Zhu, 2014)	Japan	To investigate the estrogenic activity of flavonoids and their impact on various biological processes.	Flavonoids, as phytoestrogens, impact various biological processes and signaling pathways, influencing health and development.
(Dean, Murphy and Burdette, 2017)	USA	To explore the interaction of flavonoids with non-estrogen nuclear receptors and their effects on estrogenic activity.	Flavonoids can act as agonists or antagonists on PR, AR, and AhR receptors, with effects influenced by factors like concentration and cellular context.

(Křížová <i>et al.</i>, 2019)	Czech Republic	To review the properties and health effects of isoflavones, focusing on their estrogenic activity.	Isoflavones, found in soybeans, provide chemoprotective benefits but can also act as endocrine disruptors, posing potential risks.
(Ohlsson <i>et al.</i>, 2010)	Sweden	To examine the effects of isoflavones daidzein, genistein, and apigenin on steroid hormone secretion in human adrenocortical cells.	Daidzein and genistein inhibit cortisol and testosterone secretion, and their mixture causes additive inhibition of cortisol, aldosterone, and testosterone.
(Wang <i>et al.</i>, 2021)	China	To investigate the effects of apigenin on male reproductive health.	Apigenin reduces spermatogonial proliferation and increases apoptosis, with effects mediated through the Prmt7/Akt3 pathway.

1.1.4 Bisphenol A

The most studied Endocrine disruptor chemical is Bisphenol A (BPA), a confirmed xenoestrogen which exceeds an annual production of 3.8 million tons (Geens *et al.*, 2011). It is widely applied for the production of synthetic polymers such as polycarbonates, epoxy resins and thermal paper (Björnsdotter *et al.*, 2017). The later are part of reusable bottles, kitchen utensils, protective coating of canned foods, receipts, toys, water pipes and electronic equipment (Hoekstra and Simoneau, 2013). The broad range of the production of these chemicals results in a high presence of BPA in food and drinking water, making the exposure to these chemicals exclusively anthropogenic. Polycarbonates bottles, known as water containers, present a source of migration of BPA to water and consequently, to the human body. Studies have shown that the temperature and the prolonged use of the infant bottles enhance the release of BPA, specifically in mean concentrations of 0.03ppb at 40°C and 0,13 ppb at 95°C, meanwhile after half a year of usage the presence of the chemical increased up to 0.18ppb and 18,47 ppb those temperatures respectively (Nam *et al.*, 2010).

The presence of BPA diglycidyl ether in canned food is correlated to the epoxy resin coating of these cans to prevent metal from corrosion, metal contamination of the food as well as prolonged storage. Data shows that as much as 4 to 23 ug of BPA is released from lacquers per piece (Vandenberg *et al.*, 2007). Pasteurization, a high temperature treatment of canned food for the preservation of long-term storage, is indeed responsible for a release estimated at 18 times faster from the epoxy resin coating (Kang *et al.*, 2003). The group confirmed that non pasteurized cans released significantly lower concentration of BPA (0.06 ng/cm²) compared to the pasteurized cans at 100 degrees (32 ng/ cm²).

Occurrence of BPA in ecosystem has been investigated as well throughout the past years. High BPA concentrations were measured in Elbe River in Germany, with values as high as 4-92 ug/dm³ in water and 10-380 ug/kg in sediments (Stachel *et al.*, 2003). Lee and colleagues studied the levels of BPA in 16 major rivers of Taiwan, coming to conclusion that factories producing BPA containing products are responsible for the highest value measured in water, ranging from 0.01 to 44.65 ug in dm³ in water and 0.37 to 491 ug/kg in sediments. Leachates of landfills used for the management of waste containing BPA have shown concerning levels of the latter, ranging from 740ug/dm³ in landfills in OSAKA, to 3.61 mg/dm³ in Germany and the highest concentration scored from a large-scale study in Canada where a mean of 36.7 mg/kg BPA was detected in raw sewage sludges (Kawagoshi *et al.*, 2003). As a result of all the above-mentioned sources of exposure to BPA, there have been numerous studies aiming to understand its repercussions and accumulation in our body by measuring its concentration in body fluids.

Analysis conducted from the centers for Disease control in USA, enrolling 2500 participants, detected levels of BPA in 92.6% of them, with children being exposed to higher concentration (4.5 ug/dm³) compared to adults (2.5 ug/ dm³) Markis and colleagues observed a positive correlation between the consumption of water from polycarbonate containing bottles and BPA concentration levels in urine in females, with an increase in summer season due to the high temperatures and UV ratio as explained before. Similarly, a study recorded a 1000% increase of urinary BPA as a result of 1 serving of canned soup in a time-span of 5 days (Carwile *et al.*, 2011).

Conjugated BPA concentrations (BPA-glucuronide or BPA-sulfate) were higher in the blood of pregnant women compared to non-pregnant women, with a fourfold difference (Vandenberg *et al.*, 2012a). There is evidence that human fetus is exposed to BPA, exerting higher levels of 15-18 weeks of gestation with a decline in concentration in late gestation. Study suggests that lower metabolic clearance and the conversion of BPA to its conjugates by fetal liver are responsible for such difference in fetal development (Ikezuki *et al.*, 2002). A study confronted the concentration of BPA in serum and follicular fluid from 95 women undergoing *in vitro* fertilization (IVF) and living in 2 distinct regions in Italy, one group from a high environmental impact and the other from low environmental index. The results confirmed the hypothesis that BPA concentration was higher in the first group compared to the other (Raimondo *et al.*, 2024). Such studies have shed light on a possible correlation of the levels of BPA in serum or follicular fluid and the outcomes of IVF. Yenigül and colleagues report a lower clinical pregnancy, live birth probability and reduced embryo quality in women undergoing Intracytoplasmic sperm injection (ICSI) in which BPA levels were high in serum and follicular fluid (Yenigül *et al.*, 2021). Similarly, another study comparing the oocyte and embryo quality between two groups of women divided regarding the quality of the lifestyle, including significant different BPA concentrations in follicular fluid observed the same trend (Aftabsavad *et al.*, 2024).

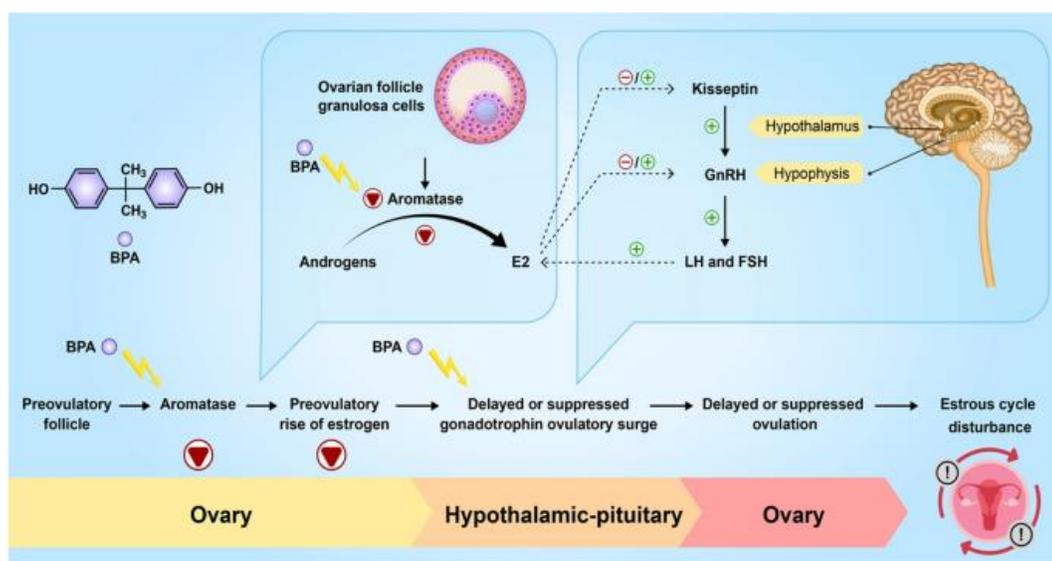


Figure 1. This figure summarizes the effect of BPA in steroidogenesis (Chaichian *et al.*, 2024).

A correlation between BPA and Polycystic ovary syndrome (PCOS) has been observed as studies suggest that BPA levels are higher in women with this condition (Tarantino *et al.*, 2013; Akin *et al.*, 2015; Hossein Rashidi *et al.*, 2017; Konieczna *et al.*, 2018). The negative effect of BPA in ovarian hypoandrogenism as suggested by the correlation between the chemical and androgen levels, highlights a contribution by the latter to the progression of such condition (Figure 1).

Recently, *in vitro* data suggested that BPA induces autophagy of granulosa cells, which consequently affects the levels of steroid hormones produced from these cells (Lin *et al.*, 2021). Clinical data from this study concurred with other observations in which BPA exposed patients had a lower E2 peak, lower number of retrieved oocytes as well as oocyte quality (Ehrlich *et al.*, 2012). BPA was banned in the European Union in 2011 after extensive scientific data showing its negative effects on health. Soon enough, the focus of the scientific community shifted to its substituted chemicals such as Bisphenol S (BPS) and Bisphenol F (BPF). Although these alternatives are advertised as the healthy option and used in products labeled as “BPA free”, their safety is yet to be proven. Rochester and Bolden reviewed the results from several studies in literature and stated that the estrogenic effects of BPS and BPF are equally potent as BPA. Same outcome results as well for androgenic, antiandrogenic, antiestrogenic effect (Rochester and Bolden, 2015).

Table 2. The table summarizes the literature data and describes the effect of BPA (N/S, non-specified).

Authors	Country	Purpose	Summary points
(Kawagoshi <i>et al.</i> , 2003)	Japan	To investigate the estrogenic activity of landfill leachate, focusing on bisphenol A as the primary contributor.	BPA is the main contributor to estrogenic activity in landfill leachate.
(Stachel <i>et al.</i> , 2003)	Germany	To analyze bisphenol A (BPA) and other related compounds in water and freshly deposited sediment samples from the River Elbe and its tributaries to assess contamination levels.	BPA concentrations in the River Elbe and its tributaries were found to be elevated, potentially harmful to aquatic organisms.

(Lee <i>et al.</i> , 2013)	Taiwan	To investigate BPA concentrations in water and sediments of Taiwanese rivers and their association with water quality parameters and pollution sources.	BPA concentrations in Taiwanese rivers are primarily influenced by industrial discharge and household waste.
(Makris <i>et al.</i> , 2013)	Cyprus	To investigate summer BPA exposure from polycarbonate bottles, focusing on urinary BPA levels.	The study highlights a significant increase in urinary BPA levels in women during summer, linked to polycarbonate-packaged water consumption, exacerbated by high temperatures and UV exposure.
(Calafat <i>et al.</i> , 2008)	USA	To assess exposure to bisphenol A (BPA) in the U.S. general population.	For disease control in the USA, enrolling 2,500 participants, BPA was detected in 92.6% of them, with children exposed to higher concentrations.
(Carwile <i>et al.</i> , 2011)	USA	To assess the impact of consuming fresh versus canned soup on urinary bisphenol A (BPA) concentrations.	Consumption of canned soup resulted in a more than 1000% increase in urinary BPA concentrations, emphasizing the significant impact of canned food on BPA exposure.
(Vandenberg <i>et al.</i> , 2012)	USA	To examine the differences in BPA concentrations between pregnant and non-pregnant women and its potential health risks.	Conjugated BPA concentrations were higher in the blood of pregnant women compared to non-pregnant women, showing a fourfold difference.
(Ikezuki <i>et al.</i> , 2002)	Japan	To measure BPA contamination in human biological fluids from various stages of pregnancy and in ovarian follicular fluid.	The study found significant BPA accumulation in early fetuses, particularly in amniotic fluid at 15-18 weeks, highlighting substantial prenatal exposure to BPA.
(Raimondo <i>et al.</i> , 2024)	Italy	To study BPA levels in serum and follicular fluids of women undergoing IVF in HEI and LEI areas.	Higher BPA levels were found in women from HEI areas, with different distribution patterns between fluids.
(Yenigül <i>et al.</i> , 2021)	Turkey	To examine the effect of BPA levels in maternal fluids on embryo quality and ICSI outcomes in women with unexplained infertility.	Higher BPA levels were linked to poorer embryo quality, lower pregnancy rates, and fewer live births.

(Aftabsavad <i>et al.</i> , 2024)	Iran	To study the relationship between BPA levels in follicular fluid, oocyte quantity, and embryo quality in women with poor ovarian response.	Higher BPA levels were linked to fewer mature oocytes and lower embryo quality.
(Konieczna <i>et al.</i> , 2018)	Poland	To measure serum BPA levels in women with PCOS and evaluate its impact on hormonal and metabolic profiles.	Higher serum BPA levels in women with PCOS were linked to increased testosterone and androgen levels, suggesting a role in ovarian hyperandrogenism.
(Ehrlich <i>et al.</i> , 2012)	North Carolina	To study the effect of BPA levels in oocyte quantity and quality.	BPA exposed patients had a lower E2 peak, lower number of retrieved oocytes as well as oocyte quality.
(Rochester and Bolden, 2015)	N/S	To assess the effects and hormonal activity of BPA substitutes BPS and BPF compared to BPA.	BPS and BPF have hormonal activities similar to BPA, causing endocrine-disrupting effects.

1.1.5 Phthalate

About 80 to 85 percent of the plasticizers are phthalate esters (Begum and Carpenter, 2022). Their use aims to provide flexibility, malleability and tolerance to temperature changes to numerous products in which they are added. Their presence in products varies mainly from their molecular weight. High molecular weight phthalates, such as DEHP, DINP, DIDP and DOP are used in construction, toys, food package etc (Arbuckle *et al.*, 2016; Kasper-Sonnenberg *et al.*, 2024). Meanwhile, low molecular weight phthalates such as BBZP, DIBP, DBP and DEP are found in varnishes, sealants, as well as used as solvents in cosmetics, creams, shampoos and candles (Koo and Lee, 2004; Kim *et al.*, 2020). The most common member of phthalates, Bis(2-ethylhexyl) phthalate (DEHP) is found in waterproof clothing and medical devices. Due to the lack of chemical bonds of phthalate with the polymers, changes in environmental conditions such as temperature, pH, pressure or radiation can accelerate the release of these chemicals from the main source and leach into environment (Benjamin *et al.*, 2017a). Similar to BPA, Phthalates are present in food, water, air, soil and dust. Mainly sources of exposure are food and drinks, due to their presence in bottles, wrappers, their release in microwaves dishes, cooking aids etc.

In addition, dermal contact plays another important role in their exposure since cosmetics, perfumes, clothes and toys have resulted products containing traces of phthalates. A study measured phthalate levels in skin wipes from 30 volunteers, covering areas like the forehead, hands, and calves. Dermal exposure ranged from 0.129 to 8.25 $\mu\text{g}/(\text{kg}\cdot\text{day})$, with seven phthalates detected. The highest levels were found on the forehead and instep, and exposure varied based on habits and gender (Zhao *et al.*, 2022). Last but not least, inhalation may represent a lower volume of exposure but more constant (Benjamin *et al.*, 2017b). A study examined inhalation and dermal uptake of D4-DEHP and D4-DEP phthalates in controlled conditions. Inhalation was the primary route of exposure for both phthalates, with average uptake rates of 0.0014 $\mu\text{g}/\text{kg bw}/\mu\text{g m}^3/\text{h}$ for D4-DEHP and 0.0067 $\mu\text{g}/\text{kg bw}/\mu\text{g m}^3/\text{h}$ for D4-DEP, highlighting the importance of inhalation in phthalate exposure (Andersen *et al.*, 2018). Phthalates, which are hydrophobic prior to the ingestion from the human body, will be hydrolysed by esterase or lipase into their metabolite's monoesters within 2h of their entry in bloodstream (Koch *et al.*, 2006). Secondly, these metabolites will be excreted after their transformation into sulpho or glucuro conjugates.

DEHP is linked to several reproductive health problems, including developmental defects in males, endometriosis and miscarriage in women, placental complications and reduced sperm motility (Sheikh, 2016). Data suggests that DEHP, in all three isoforms can bind to progesterone receptor and activate PPARs (Sheikh, 2016). PPAR and phthalate complex can interact with Follicle-Stimulating Hormone (FSH) and suppress estradiol synthesis (Latini *et al.*, 2008). DEHP disrupts FSH-stimulated steroidogenesis in human cumulus granulosa cells by inhibiting the cAMP and the extracellular signal-regulated kinase (ERK1/2) signaling pathways. This leads to reduced steroidogenic acute regulatory protein (StAR) and Cytochrome P450 (CYP19A1) expression, decreased progesterone and estradiol production, and highlights critical insights into how DEHP impacts hormone-regulated processes in the female reproductive system (Testic *et al.*, 2023). Presence of phthalate metabolites was detected in the follicular fluid of more than 86 percent of 641 women undergoing IVF. This presence was negatively correlated with the number of retrieved oocytes, mature oocyte and 2PN zygotes. There was although, no correlation between this presence and pregnancy outcome (Yao *et al.*, 2023). The presence

of these metabolites has been verified as well by studies in Italy, Israel and China. Paoli and colleagues enrolled 122 women undergoing ART treatment, in which Monobutyl phthalate (MBP) and Monoethylhexyl phthalate (MEHP) were above the limit of quantification on both blood and follicular fluid in 97.5 and 77% of subjects. While no significant associations were found between phthalates and most clinical features, follicular MBP levels were linked to irregular menstrual cycles, suggesting a potential impact on reproductive health. (Paoli *et al.*, 2020). A case study examined the relationship between phthalate metabolite levels and inflammatory cytokines, which are crucial for maintaining a healthy ovarian environment, in the follicular fluid of 76 women undergoing IVF. Monoethyl phthalate (MEP) was associated with increased IL-6, indicating a pro-inflammatory response, while mono-benzyl phthalate (MBzP) and mono(2-ethylhexyl) phthalate (MEHP) were linked to decreased MCP-1, potentially damaging immune signaling (Wang *et al.*, 2023)(Figure 2).

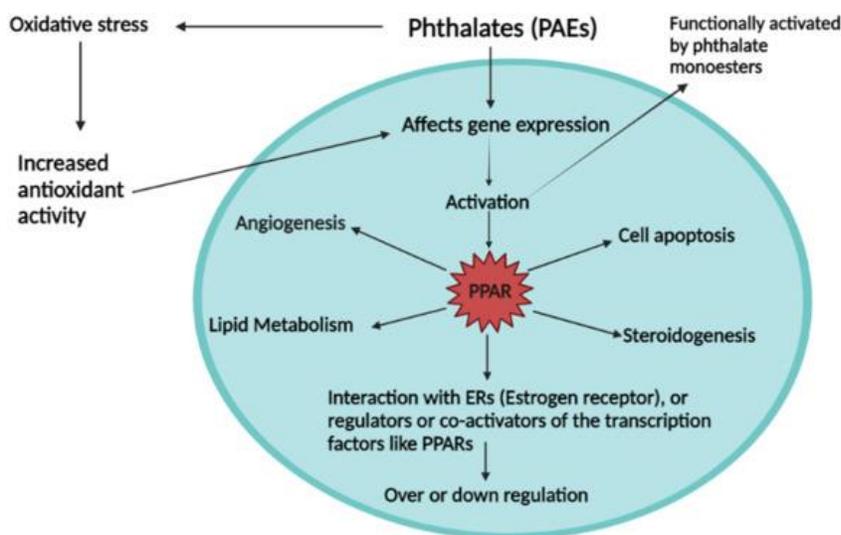


Figure 2. Figure represents the possible mechanism of the effect of Phthalates in granulosa cells (Sree *et al.*, 2023).

There are several studies related to the presence and exposure to phthalates and their association with pregnancy problems. A prospective cohort study investigated the association between phthalate exposure and maternal thyroid hormone levels during pregnancy analyzing urine and blood samples from 672 women. Significant associations were found between phthalate exposure and thyroid hormone alterations, including increased thyroxine (FT4) and

thyroid stimulating hormone (TSH) levels and changes in the TSH/FT4 ratio, particularly during the second and third trimesters. These findings suggest that phthalate exposure during pregnancy may disrupt maternal thyroid function, potentially impacting fetal growth and development (Al-Saleh et al., 2024). Broe and colleagues on a case-study examined the effects of phthalate exposure from pharmaceutical drugs on premature birth. Pregnancies exposed to drugs containing phthalates were compared to those phthalate-free ones. Results showed that third-trimester pregnancy exposed to Ortho-phthalate and phthalates polymers was associated with major risk of premature birth (Broe et al., 2019). Another study involved 1,228 women attempting pregnancy explored the impact of preconception phthalate exposure on fecundability, pregnancy loss, and biological markers. It was revealed that higher levels of certain phthalate metabolites (mono-(2-ethylhexyl) phthalate, mono-butyl phthalate, and mono-benzyl phthalate) were linked to lower fecundability but not pregnancy loss. Phthalate exposure was also associated with increased inflammation, oxidative stress, and altered reproductive hormones (Nobles *et al.*, 2023).

Lee and colleagues investigated the link between prenatal phthalate exposure and DNA methylation in cord blood. Phthalate levels were measured in maternal and neonatal urine samples and DNA-methylation was analyzed in cord blood. In neonates was found an important association between phthalates levels and methylation, even a sex-specific effect with MnBP affecting methylation mostly in female infants (Lee *et al.*, 2023). *In vitro* studies have provided impactful insight regarding phthalates impact in fertility, where DEHP was shown to decrease significantly levels of E2 and the expression of CYP19A1 transcripts as well as the protein, in rats. Similarly, Gupta and colleague reported that DEHP (1-100 ug/ml) and its metabolite MEHP (0.1-10 ug/ml) caused a decrease in CYP19A1 expression and as a result a decrease in E2 production. They also showed an inhibition in antral follicle growth.

Table 3. The table summarizes the literature data and describes the effect of phthalates.

Authors	Country	Purpose	Summary points
(Sheikh, 2016)	N/S	To explore the binding interactions of DEHP phthalate stereoisomers	DEHP is linked to several reproductive health problems. All DEHP

		with the progesterone receptor (PR).	stereoisomers can bind to PR, with the RR stereoisomer showing the strongest binding potential.
(Tescic et al., 2023)	Serbia	To investigate how DEHP affects FSH-stimulated steroidogenesis in human cumulus granulosa cells.	DEHP disrupts FSH-stimulated steroidogenesis by inhibiting cAMP and ERK1/2 pathways, reducing hormone production and affecting reproductive processes.
(Yao et al., 2023)	China	To investigate the associations between phthalate metabolite concentrations in follicular fluid and IVF/ICSI outcomes, including oocyte yield, fertilization, and pregnancy success.	The presence of phthalate metabolites in follicular fluid was negatively correlated with oocyte yield and maturation but showed no significant impact on pregnancy outcomes.
(Paoli et al., 2020)	Italy	To assess the presence of phthalates and BPA in the blood serum and follicular fluid of women undergoing ART and their associations with clinical features.	Phthalates were detected in blood and follicular fluid, with no significant associations found with clinical features, except for follicular MBP linked to irregular cycles.
(Wang et al., 2023)	China	To study the relationship between phthalate metabolites and inflammatory cytokines in follicular fluid.	Mono-ethyl phthalate was associated with a pro-inflammatory response, while other phthalate metabolites were linked to decreased MCP-1, weakening immune signaling.
(Al-Saleh et al., 2024)	Saudi Arabia	To investigate the association between phthalate exposure and maternal thyroid hormone levels during three trimesters of pregnancy.	There is a notable correlation between phthalate exposure and thyroid hormone alterations, potentially impacting fetal growth and development.
(Broe et al., 2019)	Denmark	To investigate the potential effects of phthalate exposure from pharmaceutical phthalate exposure from pharmaceutical drugs on preterm birth.	Results showed that third-trimester pregnancy exposed to orthophthalate and phthalates polymers was associated with major risk of premature birth.
(Nobles et al., 2023)	USA	To explore the impact of preconception phthalate exposure on fecundability, pregnancy loss, and biological markers.	Higher levels of certain phthalate metabolites were associated with lower fecundability but not pregnancy loss.

(Lee <i>et al.</i> , 2023)	South Korea	To investigate the link between prenatal phthalate exposure and DNA methylation in cord blood.	In neonates was found an important association between phthalates levels and methylation
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1.1.6 Perfluoroalkyl substances (PFASs)

PFAS are another class of man-made chemicals, with almost 4730 chemicals classified into these group. The number, complexity and the changing profile of these chemicals contribute to the undergoing challenges on the characterization of their properties as endocrine disruptors (European parliament). Their presence on products, as well as human exposure to this chemical is widely broad. They are present in cosmetics, paints, firefighting foams, GORE-TEX textiles, herbicides. Furthermore, throughout the years, the cautions and alert to these chemicals have contributed to further investigation into products which are contaminated from sources containing PFOA. Consumption of fast food was positively associated with increased levels of this fluorinated organic compound. Serum levels of PFOA were found higher in people who consumed higher calories from fast food at home, meanwhile lower levels on people who consumed on site. Hypothesis suggests that food packaging is a source of PFOA to humans. These compounds were found in disposable paper bags and cutlery, used to package a variety of fast-food products. Furthermore, serum levels of these fluorinated compounds were positively correlated with the consumption of microwave popcorn. The high temperatures involved in microwaving cause the packaging materials to release significant amounts of these chemicals. Similarly, canned products also contain and release such substances during the pasteurization process due to exposure to elevated temperatures.

On 2021, the Agency for Toxic Substances and Disease Registry (ATDSR) released a toxicological evaluation which correlated the exposure to PFOA with several conditions such as pre-eclampsia, decline in birth weight, increase in serum lipids etc. Furthermore, they also documented negative effects in laboratory animals such as liver toxicity, developmental and reproductive toxicity and cancer effects.

These observations as well as a systematic review by Kirk and colleagues in 2018 which screened for the potential negative effect of PFOA related to a series of health complications

gave start to a lot of investigations who delve into these negative effects. A study from Espartero and Juhaz in 2022 documented that 221 studies in the literature have correlated PFAS with organ toxicity, starting from immune effect, respiratory effects, hepatic toxicity to reproductive and endocrine toxicity (Jane L Espartero *et al.*, 2022). One study found the presence of PFAS in follicular fluid sample of 97 Australian women experiencing infertility and undergoing Assisted reproductive technology (ART). Specifically, PFOA, PFOS, PFHXS and PFNA were present in all the samples with PFOA and PFOS reaching the highest levels measured among the PFAS investigated, specifically at 4,9 ng/mL and 2,4 ng/mL (Kim *et al.*, 2020). Same authors have proven a possible correlation between levels of PFHpA and infertility female factors such as endometriosis and PCOS. A possible interpretation for this effect might be explained with the effect that PFAS have on the interference with steroid hormone synthesis due to disruption of the function of nuclear hormone receptors.

Di Nisio *et al.*, investigated a previous hypothesis which suggested that Progesterone production is alternated by levels of PFOA. The study predicted a physical interaction between P4 and PFOA molecules by computational modeling, which they went further to confirm it *in vitro*. Although PFOA treatment alone did not exert any difference in gene expression, priming of Ishikawa cells with estrogen and further treatment with progesterone, and comparison of gene expression between these conditions and those co-incubation with PFOA, resulted in 6 genes highly differentially expressed (Di Nisio *et al.*, 2020). One of them, SULT1E1, the estrogen sulfotransferase was downregulated in cells treated with PFOA. Interestingly, this gene is a candidate for the evaluation of antiprogestin effects and is important in the pathogenesis of estrogen-dependent tumors (Pasqualini, 2009; Fischer *et al.*, 2012). Furthermore, the other 3 genes are involved in pathways important for the embryo attachment.

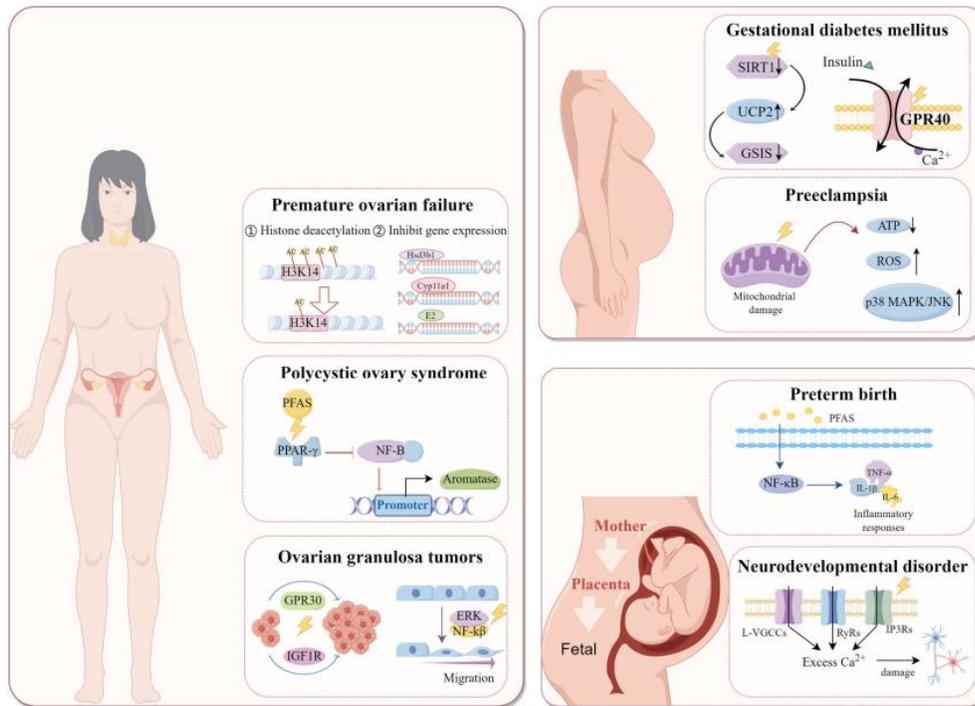


Figure 3. Figure shows the negative effect on female reproductive health of PFOA with the respective mechanism of action in cells (Qu *et al.*, 2024).

Gerwen and colleagues explored the connection between PFAS levels in plasma and thyroid cancer diagnosis. It found that higher levels of n-PFOS were associated with an increased risk of thyroid cancer, particularly papillary thyroid cancer, with a 56% higher likelihood of diagnosis for each doubling of n-PFOS. This association was consistent even for cases diagnosed more than a year after plasma samples were collected. The findings suggest a possible link between PFAS exposure and thyroid cancer (van Gerwen *et al.*, 2023).

A large study, published on 2014, included 1623 pregnant women from Greenland, Poland and Ukraine, investigated the correlation between PFOA and PFOS and menstrual cycle characteristics (Lyngsø *et al.*, 2014). They confirmed results which already were recorded from previous studies, in which they prove that menstrual cycle is vulnerable to PFOA exposure. In this case, menstrual cycle was longer in women with higher serum levels of PFOA. PFAS exposure can disrupt reproductive function through endocrine disruption, affecting various reproductive tissues, including the breast, thyroid, and hypothalamic-pituitary-gonadal axis. The chemicals may directly or indirectly impact reproductive health (**Figure 3**)(Rickard *et al.*, 2022).

A case-control study investigates the association between PFAS (perfluoroalkyl substances) concentrations and infertility related to PCOS in women. Blood samples of 180 infertile women with PCOS and 187 healthy women were analyzed for ten PFASs. While the study did not find a significant association between most PFASs and infertility, it was observed that higher plasma concentrations of perfluorododecanoic acid (PFDoA) were significantly associated with an increased risk of PCOS-related infertility. This indicates that certain PFAS compounds might negatively impact reproductive health.(Wang *et al.*, 2019). Another study investigated the relationship between PFAS (perfluoroalkyl and polyfluoroalkyl substances) exposure and self-reported infertility in women, using data from 788 participants. It found that the overall infertility prevalence was 15.54%. Various statistical models showed mixed results, with some PFASs, like n-PFOA and n-PFOS, displaying negative associations with infertility, particularly in higher exposure levels (Tan *et al.*, 2022).

Table 4. The table summarizes the literature data and describes the effect of Perfluoroalkyl substances (PFAS).

Authors	Country	Purpose	Summary points
(Jane L Espartero <i>et al.</i> , 2022)	Australia	To update knowledge on PFAS toxicity	PFAS are persistent, synthetic chemicals used in various industries and consumer products.
(Kim <i>et al.</i> , 2020)	Australia	To find associations between PFAS concentrations, age, fertilisation rates, and infertility causes in women undergoing ART.	PFAS exposure was linked to some infertility factors, but no direct relationship with fertilisation rates was found.
(Di Nisio <i>et al.</i> , 2020)	Italy	To examine how PFAS, particularly PFOA, disrupt progesterone's role in endometrial function and reproductive health.	PFAS, particularly PFOA, disrupt progesterone-mediated endometrial function, potentially affecting reproductive health.
(Lyngsø <i>et al.</i> , 2014)	Ukraine	To assess if PFOS and PFOA exposure disrupt menstrual cycles.	PFOA exposure may affect menstrual cyclicity, while PFOS exposure showed a weaker association
(Rickard, Rizvi and Fenton, 2022)	N/S	To provide an overview of how PFAS (both long- and short-chain) affect female reproductive health.	PFAS exposure can disrupt reproductive function through endocrine

			disruption, affecting various reproductive tissues.
(Wang <i>et al.</i>, 2019)	China	To evaluate the association between PFAS concentrations and PCOS-related infertility in women.	Higher levels of perfluorododecanoic acid (PFDoA) were associated with an increased risk of PCOS-related infertility, suggesting PFAS may impact reproductive health.
(Tan <i>et al.</i>, 2022)	USA	To investigate the link between PFAS exposure and self-reported infertility in women.	The study found a negative association between lower PFAS exposure and infertility, with varying effects by age and specific substances.
(van Gerwen <i>et al.</i>, 2023)	USA	To investigate the association between plasma PFAS levels and thyroid cancer diagnosis in humans.	PFAS exposure, particularly to n-PFOS, may increase the risk of thyroid cancer, especially papillary thyroid cancer.

1.1.7 Pesticides

Around 105 Substances, classified as pesticides are identified with potential role as endocrine disruptors (Mnif *et al.*, 2011). 46% of those are identified as insecticide, 21% as herbicides and 31% as fungicide. Out of this one chemical such as DDT and Atrazine have gained a specific focus since they have been forbidden to use in several countries but levels of presence in biological samples are still present. In 2019, over 4 million tons of pesticides were used in food and agriculture, according to the United Nations Food and Agriculture Organization. The regulations surrounding pesticide production and application vary significantly across regions, creating challenges in establishing uniform standards and controlling human exposure to these chemicals. Numerous studies have already demonstrated human exposure to pesticides through air, water, and soil. As a result, consuming food or liquids contaminated with these chemicals, as well as their presence in the air, can lead to continuous human exposure, raising concerns about their potential adverse effects.

Methoxychlor and triclosan, two widely used pesticides were investigated for a potential regulatory effect on estrogen receptor dependent pathways. Methoxychlor has been used as a substitution for the infamous pesticide DDT after the latter has been banned from some

countries. Triclosan, on the other hand, is present on several hygiene products such as soaps, deodorants and toothpastes due to its antibacterial effects. Interestingly, both substances increased proliferation rates on cancer cell lines compared to the estrogen treated cells. Furthermore, levels of expression of Cyclin D1 and p21, which are cell cycle related genes, were upregulated and downregulated respectively. This effects were reversed after cells were treated with ICI 182,789, an estrogen antagonist, which suggests that these adverse effects come as a results of the regulation of ER- dependent signaling (Kim *et al.*, 2014). Ketoconazole, which is a fungicide, and prometon, a herbicide has shown a different effect on E2 production from H295R cells, but both exerting a significant effect. Ketoconazole decreased E2 levels and directly inhibited aromatase activity, agreeing with previous data in the literature. Meanwhile treatment with prometon, increased aromatase activity as well as the E2 production, explaining an alteration in steroidogenesis pathway by a direct and indirect interaction with aromatase (Higley *et al.*, 2010). Some studies, have delved into the correlation between follicular fluid concentration of pesticides and indicators of female fertility such as quality of oocytes, embryo implantations rates, menstrual cycle etc.

A study investigated the levels of organochlorine pesticides (OCPs) in follicular fluid samples from 127 women (aged 20-35) undergoing assisted reproductive technologies (ART). Seventeen OCPs were analyzed, with methoxychlor being the most dominant, followed by other pesticides like heptachlor-epoxide, hexachlorocyclohexanes, endrin, and DDT. Women undergoing ART had moderate levels of organochlorine pesticides (OCPs) in their follicular fluid, with methoxychlor being the most prevalent. These pesticides, which accumulate through diet, water, and air, were present at lower levels than those reported in industrialized countries (Zhu *et al.*, 2015b). Campagna et al, were one of the first to correlate dosage of organochlorine mixture composed similarly to the ones detected in Artic, with developmental competence of porcine oocyte and the viability of cumulus cells. Such study proved a toxicological effect of these compounds, suggesting that chronically exposure can affect several endpoint such as cumulus cell viability, oocyte maturation, polyspermy and blastocyst formation (Campagna *et al.*, 2002).

Another study conducted between 2017 and 2020 on 436 women undergoing IVF/ICSI treatment found that exposure to neonicotinoid insecticides (NEOs) and their metabolites, particularly N-dm-ACE, negatively impacted reproductive outcomes. Higher NEO levels in follicular fluid were linked to fewer retrieved oocytes, mature oocytes, 2PN zygotes, and high-quality embryos. The study highlights the potential adverse effects of environmental NEO exposure on fertility treatments (Liu *et al.*, 2024). Farr and colleagues examined the association between pesticide use and menstrual cycle characteristics in 3,103 premenopausal women living on farms. It found that women who used pesticides had longer menstrual cycles and higher odds of missed periods. Additionally, women who used hormonally active pesticides had a 60-100% higher chance of experiencing longer cycles, missed periods, and intermenstrual bleeding compared to those who had never used pesticides. These findings suggest that pesticide exposure may disrupt menstrual function, with potential implications for reproductive health (Farr *et al.*, 2004).

Table 5. The table summarizes the literature data and describes the effect of pesticides.

Authors	Country	Purpose	Summary points
(Kim <i>et al.</i> , 2014)	Korea	To examine how methoxychlor and triclosan affect estrogen receptor pathways in cancer cells.	Both substances increased cell proliferation and altered cell cycle gene expression, contributing to health risks like cancer.
(Higley <i>et al.</i> , 2010)	Canada	To examine the effects of ketoconazole and prometone on estrogen production and aromatase activity in H295R cells	Ketoconazole decreased estrogen levels by inhibiting aromatase, while prometone increased both aromatase activity and estrogen production, altering steroidogenesis.
(Zhu <i>et al.</i> , 2015)	China	Analyzed OCP levels in follicular fluid from women undergoing ART to assess their impact on fertility.	Methoxychlor was the most common OCP, with moderate concentrations compared to industrialized countries.
(Campagna <i>et al.</i> , 2002)	Canada	To study the effects of organochlorine mixtures on porcine oocytes and cumulus cells	Chronic exposure to these compounds negatively affected oocyte maturation, cumulus cell viability, polyspermy, and blastocyst formation.

(Liu et al., 2024)	Korea	To study the impact of neonicotinoid insecticides on IVF/ICSI outcomes.	NEO exposure negatively affects oocyte and embryo quality in IVF/ICSI.
(Farr et al., 2004)	USA	To investigate the link between pesticides use and menstrual cycle characteristics in women living on farms.	The use of pesticides, especially those that affect hormones, is connected to changes in menstrual cycles.

1.2 The Ovary

1.2.1 Oogenesis

The ovary is a dynamic and multifaceted organ playing a central role in both gametogenesis and endocrine regulation. It is responsible for producing oocytes, which are essential for fertilization and embryonic development, and synthesizing steroid hormones such as estrogen and progesterone. These hormones regulate the menstrual cycle, maintain the reproductive tract, and prepare the body for pregnancy (Oktem and Oktay, 2008). The precursors of oocyte, primordial germ cells (PGCs), migrate towards the genital ridge which concurs with sex determination (Richardson and Lehmann, 2010). The XX gonads, lacking the Y-linked gene SRY, follow a canonical pathway which finalizes with the formation of ovaries. Subsequently, the bipotential gonad differentiates relying on somatic cell derived transcription factor such as, forkhead box LL2 (FOXL2), GATA binding protein 4 (GATA4), LIM Homeobox 9 (LHX9), Wilms Tumor 1 (WT1) and Steroidogenic factor 1 (SF1)(Frost *et al.*, 2021).

PGCs undergo mitotic divisions leading to the formation of germ cell cysts. As a result, germ cells initiate meiosis, blocked at the diplotene stage of the prophase, forming primary oocytes (Dutta *et al.*, 2016). Meiotic arrest coincides with germ cell nest breakdown which initiates follicle formation. A layer of squamous somatic cells called granulosa cells surrounds the oocyte, both being separated from interstitial tissue by basal lamina (Williams and Erickson, 2000). The primary oocyte resumes meiosis during puberty, leading to their periodic release into the female reproductive tract in a process known as ovulation (Djahanbakhch *et al.*, 2007). Further maturation of oocyte comes as a result on the stimulation of the dominant follicle by gonadotropins, such as follicle stimulating hormone (FSH) and luteinizing hormone (LH). After the first meiotic division, two cells with different cytoplasmic distribution originate, the secondary oocyte and the first polar body. The secondary oocyte undergoes meiosis II and is arrested at metaphase II, which would resume exclusively when ovulation takes place (Picton *et al.*, 1998). The follicle maturation at the same time is crucial for the oocyte to gain the ability to resume nuclear maturation and undergo fertilization and cleavage division. Such maturation, may take months to complete, as it is remarkably slow. The dimensions of the

oocyte expand from 35 to 120 μm in diameter, being almost 100 fold greater in volume, which coincides with their ability to undergo germinal vesicle breakdown (GVBD)(Gosden and Lee, 2010). At this stage, oocyte store water, lipids and ions, raise their protein content and expand in volume, changes which help guarantee the reproductive success. Structural modifications take place prior to the LH peak, with mitochondria doubled in number, redistribution of cytoplasmic organelles in the periphery, reduction of the endoplasmic reticulum, formation of perivitelline space and increased transcriptional activity. Hallmark mechanisms of fertilization such as sperm head penetration, chromatin decondensation and block of polyspermy are acquired before ovulation (Pangas and Rajkovic, 2006). Under the influence of FSH and LH, other structural and molecular changes take place. LH stimulates the continuing of meiosis, including GVBD, condensation of chromosomes and the formation of meiotic spindle near the oocyte surface. Finally, a peak in LH secretion, results with the ovulation process taking place, where the follicle bursts, releasing the oocyte, the follicular fluid and cumulus cells (M *et al.*, 2022).

1.2.2 Folliculogenesis

At puberty, the number of primordial follicles formed is about 400,000, but only 400-500 of them will complete their maturation cycle throughout reproductive age of women (Hagen-Lillevik *et al.*, 2021). Arrested primordial follicles enter the pool of growing follicles by a process called “initial recruitment” or “primordial follicle activation”. Histological features of such activation consist on a change in the shape of granulosa cells, from squamous to cuboidal, the onset of mitotic potential of the latter, which contributes on the development of the oocyte and the expression of follicle stimulating hormone receptor (FSH). During the primary follicle development, gap junctions and adhesive junctions are established between the oocyte and granulosa cells. These mechanisms of cell-cell contacts aim to exchange regulatory and nutrient molecules which are required for the oocyte development and the progress through meiosis. Connexin 37 (CX37) is a main actor involved in the establishment of such gap junction, with data stating that *cx37* deficient mice exert defects in ovary which results in failed folliculogenesis (Bachelot *et al.*, 2018). The process of recruitment continues at a constant rate

throughout the first three decades of women life, meanwhile the atresia of non-growing follicles advances throughout age, marking a decrease in fertility by the age of 30 and a substantial decline by the age of 35. FSH, cyclic AMP, activin and TGF-B are found to be activators of FSH receptor (Findlay and Drummond, 1999). Significant changes in the oocyte take place throughout the preantral stage, in which the oocyte develops a surrounding glycoprotein envelope, called zona pellucida. At this stage, the oocyte generates high amount of mRNA to support its development as well as important processes that take place later in the developmental stages.

During the next steps of folliculogenesis, there are some substantial changes at the structure of the follicles, as granulosa cells form multiple layers around the oocyte and there is an appearance of stromal like cells around basal lamina, called theca cells (Strauss and Williams, 2019). Granulosa cells change their structure to a stratified columnar epithelium. Connexin 43 (CX43) expression plays a fundamental role in the formation of the secondary follicle as it established gap junctions between granulosa cells, which is fundamental for further development. Theca cells, on the other hand, form two layers, with the inner one forming interstitial cells and the outer differentiating into smooth muscle (Aoyama *et al.*, 2019). Such events coincide with another major change in the follicle. Around the follicle, which up to this stage of development was deprived from any blood supply, starts to form blood vessels. This change in structure enables an autocrine and paracrine regulatory process.

During puberty, FSH stimulation of follicular cells induces the secretion of the follicular fluid stored inside a cavity called “antrum”. The dominant follicle displays a larger size compared to the remaining antral follicles. This size is predominantly due to the accumulation of the follicular fluid and the proliferation of granulosa and theca cells estimated at 100-fold (Williams and Erickson, 2000). Ultrasound is the gold standard procedure to count the antral follicles and estimate the ovarian reserve of women. The hormone governing the selection of the dominant follicle is FSH rise in plasma. Such a rise is closely related to the decline in the production of estradiol and inhibin A from the corpus luteum. Data states that FSH activates the Phosphatidylinositol 3-kinase (PI3K) signaling pathway in granulosa cells. Such activation

proceeds with the phosphorylation of AKT resulting in a rise of cell survival protein and inhibiting intrinsic cell death pathway. Non dominant follicles measure low fluid levels of FSH. These follicles undergo atresia, a process which is highlighted by the activation of apoptosis in the oocyte and somatic cells. Data from mouse models suggest that the oocyte itself is responsible for most of the regulation of the signaling pathway involved in recruitment and the rate of follicular growth. The Phosphatidylinositol 3-kinase (PI3K)-Akt-Foxo3a signaling pathway is a key regulator in the activation of ovarian follicles. Phosphatase and tensin homolog (PTEN) are crucial for the inhibition of such pathway. Indeed, some studies prove that loss of this lipid phosphatase in oocytes results in the global activation of primordial follicle, an event that results in the premature ovarian failure (POF) (Adhikari and Liu, 2009). Likewise, loss of function of FOXO3a, p27 and FOXL2 exert the same outcome. Another mechanism regulating follicle activation requires the mammalian target of rapamycin (mTOR) signaling pathway. The latter is inhibited by a complex of tuberous sclerosis protein TSC1 and TSC2, resulting in the regulation of such activation.

1.2.3 Steroidogenesis

Endogenous steroid hormones are considered crucial on development, growth, reproduction and systemic homeostasis (Cole *et al.*, 2019). Cholesterol is the precursor of all steroid hormones, with the synthesis of the latter taking place in specific endocrine glands. The six classes of pivotal steroid hormones are produced in: adrenal cortex (glucocorticoids and mineralocorticoids), gonads (androgens, estrogens and progestins) and kidney (calciferol) (Miller, 2013).

Theca cells are formed on the antral follicle phase of folliculogenesis, at the time where they are responsive to LH, which concurs with the activation of steroidogenic enzymes (Liu *et al.*, 2020). The structural characteristics of theca cells, such as abundant mitochondria with vesicular cristae, agranular endoplasmic reticulum and lipid vesicles are typical features of steroid secreting cells (Magoffin, 2005). The sources of cholesterol for such cells derive from different sources, such as the synthesis in endoplasmic reticulum, uptake of cholesterol by scavenger receptor B1, uptake of LDL via endocytosis and cholesterol contained inside lipid

vesicles (Miller, 2013). The later, makes up for the majority of the quantity of cholesterol uptake from lipoproteins, since the other three sources are insufficient for the demand inquired for hormone production (Gwynne and Strauss, 1982).

Mitochondria is an important site for steroidogenesis, since it contains the first enzyme important for the biosynthesis of steroid hormones (Bassi *et al.*, 2021). The residual enzymes involved in steroidogenesis are located in endoplasmic reticulum (Melchinger and Garcia, 2023). The start of the steroidogenesis takes place with the transportation of the cholesterol to the outer membrane of mitochondria (OMM)(**Figure 4**) (Rone *et al.*, 2009). Contrary to the production of polypeptide hormones, in which the secretory vesicles store large amount of hormones ready for release, granulosa and theca cells store low amounts of steroids. Hence, the production of steroids relies on a rapid response to the LH surge.

The substrate for the steroidogenesis is transported to the inner membrane of mitochondria by steroid acute regulator (STAR) (Miller, 2013). This proteins function and activation is dependent on the duration of localization on the OMM and not its cleavage from 37 to 30 DKA (Bose *et al.*, 2002). Star contains a hollow hydrophobic sterol-binding pocket which binds a single molecule of cholesterol (Tsuji-shita and Hurley, 2000; Baker *et al.*, 2005). Conformational changes take place when the protein interacts with OMM in order to accept and discharge cholesterol to the inner membrane of the mitochondria (Bose *et al.*, 2002). The expression of cholesterol side-chain cleavage enzyme p450_{scc}, encoded by CYP11A1, is exclusive for the steroidogenic cells (Monté *et al.*, 1998). Their nomenclature is due to their absorbance of light at 450nm in reduced states complexed with carbon monoxide (Gonzalez, 1988). Six of these enzymes are involved in steroidogenesis. The P450_{scc} function specifically consists on the conversion of cholesterol to pregnenolone as the first hormonally regulated step in steroidogenesis. Turnover rate per second of this reaction is six molecules of cholesterol per molecule of p450_{scc} (Miller, 2005). Mutations of CYP11A in patients, as well as the knockout model in mice demonstrate a block in steroidogenesis, highlighting the importance of the such enzyme for this pathway (Tajima *et al.*, 2001). The quantity of the production of steroids is regulated by p450_{scc}, meanwhile the type of steroid hormone produces is regulated by

downstream enzyme, such as p450c17 (Miller, 2008). Pregnenolone is converted by p450c17 (CYP17A1) to dehydroepiandrosterone (DHEA), which is a precursor of androgen. Further conversion of such product to androstenedione is facilitated by HSD3B (Nguyen *et al.*, 2012). This enzyme is involved in an alternative pathway where androstenedione is converted to progesterone and later on metabolized to androstenedione by CYP45017a and 17 β -hydroxysteroid dehydrogenase (HSD17b). Although this pathway takes place, the majority of pregnenolone is converted to androstenedione from DHEA. The final product in theca cells is testosterone which is a product of the conversion of androstenedione by HSD17b.

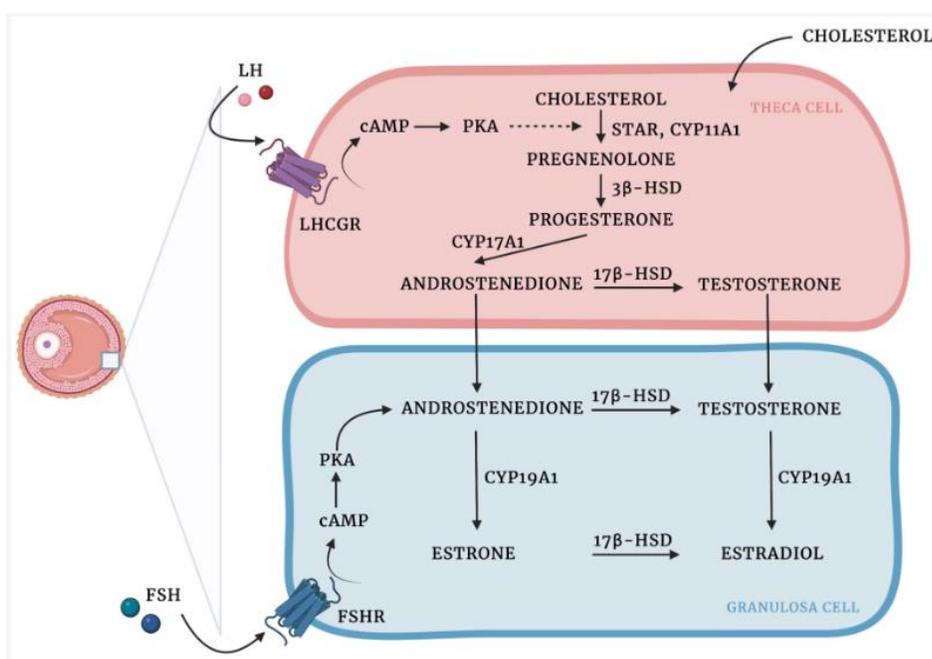


Figure 4. The theory of two Cell-2 gonadotropins-based estrogen production. LH stimulates the production of the androgen androstenedione. FSH on the other hand, is involved in the stimulation of CYP19 (aromatase) for the production of estrogen (Jozkowiak *et al.*, 2023).

An uncanonical pathway takes place in theca cells, in which 17 hydroxyprogesterone is converted to 17OH hydroxyprogesterone followed by 17OH-allopregnanolone, androstanediol and finally dihydrotestosterone.

Androstenedione and Testosterone migrate across the basal lamina and enter granulosa cells of the developing antrum (Franks and Hardy, 2018). FSH stimulates the expression of CYP19 aromatase through cAMP/protein kinase A intracellular second messenger pathway. CYP19 is

responsible for the production of estrogen by the conversion of androgen such as androstenedione and testosterone to estrone and E2. The intricacy of the expression of CYP19 aromatase stands on the fact that FSH is the primary stimulator, and factors such as insulin like growth factor (IGFs), steroidogenic factor 1 (SF-1), liver receptor homologue 1 (LRH-1), cyclic adenosine monophosphate (cAMP), peroxisome proliferator activated receptor gamma (PPAR- γ) and Gata 4 are all involved in the regulation of such activity (Fayard *et al.*, 2004; Fan *et al.*, 2005; Komar, 2005; Bennett *et al.*, 2013; Dai *et al.*, 2022). Finally, the autocrine feedback from estradiol binding to ER β , therefore increasing FSH-induced expression of CYP450, highlights an important event in folliculogenesis, that of the choice of a dominant follicle (Couse *et al.*, 2005).

1.2.4 Follicular Microenvironment

Follicular fluid (FF) is an extracellular fluid which accumulates in the antrum during development phases of the ovarian follicle. It provides the site for the maturation of the cumulus-oocyte complex and differentiation of the granulosa cells. Follicular fluid is primarily derived from plasma through the vascular compartment of the follicle wall, although some factors are secreted by the follicle itself through growth and progression. Follicular fluid forms from the blood flowing through the thecal capillaries, accumulating proportionally to the expansion of follicular antrum, as such its volume differs between dominant and subordinate follicles (Beg *et al.*, 2001). The rate of fluid accumulation, even during rapid growth, is minimal in comparison to the volume of blood flowing into the thecal capillaries (Herrmann and Spänzel-Borowski, 1998). Follicular fluid contains molecules such as proteins and hormones including FSH, LH, Growth hormones (GH), human chorionic gonadotropin (hCG), progesterone and estradiol. There are also interleukins, metabolites, such as amino acids and lipids, anti-apoptotic factors, electrolytes, anticoagulants, antioxidants, growth factors, that include epidermal growth factors (EGF) and transforming growth factor alpha (TGF- α). Another contributor in the regulation of the development of the oocyte, inside the follicle, are granulosa cells. Dependent on phases of the menstrual cycle, these cells produce estrogen prior to ovulation, and progesterone after it, becoming granulosa lutein cells (Alexopoulos *et*

al., 2000). Differentiation of granulosa cells into mural granulosa cells and cumulus cells influenced by their location during folliculogenesis, results in transcriptomic differences between such population (Latham *et al.*, 1999). Mural granulosa cells provide support for the oocyte via endocrine and paracrine pathways, meanwhile cumulus cells provide nutritional support and transport of the macromolecules (Andrei *et al.*, 2019).

1.2.5 Taste Receptors

Taste is one of the five senses for humans, with the main function consisting on the chemical analysis of the food composition. Taste receptors are responsible for the recognition of 5 different tastes: sweet, salty, sour, umami and bitter. Initially, these receptors were thought to be exclusively expressed on the taste buds located on the papillae of the tongue. Transmission of the taste signals relies on different mechanisms. Perception of sour and salty, relies on ion channels (Xiao *et al.*, 2021). Meanwhile, sweet, umami and bitter are mediated from G-protein coupled receptors (GPCRs) (Ahmad and Dalziel, 2020). These receptors are categorized into two groups: Type 1 Taste receptors (TAS1Rs) and Type 2 Taste Receptors (TAS2Rs). Three genes code for TAS1R, responsible for the detection of sweet and umami tastants. TAS1R2 and TAS1R3 genes code for two proteins which form a dimer and respond to sugar molecules and sweeteners (Behrens, 2022). Meanwhile, the transcripts from TAS1R1 and TAS1R3 are translated into proteins which form dimers responsible for the detection of l-amino acids or umami flavor (Pallante *et al.*, 2021). TAS2Rs group comprises 24 genes coding for proteins responsible for the detection of bitter tastants. Evidence of the expression of such receptors on other parts of the body instead of the conventional one, has brought propelling interest to the scientific community. Respiratory airways, gastrointestinal tract, brain, and gonads are found to express taste receptors (Li, 2013; Carey and Lee, 2019; Jiang *et al.*, 2021). There have been many different functions of these receptors in regards to the location, such as regulation of the insulin release in the brain, bladder contraction in the urothelium, and involvement in the osteogenesis and bone marrow adipogenesis (Elliott *et al.*, 2011; Kyriazis *et al.*, 2014).

Interestingly, their expression on the reproductive tract of both male and female has brought attention on their involvement on spermatogenesis in male and steroidogenesis in ovaries, even though lacking information on the exact mechanisms (Jiang *et al.*, 2021; Liu *et al.*, 2022). Data states that TAS2R3, TAS2R3, TAS2R14 AND TAS2R19 AND TAS2R43, including downstream signaling components such as α -gustducin and α -transducing are expressed in both somatic cells of the ovaries, granulosa and cumulus cells (Semplici *et al.*, 2021). These findings, based on cell type expressions of these receptors, suggest a role on steroidogenesis, but there is limited data focused on the mechanism, as well as focused on a potential involvement with the endocrine disruptors effect. Bitter compounds act as ligands which stimulate the activation of intracellular signaling upon their link to the receptor. This ligand-induced stimulation is followed by an activation of the trimeric G protein composed of α -gustducin, GB3 and GY13 forming the complex GB3Y13. The later activates phospholipase C isoform B2 (*PLC*- β 2) that follows with the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). This is followed by an activation of intracellular ion channel IP3R which allows the release of Ca^{2+} from intracellular reticulum (ER). The elevated levels of Ca^{2+} activate the Cation channel Transient receptor potential cation channel subfamily Member 5 (TRPM5) as shown in **Figure 5**.

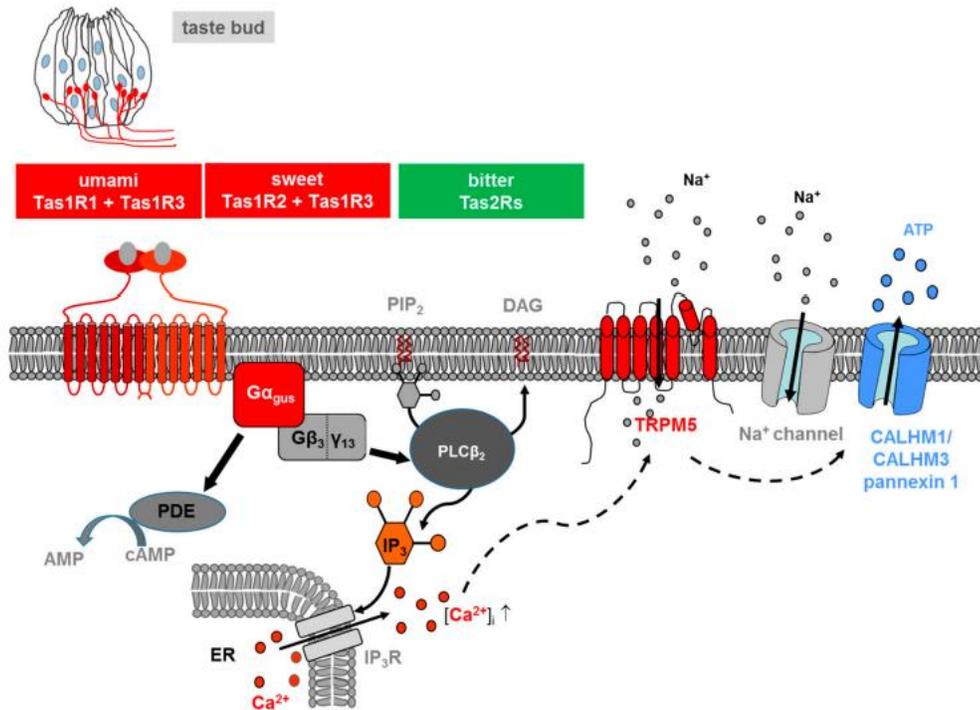


Figure 5. Signal Transduction pathway of umami, sweet and bitter stimuli in taste receptor cells on the tongue (Luddi *et al.*, 2019).

Therefore, the influx of Na $^+$ and increased concentrations of Ca $^{2+}$ depolarize the cell membrane, which is followed by a release of the ATP through pannexin 1 and calcium homeostasis modulator 1 (CALHM1). Another mechanism involving G protein a gustducin consists on the activation of phosphodiesterase (PDE) which in turn decreases intracellular levels of the second messenger cAMP by its hydrolysis to AMP.

1.3 The Endometrium

1.3.1 Cellular Composition of the Endometrium

Endometrium is a complex tissue with pivotal functions for female reproductive health such as implantation, maintaining the pregnancy and menstruation, if implantation does not occur. The growth and differentiation of endometrial tissue is governed by steroid hormones. These changes include regeneration, decidualization and finally, shedding, in the absence of pregnancy. The complex cellular makeup of the endometrium enables it to respond dynamically to the cyclical changes of the menstrual cycle, playing a critical role in embryo implantation and the maintenance of early pregnancy. Primary structural components are epithelial cells that line the surface of the endometrium and form glandular structures, specialized on production and secretion of nutrients. Another pivotal cell type are stromal cells, that not only provide a structural support but undergo a morphological and molecular modification important to support embryo implantation during pregnancy, such as decidualization. Alongside these cell type, the endometrium contains an abundance of immune cells, such as macrophages, uterine natural killer (uNK) cells, T cells, and dendritic cells, who's role includes immunosurveillance, tissue regeneration and creating the optimal environment for embryo development. Another type of cells present in this complexed structure are endothelial cells, that line the blood vessels and play an important role in angiogenesis, ensuring optimal blood supply throughout menstrual cycle. Additionally, recent studies have identified mesenchymal stem cells within the endometrium, which contribute to its regeneration after each menstrual cycle, underscoring the tissue's remarkable regenerative capacity. Any disruption in the balance of these cell types can lead to conditions such as infertility, endometriosis, or abnormal uterine bleeding.

1.3.2 Human Endometrial Cycle: Phases and Functions

Fluctuation of the levels of the two main ovarian sex steroids, estrogen and progesterone, govern these changes, which are recognized as menstrual phases. Estrogen primed endometrium coincides with the proliferative phase of the menstrual cycle. Starting at the end of menstruation, until day 14 of the menstrual cycle (ovulation), it is characterized by linear shape, an increase in endometrial thickness from 4 to 7mm and vascularization.

Secretory phase, on the other hand, starts on day 15 of the menstrual cycle, and corresponds to the luteal phase of ovarian cycle (**Figure 6**). Progesterone levels are increasing as a response to the production from the corpus luteum to sustain the released ovum. Cellular components of the endometrium include epithelial, stromal and immune-resident cells. Epithelial compartment of the endometrium is composed of luminal and glandular epithelium. Luminal epithelium is characterized from ciliated and non-ciliated, and their morphology changes throughout the menstrual cycle. Epithelial cells are responsive to estrogen, and as such, fluctuating levels of the hormone trigger changes in plasma membrane, microvilli, tight junctions and the cytoskeleton.

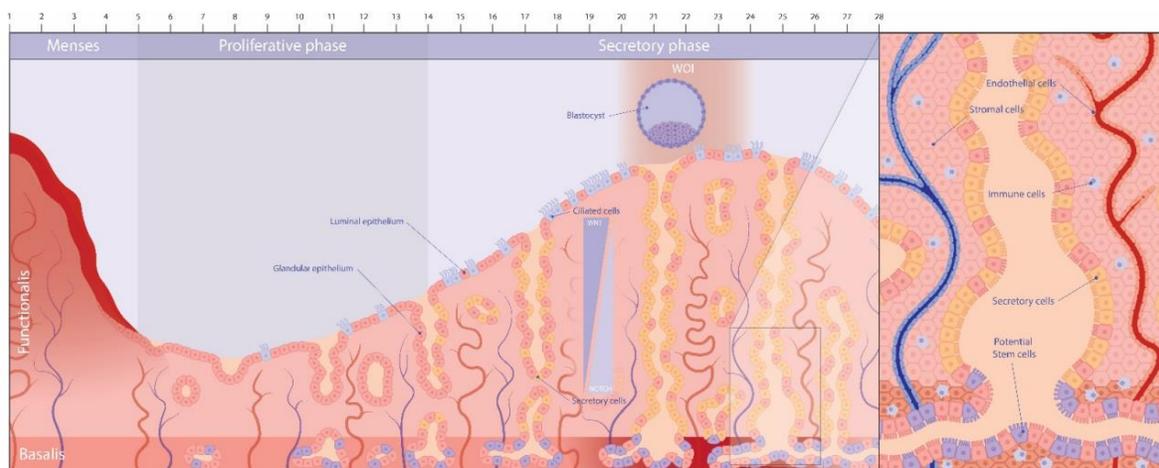


Figure 6. Visual representation of the morphology changes of the endometrium throughout the menstrual cycle (Maenhoudt *et al.*, 2022).

Ciliated cells increase in the proliferative phase, dropping then in number at the end of the secretory phase. Morphologically, during the latter phase, epithelial cells present protrusion of the plasma membrane with dimension up to 5-19 μm called “pinopodes”. During this phase,

glandular epithelium, lined by microvilli presents different cell junctions such as tight junctions, desmosomes and gap junctions. The largest portion of the endometrium is constructed by connective tissue rich in stromal cells and extracellular matrix. These cells change morphologically throughout the menstrual cycle as a result of steroid hormone regulation. They are competent for the remodeling, proliferation and the breakdown of the endometrial tissue. Another important process, during the mid-luteal phase of the menstrual cycle is the decidualization process. This process allows the transformation of fibroblast like stromal cells into secretory decidual cells. The endometrial stromal compartment plays a critical role in embryo implantation, vascular formation, and tissue remodeling throughout the menstrual cycle. Within this compartment resides a specialized population of stromal-like stem cells characterized by their self-renewal capacity and ability to differentiate into multiple lineages. These properties underpin the regenerative and adaptive nature of the endometrium. A pivotal function of these cells is their transformation into decidualized stromal cells, a process essential for implantation. Decidualization involves significant morphological and functional changes, enabling key processes such as embryo development, vascular remodeling, and immune regulation at the maternal-fetal interface. This transformation creates a supportive environment necessary for successful implantation and the early stages of placental development. Decidualization occurs during the mid-luteal phase of every ovulatory cycle, regardless of whether conception occurs.

1.3.3 Window of Implantation

The series of cyclic changes that allow endometrium to remain receptive for almost four days is known as window of implantation (WOI) and takes place after six to 8 days after the ovulation. Estrogen promotes glandular proliferation by stimulating stromal cells to produce growth factors, such as IGF1 and EGF, which act on receptors expressed by epithelial tissues (Robertshaw *et al.*, 2016). Following ovulation, the corpus luteum secretes progesterone and estrogens, which enhance the secretory activity of the endometrial glandular epithelium. Progesterone suppresses the proliferation of epithelial and stromal components, while encouraging glandular proliferation and inducing modifications in the stroma. Notably,

elevated progesterone levels lead to a reduction in the expression of estrogen receptors in both the glands and stroma, thereby diminishing the endometrium's sensitivity to estrogen produced by the ovaries (Wang et al., 2017). Even androstenedione and testosterone have an impact on implantation by inducing morphological and functional changes. The presence of pinopodes in the apical surface of the epithelium, hallmark endometrial receptivity, presented at different length, above the microvilli level of the surrounding cells. The key to uterine receptivity is the dynamic and precisely controlled molecular and cellular events that drive blastocyst growth, attachment and the subsequent events of implantation. Such events are orchestrated by paracrine signaling which involves key molecules such as prostaglandins, adhesion molecules, transcription factors, cytokines and growth factors (Governini *et al.*, 2021).

1.3.4 Endometrial Organoids

The limitations of standard 2D culture in presenting huge differences with their *in vivo* counterparts in morphological features, proliferation, differentiation and changes in cell-cell interactions and signaling pathways, have led to development of three-dimensional (3D) cell culture systems. These novel models present a promising solution to bridge the gap between research and clinical applications (Chaicharoenaudomrung *et al.*, 2019). 3D cell culture systems improve the physiological relevance of cell-based assays by better replicating the behavior of cells in their natural *in vivo* environment. This method enhances the accuracy of experimental results and provides a more realistic model for investigating cellular processes and interactions. This marks a major advancement in biomedical research, allowing for more reliable predictions of clinical efficacy and safety (Weigelt *et al.*, 2014).

3D organoids, derived from induced pluripotent stem cells, are self-organizing structures that mimic the cellular architecture and function of specific organs in a controlled laboratory environment. These models present a sophisticated option to 2D cultures, as they more accurately reflect the complexity of tissue environments. By mimicking key physiological characteristics, organoids have emerged as indispensable tools for studying organ development, disease pathways, and cellular interactions. Their applications span

regenerative medicine, cancer research, and drug discovery, providing critical insights into tissue-specific behaviors and responses (Fang *et al.*, 2023; Wu *et al.*, 2023; Gopallawa *et al.*, 2024).

Moreover, these models hold significant potential for personalized medicine by using patient-specific cells to create individualized models for therapeutic testing. In cancer research, patient-derived organoids enable scientists to anticipate patients' responses to various chemotherapy treatments, enhancing the precision of therapy. Their suitability for high-throughput drug screening minimizes dependence on animal testing while delivering more accurate human-specific data. Researchers demonstrate how stem cell-derived organoids are engineered to better replicate human tumor environments, mimicking the complexity and heterogeneity of cancers more accurately than traditional animal models. The 3D environment that closely resembles human tissue allow researchers to study drug responses without relying on animal experimentation (Hockney *et al.*, 2023; Park *et al.*, 2024). Endometrial organoids have gained significant recognition in reproductive health research, offering invaluable insights into the physiology, pathology, and hormonal regulation of the endometrium (Song and Fazleabas, 2021).

These organoids can be generated from progenitors or induced pluripotent stem cells (iPSCs), effectively recreating the complex cellular structure and functional attributes of the endometrium. They preserve and present key characteristics of *in vivo* tissues, such as organized structures and responsiveness to hormonal signals, making them valuable models for investigating conditions like endometriosis, uterine cancer, and infertility (Hibaoui and Feki, 2020). By closely replicating the endometrial microenvironment, this model enhances the ability to evaluate the efficacy and safety of potential treatments, thereby advancing reproductive medicine. In conclusion, the advent of 3D cell cultures, particularly endometrial organoids, marks a transformative leap in biomedical research, providing more accurate models for understanding complex biological processes and accelerating the development of effective therapies. Endometrial organoids serve as a robust 3D model for investigating the epithelial-endometrial interface crucial to embryo implantation (Luddi *et al.*, 2020). Recently developed human endometrial organoids provide a highly effective *in vitro* platform for

exploring the physiology and pathology of the human endometrium, as they accurately replicate both the molecular and functional attributes of the tissue (Heidari-Khoei *et al.*, 2022). This technology enables the expansion of limited starting material, greatly enhancing research potential in this area. To create these 3D cultures, cells are either embedded in or seeded onto scaffolds that mimic their natural spatial environment, particularly the structure of the extracellular matrix (ECM). Among these scaffolds, hydrogels—hydrophilic polymer networks—are the most commonly used. Their properties can be finely tuned to closely resemble the structural characteristics of native tissue ECM. These models are specifically designed to emulate organ- or tissue-specific signaling pathways, effectively recreating the niche environment of the original tissue.

2 MATERIALS AND METHODS: PART I

2.1 Human Granulosa Cell Line 5 (hGL5)

Human Granulosa Cell Line is an immortalized cell line which derives from primary luteinized GC cells after transformation with the E6 and E7 regions of papillomavirus 16 (Rainey *et al.*, 1994). Hgl5 cells were cultured in Dulbecco modified Eagle Medium (Invitrogen, Whaltman, MA, USA) containing L-glutamine (1%), penicillin/ streptomycin (1%), fetal bovine serum (10%), non-essential amino acids (1%) and ultra-serum G (Sartorius, Goettingen, Germany) (2%). Cell cultures were maintained in the incubator (5% CO₂ at 37°C).

2.2 Human Primary Granulosa Cells Collection and Isolation

Granulosa cells were isolated from the follicular fluid obtained from 60 women undergoing IVF at the Siena University Hospital. Participants gave signed informed consents for the use of these samples as well as medical data. The study complies with the declaration of Helsinki and approved by the ethical committee of the University of Siena (CEAVSE, Protocol number 18370, 2 October 2020). All the women enrolled in this study were undergoing assisted reproductive techniques (ART) due to male factor infertility. The laboratory experiments were performed under blinded conditions.

Follicular fluid sample arrives at the laboratory after the oocyte pick up procedure, and is processed by centrifugation at 500 rpm for 10 minutes. The pellet containing the cellular components such as granulosa cells, endothelial cells, red blood cells, white blood cells and macrophages was resuspended in Hanks Balanced Salt Solution (HANKS) (Sigma-Aldrich, St Louis, MO, USA), and stratified on 4 mL of 45% Percoll and centrifuged at 3500 rpm for 20 minutes. This gradient centrifugation allows red blood cells to sediments at the bottom of the tube, while granulosa cells will form a ring like shape in the middle of the volume of this solution.

Granulosa cells were retrieved and centrifuged for 10 min in 500 rpm. The pellet, is subsequently resuspended in culture medium and transferred into a Petri Dish and incubated

at 37°C and CO₂ 5% for 10 min. Macrophages adhere to the bottom of the dish, allowing a supernatant rich of granulosa cells. The latter, prior to the treatment with the selected compounds, are incubated for 24h to 48 hours in (Dulbecco's Modified Eagle Medium: DMEM), 10% Fetal bovine serum, 100mM/L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 5000 IU/mL of penicillin and 5mg/mL of streptomycin (Sigma-Aldrich, St Louis, MO, USA) and 1% essential amino acids (Sigma-Aldrich, St Louis, MO, USA).

2.3 Compounds

Compounds studied and included in this study are based on the data in literature describing them as activators of TAS2Rs and possible role as EDCs due to their phytoestrogenic effects. Daidzein, Biochanin A and Caffeine (Sigma-Aldrich, St. Luiz, MO, USA) and 3'.4'.7-Trihydroxyisoflavone (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were dissolved in dimethyl sulfoxide, ethanol and H₂O respectively. Furthermore, 3 selective antagonists of TASTE Receptors, sulfamouyl-benzoic acid (SBA), citronelle (Citro) and probenecid (PRO) were purchased from Sigma-Aldrich (St Luiz, Mo, USA). These compounds were all resuspended in DMSO and diluted in the media containing Dulbecco-s modified Eagle medium (DMEM, 1x), fetal bovine serum, L-glutamine, penicil/streptomycin, non-essential amino acid. Neither of the tested concentrations exceeded the final concentration of 0.1% of DMSO.

2.4 Cell Proliferation Assay

Cell proliferation assay was performed in order to screen for the optimal concentration of cells to plate for the experiments. Cells were plated via a serial dilution starting from 25,000 cells in a 96 well plate and incubated overnight. 10µl of CCK-8 solution (Abcam, Cambridge, Uk) was added slowly to the cells avoiding the creation of bubbles which interfere with the experiment. The plate was incubated for 4 hours, after that the absorbance was measured at 450 nm using a Dynaread microplate reader (Dynex Technologies, Czech Republic). Absorbance of 8 wells for each cell density was calculated and the average values were used to prepare the calibration curve.

2.5 Cytotoxicity Assay

The possible toxic effect of Genistein, BCA, Daidzein, 3'4'7-Trihydroxiisoflavone on the hGL5 cell viability was investigated by CCK-8 kit. Cells were treated at different concentrations of compounds taking into consideration concentrations reported previously in the literature. Similarly, antagonists of taste receptors such as PRO, SBA and Citro were screened as well for their potential cytotoxic effect in order to establish the optimal concentration for the treatment of the cells. To this purpose, CCK-8 is used according to the manufacturer instructions. Absorbance values from 4 wells from each treatment were measured and the average of these values were considered to prepare the calibration curve.

2.6 Mitotracker

After an incubation of 24 hours with the compounds, potential mitochondrial morphology changes were investigated. MitoTracker Red CMXRos[®] probes allows the diffusion of the dye across the plasma membrane and accumulation into the active mitochondria. A final concentration of 200nM of the dye was added to the cell medium and incubated for 30 minutes. Subsequently, after washed with PBS 1X, cells were fixed with ice cold methanol and incubated for 15 min at -20 °C. We proceeded with 3 washes with PBS 1x to remove any residue of methanal and subsequently 6 minutes of incubation with DAPI diluted at 1:10000 allow the staining of the nucleus. Finally, after three more washes, the slides are mounted with DABCO and observed with an inverted fluorescence microscope.

2.7 RNA Extraction and Complementary DNA Preparation

Total RNA was isolated from cells using RNeasy protect mini kit (Qiagen, Hilden, Germany), according to the manufacturer protocol. RNA extracted was resuspended in 30 µl of water and proceeded with the measurement of its concentration and purity by NanoDrop[®] N100 UV-vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 1µg of the RNA was transcribed to cDNA by iScript cDNA Clear TM cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Genomic DNA was removed by a treatment with DNase which was

inactivated by an incubation of 5 min at 25°C and consecutive 5 min at 75°C. Finally, 4 µl of IScript Reverse transcription Supermix was added to the samples and the mix was incubated for 5 min at 25°C, 20 min at 46°C and 1 min at 95 °C. Primers used in this study are listed in **Table 6**.

Table 6. List of primers used for RT-PCR.

Target Gene	Acronym	Assay ID
<i>Taste receptor, type 2, member 3</i>	<i>TAS2R3</i>	dHsaEG5003946
<i>Taste receptor, type 2, member 4</i>	<i>TAS2R4</i>	dHsaEG5003947
<i>Taste receptor, type 2, member 14</i>	<i>TAS2R14</i>	dHsaEG5004113
<i>Taste receptor, type 2, member 19</i>	<i>TAS2R19</i>	dHsaEG5003736
<i>Taste receptor, type 2, member 43</i>	<i>TAS2R43</i>	dHsaEG5004567
<i>Steroidogenic Acute Regulatory Protein</i>	<i>STAR</i>	Hs.PT.58.40560623
<i>Cytochrome P450 Family 17 Subfamily A Member 1</i>	<i>CYP17A1</i>	Hs.PT.58.20669937
Reference Gene	Acronym	Assay ID
<i>Hypoxanthine Phosphoribosyltransferase 1</i>	<i>HPRT1</i>	Hs.PT.58v.45621572

2.8 Western Blot Analysis

Western blotting was performed as previously described (Focarelli *et al.*, 2018). Briefly, 50 µg of total protein were denatured by dilution in Laemmli buffer, kept at 95 °C for 5 minutes, and separated on a 10% polyacrylamide gel using the Cell Mini Protean system (Bio-Rad). Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) using a Mini Trans-Blot apparatus (Bio-Rad).

The membrane was blocked for 1 hour in 5% nonfat dry milk and subsequently incubated overnight at 4 °C with primary antibodies diluted in 1% nonfat dry milk/TTBS (TBS containing 0.2% Tween 20). After washing in TTBS, the membrane was incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (**Table 7**). The same

nitrocellulose membrane was also probed with an anti- β -actin antibody (Microscience, Wokingham, UK), followed by incubation with the corresponding HRP-conjugated secondary antibody as an internal loading control. Immunostained bands were visualized using chemiluminescence with the ImageQuant LAS 4000 system (GE Healthcare).

Table 7. List of Antibodies used in this study.

Antigen	Donor Species	Dilution	Manufacturer	RRID
<i>TAS2R3</i>	Rabbit	1:500	Thermo Fisher Scientific	AB_2556263
<i>TAS2R4</i>	Rabbit	1:500	Thermo Fisher Scientific	AB_2201090
<i>TAS2R14</i>	Rabbit	1:500	NovusBio Laboratories	AB_11053160
<i>TAS2R19</i>	Rabbit	1:1000	Thermo Fisher Scientific	AB_962285
<i>TAS2R43</i>	Rabbit	1:1000	Thermo Fisher Scientific	AB_2815963
<i>B actin</i>	Mouse	1:2000	Bio-Rad laboratories	AB_2223350
<i>ERK</i>	Rabbit	1:1000	Cell Signaling	AB_390779
<i>P-ERK</i>	Rabbit	1:1000	Cell Signaling	AB_2650561
<i>COX-4</i>	Rabbit	1:400-1000	Thermo Fisher	AB_10987478
<i>B Tubulin</i>	Mouse	1:100	Thermo Fisher	AB_2609649
Secondary antibodies				
<i>Anti-Mouse-IgG HRP</i>	Goat	1:8000	Bio-Rad laboratories	AB_609692
<i>Anti-Rabbit-IgG HRP</i>	Goat	1:8000	Bio-Rad laboratories	AB_1102634
<i>Alexa Fluor Anti-Rabbit 488</i>	Goat	1:500	Thermo Fisher	AB_2651036
<i>Alexa Fluor Anti-Mouse 568</i>	Goat	1:500	Thermo Fisher	AB_1500898

2.9 Oil Red O Staining and Morphological Observation

To evaluate the amount of lipids accumulated in these cells we used Oil red O staining method. 0.5g of this dye was resuspended in mL of isopropanol. 30 ml of this stock was added to mL of Oro saturated solution (ddH₂O). After fixing the cells with 4% paraformaldehyde for 10 minute

and consequent washing with PBS, Oil red O (Sigma-Aldrich, St Louis, MO, USA) was added for 15 min at room temperature. After that, cells were stained with hematoxylin and observed using a phase contrast microscope (Olympus, Tokyo, Japan). The quantification of the droplet was done from a determination of OD of the supernatant at 540nm using the automatic enzyme immunoassay analyzer.

2.10 Transmission Electron Microscopy

Cells were fixed in Karnovsky fixative, prepared in 0.1M cacodylate buffer (CB), pH 7.2, for 2 hours at 4 °C. After fixation, the samples were washed in CB overnight and subsequently postfixed in 1% osmium tetroxide (OsO₄) in CB, for 1 hour at 4 °C. The samples were then washed in CB overnight, dehydrated through a series of diluted ethanol and embedded in epon-araldite resin. Ultrathin sections (70 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate, and visualized using a FEI Tecnai G2 Spirit transmission electron microscope (Hillsboro, OR, USA)(Luongo *et al.*, 2022).

2.11 Elisa Test

The secretion of P4 and E2 from the cells was measured in the culture medium of HGL5 and granulosa cells treated for 24 hours. The concentration of steroid hormones secreted by the cells in the spent culture medium was measured by Immulite 2000 (Siemens).

2.12 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Data from the experiments were tested for normality using either the Shapiro-Wilk or Kolmogorov-Smirnov tests. Depending on the results, parametric tests (ANOVA) or nonparametric tests (Kruskal-Wallis) were applied. Densitometric analyses were performed with ImageJ software, and statistical significance was defined as p-value <0.05.

3 MATERIALS AND METHODS: PART II

3.1 Endometrial Tissue Collection

Endometrial biopsies were collected from Caucasian women under the approval of the ethic committee of the South-Estonian Hospital of Võru, Estonia and in accordance with the guidelines of the Declaration of Helsinki 2000. Endometrial epithelial organoids were derived from endometrial biopsies obtained from six healthy volunteers (n=6), who provided written informed consent prior to participation. Following the isolation of epithelial and stromal cells from the biopsies, the epithelial cells were cultured for seven days to establish endometrial organoids. Upon successful generation, the organoids were preserved at -80°C until further experiments.

3.2 Endometrial Glands Isolation and Organoid Assembly

Endometrial tissues are cut using scalpels into cubes approximately 0.5 mm³. These tissue fragments are then subjected to enzymatic digestion in 20-30 mL of a solution containing 1.25 U/mL Dispase II (Sigma-Aldrich, St Louis, MO, USA) and 0.4 mg/mL collagenase V (Sigma-Aldrich, St Louis, MO, USA), dissolved in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (Biosera, Cholet, France). The digestion process occurs with gentle shaking at 37°C for a duration of 30 to 60 minutes. Following digestion, the supernatant is filtered through the 100 µm sieves (Corning, New York, USA). The sieves were inverted into a Petri dish, and the retained glandular elements were rinsed off the sieve membranes. These were then centrifuged into pellets and resuspended in ice-cold Matrigel (Corning, New York, USA) at a 1:25 ratio (vol:vol). Small 25 µL droplets of this Matrigel-cell suspension were placed into a 48-well plate (Costar, Milan, Italy), allowed to set at 37°C, and then covered with 250 µL of organoid Expansion Medium (ExM) (Turco *et al.*, 2017). The medium was refreshed every 2 days, and the cultures were passaged by manual pipetting every 7-10 days. To freeze endometrial epithelial organoids (EEOs), Matrigel was removed with Cell Recovery Solution (Corning, New York, USA), and the organoids were

resuspended in Recovery Cell Culture Freezing Medium (Thermo Fisher Scientific, Waltham, MA, USA).

For cytotoxicity assay, the EEOs were extracted from the Matrigel using Cell Recovery Solution and subjected to repeated pipetting, followed by trypsinization with TrypLE Express (Invitrogen, Waltham, MA, USA). Subsequently, the cells were washed in expansion medium and passed through a 40 μm cell strainer (Corning, New York, USA) to achieve a single-cell suspension. To ensure accuracy in cell counting, the cells were diluted with trypan blue to identify non-viable cells, and the viable cells were counted using a hemocytometer. Within the first three days of culture, the ExM was supplemented with 10 μM Y-27632, a Rho Kinase (ROCK) inhibitor, aiming to promote cell survival during single-cell dissociation and reduce apoptosis (Merck).

3.3 EEO Treatment

EEO are treated with estradiol (E2) to mimic the proliferative phase of the menstrual cycle (ppEEO), and E2, progesterone (P4), and cyclic adenosine monophosphate (cAMP) to mimic the mid secretory phase (mspEEO). Cells were treated with E2 at a final concentration of 10nm (Sigma-Aldrich, St Louis, MO, USA), progesterone was added at a final concentration of 1 μM and cAMP at 1 μM (Sigma-Aldrich, St Louis, MO, USA). Furthermore, our aim was to explore the effect that a mixture of phthalate metabolites can have in such phases. To this purpose, cells were treated with a mixture of phthalate, called “BAD Actor” mixture (Iizuka *et al.*, 2022). This composition of such mixture is shown in **Figure 7**.

BAD mixture (effect-based mixture)				Stock solution		Working concentration (Low dose)	
Parent	Metabolites	%	MW	mM	mg/ml	μM	ng/ml
DEHP	MEHHP	9.9	294	21.63	6.36	0.16	47.69
DiBP	MiBP	6.5	222	14.15	3.14	0.10	23.56
DEP	MEP	80.3	194	175.15	33.98	1.31	254.84
BBzP	MBzP	3.2	256	6.94	1.78	0.05	13.32
	SUM	99.9		217.87	45.26	1.63	33.94

Figure 7. Composition of Bad Actor Mixture (Iizuka *et al.*, 2022).

These metabolites are strongly associated with serum estradiol concentration, identified through the Weighted Quantile Sum (WQS) model ($P = 0.05$). The weights for the components of the BAD Actor mixture were as follows: MEHHP (22%), MiBP (monoisobutyl phthalate, 21%), MEP (monoethyl phthalate, 21%), and MBzP (monobenzyl phthalate, 16%). Study design is described in **Figure 8**. Concentrations of this mixture tested in our experimental design are 1x and 100x of the working concentration described in **Figure 7**.

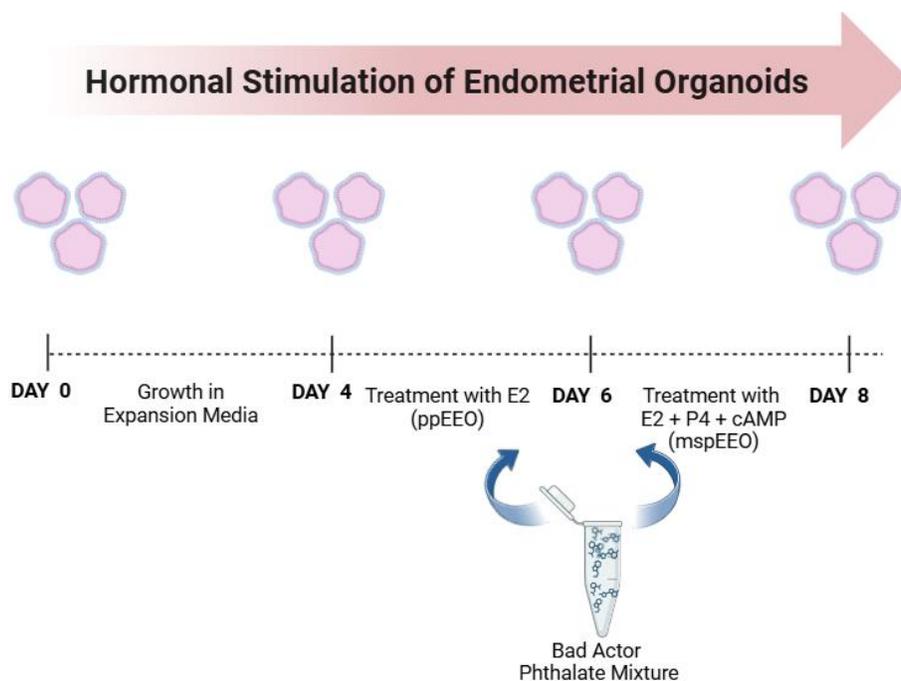


Figure 8. Illustrative description of experimental design in our study (created by Biorender).

3.4 Viability Assay

To establish the optimal number of single cells plated for cytotoxicity assay, aiming the optimal formation of EEOs in order to continue further screening, we used Resazurin based assay, following the manufacturer instructions at a final concentration of 50 μM (Sigma-Aldrich, St Louis, MO, USA). The fluorescence intensity and colorimetric absorbance measurements were carried out in Cytation 5 multi-mode readers (Biotek; Winooski, VT, USA). The settings for the fluorescence signal as well as colorimetric are as following: Readings were taken every 10 minutes for 4 hours under the following conditions: fluorescence with excitation at 540 nm, emission at 590 nm, using a monochromator, top optics, and a gain of 50; meanwhile

absorbance measured at 570 nm and 600 nm, using a monochromator with a read height of 8.5 mm (Lavogina *et al.*, 2022).

Cells were seeded in growth medium onto a 96 well plate at a density of 1000, 2000, 3000, 4000 or 5000 in triplicates. After incubation for 7 days and confirmation by microscopy of the formation of 3D organoids, the medium was removed from the cells, proceeding with a PBS wash and finally the addition of 100 μ l of the resazurin solution to each well.

After the establishment of the optimal number of cells, we proceeded to screen for the cytotoxicity of BAD Actor phthalate mixture, testing 3 approaches: Fluorometric, colorimetric and luminescence. For the colorimetric and fluorescence signals, Resazurin assay was used, meanwhile for the luminescence assay we used Cell titer Glo 3D[®] (Promega Corporation, USA). DMSO was used as a vehicle and didn't exceed final concentration of 0.5% in expansion medium. CellTiter-Glo[®] 3D Reagent was thawed at 4°C overnight. Once thawed, the reagent was placed in a 22°C water bath for approximately 30 minutes.

Equal amount of CellTiter-Glo[®] 3D Reagent to the volume of cell culture medium in each well was added (for a 96-well plate, 100 μ L of reagent added to 100 μ L of medium containing cells). Content was vigorously mixed for 5 minutes to ensure cell lysis. The plate was incubated at room temperature for another 25 minutes to stabilize the luminescent signal. Fluorescence, colorimetric and luminescence signals were measured by Cytation 5 multi-mode reader (Biotek; Winooski, VT, USA).

3.5 Sequencing and Differential Gene Expression and Enrichment Analysis

After the Exposure to all the treatment conditions, β -mercaptoethanol was added to the wells to ensure cell lysis. Total RNA was extracted using the RNeasy micro kit according to the manufacturer protocol (Qiagen, Germany). The concentration and the quality of the RNA were measured by RNA High Sensitivity Assay kit (Qubit[™], Thermo Fisher Scientific) and 4150 Tape station system (Agilent Technologies, USA). All the samples resulted with RNA integrity number higher than 8, and 300 ng were used for single ended TruSeq[™] RNA sequencing library preparation kit (Illumina, San Diego). Pooled libraries were sequenced on the Illumina NextSeq

2000 system (Illumina, USA). Data analysis was conducted using R version 4.3.3, an open-source platform for statistical analysis and data visualization. Differential gene expression analysis was performed with the DESeq2 package (version 1.36.0) (Love *et al.*, 2014) using as input counts matrix obtained from the sequencer (Love *et al.*, 2014). To reduce the complexity of the design formula and analyses, the dataset was divided into smaller subsets, each containing only the samples relevant to the specific comparison. This approach allowed for a more focused and interpretable differential expression analysis by simplifying the design model. Gene counts were filtered to exclude genes with low or no expression, applying a threshold of at least 50 counts per million (CPM) in at least three samples. This filtering step ensured that only reliably expressed genes were retained for downstream analysis. The *DESeq2* function pipeline was then employed to normalize the data, estimate dispersion, and perform the differential expression analysis. For all comparisons, the design formula used was “~ donor + condition”, which accounted for individual donor effects while focusing on the differences between conditions. Genes with a p-value <0.05 were considered significant. Enrichment analysis was performed using the ClusterProfiler package (version 4.10.1) (Yu *et al.*, 2012). Genes with p-value <0.05 from the differential expression analysis were used as input for the enrichment analysis. Gene Ontology (GO) terms categorized into cellular component (CC), biological process (BP), and molecular function (MF) (Thomas *et al.*, 2022) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (Kanehisa and Goto, 2000) were used as reference. GO terms and KEGG pathways with p-value <0.05 were considered significant. KEGG pathways were visualized using the Pathview package (version 1.42.0) (Luo and Brouwer, 2013). Pathway diagrams provided a graphical representation of the location and regulation of differentially expressed genes within biological pathways, facilitating the interpretation of functional relationships.

4 RESULTS AND DISCUSSION: PART I

This part of the study aims to screen different compounds for their potential effect as endocrine disruptors. To this end, preliminary experiments were conducted on the human ovarian immortalized cell line hGL5, which exhibits traits similar to primary follicular granulosa cells, including cell retraction upon protein kinase-A activation, the ability to synthesize progesterone and estradiol, and the expression of P450 aromatase, the key enzyme in estradiol production (Casarini *et al.*, 2017). These attributes make hGL5 as a suitable model for studying the impact of selected EDCs on steroidogenesis. Since EDCs are reported to activate type 2 taste receptors (TAS2Rs), a subclass of taste receptors expressed in various extraoral locations, hGL5 cells were utilized to explore the potential role of TAS2R in regulating steroidogenesis and delineate the molecular pathways influenced by ECD treatment. Building on the findings from the immortalized cell line, the study further aimed to assess and validate the biological effects of the natural endocrine disruptor Biochanin A (BCA) on ovarian steroidogenesis in primary human granulosa cells. The use of specific TAS2Rs confirmed and validated the molecular modifications induced by BCA, providing valuable insights into the signal transduction cascade underlining this pathway.

4.1 hGL5 Cell Line Expresses Bitter Taste Receptors

hGL5 cells were used to establish initial conditions and assess the effects of EDCs. Their rapid proliferation and ease of use present significant advantages over primary granulosa cells, which are often collected in limited numbers during oocyte retrieval, making them unsuitable for extensive gene regulatory studies and prone to biases stemming from genetic variability.

First of all, we further validated the suitability of hGL5 cells as model for human granulosa cells by confirming their expression of bitter taste receptors, consistent with our previous finding in primary granulosa cells (Semplici *et al.*, 2021). Indeed, as shown in **Figure 9A**, hGL5 expresses all TAS2R we tested, with TAS2R14 being the highest expressed (p-value <0.05). The intracellular levels of mRNA were also confirmed at protein level by Western Blot as shown in

Figure 9B. As previously observed in other studies, where TAS2R14 is the most highly expressed taste GPCR, its expression in hGL5 cells surpasses that of other taste receptors (Semplici *et al.*, 2021).

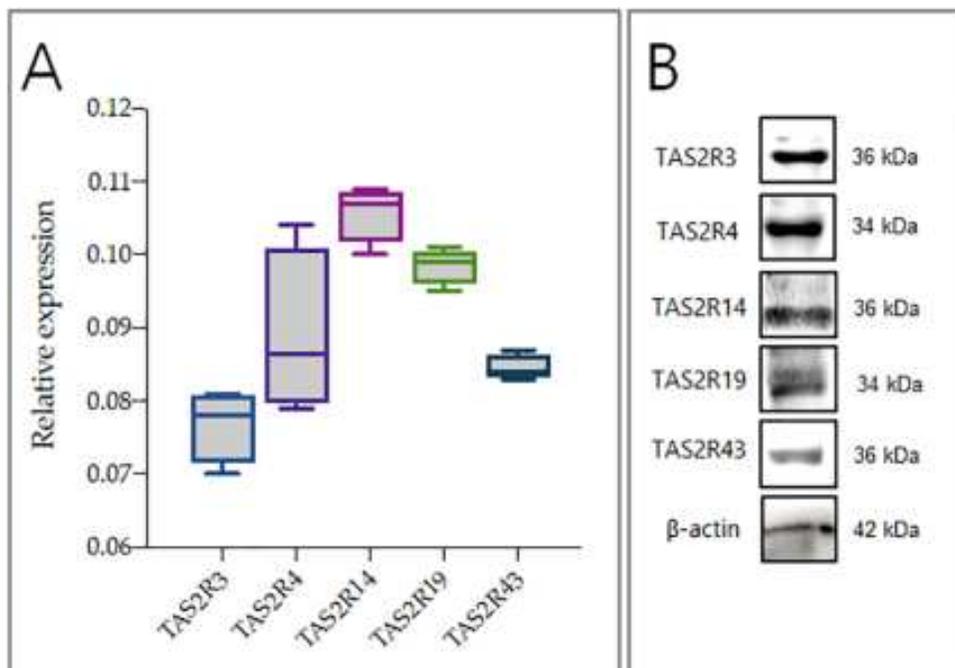


Figure 9. Expression of TAS2Rs in hGL5. **(A)** Relative expression of TAS2R3, TAS2R4, TAS2R14, TAS2R19, TAS2R43 in hGL5. Graphical diagrams are plotted as box-whisker plots, where boxes show the interquartile range with median and mean values. **(B)** Image of western blot analysis of TAS2Rs in hGL5. Equal protein loading was verified using the housekeeping β -actin.

4.2 Effect of EDCs Exposure on Viability of hGL5 Cells

A cell proliferation assay was conducted to determine the optimal cell density for *in vitro*, ensuring accurate assessment of the effective doses of the selected EDCs for subsequent experiments. According to the results presented in **Figure 10A**, the optimal cell density was 5000 cells/well obtained from the linear portion of the standard curve. hGL5 cells demonstrated sustained high proliferative activity, making them an ideal model for cell toxicity assays that require a substantial number of viable cells. Therefore, we next assessed the viability of hGL5 cells following exposure to selected EDCs known to act as specific agonists of bitter taste receptors. Based on literature and to replicate *in vivo* exposure levels, all

compounds were tested up to a maximum concentration of 1000 μM , with the exception of genistein, which was tested at a maximum concentration of 250 μM .

BCA exerted toxic effect at 200 μM (**Figure 10**), isoflavone at concentrations higher than 50 μM . Meanwhile, for caffeine, daidzein and genistein no toxic effects were observed (**Figure 10**), in agreement with other studies in the literature (Antosiak *et al.*, 2017; Meisaprow *et al.*, 2021). Based on these results, the highest non-toxic concentration of each compound was as follows: BCA: 10 μM ; Caffeine: 300 μM ; Daidzein: 500 μM ; Genistein: 5 μM ; Isoflavone: 25 μM .

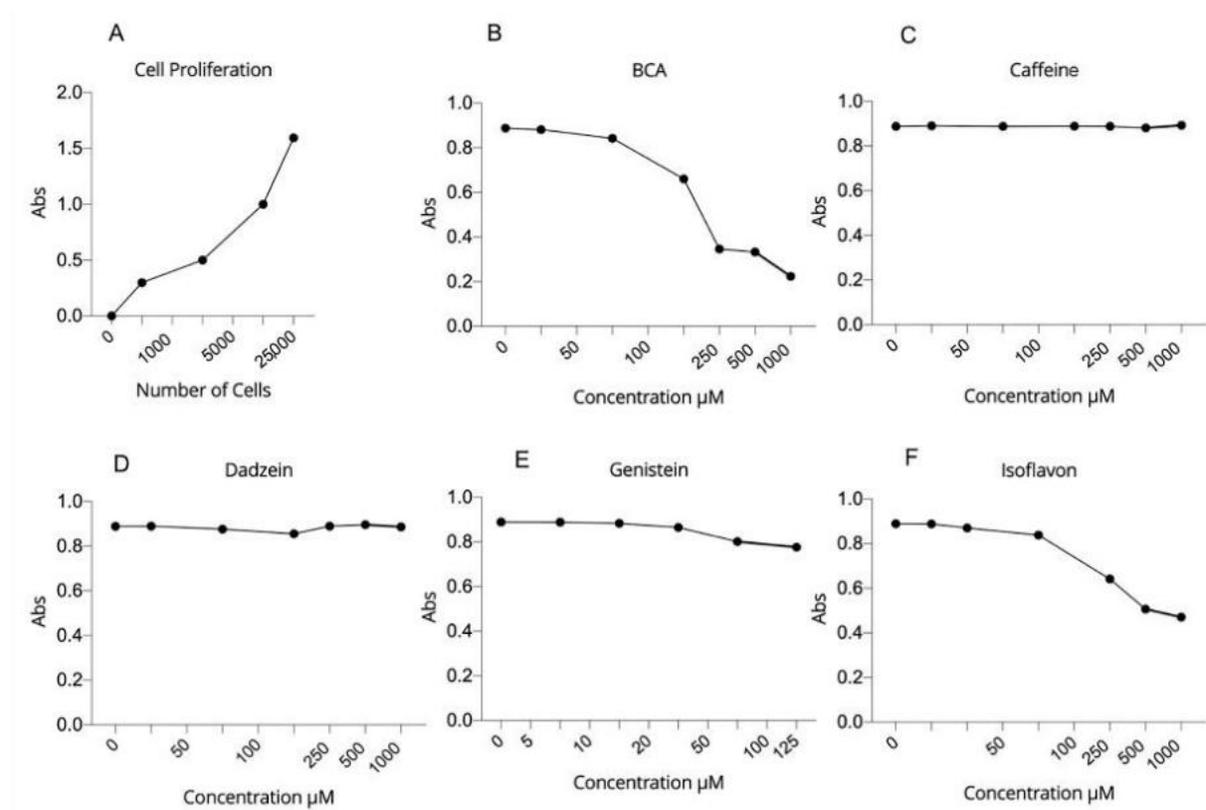


Figure 10. (A) Cell proliferation assay screening different cell concentrations plated in a 96 well plate to determine the optimal number of cells for subsequent experiments. Cytotoxicity assay was performed to assess the toxic effects of bitter compounds on hGL5 cells using the Cell Counting Kit-8 (Abcam). The non-toxic concentrations identified for each compound were as follows: 10 μM BCA (B), 300 μM caffeine (C), 500 μM daidzein (D), 5 μM genistein (E), and 25 μM isoflavone (F).

4.3 EDCs Act as Agonists on TAS2Rs Affecting hGL5 Mitochondrial Footprint

A previous study reported that TAS2R agonists, such as chloroquine and quinine, induce human airway smooth muscle cell death by affecting mitochondria structure and function (Pan

et al., 2017). Based on this observation, we used MitoTracker™, a fluorescent dye that labels mitochondria based on the membrane potential in living cells, to study mitochondrial shape and dynamics in hGL5 treated with TAS2R agonists. The mitochondria stained in living cells were then analyzed by Mitochondrial Network Analysis (MiNA). This toolset, designed for semi-automated analysis of mitochondrial morphology provides deeper insights into how mitochondrial dynamics are connected to cellular health (Valente *et al.*, 2017). The software processed the MitoTracker Red images by converting them into skeletonized forms and generating a dataset of mitochondrial parameters, including the total number of individuals and networks, mean branch size, mean branch length and network dimensions. Using MiNA macros, we analyzed the dynamic behavior of mitochondria in hGL5 cells exposed to TAS2Rs agonists. Treatment with BCA significantly increased the median branch length (p-value <0.05; data not shown), leading to an increased mitochondrial footprint (p-value <0.05), which represents the area occupied by mitochondrial structures. In contrast, caffeine treatments in hGL5 caused mitochondrial compaction, loss of branches and a clear reorganization of the mitochondria distribution. This resulted in a significant reduction in the mitochondrial footprint (p-value <0.05) (**Figure 11**), indicative of mitochondrial fragmentation. No significant modification occurred in mitochondria of hGL5 treated with other compounds.

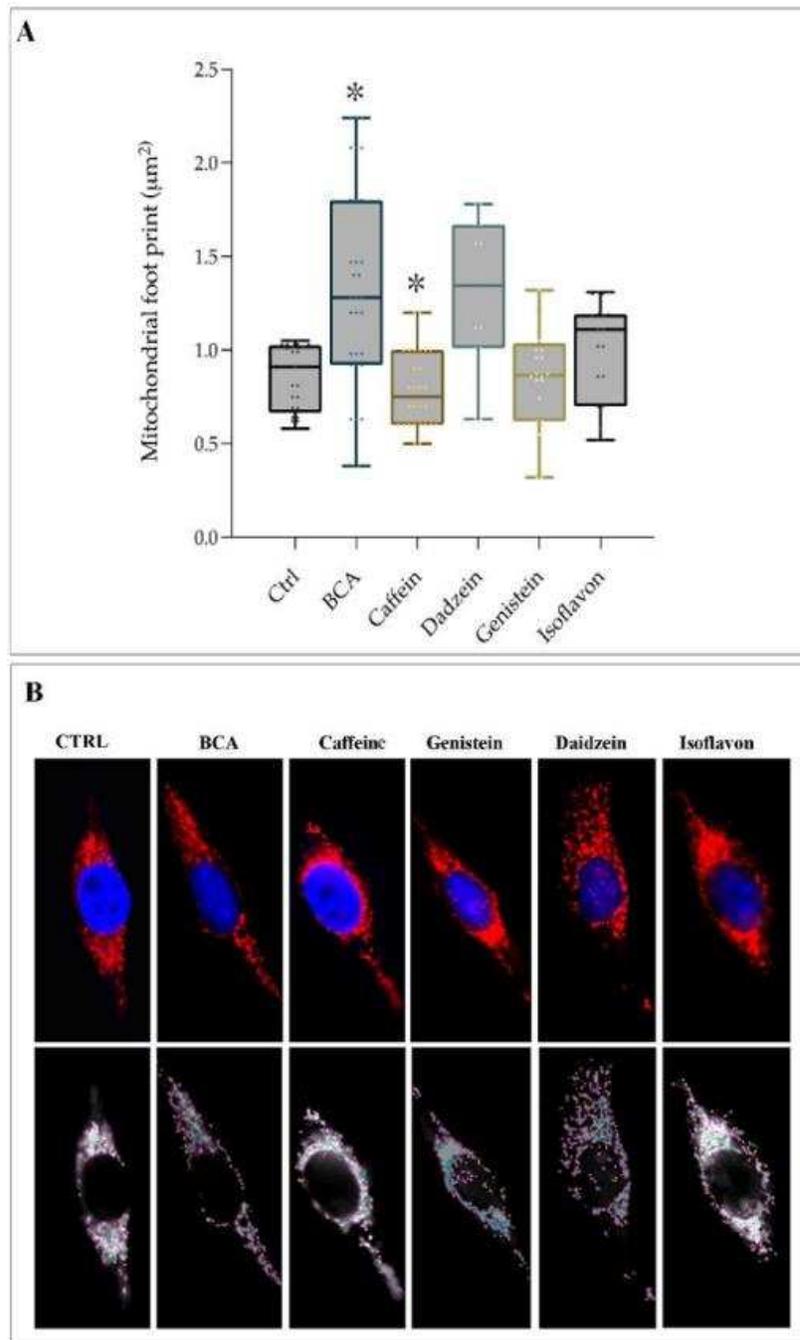


Figure 11. Results of mitochondrial network analysis in hGL5 cells. **(A)** Summary Box plots display the median (horizontal lines), interquartile range (box), and values within 1.5 times the interquartile range (vertical lines). Statistical comparisons were conducted to compare cells treated with different agonist. (*p-value <0.05) **(B)** Mitochondria footprint (red) of cells exposed to various compounds. Nuclei were DAPI-stained (blue). CTRL refers to untreated cell (control). Magnification: 630x. Staining images represent five independent experiments.

Electron microscopy validated the morphological changes we observed by fluorescence probing. In BCA stimulated cells, mitochondria display an increased diameter and an elongated

tubular shape, compared to the round mitochondria observed in the control cells (**Figure 12**). Additionally, the ultrastructure revealed large portions of filamentous ER located near enlarged mitochondria in steroid-producing cells. In contrast, smaller and round mitochondria were observed in hGL5 cells treated with caffeine (**Figure 12**), consistent with results from mitochondrial footprint analysis (**Figure 11**).

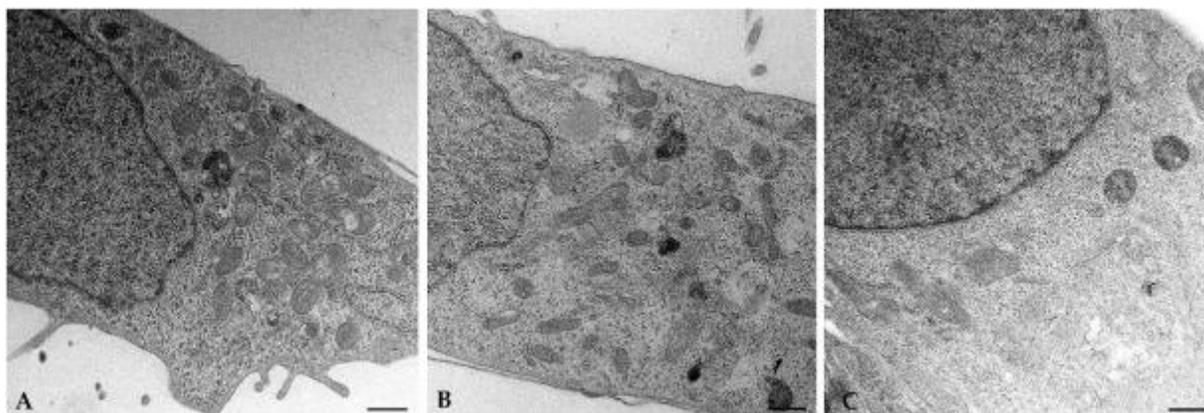


Figure 12. Representative transmission electron microscopy (TEM) images of untreated hGL5 cells (**A**), cells treated for 24h with BCA (**B**) 24h caffeine-treated hGL5 (**C**). Scale bar: (A, C), 500 nm; (B), 200 nm. Image representative of three independent experiments.

These morphological changes, previously associated to steroid hormone biosynthesis in steroidogenic cells, (Sreerangaraja Urs *et al.*, 2020; Bassi *et al.*, 2021), suggest that EDC exposure may disrupt these critical processes. Indeed, mitochondria plays a central role in steroid hormones biosynthesis, relying on dynamic processes such as fission and fusion, which are hallmark features of steroidogenic cells. Mitochondrial fusion, associated with an increased mitochondrial footprint, typically supports steroid hormone production, whereas mitochondrial fission is reduced during this process (Park *et al.*, 2019).

4.4 BCA and Caffeine Affect TAS2Rs Relative Abundance

According to our results showing BCA and Caffeine as the most effective agonists of TAS2Rs, we investigated the expression of each TAS2Rs after the treatment with their agonists. As shown in **Figure 13**, BCA at a concentration of 10 μ M increased the expression of TAS2R14 and TAS2R3 while decreasing the expression of TAS2R19. Meanwhile, caffeine increased the

relative abundance of TAS2R14 while significantly decreases the expression of TAS2R3, TAS2R19 and TAS2R43.

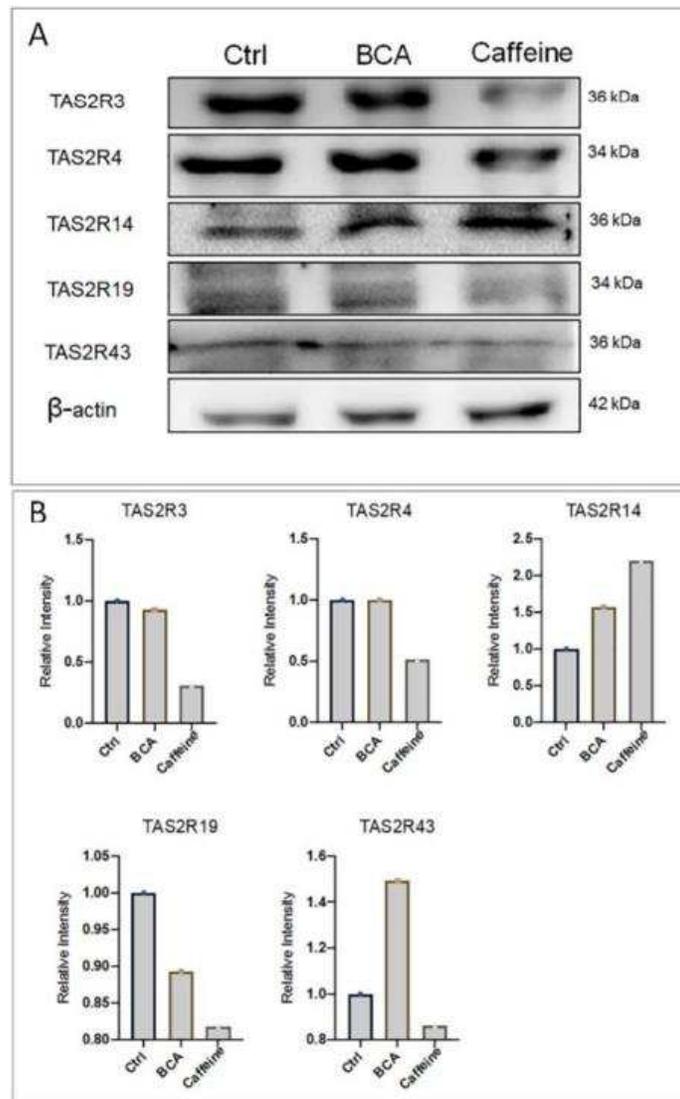


Figure 13. Expression of TASR proteins in hGL5 cells. **(A)** Western blot analysis of TAS2Rs on cells treatment with BCA and caffeine. β -actin was used as a loading control. **(B)** Relative quantification of western blotting band intensities.

4.5 BCA and Caffeine Affect Intracellular Lipid Storage and Steroid Secretion

Previous data suggest that a well-established link exists between taste receptors and steroid hormones. To investigate whether the effects of BCA and caffeine (both recognized TAS2R agonists) on mitochondria may influence steroidogenesis, we assessed the morphology and

the load of lipid droplets in hGL5 cells. These lipid droplets are functionally important in steroidogenesis as they serve as a reservoir of cholesterol, the essential substrate for the synthesis of steroid hormones. The morphology of lipid droplets in hGL5 cells treated with caffeine and BCA was assessed using Oil Red O staining. Morphological changes are evident as lipid droplets merge, resulting in size alterations post fusion (**Figure 14**). In cells treated with caffeine were observed smaller and spherical lipid droplets. Meanwhile, with BCA-treated cells formed relatively larger lipids droplets, which appeared spherical or irregular in shape (**Figure 14C**). To further asses intracellular lipid levels, we performed Oil Red O staining and extraction. As shown in **Figure 14D**, hGL5 cells cultured with caffeine for 24 hours exhibited lower lipid accumulation compared to controls. After Oil Red O staining, the color of lipid droplets in the control group was visibly brighter than in the BCA and caffeine-treated groups. Since the intensity of the color correlates with triglyceride and cholesterol levels within lipid droplets, these results indicate that lipid content was higher in the control group than in the treated cells. Statistical analysis using Tukey's Multiple Comparison test revealed a significant reduction in lipid content in caffeine-treated cells compared to both the control and BCA groups (p-value <0.001).

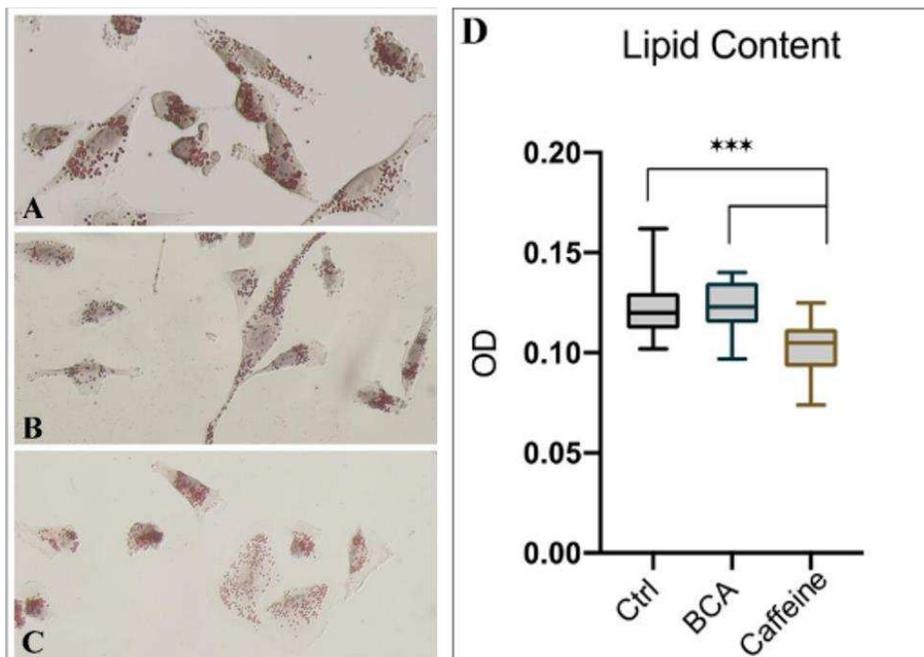


Figure 14. Lipid droplets analysis. Morphological characteristics of lipid droplets in cultured hGL5 cells. **(A)** Untreated samples. **(B)** 24 h, BCA-treated cells. **(C)** Caffeine-treated cells. Magnification: 400×. **(D)** Lipid content quantification by Oil red O extraction. *** Ctrl vs Caffein and BCA vs Caffein: significance p-value <0.001.

The increased lipid accumulation after BCA exposure aligns with the observed increase in mitochondrial footprint. Notably, an increase in intracellular lipid content has been associated with follicle growth, showing an exponential rise in large antral follicles and cultured granulosa cells (Sharma *et al.*, 1996; Gao *et al.*, 2019). Finally, we measured the secretion of steroid hormones, estrogen and progesterone, in the culture medium of control and treated cells with agonist after 24 hours incubation. As shown in **Figure 15**, E2 secretion was elevated In BCA treated cells, meanwhile exhibiting a decrease in P4.

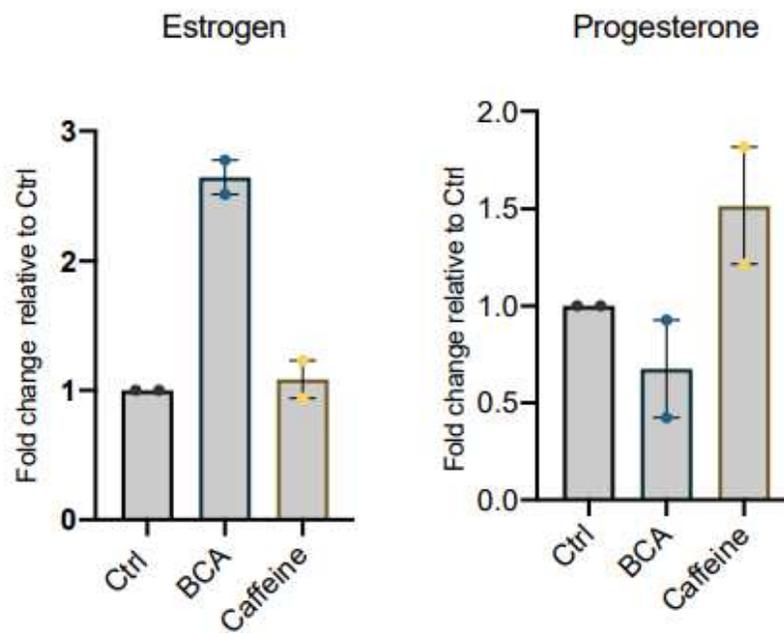


Figure 15. The effect of BCA and caffeine on P4 secretion from hGL5. The cells were cultured for 24 h with BCA 50 μ M or caffeine. Data are expressed as a fold change relative to the control culture (set as 1).

BCA increased E2 secretion (p-value <0.05), while caffeine did not; conversely, BCA induced a decrease, although not statistically significant, in P4 secretion. These findings are consistent with a study demonstrating a direct correlation between serum estradiol levels, mitochondrial mass, and mitochondrial membrane potential in primary human cumulus cells (Sreerangaraja Urs *et al.*, 2020). In addition, previous studies have highlighted a dose-dependent effect of

various isoflavones. Additionally, a survey of over 30 isoflavones and related compounds inhibit key enzymes in neurosteroid and steroid hormone biosynthesis, with their impact on steroidogenesis being dose-dependent, suppressing at lower doses and promoting at higher ones (Wong and Keung, 1999; Almstrup *et al.*, 2002).

4.6 Primary human granulosa cells: BCA affects TAS2R14 and TAS2R43 expression and regulates steroidogenesis

Firstly, we assessed the cytotoxicity of BCA in human primary GCs isolated from the follicular fluid of women undergoing IVF. No toxic effects at the tested concentrations. Consequently, we proceeded with treatment at the optimal concentration of 10 μ M, and explored its potential association with TAS2R expression levels. As shown in **Figure 16A, B**, an increase in expression was observed for *TAS2R14* and *TAS2R43*. This effect was suppressed by sulfamoylbenzoic acid (SBA), the selective antagonist of TAS2R14 as well as PRO and citronelle (CITRO), two selective antagonists of TAS2R43. Our findings also demonstrate that PRO and CITRO effectively inhibit *TAS2R14*, while SBA can oppose the effect of BCA in inducing *TAS2R43* expression.

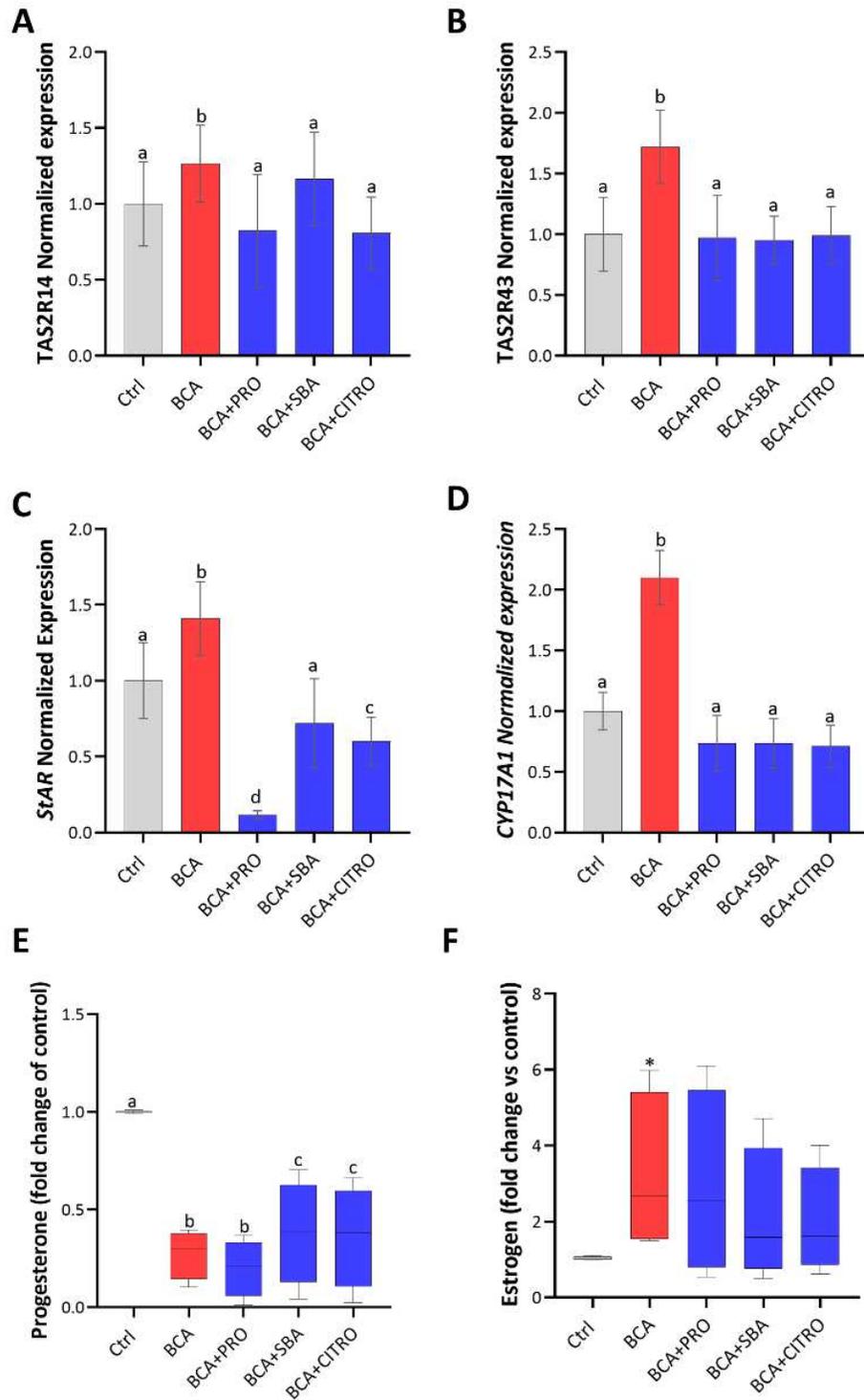


Figure 16. Expression of TAS2R in granulosa cells treated with BCA, BCA+ PRO, BCA+ SBA and BCA+ Citro (**A-D**). (**E**) The effect of treatments on progesterone secretion and (**F**) estrogen secretion from granulosa cells. Data are expressed as a fold change relative to control (set as 1).

All antagonists (PRO, SBA, CITRO) significantly decreased TAS2Rs expression, thus confirming this EDC directly affects the TAS2Rs pathway. Previous report has shown that saccharin, an agonist for TAS2Rs receptors can modulate expression of enzymes involved in steroidogenesis (Jiang *et al.*, 2021). Therefore, we investigated the expression of *StAR* and *CYP17A1*, critical enzymes for steroidogenesis pathway, involved in the transport of cholesterol to the inner mitochondrial membrane and the other converting pregnenolone to 17 α -hydroxypregnenolone. BCA treated cell exhibited a 70% increase in *StAR* mRNA levels compared to control (p-value <0.05). Similarly, the expression of *CYP17A1* was two-fold higher on BCA treated cells compared to the control. Meanwhile, when the antagonist treatment was introduced along with the BCA treatment, the expression of both genes was significantly reduced (p-value <0.05). By using specific TAS2R antagonists, we showed that BCA effectively activates TAS2Rs in primary GCs, leading to a marked upregulation of the steroidogenic enzymes *StAR* and *CYP17A1*.

After 24-hour exposure to BCA and TAS2R antagonists, we measured the secretion of steroid hormones in the cell culture medium (**Figure 16E, F**). BCA treated cell secreted a significantly lower level of progesterone (p-value <0.001), an effect that was significantly counteracted by SBA and CITRO, but not by PRO (**Figure 16E**). In contrast, there was a substantial increase in estrogen secretion (p-value <0.001), which was partially reduced by the antagonist SBA and CITRO (**Figure 16F**). This disparity in hormone production by BCA stimulation may suggest a possible biphasic effect on steroidogenesis. Such effect is observed in bovine granulosa cells, where Biochanin A exhibits a dose-dependent effect on steroidogenesis, enhancing progesterone production by 50% at 185 nmol/L while inhibiting it at concentrations above 176 nmol/L (Kaplanski *et al.*, 1981). Our results demonstrate a comparable effect on steroidogenesis with BCA at a concentration of 10 μ M, further supporting these observations. Notably, the effects of BCA are reversed by selective TAS2R inhibitors, confirming that its actions are mediated through bitter taste receptors. While the exact role of ovarian TAS2R activation in steroidogenesis remains unclear, existing evidence suggests that these receptors are involved in regulating hormone synthesis and balance by modulating steroidogenic pathways in various cell types (Jiang *et al.*, 2021; Liu *et al.*, 2022). For example, studies indicate

that the activation of bitter taste receptors may inhibit progesterone production, potentially through NO/cGMP and apoptotic signaling pathways (Jiang et al., 2021). In a murine PCOS model, oral administration of KDT501, a ligand for the TAS2R4 orthologue, effectively alleviated PCOS-related endocrine and metabolic disturbances and restored reproductive function, outperforming the effects of the PPAR γ agonist. Specifically, KDT501 reduced testosterone and androstenedione levels without significantly affecting LH or FSH, while also decreasing hepatic lipid accumulation and body fat (Wu *et al.*, 2019). Interestingly, treatment of control and PCOS rats with the artificial sweetener saccharin sodium, which activates sweet (T1R3) and bitter taste receptors (T2R3), resulted in alterations in steroidogenesis and hormonal imbalance. Such treatment promoted the expression of steroidogenic-related factors *StAR*, *CYP11A1* and *17 β -HSD* as well as increased levels of testosterone, estrogen and progesterone (Magdy *et al.*, 2024).

Multiple studies have demonstrated the involvement of ERK1/2 in the regulation of progesterone production in granulosa cells. To further explore the role of BCA on modulating steroidogenesis, we examined ERK1/2 activation by immunoblotting (**Figure 17A, B, C, D**) using a phospho-specific antibody that detects dually phosphorylated ERK1 and ERK2, a standard approach for indirectly assessing ERK activity. As illustrated in **Figure 17D**, levels of activated ERK (pERK) significantly decreased after treatment with BCA (p-value <0.05). This result aligns with previous studies indicating that ERK1/2 is essential for regulating progesterone production in granulosa cells (Seger *et al.*, 2023). Notably, both ERK and PKA are vital mediators of efficient steroidogenesis, with ERK playing a key role in positioning StAR correctly on the mitochondrial membrane (Duarte *et al.*, 2012; Jefferi *et al.*, 2022). The observed decrease in ERK levels may help explain the reduction in progesterone production, despite an increase in *StAR* transcription. Furthermore, our study revealed a significant decrease in intracellular cAMP levels, a critical second messenger in LH-induced steroidogenesis.

Furthermore, intracellular cAMP levels, a key second messenger involved in steroidogenesis signaling, were reduced in BCA-treated cells (**Figure 17E**). This reduction was partially reversed by the treatment with TAS2R antagonists. These results align with data suggesting that TAS2R

activation stimulates PDE activity and confirm that BCA, through TAS2R receptors, can modulate steroidogenesis in granulosa cells (Jeruzal-Świątecka *et al.*, 2020). These findings collectively emphasize BCA's involvement in modulating key signaling pathways necessary for optimal steroidogenesis, likely through its interaction with TAS2R receptors.

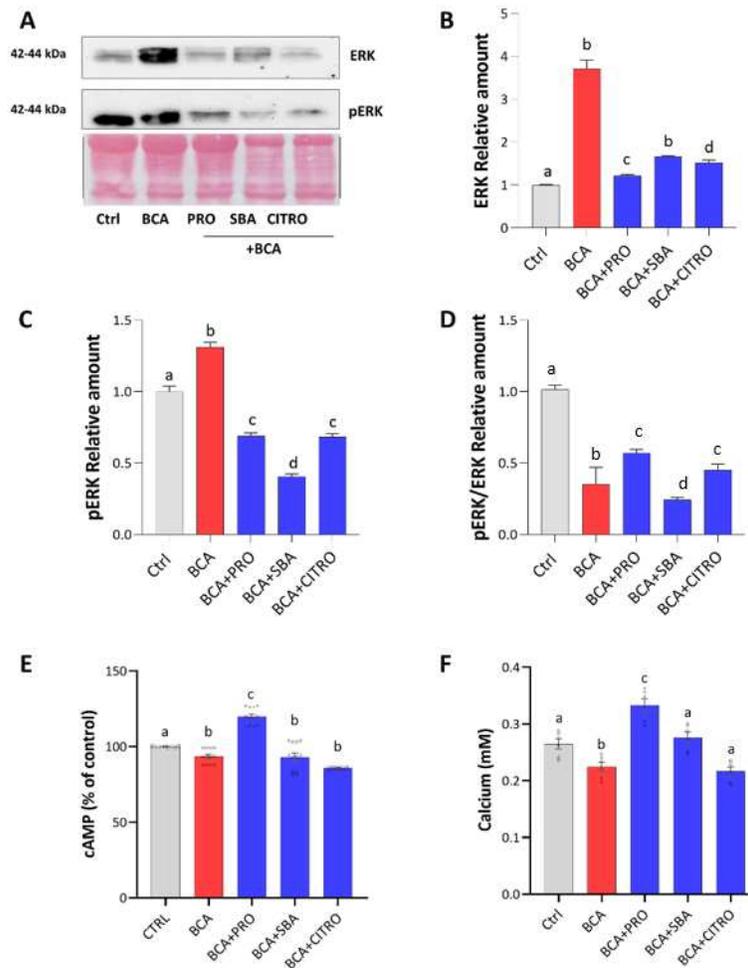


Figure 17. This figure illustrates experimental results showing ERK1/2 signaling, cAMP levels, and calcium concentrations under BCA, BCA+ PRO, BCA+ SBA, BCA+ CITRO treatment conditions. **(A)** The western Blot displays the total levels of ERK1/2 (upper band) and pERK (lower band). **(B)** Relative Quantification of ERK1/2 levels **(C)** Relative Quantification of pERK levels **(D)** Relative quantification of pERK/ ERK ratio **(E)** Levels of cAMP expressed as percentages compared to the control **(F)** Calcium concentration measured and presented as absolute values (mM).

4.7 BCA affects Lipid droplet (LD) homeostasis

Treatment of granulosa cells with BCA, caused an increase, in the cytoplasmic accumulation of intracellular lipids after the staining with OilRed O, a selective stain of neutral lipids, despite not significant (**Figure 18**). Meanwhile, treatment with selective antagonists of TAS2R43 and BCA, resulted in a decrease in lipid content, as shown in **Figure 18**. In contrast, CITRO, didn't counteract the lipid-accumulation effect of BCA. Furthermore, lipid droplet morphology analysis in BCA-treated GCs showed smaller, predominantly spherical lipid droplets (p-value <0.001) (**Figure 18**). The size of lipid droplets was significantly increased following the treatment with TAS2Rs antagonists (p-value <0.001). The decrease in lipid droplet size after BCA treatment is consistent with its established ability to inhibit lipid accumulation (Yu *et al.*, 2020). This observation further highlights the role of TAS2Rs in regulating cellular metabolism and energy dynamics. Specifically, it suggests that BCA may influence lipid storage or utilization, potentially affecting cellular energy balance and steroidogenesis (Talbot *et al.*, 2020). In this context, TAS2R138 has been shown to facilitate the degradation of lipid droplets in neutrophils, providing additional evidence for its role in lipid metabolism (Pu *et al.*, 2021).

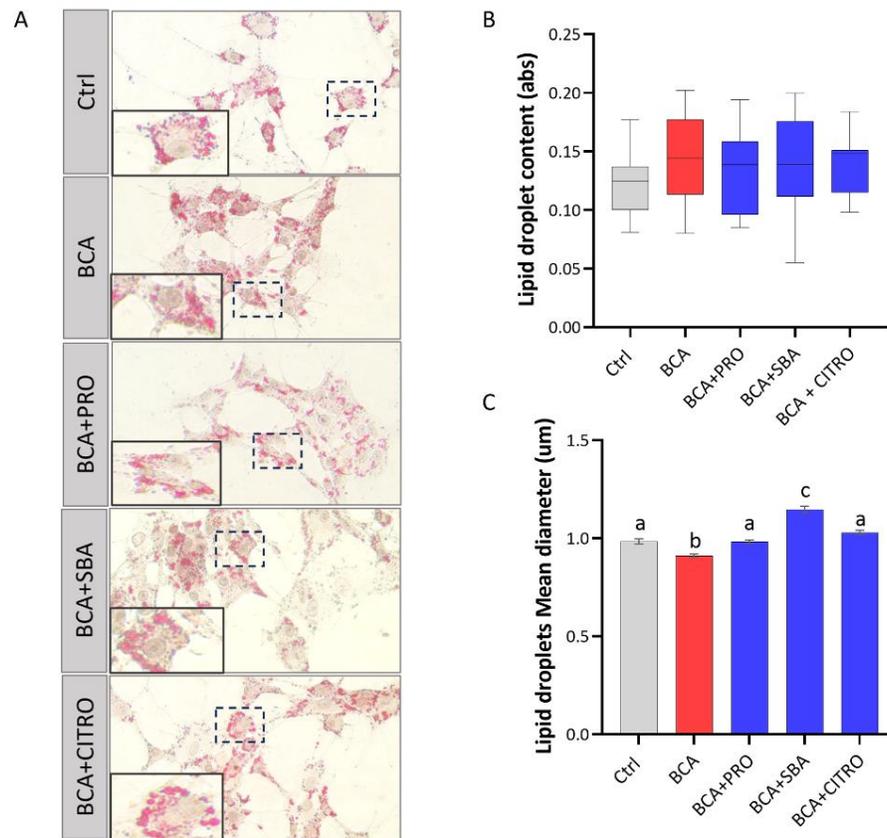


Figure 18. Lipid droplets analysis. Morphological characteristics of lipid droplets in human granulosa cells. **(A)** Morphological observation of CTRL, BCA, BCA+ PRO, BCA+ SBA, BCA+ CITRO treatment conditions **(B)** Absorbance values after quantification of lipid droplet content **(C)** Lipid droplet parameters (mean diameter) measurements in μM , compared to the control.

4.8 TAS2R antagonist reverts the effect of BCA on mitochondrial dynamics.

The Mitochondrial Analyzer is a software which detects morphological and structural features of mitochondria, providing an accurate assessment of mitochondrial organization. The latter is of important interest due to the fact that is the site where steroidogenesis takes place. Using this tool allowed us to observe that the number of mitochondria per cell remained equal throughout all treatments, while BCA treatment significantly increased the mean mitochondrial area (p-value <0.001) compared to the control, indicating activation of mitochondrial fusion (**Figure 19A, B**). This effect was effectively reversed by TAS2Rs

antagonists (p-value <0.001), which, counteracted the BCA-induced changes and appeared to inhibit mitochondrial fusion (Figure 19A, B).

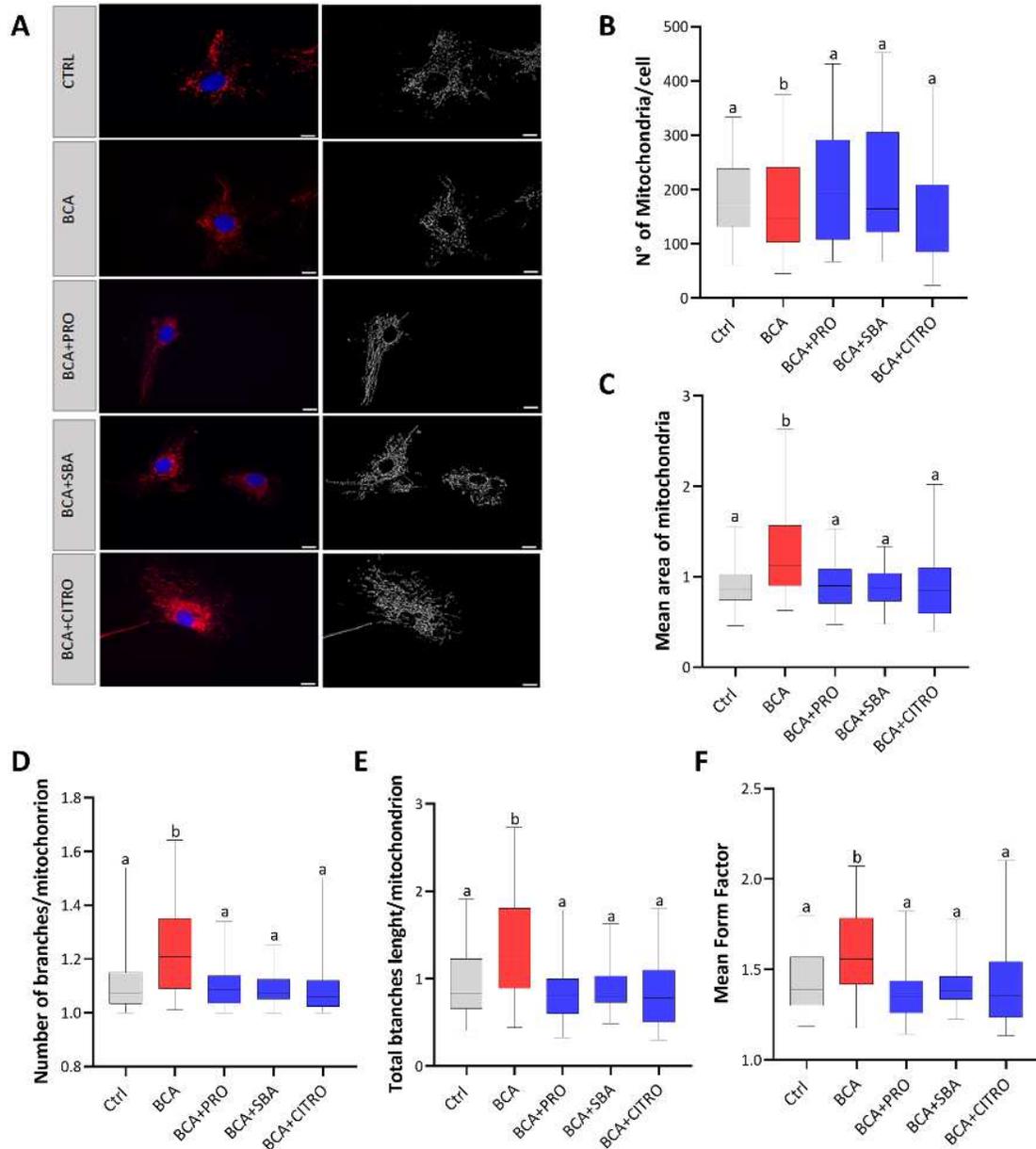


Figure 19. Results of mitochondrial network analysis in human granulosa cells. **(A)** Mitochondria footprint (red) of cells exposed to various compounds. Nuclei were DAPI-stained (blue). CTRL refers to untreated cell (control). Magnification: 630x. Staining images represent five independent experiments. **(B)** Quantification of the number of mitochondria per cell. **(C)** Evaluation and quantification of mean area of mitochondria **(D)** Measurement of network complexity by quantification of mitochondrial branches **(E)** Measurement of network connectivity by quantification of total branch length per mitochondria **(F)** Mean form factor quantification measurement to evaluate elongation and shape.

Another important parameter to consider while investigating changes in mitochondrial morphology is the alterations in mitochondrial networks, as these network modifications are closely linked to energy metabolism (Shin *et al.*, 2016). BCA treatment significantly increased both the number of branches and the total branch length per mitochondrion, indicating enhanced network complexity (**Figure 19C, D**; p-value <0.001). Conversely, antagonist treatment led to a significant reduction in both branch number (**Figure 19A**; p-value <0.05) and total branch length (**Figure 19C, D**; p-value <0.01).

To assess the overall complexity of the mitochondrial network, we calculated the average form factor, where higher values reflect more intricate networks, meanwhile lower values indicate simpler ones. As illustrated in **Figure 19**, the average form factor is significantly elevated in BCA-treated GCs, corroborating the increase in network complexity observed (**Figure 19E**; p-value <0.001). Notably, the antagonists reversed the effects induced by BCA, leading to a significant reduction in mitochondrial network complexity as well as a higher level of potential energy. Using Mitochondrial Analyzer, we found that BCA-treated cells displayed greater mitochondrial network complexity, suggesting an increase in mitochondrial fusion a crucial process for steroid production (Duarte *et al.*, 2012) Mitochondrial morphology changes have shown to be correlated with oxidative phosphorylation activities as well as energy efficiency (Glancy *et al.*, 2020). Meanwhile, fission is positively correlated with increased ROS production (Park *et al.*, 2011).

To confirm this correlation in our cells, we measured ROS level in treated GCs. The results, shown in **Figure 20** indicate that BCA treatment reduce ROS production. This effect is, partially counteracted by TAS2R-selective antagonists, although these changes do not reach the statistical significance (**Figure 20A**).

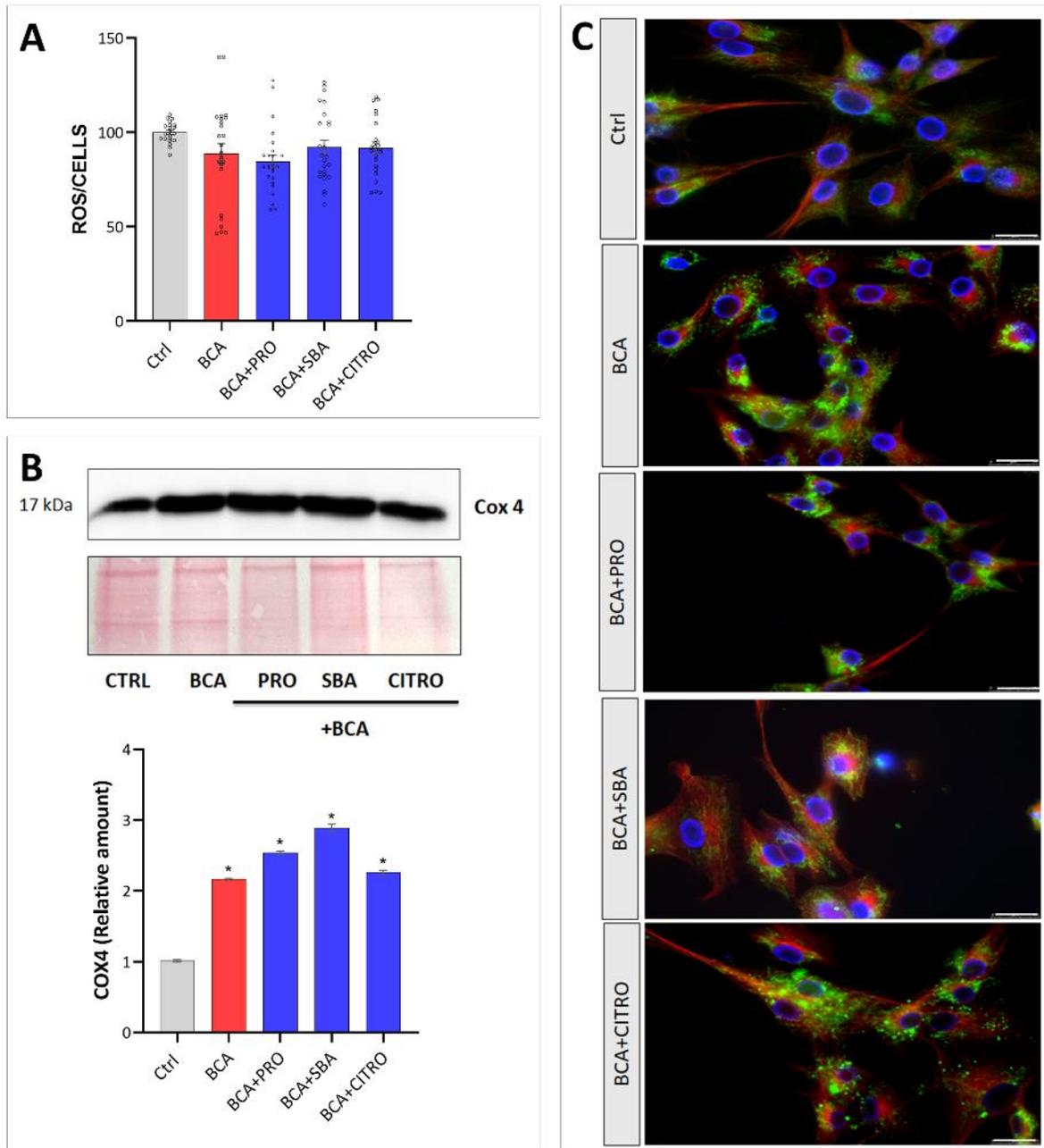


Figure 20. (A) Measurement of ROS levels in human granulosa cells throughout different treatments. (B) Western Blot displaying COX4 expression (C) Representative fluorescence microscopy images showing COX labeling (green) in cells under different conditions.

The production of ROS is strongly linked to COX4 levels, a critical marker of mitochondrial function. COX4 modulates BMI1 expression by decreasing mitochondrial ROS production (Oliva *et al.*, 2015). Immunoblot analysis of GC showed elevated COX4 expression, with BCA treatment significantly enhancing its relative abundance (Figure 20); this effect was not

reversed by antagonist treatment. Immunofluorescent staining revealed that COX4 was tightly clustered around the perinuclear region in untreated cells, whereas BCA treatment promoted mitochondrial expansion throughout the cytoplasm (**Figure 20**). It has been reported that cells with hyperfused mitochondria exhibit higher respiratory activity and increased oxidative capacity, while fragmented mitochondria are associated with lower energy demand, reduced respiratory activity, and increased cellular stress (Westermann, 2012; Youle and van der Bliek, 2012). A well-developed mitochondrial network, such as the one induced by BCA treatment, is linked not only to mitochondrial fusion and enhanced OXPHOS capacity but also to changes in mitochondrial dynamics, such as density, number, and spatial distribution, that can affect mitochondria-driven ROS propagation (Liesa and Shirihai, 2013). Our results showed that BCA treatment decreases ROS production, an effect that is partially reversed by TAS2R selective antagonists, though this difference did not reach statistical significance.

5 Results and Discussion: PART II

The second part of this thesis aimed to develop an innovative approach for evaluating the impact of endocrine-disrupting molecules on female fertility. To achieve this, 3D organoids derived from endometrial biopsies were used to investigate molecular pathway alterations induced by these compounds during the proliferative and secretory phases of the menstrual cycle, paying particular attention to the window of implantation. First, we characterized using RNA sequencing (RNA-seq) the gene expression fingerprinting of EEO induced *in vitro* to mimic the different phases of the endometrial cycle. The proliferative phase was induced by treating EEO with estradiol (E2), while the mid-luteal phase of the secretory cycle, representing the window of implantation, was modeled using a combination of E2, progesterone (P4), and cAMP. Next, we optimized a cytotoxicity assay in the EEO model, determining the optimal cell density and selecting the most suitable viability assay for the experimental design. The organoids were then exposed to a phthalate mixture to replicate the complex *in vivo* exposure scenario, where the human body encounters multiple chemicals simultaneously. This approach overcomes the limitations of studying individual chemicals in isolation. Finally, RNA-seq provided an overview of changes under different treatments, offering valuable insights into the molecular mechanisms underlying endocrine disruptor exposure.

5.1 Establishment and characterization of EEOs as a suitable model for *in vitro* analyses

Endometrial fragments, after proper processing and 7 days of culture under the described conditions, formed organoid-like structures that grew in size (**Figure 21**). Once formed, these organoids were dissociated into single cells for passaging and replating. The resulting expanded endometrial organoids (EEOs) preserved their structural and morphological integrity for up to 15 passages.

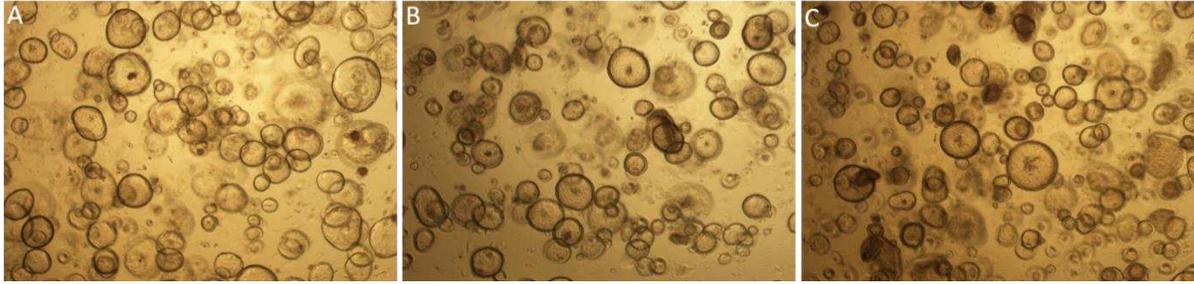


Figure 21. Representative image depicting the formation of endometrial epithelial organoids (A-C). Organoids are cultured in Matrigel matrix and are observed at 40x magnification under a bright field optical microscope.

To replicate the uterine microenvironment, undergoing specific changes in response to ovarian hormones, EEOs were treated with E2 to simulate the proliferative phase (ppEEO) and with E2, P4, and cAMP to mimic the mid-secretory phase, that is the window of implantation (mspEEO). To validate these *in vitro* conditions, RNA sequencing (RNA-seq) was performed to comprehensively characterize the resulting gene expression profiles. Differential expression analysis identified 294 genes altered between the two phases, with 228 upregulated and 66 downregulated genes in mspEEO compared to ppEEO, highlighting distinct transcriptomic changes associated with the menstrual cycle phases. Among the upregulated genes (1.41-fold increase), was monoamine Oxidase A (MAOA), known for its critical role in establishing endometrial receptivity during the window of implantation. Studies shown MAOA expression is significantly higher in mid-secretory phase than in the early secretory phase, likely regulated by progesterone stimulation, aiding endometrial preparation for implantation (Choo *et al.*, 2023). Elevated MAOA transcript and protein levels during the receptive phase have been linked to successful implantation, while reduced levels are associated with repeated implantation failure. Though its precise role remains unclear, MAOA's marked increase during WOI suggests it may regulate oxidative stress, hormonal signaling, or metabolic adaptation (Henriquez *et al.*, 2006).

As shown in **Figure 22**, Gene Ontology (GO) analysis, analysis provided valuable insights into the pathways differentially regulated by treatments mimicking menstrual cycles. The Cellular Component analysis, revealed significant enrichment (p -value <0.0001) in pathways associated with extracellular matrix, cell surface, cell-cell junction and focal adhesion. These findings suggest structural changes between the proliferative and secretory phases of the

menstrual cycle. During secretory phase, alterations in the extracellular matrix reflect endometrial remodeling, essential for tissue growth, repair and preparation for potential implantation. Changes at the cell surface align with literature, indicating that epithelial and stromal cells coordination promote tissue regeneration. Furthermore, variations in cell-to-cell junctions suggest dynamic remodeling of junctional complexes, with increased stability during the proliferative phase and disruption during the secretory phase to facilitate cellular migration and trophoblast invasion.

The second chart, as shown in **Figure 22**, presents results from the GO Molecular Function, which focuses on the specific biochemical activities of gene products at the molecular level. Key pathways identified in our analysis include extracellular matrix structural component, calcium dependent protein binding, structural molecular activity and cell-cell adhesion mediator activity, all of which are highly significant (p-value <0.008). As previously described, changes in extracellular matrix structural components are characteristic of changes in menstrual cycle, since studies suggest that their regulation follows a cyclic pattern, driven by steroid-induced remodeling function in the endometrium. (Diaz *et al.*, 2012). Concurrently, dynamic changes in cell to cell adhesion mediator activity, such those involving molecules like β -catenin, play a dual role in maintaining the structural integrity of adhesion complexes and regulating intracellular signaling pathways critical for cellular functions. β -catenin is expressed in both phase of the menstrual cycle and is known to contribute to endometrial receptivity. Furthermore, other adhesion molecules such as L selectins, exhibit cyclical expression pattern. Studies report increased L-selectin levels during the early and mid-secretory phases, underscoring their role in preparing the endometrium for implantation (Diaz *et al.*, 2012; Margarit *et al.*, 2009).

ppEEO vs mspEEO

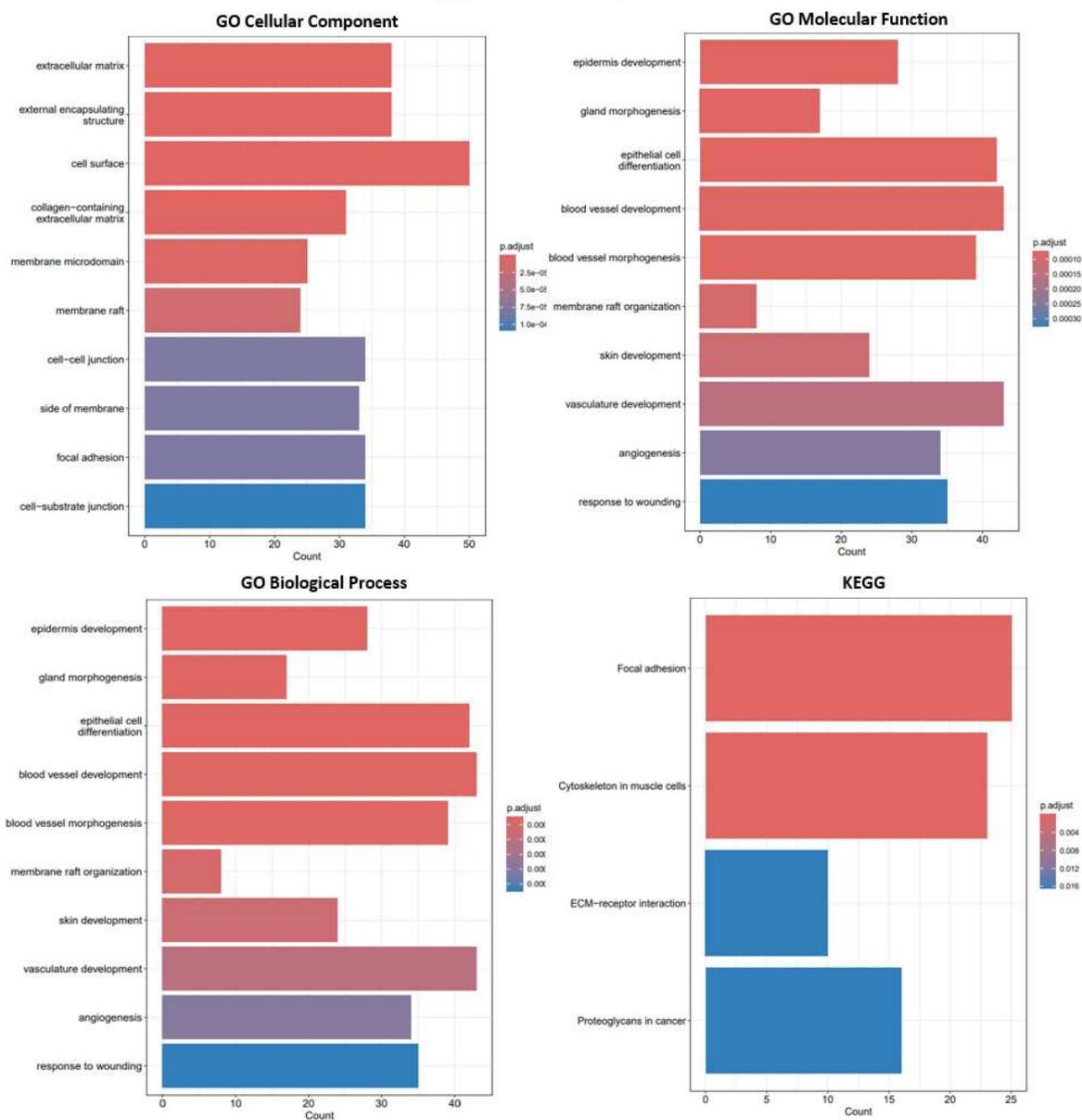


Figure 22. Picture shows 4 different biological pathway enrichment analysis based on differentially expressed genes confronting ppEEO vs mspEEO groups. Pathways are derived from Go cellular component, Go Molecular function and Go Biological Process. On the y axis are listed the biological pathway, ordered by significance (**p-value** <0.05) and on the x axis the number of DEG genes between the 2 conditions for each pathway.

Go Biological Process analysis, categorizes and interprets differentially regulated genes within specific biological processes (**Figure 22**). The pathways identified, all with p-value <0.0008, include epidermis development (most significant), epithelial cell differentiation, gland

morphogenesis and blood vessel development. These processes reflect the structural and morphological changes that distinguish the phases of the menstrual cycle.

During the secretory phase, progesterone drives epithelial cells toward differentiation, preparing them for secretory activity essential for establishing a receptive environment for implantation. For instance, glandular epithelial cells differentiate into secretory cells, producing nutrients and growth factors required for embryonic support. These findings align with studies by Tsoleva and colleagues (2022), who demonstrated that organoid models derived from isolated endometrial epithelial cells adapt to differentiation signals based on Wnt/Notch stimulation. Specifically, Wnt signaling increased ciliated cell proportions, while Notch signaling enhanced secretory cell proportions in organoids treated with progesterone (Tsoleva *et al.*, 2022). Additionally, KEGG pathway corroborates the GO enrichment findings, highlighting focal adhesion as the most significantly altered pathway (25 DEG genes) followed by the extracellular matrix-receptor interaction pathway (more than 10 DEG genes). These pathways further emphasize the dynamic remodelling processes occurring in the endometrium across menstrual cycle phases.

The heatmap illustrated in **Figure 23**, depicts the expression profiles of differentially expressed genes associated with the extracellular matrix pathway as the most significant pathway altered between two conditions, ppEEOs and mspEEOs. There are 11 genes differentially expressed, forming clearly distinguishable clusters. These clusters between the two treatments indicate the up regulation of several genes in the Extracellular Matrix pathway, while others are down-regulated. All the identified genes play a role in Extracellular Matrix pathway, which is a key pathway involved in epithelial endometrial differentiation throughout the menstrual cycle.

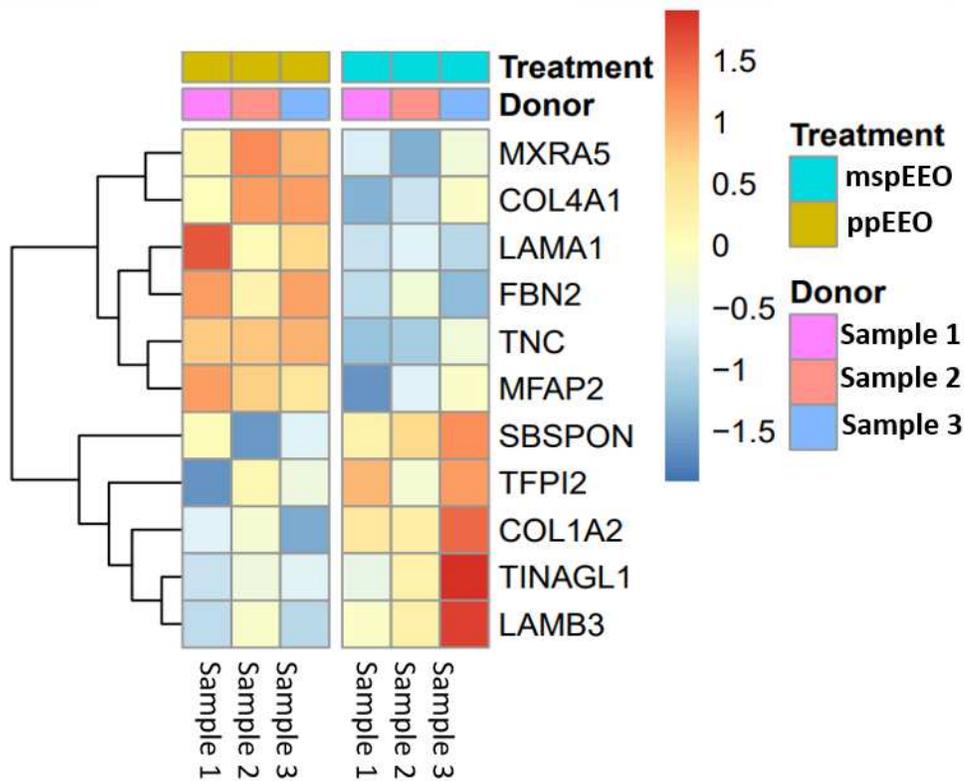


Figure 23. Heatmap illustrated the expression patterns of genes significantly differentially expressed involved in Extracellular Matrix pathway between ppEEO vs mspEEO. Colors indicate the fold-change in expression between two group: Blue indicating down regulation; Red indicates up-regulation. The heatmap rows represent genes, while the columns correspond to samples.

5.2 Optimization of *In Vitro* Parameters for Using EEOs as a Platform to Screen EDC

We first performed a proliferation assay for our EEOs by using Resazurin Viability assay, a colorimetric assay which emits fluorescence as well, allowing for two different measurements. Endometrial epithelial single cells were plated at different cell concentrations, of 1000, 2000, 3000, 4000, and 5000 cells per well. Each concentration was plated in triplicates and incubated for 7 days to allow the formation of 3D organoid structures. This test allow both fluorescent intensity and colorimetric absorbance analysis, which can be repeated over a period of 4 hours, making it a versatile and sensitive approach for measuring cell proliferation.

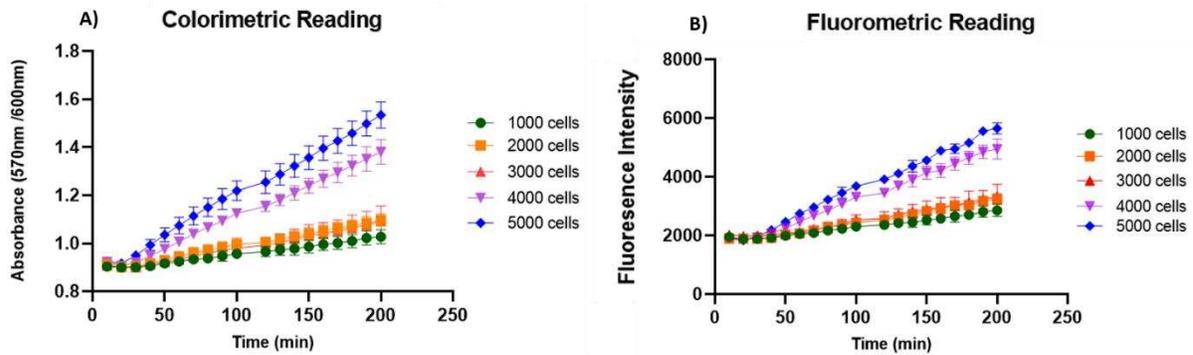


Figure 24. Cell proliferation assays using Resazurin viability test. Epithelial endometrial organoid cells were plated on a concentration from 1000 up to 5000 cells and the graphs illustrates the linear range in time of: **A)** Colorimetric absorbance **B)** Fluorometric intensity.

Fluorescent intensity and absorbance ratios were measured every 10 minutes over a total of 200 minutes. Both fluorimetric and colorimetric assays demonstrated clear, logical dependencies on cell number and time (**Figure 24**). The results indicate a strong correlation between the number of cells plated and the signals intensity, supporting assay's accuracy in assessing cell proliferation and its validity for subsequent experiments. Data indicated a linear increase in both fluorescent intensity and absorbance ratio over time. Based on these analysis it was concluded that the optimal cell density for our experimental design lies between 3,000 and 5,000 cells per well. This range ensures accurate and reliable measurements while minimizing variability in the results as well as consistent and reliable results by minimizing cell culture challenges such as nutrient limitation or space restriction, which can affect the assay's validity. Finally, we determined the concentration of 4000 cells per well as the optimal concentration for subsequent cytotoxicity assay.

Considering that EEOs are plated in Matrigel matrix, we aimed to investigate whether this structural encapsulation would affect absorbance measurements in the assay. To address this, we compared absorbance values between readings of EEOs in dissolved matrigel before the proliferation assay, and the intact Matrigel structure containing EEOs. Fluorescent intensity and absorbance ratios were measured every 10 minutes over 200 minutes using a spectrophotometer. Also in this case, both assays demonstrated clear, logical dependencies on cell number and time. These results show a clear positive correlation between cell number

and absorbance, consistent with the non-dissolved condition, though absorbance values were generally lower in the dissolved state, as shown in **Figure 25**.

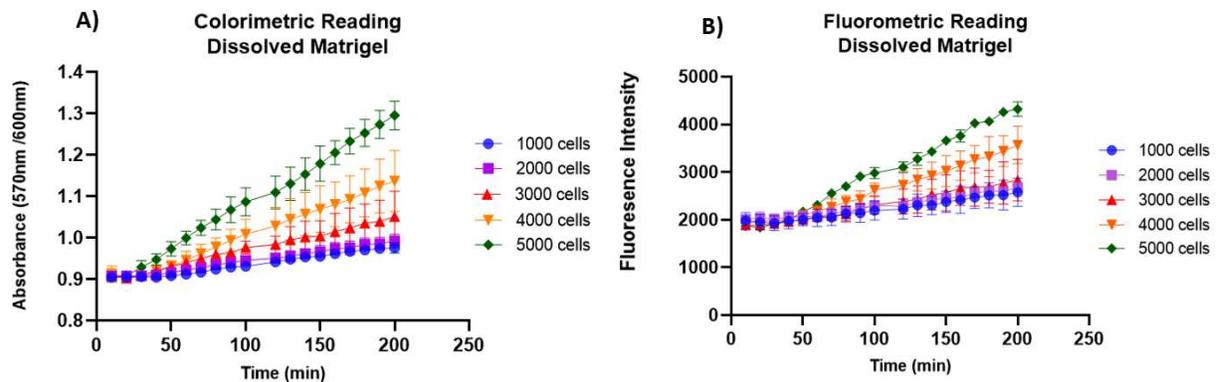


Figure 25. Cell proliferation assay using Resazurin Viability test. Matrigel was dissolved before the measurement. Epithelial endometrial organoid cells were plated on a concentration from 1000 up to 5000 cells per well and the graph illustrates: **A)** the colorimetric absorbance and **B)** the fluorescence intensity values.

There are some differences in values on the colorimetric absorbance and the fluorescence intensity between the two conditions taken into consideration. Comparing the values obtained for both conditions, non-dissolved Matrigel and dissolved Matrigel (**Figure 25**), it is observed that both fluorescent intensity and absorbance ratios were higher in samples containing non-dissolved Matrigel compared to their dissolved counterparts. This suggests that the structural integrity of Matrigel does not influence negatively the assay's sensitivity and signal strength.

Once the cell number (4,000 cells per well) and the role of Matrigel were determined, we tested two concentrations of BAD Actor phthalate mixture based on literature, 1x and 100x mixture concentrations, using Resazurin assay. As previously described, this simple and versatile assay allows for daily quantification of cytotoxic effects, enabling more comprehensive temporal analyses.

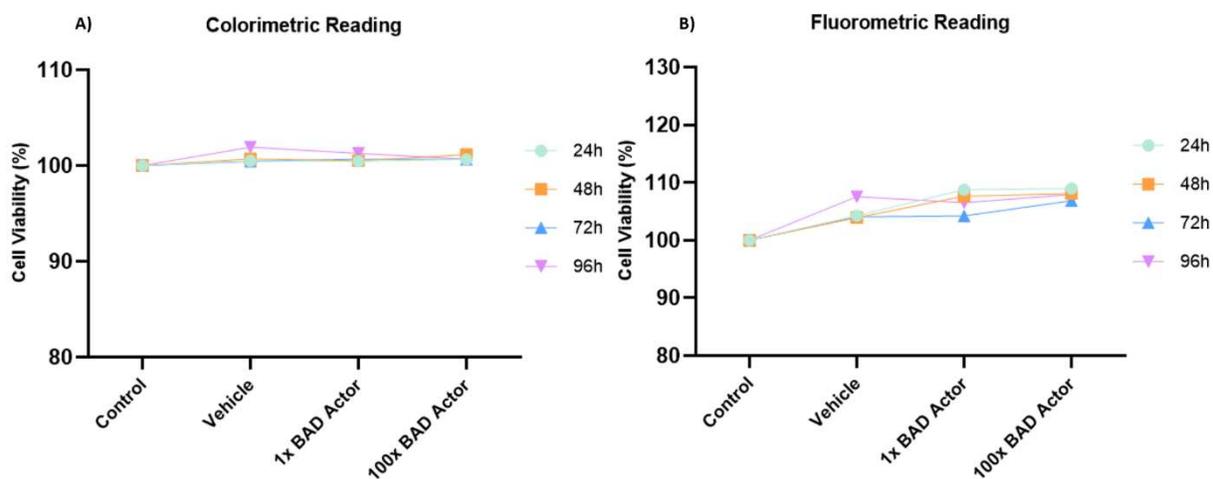


Figure 26. Graph represent the measurement of (A) Colorimetric absorbance; (B) Fluorescence intensity measurement. Colors represent measurements in four different days: 24h (green); 48h (orange); 72h (blue); 96h (violet). DMSO at 0.1 % was used as vehicle.

Repeated cytotoxicity measurements over four days provided valuable insights into whether any of the treatments planned in our experimental design would exhibit toxicity to the cells at any time during the experimental period. As shown in **Figure 26**, none of the treatment conditions demonstrated viability measurements below those of control. Moreover, all time points exhibited comparable values across treatments, indicating that repeated assessments using the Resazurin assay consistently maintained fluorescence and colorimetric absorbance intensity without noticeable decline. This suggests that BAD Actor phthalate mixture at a concentration of 100x is not toxic to the cells, which suggest that further treatment of EEOs at this concentration would not exert toxicity.

5.3 Modulation of Molecular Pathways by Phthalate Mixture

To investigate potential role of BAD Actor phthalate mixture in modulating molecular pathways critical for endometrial function, we investigated transcriptomic changes between two mimicked phases of the menstrual cycle under the treatment with BAD Actor phthalate mixture for 96 hours. When comparing mspEEO to ppEEO after phthalate treatment, we identified 460 upregulated genes and 179 downregulated genes. Notably, 327 upregulated genes and 160 downregulated genes were unique in the mspEEO-EDC vs ppEEO-EDC comparison, while absent in the mspEEO vs ppEEO comparison described in the previous

section. This highlights a significant set of differentially expressed genes exclusive to this comparison, indicating potential involvement in distinct pathways or biological processes that may follow a different trend compared to the other comparison. To investigate further, we conducted an in-depth analysis using Gene Ontology (GO) annotation. The GO categories for Molecular Function, Biological Process, and Cellular Component identified pathways consistent with those identified in the ppEEO vs mspEEO comparison group. Notably, pathways such as focal adhesion, cell adhesion molecular binding, and epithelial cell differentiation emerged as the most significantly enriched, suggesting structural alterations that mirror the transition between the proliferative and secretory phases of the menstrual cycle. These changes align with changes observed in cells unexposed to the phthalate mixture. Additionally, KEGG pathway analysis revealed significant enrichment in the MAPK and ERK signaling pathways, with over 25 DEGs identified (**Figure 27**). Interestingly, this pattern was not detected in the KEGG analysis for the ppEEO vs mspEEO comparison, indicating distinct molecular signatures.

ppEEO + BAD Actor vs mspEEO + BAD Actor

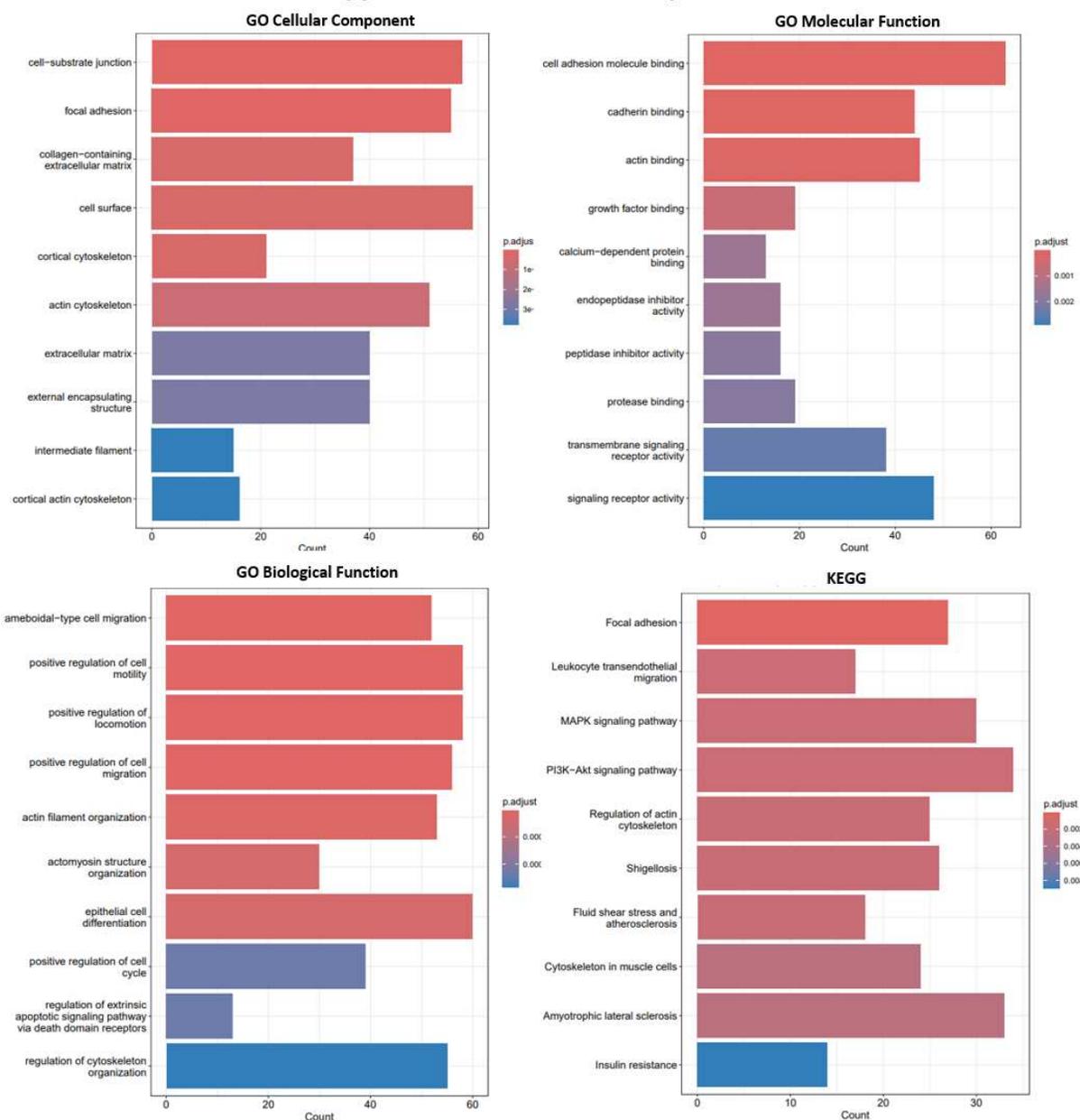


Figure 27. Illustration shows 4 different biological pathway enrichment analysis based on differentially expressed genes confronting ppEEO + BAD Actor vs mspEEO + BAD Actor. Pathways are derived from Go cellular component, Go Molecular Function and Go Biological Process. On the y axis are listed the biological pathway, ordered by significance (p-value <0.05) and on the x axis the number of DEG genes between the 2 conditions for each pathway.

The MAPK pathway, which is pivotal in regulating cellular processes such as proliferation, differentiation, apoptosis, and immune responses, is disrupted by numerous EDCs across various experimental models (Morrison, 2012; Yue and López, 2020). BPA, a widely studied

estrogenic EDC, was found to modulate immune responses by activating MAPK pathway. In a study involving microglial cells exposed to BPA, cytokine regulation was mediated through ERβ signaling, MAPK activation, and NF-κB signaling. Additionally, other estrogenic EDCs, such as nonylphenol (NP) and 4-octylphenol (4-OP), also triggered ERK phosphorylation in MCF-7 cells (Zhu *et al.*, 2015a). A second study investigated the effects of di-n-butyl phthalate (DBP) on MAPK signaling in a murine *in vivo* model of testicular pathology. The phosphorylation ratios of ERK1/2, JNK, and p38 were significantly increased, suggesting that DBP exposure perturbs spermatogenesis and germ cell development by dysregulating the MAPK pathway (Wang *et al.*, 2019a). These findings collectively underscore the influence of EDCs on MAPK signaling, mediated through both classical and non-classical estrogen receptor pathways.

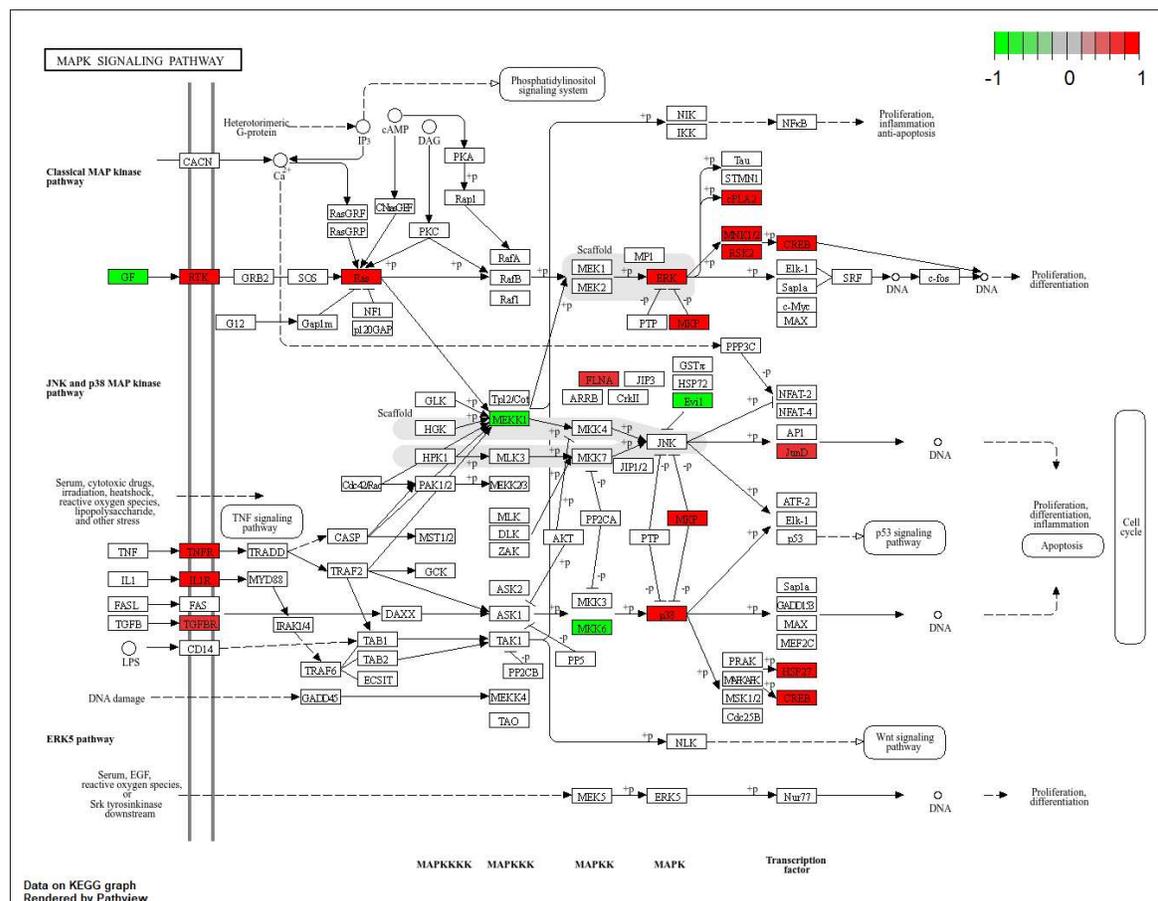


Figure 28. This figure illustrates the MAPK (Mitogen-Activated Protein Kinase) signaling pathways, rendered by Pathview using KEGG data, encompassing the classical MAP kinase pathway, JNK and p38 MAP kinase pathway, and ERK5 pathway. Key proteins and interactions are depicted, highlighting their roles in cellular processes such as proliferation, differentiation, and apoptosis. The nodes are color-coded based on their activity levels (-1 to 1),

where red represents upregulated components and green indicates downregulated components under experimental conditions.

Upregulation of *MEK1* and *ERK*, as illustrated in **Figure 28** suggests enhanced signaling activity, while the downregulation of *MAP3K6* indicates reduced input in stress-related pathways. Upregulation of *Heat Shock Protein 27 (HSP7)* is often linked to cellular stress responses, such as heat shock, oxidative stress, and inflammation. This protein can stabilize cytoskeletal components and contribute to anti-apoptotic processes (Paul *et al.*, 2002; Venugopal *et al.*, 2019). *MAPK-Activated Protein Kinase 2/3 (MK2/3)* resulted upregulated in our analysis as indicated in **Figure 28**. These kinases are part of the p38 MAPK pathway and are involved in inflammatory responses and stress signaling (Cuenda and Rousseau, 2007).

KEGG analysis, as illustrated in **Figure 27**, revealed significant alterations in the phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) pathway as well, with over 25 genes showing notable changes. This pathway is a critical regulator of cellular processes, including protein synthesis, cell cycle progression, proliferation, apoptosis, and cytokine stimulation during stress. Activated by PI3K, Akt functions as a central mediator of PI3K-initiated signaling by phosphorylating downstream targets such as pro-apoptotic proteins, transcription factors, and other kinases. It plays an essential role in protecting cells against oxidative stress, inhibiting apoptosis, and promoting cell survival (Bao *et al.*, 2017; Wu *et al.*, 2020). However, emerging evidence suggests that EDCs such as di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and their mixtures, can significantly alter the PI3K/Akt pathway, leading to disruptions in cell homeostasis.

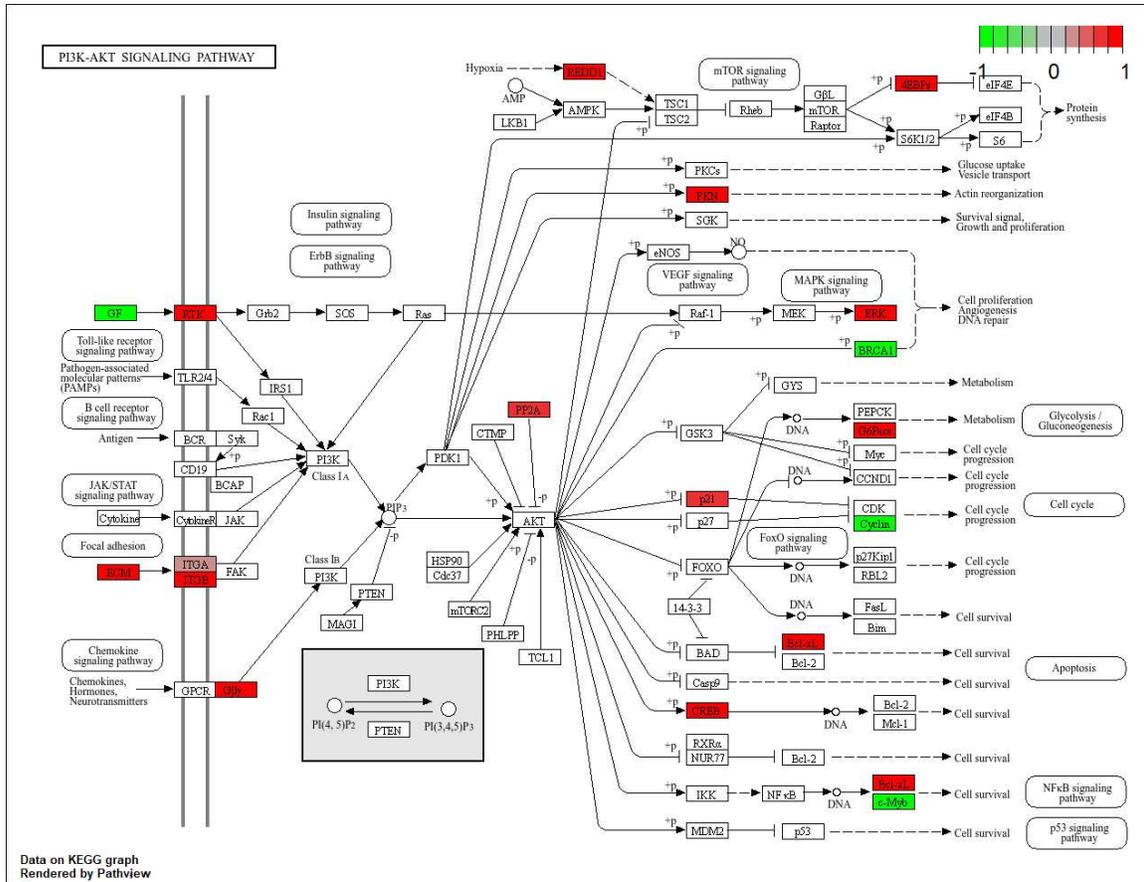


Figure 29. This figure illustrates Differential gene expression in the PI3K-AKT signaling pathway, rendered using Pathview with KEGG pathway data. Key proteins and interactions are depicted, highlighting their roles in cellular processes such as proliferation, differentiation, and apoptosis. The nodes are color-coded based on their activity levels (-1 to 1), where red represents upregulated components and green indicates downregulated components under experimental conditions.

The central role of PI3K and AKT in regulating cell survival, proliferation, metabolism, and apoptosis is illustrated in **Figure 29**, where key nodes such as *AKT*, *BRCA1*, and *c-Myb* show significant changes in expression, reflecting pathway alterations. Interactions with pathways such as mTOR, NF κ B, and MAPK are also shown, demonstrating extensive crosstalk. The observed gene expression profile suggests potential hyperactivation of survival pathways and dysregulation of apoptosis.

Experimental studies in INS-1 cells, a widely used pancreatic beta-cell model, demonstrated that DEHP, DBP, and their mixtures decreased the expression levels of PI3K110 and p-Akt473 proteins compared to the control group. Notably, the combination of DEHP and DBP exhibited a more pronounced inhibitory effect on the (PI3K/Akt) pathway than either chemical alone.

These findings suggest that EDCs can regulate the PI3K/Akt pathway, thereby impairing the cell's ability to counteract oxidative stress and survive (Li *et al.*, 2021).

5.4 Effect of the "Bad Actor" Mixture on the Transcriptome of EEOs: Focus on the Mid-Secretory Phase

Building on the intriguing results from the transcriptomic comparison between the mspEEO-EDC and ppEEO-EDC groups, we focused on the specific modifications induced by the "Bad Actor" phthalate mixture in EEOs mimicking the mid-secretory phase. To explore the effects of these phthalates during the secretory phase of the menstrual cycle, we first conducted pathway enrichment analysis to identify potential pathways regulated by the EDCs. For this, we utilized four distinct pathway enrichment tools to gain a comprehensive understanding of the affected pathways.

As shown in **Figure 30**, the respiratory chain complex pathway emerged as the most significant cellular component in our GO cellular component analysis, with the lowest p-value of 0.005. Changes in the expression of components in the respiratory chain complex pathway suggest potential mitochondrial dysfunction, impaired oxidative phosphorylation as a possible compensation for oxidative stress (Reddam *et al.*, 2022). The second most significantly altered pathway between both conditions is the respirasome, which refers to the organization of respiratory complexes in the inner mitochondrial membrane to form supercomplexes that improve mitochondrial function. Disruption in the organization of supercomplexes can lead to increased levels of ROS (Winge, 2012). Similarly, alterations in genes encoding proteins located in the inner mitochondrial membrane, point to potential outcomes such as mitochondrial membrane instability, which may ultimately lead to apoptosis (Castedo *et al.*, 2002). This pathway includes the highest number of differentially expressed genes, with more than 20 identified. The GO Molecular Function analysis, identified oxidoreduction driven active transmembrane transporter activity, electron transfer activity and primary active transmembrane transporter activity as the most significant pathway (**Figure 30**). Alterations in oxidoreduction driven active transmembrane transporter activity could indicate disruption in

energy production, metabolic balance as well as oxidative stress response. Changes in the expression of genes coding for products involved in electron transfer activity suggest that these structures may impair their function due to their sensitivity to ROS-induced damages (Musatov and Robinson, 2012; Kovacic and Jacintho, n.d.).

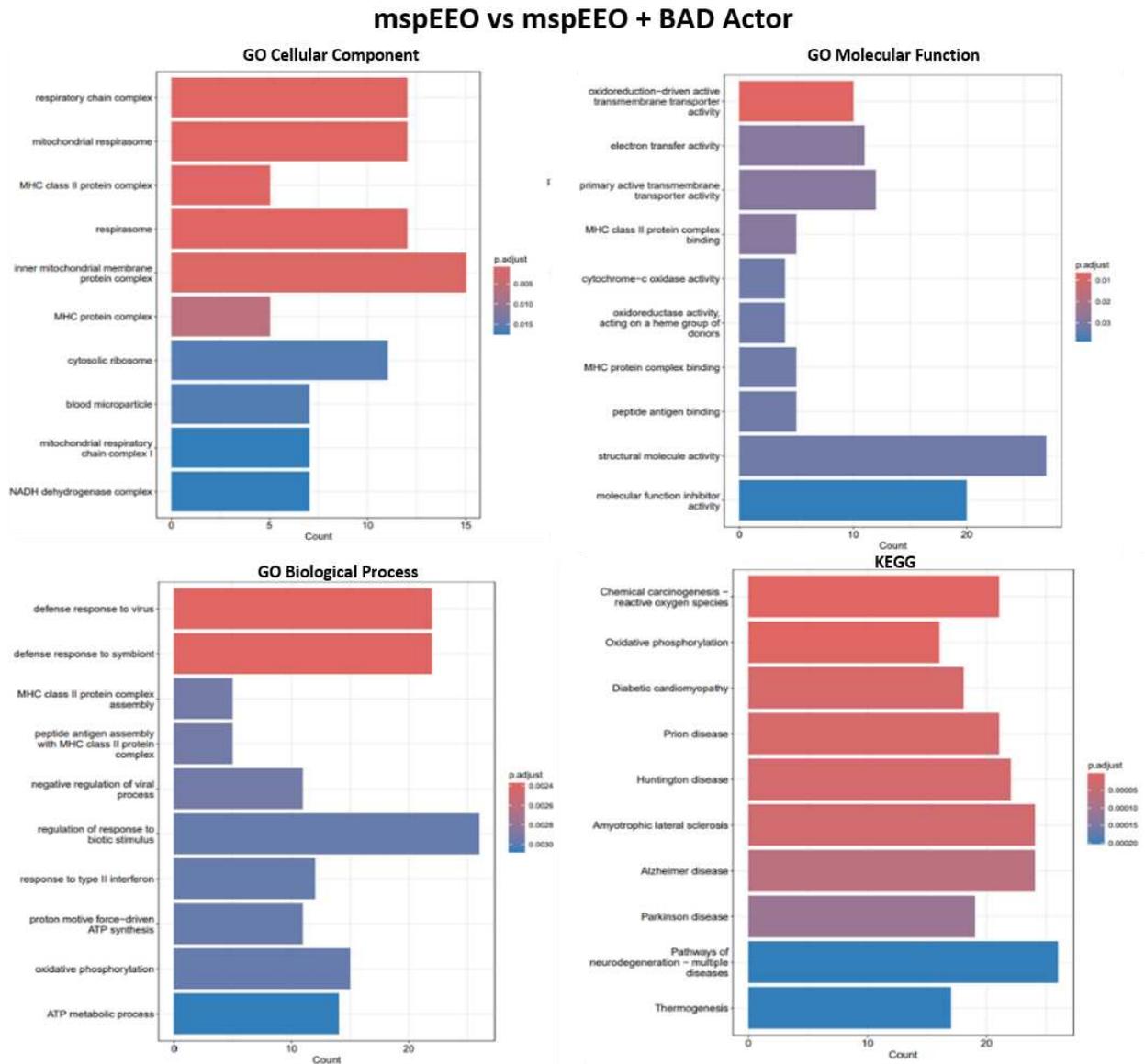


Figure 30. Picture shows 4 different biological pathway enrichment analysis based on differentially expressed genes between two treatment groups: mspEEO + BAD Actor and mspEEO. Pathways are derived from Go cellular component, Go Molecular function, Go Biological Process and Kyoto Encyclopedia of Genes and Genomes (KEGG). On the y axis are listed the biological pathways, ordered by significance (p-value <0.05) and on the x axis the number of DEG genes between the 2 conditions for each pathway.

The Go Biological Process analysis, consistent with the other analyses, identified oxidative phosphorylation and ATP synthesis as key pathways altered between the two treatments (**Figure 30**). While not having the highest enrichment score, both pathways showed significant changes, with more than 10 genes differentially expressed. These alterations, as described earlier, suggest a shift in cellular energy metabolism and potential response mechanism triggered by increased reactive oxygen species. Reactive Oxygen species pathway, resulted with the highest enrichment score as the most significantly altered in our KEGG analysis as shown in the **Figure 30** Followed by oxidative phosphorylation, both pathways presented more than 15 genes DEG between two conditions and highly significance (p-value <0.0001).

By integrating the results from all these GO analyses, we identified “oxidative phosphorylation” as the pathway with the most significant changes in the activity between these two conditions. To further explore the possible mechanisms involved into a possible electron transfer and oxidative stress induced by the mixture of phthalates, we further focused on this pathway. By integrating the results from all the GO analyses, we identified oxidative phosphorylation as the pathway with the most significant changes in activity between the two conditions. **Figure 31** provides a graphical visualization of the oxidative phosphorylation pathway, generated from the KEGG analysis results. This representation offers a clearer understanding of the spatial organization of the products encoded by the genes whose expression was altered by phthalate treatment. The nodes are color-coded based on their regulation, with red indicating upregulation and green indicating downregulation in phthalate-treated samples. The visualization is organized to highlight each complex embedded in the inner mitochondrial membrane, which plays a key role in oxidative phosphorylation, facilitating a more detailed understanding of the pathway.

The *NDUFV1*, *NDUFS7*, as well as *NDUFA11* genes are upregulated in phthalate treated organoids, and encode for subunits of the NADH dehydrogenase, the first enzyme of the mitochondrial electron transport chain (Complex I). Interestingly, data shows that this site is highly involved in ROS production, suggesting that results from GO analysis concord with data in the literature. (Hirst, 2013; Varghese *et al.*, 2015).

COX2, *COX7A* and *COX7B*, coding for subunits of Cytochrome c oxidase (Complex IV), were downregulated in mspEEO + BAD Actor group. This complex facilitates the formation of H₂O molecules, as it is the last step of the electron transfer, by transferring electrons from the reduced Cytochrome C, to oxygen. Furthermore, alongside other proton pumps such as complex I and III, it generates a proton gradient which drives ATP synthesis. Similarly to the other complexes dysregulation, impaired function of such complex can results in pathological conditions as well as production of mitochondrial ROS (Srinivasan and Avadhani, 2012). Interestingly, upregulation of *COX8*, another gene coding for Complex IV subunit, remains unclear.

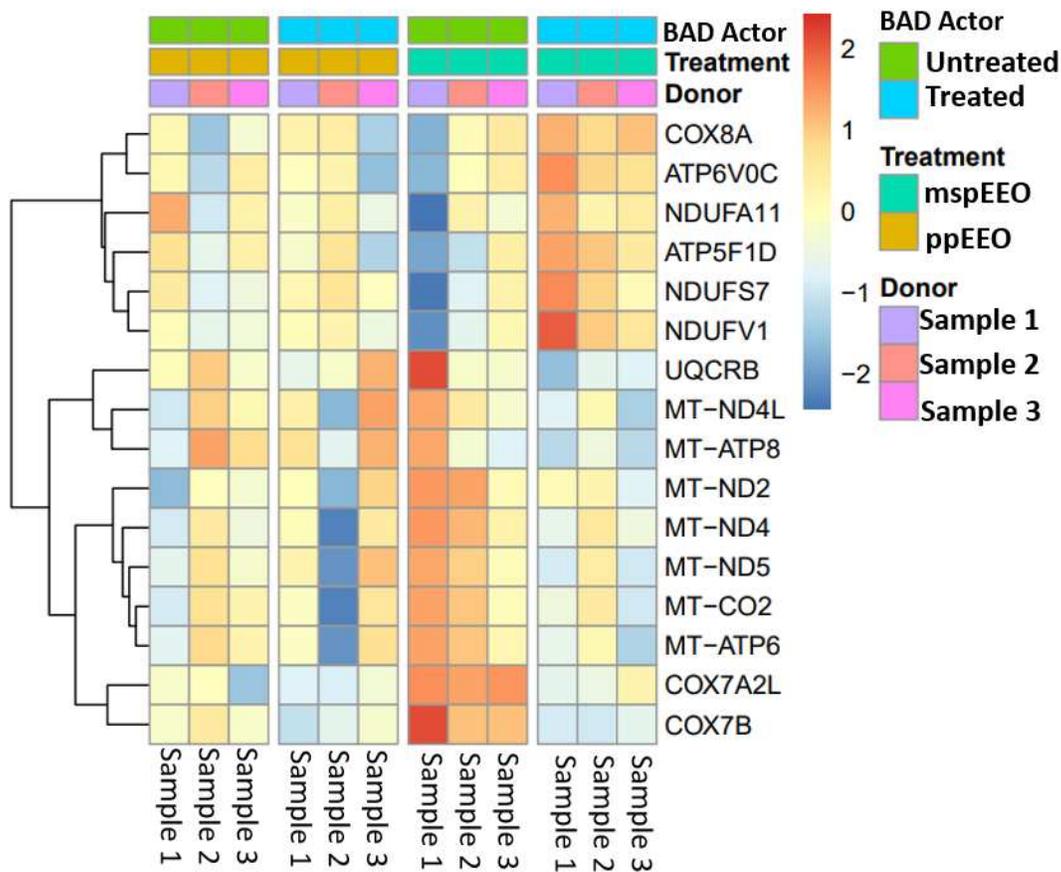


Figure 32. Heatmap illustrated the expression patterns of genes significantly expressed in the oxidative phosphorylation pathway between four different treatment groups. Colors indicate the fold-change in expression between two group: Blue indicating down regulation; Red indicates up-regulation. The heatmap rows represent genes, while the columns correspond to samples.

Figure 32 illustrated the heatmap highlighting the gene expression differences in the oxidative phosphorylation pathway. The observed difference in clusters of mitochondrial genes differentially expressed between mspEEO treated and untreated groups, potentially suggests an adaptive or stress response. As explained above, several mitochondrial complex I-related genes such as *NDUFS7* and *NDUFA11* exhibit similar expression trends, particularly under phthalate mixture exposure, reflecting their shared roles in mitochondrial respiratory function. Genes related to mitochondrial ATP synthesis such as *MT-ATP6* and *MT-ATP8* also form clusters, showing variable responses to treatments. Genes such as *NDUFV1*, *MT-ND5*, and *COX8A* show a noticeable difference in expression between the two groups.

6 Conclusion

Our research provides significant insights into the impact of endocrine disruptors (EDCs) on female reproductive physiology, focusing on both ovarian and endometrial functions. In the ovarian context, we explored the effects of the natural compound biochanin A (BCA) on granulosa cell function, using a robust in vitro model. Our findings demonstrated that BCA, along with caffeine, not only increased the abundance of TAS2R14 but also altered mitochondrial function, as reflected by changes in mitochondrial footprint and branch length. This was accompanied by a shift in steroid metabolism, where BCA promoted estrogen secretion while reducing progesterone production. Furthermore, BCA and caffeine influenced lipid droplet dynamics, suggesting their role in supporting granulosa cell differentiation and energy provision for follicle development. In human primary granulosa cells, BCA activated TAS2Rs, resulting in the upregulation of steroidogenic enzymes, such as StAR and CYP17A1, and a consequent increase in estrogen production. Importantly, TAS2R antagonists confirmed the central role of these receptors in mediating the observed effects. These findings emphasize the importance of TAS2R receptors in regulating steroidogenesis and mitochondrial function, offering valuable insights into how natural EDCs like BCA may influence female fertility.

In parallel, we investigated the effects of EDCs on the endometrium using a 3D model derived from endometrial biopsies. This model allowed us to examine molecular pathway alterations during the proliferative and secretory phases of the menstrual cycle, influenced by a phthalate mixture. Our data confirmed the robustness of the resazurin viability assay in matrigel-embedded organoids, ensuring that structural encapsulation did not compromise assay sensitivity. By mimicking the uterine microenvironment with specific hormonal treatments, we gained transcriptomic insights into the dynamic changes within the endometrial environment. These findings highlighted distinct molecular signatures linked to the menstrual cycle and underscored the utility of 3D organoids for studying endometrial physiology and the effects of EDCs. We also revealed that EDCs significantly modulate MAPK and ERK signaling pathways, suggesting their central role in mediating the effects of both hormonal and environmental stimuli.

Together, these complementary studies provide a comprehensive framework for understanding how EDCs influence both ovarian and endometrial physiology. They underscore the critical role of signaling pathways in female fertility and highlight the potential of advanced 3D organoid models for elucidating the complex interplay of these pathways. This work offers a foundation for exploring strategies to mitigate the adverse effects of environmental disruptors on reproductive health.

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