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EMERGING CONTAMINANTS AND HEALTH: POTENTIAL EFFECTS OF ENDOCRINE DISRUPTORS ON HUMANS AND THE ENVIRONMENT

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Summary

Abstract

Endocrine disruptors (EDs) constitute a critical global health challenge. Their ubiquitous environmental presence and ability to perturb intricate hormonal signaling are increasingly implicated in adverse neurological outcomes.

This investigation examined the neurotoxic potential of six representative EDs -- pesticides (atrazine, cypermethrin, vinclozolin), industrial agents (diethyl phthalate, PFOS), and a pharmaceutical (17α-ethinyl estradiol) -- utilizing the SH-SY5Y human neuroblastoma cell line. Cellular viability, reactive oxygen species generation, and microRNA (miRNA) expression profiles were meticulously assessed. Exposure to pesticides elicited specific miRNA modulation (miR-200a-3p, miR-146b-5p, miR-29b-3p), targeting the pivotal PI3K/Akt/mTOR cell proliferation pathway. Molecular analysis revealed significant upregulation of key pro-proliferative markers (ADM12, Bcl-2, MMP2, CDK6, HDAC4, p-Akt/mTOR) alongside p53 downregulation. Conversely, diethyl phthalate, PFOS, and 17α-ethinyl estradiol induced a distinct miRNA downregulation (miR-18b-5p, miR-133b, miR-200a-3p, miR-653-5p), suggesting involvement of the Ras signaling cascade, with subsequent validation confirming modulation of pathway components (CDK6, SOS1, PTEN) and marked Ras pathway activation via Western Blot, particularly with 17α-ethinyl estradiol.

The months spent at BioBasic Europe enabled a crucial step in bridging *in vitro* findings to human relevance. Specifically, prevalent ED sunscreen components, namely ethylhexyl salicylate and avobenzone, were identified in commercially available products and subsequently tested for their toxicity. Furthermore, nickel levels in these consumer products were also assessed to ascertain its presence.

Collectively, these findings underscore the neurotoxic capacity of EDs through the dysregulation of gene expression and critical signaling pathways, warranting further mechanistic elucidation to inform effective preventative strategies against chronic environmental exposure.

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1 Introduction

1.1 Endocrine disrupting molecules

Environmental pollution is an increasing and concerning effect of human consumerism, industrialization, and life quality enhancement.[1] Emerging pollutants (EPs) constitute a diverse class of compounds investigated for their detrimental impact on environmental and human health, even at low concentrations. This category includes a wide array of substances, such as industrial chemicals, synthetic pharmaceuticals, hormones, pesticides, and personal care products, all of which are routinely produced and released into the environment.[2] Many of these molecules are classified as Persistent Organic Pollutants (POPs), due to their resistance to environmental degradation.[3] The main issue about these compounds is the lack of regulation on their production, use, and disposal, hence leading to unknown and dangerous effects on the population worldwide.[14] Another challenge associated with EPs is the broad spectrum of compounds present within this category; however, among them, endocrine disruptors (EDs) are particularly notable for their potential risks.[5] The term 'endocrine disruptors' was first coined in the 1990s, and over the years, various global organizations have provided increasingly detailed definitions to describe these compounds.[6] Only in 2011, the World Health Organization (WHO) introduced the official definition of EDs as 'exogenous substance or a mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub) populations' (WHO, 2011). These agents can directly or indirectly interfere with the hormone system, inducing effects not only on the exposed subjects, but also on their offspring.[7] The endocrine system is responsible for regulating physiological functions through organs and glands (such as, for instance, the hypothalamus, the thyroid gland, pancreas, and gonads) that produce and secrete hormones, messengers released into the bloodstream as a result of endogenous and exogenous stimuli.[8] It is, therefore, evident and alarming that alterations in the endocrine system can produce harmful effects throughout the entire organism. In this scenario, EDs can interfere directly in the hormone synthesis, secretion, metabolism, elimination, and transport. However, the main mode of interaction with the endocrine system is their ability to mimic the action of endogenous

hormones, either agonist or antagonist, on the receptor, thereby inducing unwanted effects.[9]

Over 4,000 are the molecules nowadays considered EDs, and the numbers are increasing due to the continuous and tremendous production and utilization of these molecules, or the products that contain them.[7] Some EDs are from natural sources, such as the phytoestrogen, originated by plants, or mycotoxins, from various types of fungi. Nevertheless, the biggest class of EDs is represented by the chemical EDs, molecules produced by human activity and spread in the environment through, for example, the emission of toxic gasses from industries and car pollution, the adoption of fertilizers in the agricultural field, and also the runoff from urban sites and factories in rivers and lakes.[7] They can also be found in a huge variety of everyday products.[6] Radiation, artificial light, stress, and temperature are part of this category as well, having the ability to interfere with the hormonal system.[7]

Since pharmaceuticals, pesticides, plasticizer, and many other molecules have EDs properties, they are widespread in the environment and can be easily detected in soil, air, and both surface and ground water.[10] The primary route of human exposure to these substances is ingestion of contaminated food, followed by inhalation of harmful gases, and skin contact. On the other hand, fetuses and infants, who are the most vulnerable groups to exposure to these molecules, come into contact with them through the placenta and breast milk, respectively.[7]

According to the Scientific Statement of the Endocrine Society, EDs exposure can affect female and male reproductive systems and metabolism, as well as the nervous, cardiovascular, immune, and endocrine systems, and induce obesity and numerous cancer types.[11] It has been established that endocrine and nervous system are deeply interconnected, generating the neuroendocrine system. On the other hand, the neuroendocrine system works side by side with the immune system.[12] It is therefore clear that EDs pose a tremendous risk for the population worldwide, as well as for all the environments around the planet.

1.2 Regulatory Landscape of Endocrine Disruptors

During the 1990s, following significant cases that suggested a connection between environmental chemicals and reproductive health issues in both humans and wildlife, EDs became a key concern for the public and regulatory bodies.[13] To manage the risks posed by EDs, the European Union (EU) has established a fairly comprehensive set of legal rules such as Regulation 528/2012 on biocidal products, Regulation 1107/2009 concerning plant protection items, Regulation 1223/2009 for cosmetic goods and the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation 1907/2006.[14] The initial scientific standards for identifying endocrine-disrupting characteristics were established within the regulations concerning Biocides and Pesticides, later detailed in Commission Delegated Regulation (EU) No 2017/2100 and Commission Regulation (EU) 2018/605, respectively. These criteria, drawing upon the WHO definition of an ED, serve as a foundational point for creating a unified strategy for ED identification across various EU sector-specific laws.[15]

However, the identification of molecules showing EDs properties posed immediate and unique challenges. Their effects are often subtle and difficult to detect using standard toxicology, the nature of the impact varies with the timing of exposure, and adverse health outcomes can be significantly delayed. Unlike direct toxicities, universally accepted 'endocrine disruption' endpoints are lacking, and many hormone-related biological responses can also be influenced by non-endocrine mechanisms.[13] Consequently, no single test can definitively classify a chemical as an ED for regulatory purposes, leading to the requirement of evaluating data from various testing methods. This involves integrating information about how a chemical interacts with a biological target (such as a receptor or enzyme) and how that interaction manifests at more complex biological levels (like organs or the entire organism).[16] To introduce an additional layer of complexity, international regulations concerning EDs show some variation: while some consider effects solely on human health, others include impacts on wildlife. Despite the need to connect the mechanism of action to the observed effect, some regulatory systems don't mandate in vitro data, which can make proving an endocrine-specific mechanism difficult. In certain regions and industries, identifying EDs is further complicated by restrictions on animal testing for chemical safety

assessment, such as the EU Cosmetics Regulation (1223/2009). Moreover, regulatory bodies have different options for requesting additional tests and methods for assessing a chemical's potential to disrupt endocrine function. No single test can fully capture both the mechanistic effects of chemicals and the real-world effects relevant for regulatory decisions.[13] The current process for identifying EDs is therefore time-consuming, resulting in the assessment of only a limited number of substances annually.[17]

In 2018, the European Community (EC) released a communication titled '*Towards a comprehensive European Union framework on endocrine disruptors*', followed by a 'fitness check' to evaluate the methods used for assessing and managing EDs across various EU laws. This evaluation involved consultations with the public and stakeholders. Based on these consultations, amendments to REACH were suggested, emphasizing the necessity of generating data specifically for EDs identification.[16] The incorporation of existing standards into the approval process for active substances, safeners, and synergists has been significantly optimized by the collaborative 'ED guidance document' from the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) proposed in 2018.[17]

In 2020, the EU further introduced its 'Chemicals Strategy for Sustainability' under the broader Green Deal (European Commission, 2020). This strategy strongly emphasizes EDs, highlighting the need for improved identification through increased testing and aiming to prohibit their use in all consumer goods by classifying them generically as 'Substances of Very High Concern'.[17] These ED-focused information requirements are currently under discussion and drafting.[16] The potential changes outlined in this Strategy include: (i) a mandatory testing requirement for at least seven in vitro endocrine activity endpoints for all chemical registrations, (ii) a revised classification system for 'EDs', and (iii) a 'generic approach' that would ban all EDs from consumer products, categorizing them as harmful chemicals 'requiring special attention' without specific regard to dosage or exposure levels.[17] A key element of this strategy is the "one substance, one assessment" approach. This concept aims to improve the uniformity, clarity, and efficiency of chemical safety assessments within the EU's legal framework (European Commission, 2024).[15]

1.3 Categories of endocrine disrupting molecules

Given the considerable heterogeneity and structural diversity of the molecules within the group of EDs, as illustrated in Figure 1, their characterization presents a significant challenge.[18]

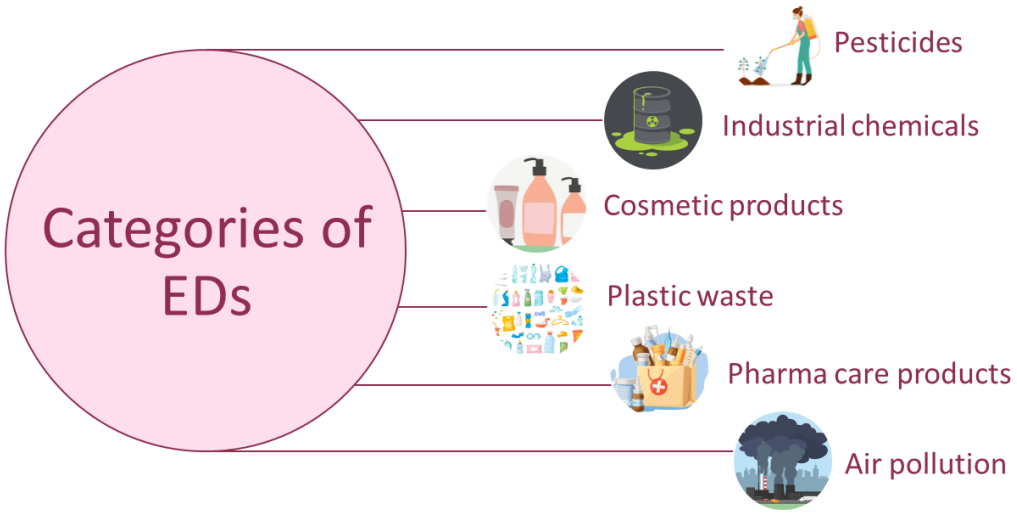

Figure 1: Most important EDs categories

Molecules with endocrine-disrupting activity typically exhibit low molecular weights, generally around 1,000 Da or less. Many of these compounds also possess a phenolic moiety analogous to that found in numerous endogenous steroid hormones. But, beyond this commonality, these molecules display considerable variability in their properties. Additionally, it is noteworthy that a single molecule may produce divergent effects at different concentrations, with its impact on a tissue depending on the tissue's developmental state. Consequently, EDs are sometimes referred to as 'chemical chameleons' due to their variable biological effects.[19] This makes it impossible to classify them based on their chemical structure.[20] Therefore, EDs are categorized based on their origin, whether natural or synthetic, and their application, such as pesticides, sunscreens, pharmaceuticals, and industrial products.[18]

1.3.1 Pesticides

'Pesticide means any substance or mixture of substances or biological ingredients intended for repelling, destroying, or controlling any pest or regulating plant growth' (The Food and Agriculture Organization of the United Nations, 2021). This term

describes a broad class of molecules used to protect crops or ornamental plants from microorganisms, bacteria, fungi, pest plants, and even insects and animals, and to help them grow and flourish. The primary legal structure governing the market introduction of any pesticides is Regulation (EC) No 1107/2009, which outlines the requirements and procedures for the approval and subsequent authorization of active substances. The Biocide Product Regulation (EU Regulation No 528/2012), on the other hand, rules the use of biocides. For active substances to be utilized within the pesticides class, they are required to meet specific approval standards, and conversely, these molecules are prohibited from being marketed or employed without prior authorization within an EU Member State.[21] Generally, authorization for use in pesticides and biocides is denied if a substance is shown to be an endocrine disruptor (COM/2018/734 final).[22]

Depending on the target organism of their action, they are referred to as fungicides, herbicides, insecticides, rodenticides, or general pesticides.[23] Other than by their target species, pesticides can be classified by their chemical structure, or by their mode of action. Organic and inorganic pesticide characterization is the most common.[24]

Data show that more than two million tons of pesticides are in use worldwide and this employment is essential to ensure the availability of food reserves to meet the needs of the growing population.[25] The use of pesticides is also crucial in the fight against vector-borne diseases, such as malaria and dengue.[26] On the other hand, the overuse of pesticides, without strict regulation or control, has led to alarming effects:[27] It has been confirmed that exposure to various pesticides can cause allergies, asthma, and irritation, hormones dysregulation, cancer, and low birth weight, birth defects, and fetal death if pregnant women are exposed.[28]

Of interest is the synthetic herbicide atrazine ((ATZ), 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), applied on different crops, like sugarcane, corn, sorghum, and forest trees to eradicate grass, broadleaf, and weeds.[29] ATZ stands out as the second most widely used herbicide in the United States, also extensively exploited in China and many emerging economies, primarily due to its low production costs,[30] and its annual use is estimated to be 70,000-90,000 tons worldwide.[31] However the usage of ATZ was banned by the EU in 2004, and even before in Italy, in 1992, its broad use in other countries has led to its detection in water reserves and soils worldwide.[32] The levels of ATZ differ across countries due to the varying usage of this compound.

In general, concentrations in water range from 0.02 ng/L to 80 μg/L, while in soils they range from 0.99 ng/g to 19.59 μg/g. Moreover, its half-life is approximately 57 weeks, although residues of ATZ and its metabolites have been found up to 22 years later.[33] Because of that, ATZ is considered a POP.[29] The WHO has declared that the safe threshold of ATZ in drinking water is 100 μg/L.[34] Although, since this molecule shows a high chemical stability and a high water solubility, it has been found at concentrations greater than those approved for use.[31]

Also vinclozolin ((VNZ) 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione), a dicarboximide fungicide introduced in 1981, is of interest due to his wide use on fruits, vegetables, ornamentals, and vines (being the most frequently used fungicide by the wine industry) to prevent decay.[35] *In vitro* and *in vivo* studies have demonstrated the anti-androgenic activity of VNZ, placing it in the group of molecules having endocrine disruptor-related activity.[36] Because of that, VNZ has been first restricted in 2004 by the Environmental Protection Agency (EPA) in the United States, while in 2006 regulations have been applied by other countries as well, including Finland, Norway, Denmark, and Sweden.[37] Finally, in 2007 the EU banned its usage.[38] Despite these limitations, EPA calculated a daily population exposure to VNZ of 2.933 μg/kg.[35] Moreover, VNZ is ubiquitously present in global water reservoirs, having been detected at concentrations ranging from 0.1 to a maximum of 52 μg/L.[39]

Cypermethrin ((CYP) (*S*)-α-cyano-3-phenoxybenzyl (1R, cis)-3-(2,2dibromovinyl)-2,2 dimethyl cyclopropane carboxylate) is a type II pyrethroid insecticide widely used in the world for his broad spectrum and high efficiency. It's able to induce paralysis by inhibiting the sodium channels in the central nervous system (CNS) of the pests. However, studies demonstrate that CYP can cross the blood–brain barrier of non-target species as well, leading to neurotoxicity and behavioural problems. At the same time, it can affect also the reproductive system, especially in males, thanks to its anti-androgen activity.[40] This class of compounds has been utilized since the 1980s because of their low cost and reduced toxicity compared to other pesticides on the market. However, such extensive use has led to the pervasive spread of CYP across all environmental matrices: residues of CYPs have been found not only in agricultural soils, but also in the rivers and ground water close the agricultural districts [20], resulting in constant exposure for the global population.[41] Its concentration in the water systems ranges from

0.01 to 9.80 µg/L but the runoff from agricultural sites has been estimated to be around 194 µg/L. Additionally, high concentrations have been detected in sediments as well as in the body fluids of vertebrates and invertebrates. Notably, elevated levels of CYP, up to 31 µg/mL, have been found in breast milk and blood of
individuals exposed to this molecule.[41] Despite numerous studies confirming various toxicity mechanisms associated with CYP, including several linked to endocrine disruption,[42] its use has not been entirely prohibited within the EU. Indeed, Regulation (EC) No 1107/2009 of the European Parliament and of the Council permitted this substance solely as an insecticide, albeit categorizing it as a candidate for substitution. In 2021, Commission Implementing Regulation (EU) 2021/2049 re-approved these guidelines, allowing its continued use under specific conditions. In contrast, alpha-CYP, a specific isomer of the pyrethroid insecticide, failed to meet the legislative requirements, resulting in a prohibition of its use enacted in June 2021.

1.3.2 Industrial chemical agents

In everyday lives, each of us encounters numerous synthetic chemicals through food, cosmetics, cleaning products, medications, and other environmental sources.[43] The REACH regulation (EC, 2006), applicable to most chemical substances produced, imported, sold, or utilized within the EU in quantities exceeding one tonne annually, is designed to secure a high degree of safety for both human health and the environment. Entities that manufacture or import chemicals are obligated under REACH to supply standard information, encompassing data on their physical and chemical characteristics, toxicity, ecotoxicity, and environmental behavior.[16] However, despite many of these molecules being classified as EDs, their use remains widespread across many products utilized by the general population.[43] As an example, phthalates, diesters of phthalic acid, have been deeply studied for their EDs properties. These molecules are categorized based on their molecular weight: high molecular weight phthalates are primarily used to enhance the flexibility and durability of plastics, while low molecular weight phthalates are found in personal care products, cosmetics, insecticides, and food packaging materials. [44] Their production rose from 2.7 million to approximately 6 million tons annually between 2007 and 2017, making them ubiquitous environmental

contaminants.[45] Phthalate esters may exhibit both estrogenic and antiandrogenic activity through interactions with androgen (AR) and estrogen (ER) receptors.[46] Notably, diethyl phthalate (DEP) is one of the most used phthalates,[44] been identified in numerous personal care products, particularly in cosmetics, with concentrations ranging from 80 µg/g to 60 mg/g. This molecule has also been detected in certain child-care products with maximum concentrations of 270 µg/g.[47] In the environment, DEP has been estimated to range from 30 ng/L to 390 µg/L in aquatic ecosystems globally, while residues in terrestrial environments range between 0.3 µg/g and 4 µg/g.[48]

Per- and polyfluoroalkyl substances (PFASs) constitute a diverse group of over 4,000 fluorinated compounds extensively utilized in various applications, such as disposable food packaging and non-stick cookware, as well as in outdoor equipment, carpets, and water-based foam formulations. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are among the most prevalent and extensively researched PFAS, classified as POPs. Although their manufacture and use have been restricted in 2009 under the Stockholm Convention to mitigate human and environmental health risks, these substances continue to be detected across various environmental and biological matrices due to their extensive historical use and inherent chemical stability.[49] Indeed, PFOS is still detected in various matrices, including drinking water, lakes, seas, soils, and even in biota and in the blood of animals and humans. This is attributed to its prolonged environmental and biological half-life, which can extend up to 6 years.[50] Its high-water solubility, low vapor pressure, and low absorptivity contribute to its presence in water bodies, with concentrations ranging from ng/L to µg/L.[51]

1.3.3 Pharmaceuticals

Diethylstilbestrol (DES) is one of the most well-known pharmaceuticals with endocrine-disrupting action. Synthetized in 1938, this estrogen was prescribed to over ten million pregnant women to avoid miscarriages or preterm labor.[52] However, in 1971, the Food and Drug Administration (FDA) prohibited the use of this drug during pregnancy after evidence emerged that the daughters of women who had taken DES exhibited a significantly increased incidence of clear cell adenocarcinoma of the vagina,

a rare form of cancer. Moreover, women who were administered the drug demonstrated not only an elevated incidence of infertility but also a higher prevalence of complications during both pregnancy and delivery.[53]

Another molecule of interest is 17α-ethinyl estradiol (EE_2), a synthetic estrogen widely used in oral contraceptives, often in combination with progestin molecules.[54] EE_2 is also employed in farm animals and aquaculture. Its detection in domestic sewage and effluent bodies is exacerbated by its extensive use by the population.[55] It has indeed been estimated that a woman using oral contraceptives excretes 35 mg of EE_2 in her urine daily[56], while environmental concentrations of EE_2 have been found to range from 0.2 ng/L to 100 ng/L in different matrices.[57] Even at these low concentrations, adverse effects on aquatic biota have been documented, caused by the toxicity of EE_2 and its endocrine-disrupting properties. Moreover, this molecule demonstrates significant environmental persistence, largely attributable to its significant resistance to degradation in wastewater treatment systems and its extensive and continuous global usage.[58]

1.3.4 Sunscreen filters

In recent years, increased public concern over skin cancer has driven a rise in sunscreen use. Sunscreen agents are compounds incorporated into formulations such as creams, lotions, oils, and sprays that enable tanning while simultaneously protecting the skin from UVA and UVB radiation.[59] Sunscreen agents are categorized into two types: organic filters, which absorb light through their chemical structure, and physical filters, represented by zinc oxide and/or titanium dioxide, which reflect and scatter light to prevent damage on the skin.[60] Sunscreen filters are classified as cosmetic products. The marketing of cosmetic products within the EU is governed by Regulation (EC) No 1223/2009, which establishes lists defining permissible and forbidden substances. These lists also specify concentration limits for certain allowed ingredients, ensuring consumer safety.[61]

Despite the critical role of these compounds, recent concerns have emerged about their security. Blood concentrations surpassing the FDA's safety threshold of 0.5 ng/mL have been observed in individuals participating in clinical trials, causing evaluations of the

actual extent of absorption of these compounds.[62] Once accumulated in tissues, sunscreen filters can interfere with the nervous and endocrine systems, leading to imbalances in exposed human population. Concerns regarding the toxicity of sunscreen filters induced the US FDA in 2021 to require further safety evaluations, necessary for the authorization of new compounds and for the continued use of those already approved on the market.[63] Moreover, in 2018, the state of Hawaii banned the sale of sunscreen products containing oxybenzone and octinoxate, organic UV filters with demonstrated endocrine-disrupting properties that can induce harmful effects on coral reefs and marine ecosystems. Indeed, exposure to sunscreen filters is not only hazardous to humans: an increasing number of studies have shown that these molecules can adversely affect wildlife, particularly marine species, leading to impacts on development, reproductive and nervous systems, and tumor growth. Additionally, marine flora is also vulnerable to the effects of these filters, as evidenced by the increasing phenomenon of coral bleaching which is causing the loss of colour in some of the world's most breathtaking coral reefs.[64]

Among the molecules with sunscreen filtering properties, avobenzone ((AVO) butyl methoxydibenzoylmethane) is one of the most widely utilized globally, with allowed concentrations in formulations of less than 3% in the US and less than 5% in Europe.[65] AVO has been detected in the surface waters of several countries at concentrations ranging from 1.37 to 721 ng/L, while in sediments, this sunscreen filter has been found at concentrations ranging from 4.13 to 33.48 ng/g.[66] Interestingly, AVO has also been identified in various marine species, with concentrations reaching up to 21 ng/g lipid weight, suggesting its significant environmental persistence and bioaccumulation potential.[67] Recent studies have confirmed several toxic effects of AVO, including its ability to induce oxidative stress and exert androgenic, estrogenic, and particularly thyroid hormone-disrupting effects, classifying it as an ED.[68]

Another widely used molecule for its sunscreen filtering properties in personal care products is ethylhexyl salicylate ((EHS), 2-Ethylhexyl 2-hydroxybenzoate). Despite its low water solubility, this compound has been detected in seawater, surface water, beaches, aquatic ecosystems, and even in soil, making it an emerging environmental contaminant.[69] The concentration ranges at which this filter has been identified globally in surface waters vary from 17 ng/L to 1.2 µg/L. Due to bioaccumulation, EHS has been

found in European fish at concentrations as high as 72 μg/kg. Reports have indicated adverse health effects on humans associated with EHS: recent *in vitro* assessments have shown that EHS exhibits hormonal activity, including estrogenic or antiestrogenic effects, as well as antiandrogenic and partial androgenic activities.[70]

1.3.5 Heavy metals

The term 'heavy metals' refers to a group of metals and metalloids characterized by an atomic density greater than 4 g/cm³.[71] These substances are primarily found in rocks, marine sediments, and volcanic lava, but the majority are released into the environment by technological industries.[71] Heavy metals are fundamental for the physiological functioning of the body: iron, zinc, and copper are considered 'essential' as they participate in cellular growth and development, DNA synthesis, and cellular respiration.[72] On the other hand, excessive accumulation of heavy metals has been shown to be toxic to humans, in both acute or chronic exposure, leading to cellular death and deterioration of organs such as the kidneys, liver, heart, and even the brain. These effects are primarily attributed to the heavy metals' ability to generate reactive oxygen species (ROS), resulting in significant oxidative stress at the cellular level.[73] Additionally, some heavy metals can interact with endogenous hormones and their receptors. For this reason, they are classified as metalloestrogens.[74] Nickel (Ni), cadmium, lead, mercury, arsenic, copper, zinc, and manganese are the heavy metals most thoroughly characterized as EDs.[75]

1.3.6 Natural substances

Phytoestrogens are plant-produced non-steroidal polyphenols discovered in the late 19th century. Their structural and size similarity to the endogenous 17β-estradiol enables them to exert estrogenic or antiestrogenic effects in mammals, which can contribute to the plant's defence against harmful animals or insects.[76] Specifically, phytoestrogens show a high binding affinity for both nuclear and membrane estradiol receptors, as well as the capacity to stimulate estrogen-dependent gene transcription.[77] Human exposure, that primarily occurs through the ingestion of fruits, vegetables, and foods containing their derivatives, can lead to endocrine modulation, which, in certain cases, may be

detrimental to health.[78] The four main classes of phytoestrogens are flavonoids (again divided into flavonols, isoflavones, and prenylflavonoids), lignans, coumestans, and stilbenes. The division is based on their chemical structure.[76]

Mycotoxins are highly toxic metabolites produced by fungi that pose significant risks to vertebrates. Their presence in agricultural soil or directly on crops leads to exposure of both humans and animals consuming these products, resulting in serious intoxications known as mycotoxicosis.[79] These contaminants are considered EDs. A notable example is zearalenone, which, due to its structural similarity to the natural hormone 17β-estradiol, is capable of activating estrogen receptors and inducing adverse effects on the reproductive system.[80]

1.4 Environmental and human exposure

Industrialization, consumerism, and population growth have led to an exponential increase in the manufacture and use of various products.[81] The demand for more food has driven the extensive use of pesticides in agriculture and estrogenic drugs in farm animals.[27] Additionally, an increasing number of pharmaceuticals and personal care products are used by the population and subsequently discharged into urban wastewater through shower and toilet drains. Numerous swimmers, covered in sunscreen lotions, enter the water, causing these chemicals to disperse into seas and oceans.[64] Industries operate continuously, releasing their production waste into rivers and lakes, while also contributing to air pollution, which is further exacerbated by the growing number of automobiles and transportation vehicles.[82] Consequently, EDs have become pervasive across the environment, detectable in various matrices such as soil, air, and most notably, water.[25] Once released into the environment, these molecules exhibit pseudo-persistence and resist rapid degradation. Therefore, ecosystems and wildlife are among the first to suffer from the effects of EDs.[83]

The primary route of exposure for the general population and the fauna is through the ingestion of foods containing these molecules, their residues, or their metabolites. For example, it has been estimated that over 80% of pesticide residues are ingested by humans through food consumption.[23] Ingestion also involves industrial products, such as phthalates or PFAS, used as plasticizers, which can leach from plastic containers used for food storage,[84] as well as microplastics and heavy metals consumed by marine

life that is subsequently ingested by the population.[75] This process is known as bioaccumulation, which refers to the gradual accumulation of substances in an organism that cannot effectively eliminate them. An example is fish that ingest large quantities of pollutants without being able to process or digest them. When humans consume these fish, they are exposed to these substances as well. This phenomenon is referred to as biomagnification and describes the increase in concentration of toxicants as they are transferred through successive trophic levels, with larger organisms ingesting smaller ones, thereby accumulating higher levels of toxins.[85] When discussing EDs, these processes are prevalent due to the highly lipophilic nature of these molecules, which leads to their bioaccumulation in the adipose tissue.[86]

The consumption of contaminated water is another source of exposure to EDs for both humans and wildlife. The primary sources of these contaminants in aquatic environments include effluents from domestic wastewater treatment plants, industrial discharges, agricultural runoff, and the improper disposal of medical waste,[87] highlighting the limitations of conventional water treatment systems in effectively removing these pollutants.[88] When referring to sunscreen filters, another pathway through which these substances can enter aquatic environments is via swimmers. Indeed, it has been estimated that in 2020, 1.5 billion tourists visited coastal destinations, leading to the release of at least 25% of the sunscreen used into aquatic ecosystems.[64]

Inhalation is another significant route of exposure. Many pesticides are applied to crops using volatilization techniques, leading to initial inhalation by agricultural workers, as well as dispersion into the air, which facilitates their transport over considerable distances from the fields, thus reaching the general population.[25] Dioxins, compounds categorized as POPs and classified as EDs, as well as a group 1 carcinogen,[89] are also widely present in the air, as they are released into the environment through combustion processes.[90] Some EDs found in personal care products in spray form, such as phthalates and sunscreen filters, can be inhaled by consumers due to their vaporized state.[91]

Lastly, dermal contact represents a significant exposure pathway for EDs. Personal care products and sunscreen filters pose the most immediate risk due to their direct application on the skin; however, the general population can experience dermal exposure to any category of EDs that are released into the environment.[64]

1.5 Potential health implications

EDs are increasingly acknowledged as significant and urgent threats to public health, with the potential to become one of the foremost environmental risks on a global scale.[92] Although epidemiological data have shed light on the correlation between exposure to EDs and the development of numerous diseases, it remains impossible to fully characterize the impact of these molecules on the environment and human populations. Similar to endogenous hormones, EDs act at very low concentrations, causing harmful effects even at the levels found in the environment.[93] It is particularly notable that the most pronounced and toxic effects are frequently observed at low doses of exposure, whereas higher concentrations can lead to attenuation or elimination of these effects. This apparent discrepancy may be attributed to variations in tissue sensitivity and responsiveness to EDs, or to the presence of distinct receptor subtypes within the same tissue that exhibit differential activation thresholds for various ED doses. [94] Another characteristic of EDs is known as the 'cocktail effect,' as multiple studies have demonstrated that simultaneous exposure to diverse compounds can enhance endocrine-disrupting activity compared to what would be observed if the exposure was to a single toxicant.[95] Additionally, there is an individual variability in exposure to different types and quantities of EDs, which can interact synergistically and exert toxic effects depending on the health status, age, sex, life style and eating habits, occupation, and genetic profile of the exposed individual.[94] Moreover, the window of exposure is crucial. The WHO has stated that fetuses, newborns, and children during puberty (Figure 2) are the most at risk due to the immaturity of protective systems, such as the blood-brain barrier and DNA repair mechanisms, which are still in the process of development. Additionally, while endogenous hormones may have reduced bioavailability, especially in the first periods after birth, EDs are more readily available in the body, which can result in improper hormone signaling.[93] EDs can be transmitted from the mother to the fetus through the placenta or to the neonate via breast milk, where these lipophilic molecules accumulate. Exposure during these critical developmental periods, where organs and systems are forming and differentiating, can have significant and potentially damaging effects. Furthermore, many of the harmful effects caused by exposure to EDs have a latency period and may not become visible or

recognizable for many years. Significant toxicity has been detected in adult populations as a result of prolonged and continuous exposure to multiple EDs.[96]

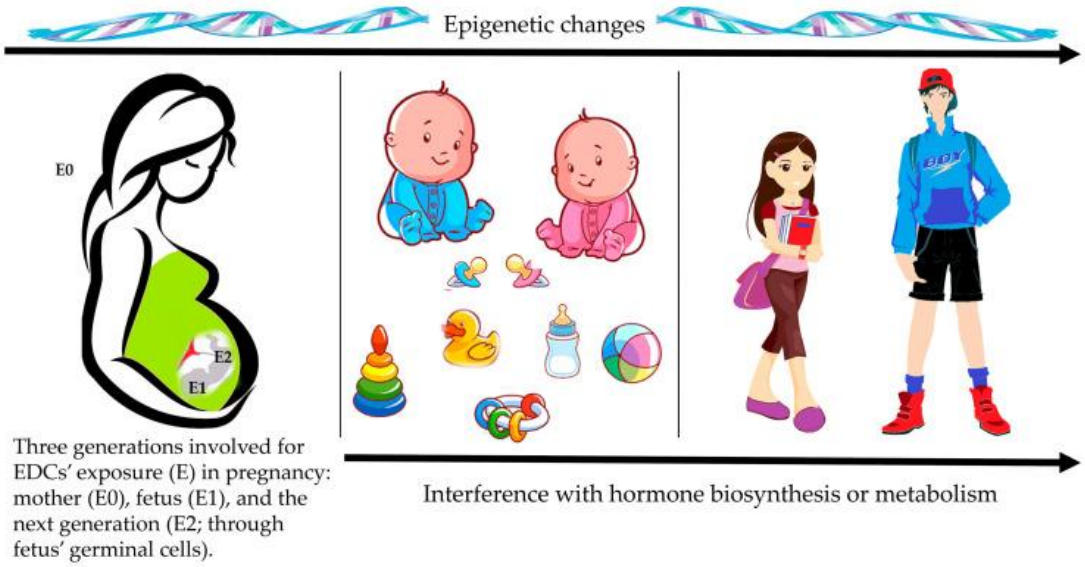

Figure 2: Critical developmental windows, such as in utero, early postnatal periods, and puberty, exhibit heightened sensitivity to EDs exposure.[97]

While significant emphasis has been placed on studies investigating the neurodevelopmental effects of EDs, the impact of exposure to these molecules on the adult CNS remains less understood. It is crucial to consider that age-related decline in endogenous hormones can render the nervous system more susceptible to the effects of EDs, potentially leading to cognitive and behavioral alterations.[98] It is also important to note that AR and ER receptors are highly expressed in the nervous system, particularly in the brain. This abundance facilitates the toxic effects of molecules with endocrine-disrupting activity.[99]

Pesticides are essential tools for both crop health and human well-being. However, the issue with these molecules, particularly insecticides and rodenticides, is that their targets are often not species-specific. This results in non-target species also suffering from the detrimental effects of these chemicals.[100] Due to its endocrine-disrupting activity, ATZ most studied effects are on the reproductive system: exposure can lead to developmental defects, hormonal imbalances, disrupted female reproductive cycles, and impaired function of both male and female gonads.[101] Also exposure to the fungicide VNZ has been associated with issues in the development of the reproductive system and sexual organs, reduced sperm motility, as well as the development of pulmonary and renal fibrosis, behavioral alterations and motor inhibition in mammals,

fish, and birds. Moreover, transgenerational epigenetic effects have been observed in studies of pregnant rats exposed to VNZ during gonadal development.[102] The influence of various industrial EDs on human health is also notably profound.[103] More specifically, exposure to PFOS and PFAS has been linked to a spectrum of adverse health effects impacting the immune, nervous, reproductive, hepatic, and cardiovascular systems.[104] Concerning DEP, its widespread use in personal care products has been linked to multiple cases of dermatological irritation.[105] These outcomes are primarily identified in females, likely due to their higher usage of personal care products.[10] Another category of substances within the personal care products group is sunscreen products. Despite their primarily topical application, significant amounts have also been detected systemically, in the blood, urine, and even in breast milk, due to skin absorption and their presence in drinking water, air, cosmetics, and plastic packaging.[106] *In vivo* and *in vitro* studies have demonstrated that, at the systemic level, sunscreen filters can induce multiple alterations, with particularly alarming consequences for pregnant women and their babies.[107] It is also important to consider the adverse effects these molecules have on the ecosystem. High concentrations of sunscreen filters have been found in marine wildlife, as well as in birds and terrestrial mammals, where they can bioaccumulate and increase their toxicity.[108] Due to their endocrine-disrupting activity, filters, particularly organic ones, can induce toxic alterations in molluscs, fish, and dolphins.[107] Ecosystems globally are affected by the presence of EDs. The most striking example of the marine toxicity of sunscreen filters is coral bleaching caused by DNA damage.[108] It is crucial to remember that the harmful effects of EDs extend beyond human populations: animals and ecosystems are also deeply damaged by exposure to these compounds.

1.6 Mechanism of action

Elucidating the mechanisms through which EDs operate, as well as assessing their role in contributing to specific health problems, is a matter of significant concern for public health and environmental science, as it is crucial for developing effective strategies for risk assessment and mitigation.[109] Despite the extensive efforts by the scientific community to fully elucidate the mechanisms through which these substances exert their effects, significant uncertainties remain due to the ability of EDs to act through multiple pathways, targeting various biological systems and thus leading to diverse outcomes.[110] The characterization of the mechanisms of action provided by La Merrill et al. in 2020 is among the most comprehensive, as illustrated by the diagram presented in Figure 3.

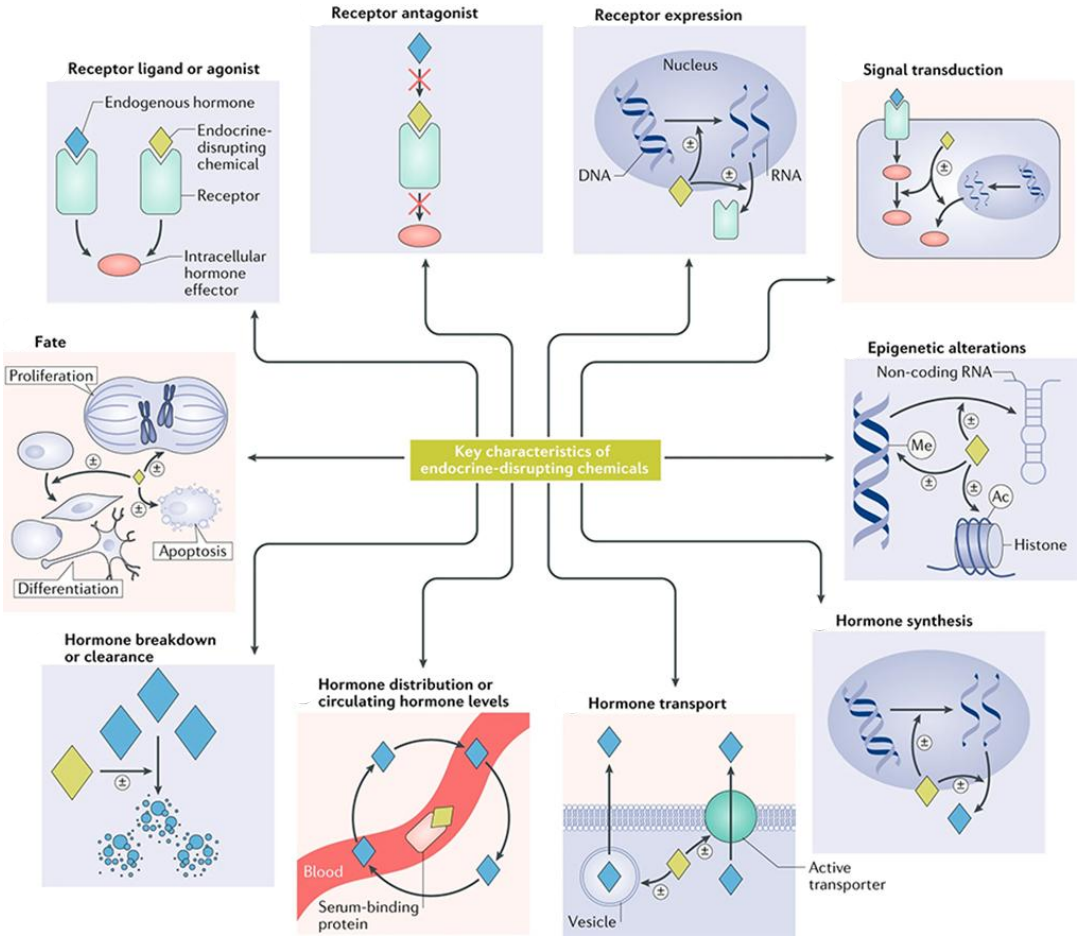

Figure 3: EDs key mechanisms of action.[111]

Interaction with hormonal receptors

The primary mechanism of action of EDs molecules is via interaction and activation of hormonal receptors, through which they can elicit non-physiological systemic responses. The receptor's lower sensitivity to these compounds is offset by their elevated concentrations, due to their high environmental prevalence and their prolonged half-life within the body.[112] An example is given by PFAS molecules, particularly PFOS, which are capable of binding to the ER, inducing gene transcription and resulting in the aberrant modulation of cellular functions.[113] Among the novel molecules that mimic hormones and can bind to their respective receptors, some can obstruct the receptor's transition to its active conformation or they can freeze the receptors in their inactive state, thereby antagonizing the actions of endogenous hormones. VNZ and CYP specifically act as anti-androgens by inhibiting the activity of AR, leading to disruptions in the reproductive system, and particularly affecting male reproductive function.[114]

Modulation of receptor expression

Modulation of receptor production, either stimulation or inhibition, is another mechanism through which these molecules can exert their endocrine-disrupting effects. It is noteworthy that, especially in the case of receptor production inhibition, EDs do not need to have a structure similar to that of endogenous hormones, because these processes are regulated at other levels.[112] Expression of mineralocorticoid receptors, for example, has been reduced following exposure to phthalates, as demonstrated by studies in adult mice.[111]

Engagement with endocrine signaling pathways

Interaction with molecules involved in receptor-activated pathways is another mechanism of action for EDs, where they interfere with a multitude of potential downstream targets within the hormonal pathway, making it extremely challenging to identify all of them. However, it is crucial to recognize that this mechanism can lead to both non-endocrine and toxic effects. As an example, the herbicide ATZ has been studied for its ability to interact with the hypothalamic-pituitary-gonadal axis, leading

to the inhibition of luteinizing hormone release through its interaction with the neuropeptide kisspeptin.[115]

Interaction with the biosynthesis and catabolism of endogenous hormones

Certain molecules are capable of directly modulating hormone biosynthesis or catabolism. In these instances, the molecules may exhibit substantial structural divergence from endogenous hormones, as they are not required to compete with them.[112] Numerous phytochemicals have been studied for their capacity to interfere with hormonal synthesis. For example, ATZ exposure has been shown to decrease testosterone levels while increasing estradiol, estrone, and progesterone levels in various cell types. Similarly, CYP has been reported to increase levels of estrogen, cortisol, aldosterone, luteinizing hormone, follicle-stimulating hormone, and also testosterone.[116]

Hormonal transport modulation

Endogenous hormones circulate in the body at very low concentrations in the bloodstream; some travel in a 'free' state, while others are bound to transport proteins such as glycoproteins or transcortin. EDs can interfere with both the binding of these hormones to their transport proteins, by attaching themselves and blocking the site for hormones, and the production of these proteins, altering hormone levels in the bloodstream.[117]

Epigenetic alterations

Finally, a crucial mechanism through which EDs can exert their toxicity is by epigenetic modulation, including DNA methylation, histone acetylation, and non-coding RNAs expression.[118]

1.7 Regulation of genomic expression

The precise mechanisms through which EDs exert their phenotypic effects are not fully elucidated; however, accumulating evidence suggests that epigenetic modifications play a significant role in the induction of toxicity in different systems. Epigenetics is a

field of biology that examines the causal relationships between genes and their expression. It involves the study of inheritable changes that occur without alterations to the DNA sequence.[93] Alarmingly, not only are epigenetic modifications preserved into adulthood, but it has also been demonstrated that these modifications can be transmitted to the progeny, as showed in Figure 4. Indeed, *in vivo* studies have demonstrated effects transmitted to the offspring of parents exposed to various EDs, revealing transgenerational health impacts and potential long-term consequences on developmental and reproductive outcomes.[118]

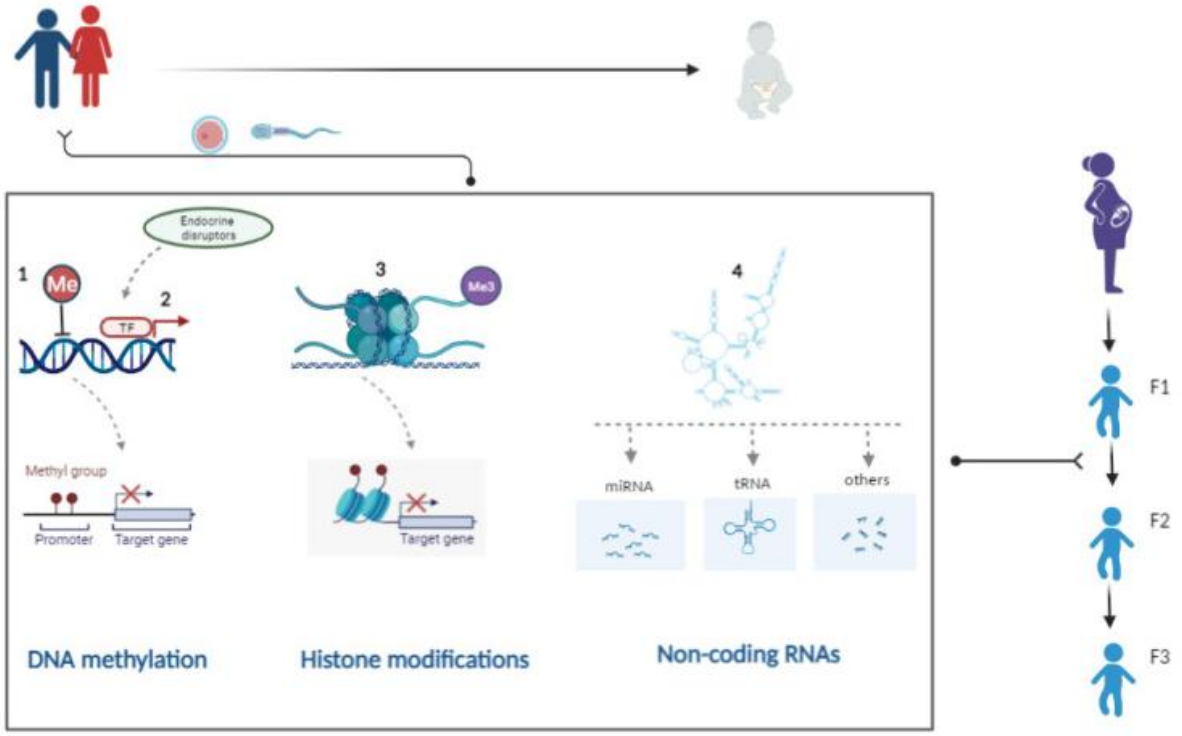

Figure 4: Mechanisms of epigenetic inheritance in the germline. The transmission between generations can induce disease susceptibility in the progeny.[119]

In this regard, pesticides have been extensively studied due to the observation of increased cancer incidence, such as leukemia and neuroblastoma, among offspring of parents who, particularly for occupational reasons, have been exposed to these EDs.[120] EE_2 has also been shown to induce transgenerational effects in the gonads, liver, and brain as evidenced by multiple *in vivo* studies.[121] Research involving zebrafish exposed to sunscreen filters has demonstrated the induction of oxidative stress, developmental malformations, and thyroid toxicity in the progeny of exposed organisms. These findings provide evidence that certain sunscreen agents may also elicit detrimental effects through epigenetic mechanisms.[122]

1.7.1 miRNAs

Epigenetic modifications include the regulation of micro RNAs (miRNAs) expression. miRNAs are short sequences, approximately 18 to 25 nucleotides, of non-coding, single-stranded RNA capable of inhibiting messenger RNA (mRNA) translation.[123] These molecules are generated in the cell nucleus, where they are initially transcribed by the enzyme RNA polymerase II. Subsequently, following capping, splicing, and polyadenylation, primary miRNAs (pri-miRNAs) are formed, which display one or more hairpin structures. Then, the RNase named Drosha, along with its cofactor DGCR8, processes the pri-miRNAs into pre-miRNAs, consisting of 70-100 nucleotides. Exportin-5 then transports them into the cytoplasm through the nuclear pores. Here, the RNase Dicer cleaves the pre-miRNA, while helicases unwind the double helix to produce a single strand, which is the mature miRNA. Finally, to exert its function, the mature miRNA must be incorporated into the RISC complex, where it associates with the Argonaute protein (Ago-2), which then targets the mRNA. The complex binds to the 3′-untranslated region (3′UTR) of the mRNA, through which it can regulate gene expression (Figure 5).[124]

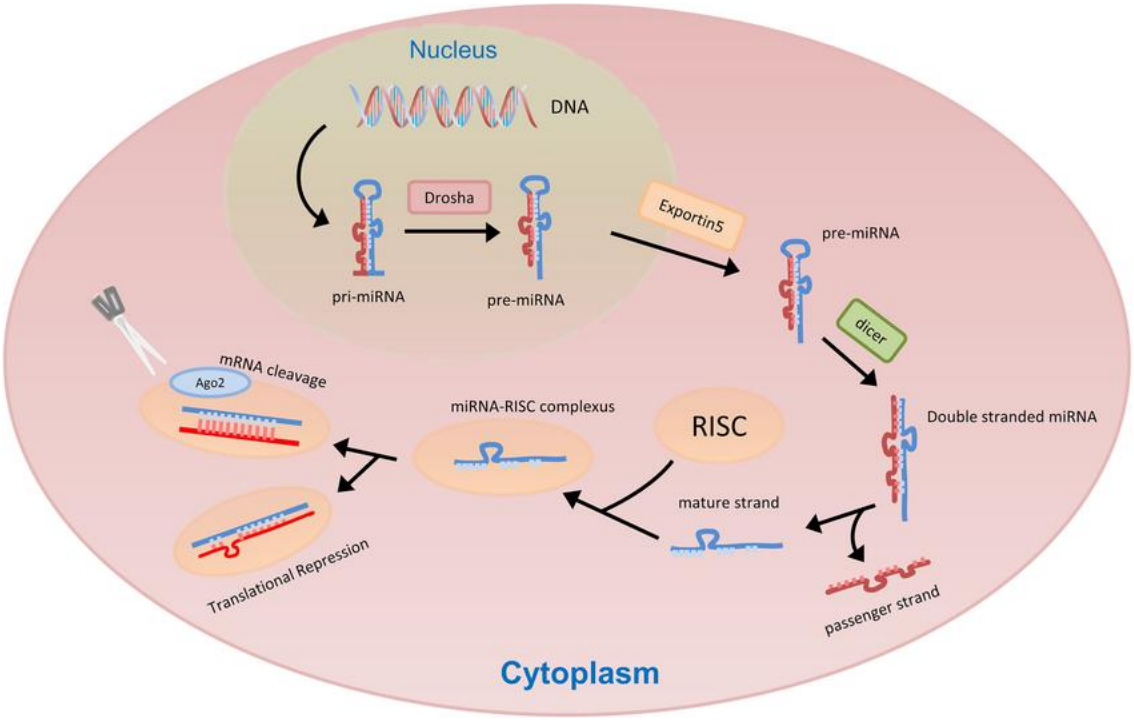

Figure 5: Mechanisms of endogenous miRNAs biogenesis and their role in gene expression regulation.[125]

However, it has been discovered that in humans, 60% of these interactions occur even without perfect complementarity between the two parts.[126] For this reason, a single miRNA can have numerous mRNA targets and, simultaneously, multiple miRNAs can target the same mRNA. This can lead to the modulation of the same pathway at different points. Indeed, miRNAs can regulate all cellular processes, including metabolism, differentiation, proliferation, cell death, and adaptation to environmental stimuli.[127]

The expression of numerous miRNAs is modulated following exposure to various EDs, leading to alteration in the body homeostasis. As an example, miR-200 family, which includes miR-200a-3p, has been linked, through studies on zebrafish, to the modulation of neurogenesis as well as somatic growth, underscoring its critical role in the regulation of reproductive and neural development. These miRNAs directly target genes involved in the hormone-insulin-like growth factor pathway. Similar effects have been observed in murine models, where miR-200 is essential for the regulation of ovulation and, consequently, fertility in female rats. This process occurs through the inhibition of luteinizing hormone synthesis via the zeb1 gene.[128] Modulation of miR-653 expression has been observed in various pathologies and, in particular, has been linked to the progression of certain cancers by targeting key genes.[129] Significant effects are also observed in the CNS. The downregulation of miR-18b-5p, by modulating the expression of the HIFα gene, can contribute to the progression of Amyotrophic Lateral Sclerosis,[130] while studies on Alzheimer's patients have found a downregulation of miR-29b-3p.[131] Finally, the miR-146 family has immunomodulatory activity: their overexpression has been observed during neuroinflammatory processes in microglial cells of adult brains.[132]

1.8 Overview of the Ras and PI3K/AKT/mTOR Pathways

The Ras pathway is involved in the regulation of basal cellular processes, controlling proliferation, metabolism, and differentiation in response to extracellular signals.[133] Notably, the mutation of activation of this pathway has been recognized as the most common oncogenic driver across different cancer types,[134] with 33% of Ras mutations detected in all human cancers.[133]

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) pathway, is also crucial for cellular growth, survival, metabolism, and responses to stresses. Abnormal activation of this pathway is observed in nearly all solid tumors.[135] Moreover, through its regulatory role in cell proliferation, this pathway plays a crucial role in neuronal apoptosis. Doing so, it contributes to the protection of the brain by modulating cellular processes that influence neuronal survival and death, thereby supporting overall brain health and function.[136] These two pathways are intricately connected and mutually influence each other, as shown in Figure 6.

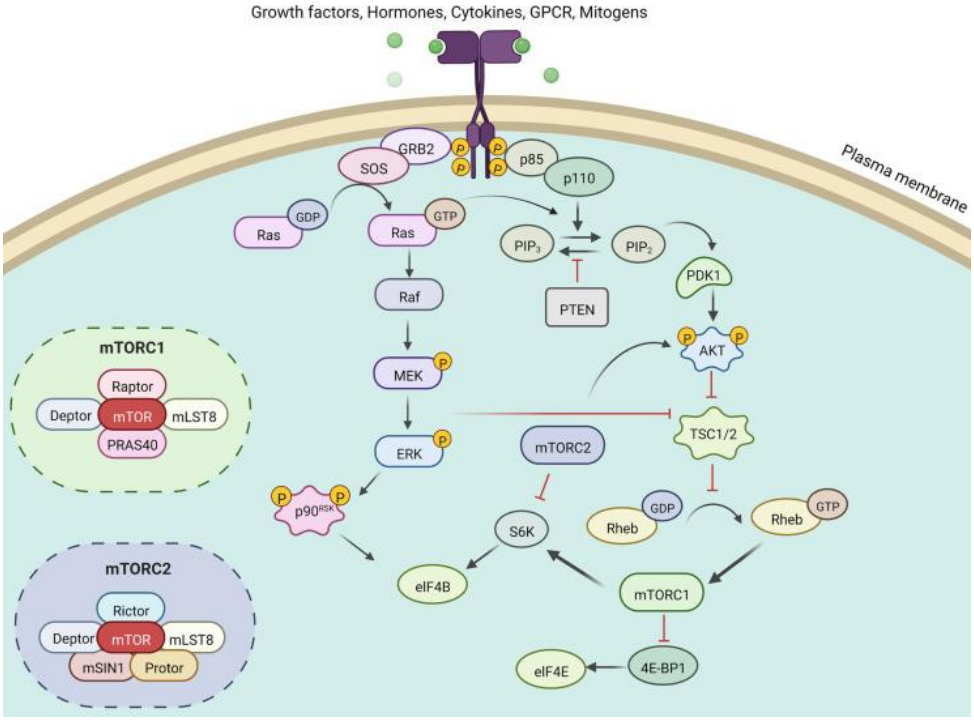

Figure 6: Schematic representation of Ras and PI3K/AKT/mTOR Pathways.[137]

Some genes are common to both pathways and the Ras protein has the capability to activate both. Additionally, if one of these pathways is suppressed, the other can compensate for the resulting deficits, having various nodes able to interact with each other.[138] Modulation of these pathways has been observed following exposure to various endocrine EDs, including phthalates, sunscreens, and many others. It is therefore of considerable interest to investigate the effects on the pathways to elucidate the underlying mechanisms of action of these environmental pollutants.

Understanding how these EDs influence such critical signaling pathways can provide insights into their potential impact on cellular processes and contribute to the development of strategies for mitigating their adverse effects.

2 Aim

EDs are emerging compounds, ubiquitous in the environment, that have the potential to induce toxicity upon contact with living organisms and, at the same time, pose a significant threat to the ecosystem.[5] In particular, these molecules can interact with the synthesis, metabolism, action, and degradation of endogenous hormones and their respective receptors, inducing numerous and significant effects on the physiology of the organism.[7] EDs enter the environment through various pathways, including the direct use of these molecules in nature, residues in industrial and domestic wastewater, and the continuous production of smog in urban areas.[7] Consequently, the population is continuously exposed to a broad array of different substances through the ingestion of contaminated food or water, via inhalation of the volatile EDs, or through direct topic contact. These chemicals can exert toxic effects even at very low concentrations, posing a significant health risk.[94] Indeed, numerous *in vitro* and *in vivo* studies have established a significant correlation between exposure to various EDs and the development of several types of health disorders, including, but not limited to, various kinds of cancers, allergic reactions, and developmental defects on multiple systems. Studies on ED exposure primarily focused on reproductive health, yet emerging data underscore their impact on neurodevelopment and neuroendocrine regulation.[11]

The aim of the current project was to evaluate the effects of subtoxic exposure to various classes of EDs, with a particular focus on their toxicity to the adult CNS. Indeed, while the neurodevelopmental effects have largely been addressed, the potential impact of EDs on the adult CNS remains relatively unexplored.[98]

In the first part of this study, epigenetic mechanisms through which EDs exert their neurotoxic effects, including the identification of key cellular pathways that are modulated upon EDs exposure, were elucidated. The study focused on chemicals characterized by high production volumes, environmental persistence, and widespread presence in consumer goods. Therefore, in the first part of this project, a diverse panel of compounds, such as pesticides (ATZ, VNZ, CYP), industrial chemicals (DEP, PFOS), and pharmaceuticals (EE_2) was selected. The SH-SY5Y human neuroblastoma cell line was employed as an *in vitro* model, offering a balance between experimental tractability and physiological relevance. Despite inherent limitations,

this cell line was chosen for its reproducibility, its suitability for controlled dosing regimens, and its capacity to differentiate into mature neuronal phenotypes, providing a valuable *in vitro* system for studying adult neurotoxicity.

In order to investigate the effects of subtoxic exposure relevant to human conditions, cells were treated with a range of concentrations of the six selected EDs. Cell viability and oxidative stress assays were conducted to determine the optimal exposure concentrations.

To investigate the potential mechanisms underlying ED-induced neurotoxicity, the study focused on epigenetic alterations, particularly miRNA expression. This approach was motivated by the well-established role of miRNAs in regulating gene expression and their involvement in various neurological disorders, including neurodegenerative diseases.[123] Furthermore, previous studies have demonstrated that EDs can modulate miRNA expression, thereby contributing to their adverse effects on the organism.[118] miRNA profiling revealed significant alterations in miRNA expression following exposure to the selected EDs. Subsequently, bioinformatic analysis proved crucial in selecting relevant miRNAs involved in neurodegeneration, neurodevelopment and other CNS processes, and their target genes. Quantitative real-time PCR (qRT-PCR) was exploited to validated these findings and demonstrate significant alterations in genes involved in key signaling cascades, such as Ras and PI3K/Akt/mTOR, implicated in cell survival, proliferation, differentiation, and metabolism. Alterations in these pathways have been linked to various pathologies, including numerous cancers. [138] Western Blot analysis confirmed the observed changes in protein expression levels, providing further evidence for the impact of EDs exposure on these critical cellular processes.

The second phase of the experimental work was conducted at BioBasic Europe S.r.l., a leading company specializing in safety and efficacy assessments for dermo-cosmetic products, medical devices, and dietary supplements. Their laboratories in Pavia conduct quality testing on a diverse range of products available on the market. This enables them to maintain extensive databases on the most commonly used molecules and their subsequent release into the environment. Among these are sunscreens, which contain essential molecules for UVA and UVB protection; however, many of these substances are known EDs. Specifically, a screening of the sunscreens analysed by the company was conducted to identify and quantify the EDs present. Analysis revealed that organic UV filters EHS and AVO were the sunscreens

with endocrine-disrupting activity that show the highest prevalence in commercial products. Consequently, a decision was made to investigate the neurotoxic effects of these two molecules using the SH-SY5Y cell line. This involved analysing gene and protein expression changes following exposure to these compounds. Additionally, the cutaneous toxicity of EHS and AVO was assessed using the HaCaT human keratinocyte cell line. This choice was motivated by the primary route of exposure to sunscreens being the skin, thus warranting an investigation of their potential dermal toxicity.
Finally, the company also quantified Ni, a well-known endocrine-disrupting metal, in cosmetic products. Through the analysis of their extensive database, the levels of Ni in commercially available products were determined.

This multifaceted approach allowed for a comprehensive assessment of the impact of some molecules with endocrine disrupting activity on cellular function and provided insights into their potential neurotoxic effects. These analyses have identified the capability of some EDs to modulate Ras and the PI3K/Akt/mTOR pathways in a differentiated model of CNS cells, posing the basis for understanding the role of exposure to EDs in adult life.

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3 Materials and Methods

3.1 EDs sourcing and preparation

The compounds ATZ (CAS Number:1912-24-9), VNZ (CAS Number: 50471-44-8), CYP (CAS Number: 52315-07-8), EE_2 (CAS Number: 57-63-6), DEP (CAS Number: 84-66-2), and PFOS (CAS Number: 1763-23-1) were purchased as pure substances [1M] from Merck Life Science S.r.L. (Milan, Italy). On the other hand, AVO (CAS Number:70356-09-1) and EHS (CAS Number: 118-60-5) were purchased as pure substances [1M] from Fluorochem Ltd (Glossop Derbys, United Kingdom) and generously provided by BioBasic Europe. Stock solutions [100 mM] were prepared by dissolving the parent compound in dimethyl sulfoxide (DMSO, Merck Life Science S.r.L.), filtered, aliquoted into 1.5 mL Eppendorf tubes, and stored at -20°C. Working solutions were prepared by diluting the stock solutions in DMEM culture medium supplemented with 2% FBS, without phenol red (Euroclone S.p.A, Pero, Italy).

3.2 Cell culture

In vitro experiments were conducted on SH-SY5Y human neuronal cells, purchased from the Lombardy and Emilia-Romagna Experimental Zootechnic Institute (Brescia, Italy), and on HaCaT human keratinocytes, generously provided by Professor Andrea Tarozzi from the Scienze per la Qualità della Vita (QUVI) Department at the Alma Mater Studiorum, University of Bologna. Both cell lines were cultured as a monolayer in 100 mm diameter cell culture dishes, in DMEM supplemented with 10% FBS, previously heat-inactivated at 57°C for 30 minutes, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (Euroclone S.p.A). The cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO_2). Subcultures were prepared by detaching the cells using a 0.02% EDTA-trypsin solution (Euroclone S.p.A). This process was repeated twice a week using phenol red-containing DMEM. Subsequently, before setting up the plates or dishes used in the experiments, the cells were transferred to phenol red-free DMEM to avoid interactions with ER. Indeed, phenol red is a pH indicator commonly used in cell culture media, as it gradually changes colour from yellow to red with increasing pH between 6.8 and 8.2. It has been demonstrated that phenol red, at the concentration normally used, exhibits estrogenic

activity.[139] Given the interaction that EDs may have with the estrogenic system, phenol red was excluded in subsequent experimental trials. To conduct the experiments, the cells were detached and counted using a Burker chamber, following staining with eosin (Merck Life Science S.r.L.).

3.3 Neuronal cells differentiation

SH-SY5Y cells were seeded in a 6-well plate at a concentration of 6×10^4 cells/well and in a 96-well plate at 2×10^3 cells/well in DMEM with 10% FBS without phenol red. After 5 hours, the medium was removed, and the cells were treated with a working solution of retinoic acid (Merck Life Science S.r.L.) at a concentration of 10 µM in complete culture medium (DMEM with 2% FBS without phenol red). The treatment was repeated three times, every 48 hours.

3.4 Cells plating and EDs treatment

For undifferentiated SH-SY5Y cells, in order to assess cell viability, 96-well plates were set up with 1 x 10^4 cells/well, while for the evaluation of ROS formation, 96-well plates were prepared with 2 x 10^4 cells/well. Cell cultures were treated with the test substances 24 hours after plating. To assess cell viability via the MTT test, later discussed, cultures were treated with different concentrations of ATZ, CYP, VNZ, EE_2, PFOS, and DEP, specifically 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM, for 24, 48, or 72 hours in DMEM without phenol red supplemented with 2% FBS at 37°C in 5% CO_2. To evaluate ROS formation, undifferentiated SH-SY5Y cells were treated with different concentrations of the same molecules, specifically 1 nM, 10 nM, 100 nM, and 1 µM, once again for 24, 48, or 72 hours in DMEM without phenol red supplemented with 2% FBS at 37°C in 5% CO_2. Given the absence of observed differences across the three analyzed time points, subsequent assays were conducted solely at the 48-hour exposure interval.

In the case of differentiated SH-SY5Y cells, to evaluate cell viability via the AlamarBlue HSTM assay, 2×10^3 cells/well were plated in a 96-well plate and, after differentiation, exposed to ATZ, CYP, VNZ, EE_2, PFOS, and DEP at the concentration of 100 nM or 1 µM for 48 hours at 37 °C in 5% CO_2. On the other hand, to assess the production of ROS, 6×10^4 cells/well were differentiated and then treated with the same experimental design exploited for the Alamar assay. The same conditions were used in SH-SY5Y differentiated cells to

analyse the effects of AVO and EHS exposure after 48 h, at concentrations ranging from 100 µM to 1.5 nM.

To determine the cell viability and the ROS formation, 2 x 10^4 and 3 x 10^4, respectively, HaCaT cells were plated in 96 well plates and after 24 hours were exposed to AVO and EHS at concentrations ranging from 100 µM to 1.5 nM for 48 hours in DMEM without phenol red supplemented with 2% FBS at 37°C in 5% CO_2.

For pellet collection, SH-SY5Y cells differentiated in 4 wells of a 6-well plate, treated with the same ED (ATZ, CYP, VNZ, EE_2, PFOS, DEP, AVO or EHS) at the same concentration (100 nM or 1 µM and 10 nM, 100 nM, or 1 µM only for AVO and EHS) for 48 hours in DMEM without phenol red supplemented with 2% FBS at 37°C in 5% CO_2, were pooled together to obtain the pellet.

To collect the HaCaT cell pellet, 100 mm dishes containing 1 x 10^6 cells each were prepared. After 24 hours from plating, the dishes were treated with AVO and EHS at concentrations of 10 nM, 100 nM, and 1 µM for 48 hours in DMEM without phenol red supplemented with 2% FBS at 37°C in 5% CO_2. After the 48-hour treatment, the cell pellet was collected.

For each experiment, control wells, referred to as vehicle (Vh), were included. These wells were treated with culture medium containing sterile DMSO (<0.1%) and incubated for 48 hours under the same conditions as the cells treated with EDs.

3.5 Determination of cellular viability

Cell viability was assessed by the reduction of MTT to insoluble formazan.[140] Briefly, cells plated in a 96-well plate were deprived of their medium (where they had been incubated with EDs) and placed for 2 hours in the incubator with a tetrazolium dye MTT (3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, St Louis, MO, USA) in Hanks' Balanced Salt Solution (HBSS, Euroclone S.p.A.) (0.5 mg/mL). After washing with HBSS, the formed crystals were dissolved in isopropanol (Sigma-Aldrich), and the amount of formazan was quantified using a multilabel plate reader (GENios, TECAN®, Mannedorf, Switzerland) at 570 nm, with reference wavelength of 690 nm. The results are expressed as the mean ± SD of the percentage viability relative to Vh-treated cells (DMSO < 0.1%).

The AlamarBlue HSTM assay (Thermo Fisher Scientific, Waltham, MA, USA), which utilizes the reduction of resazurin to resorufin,[141] was also used to assess cell viability. Following treatment with the EDs, 10 µL/well of cell viability reagent was added directly to the cell medium and the cells were incubated for 1 hour at 37°C with 5% CO_2. The amount of resorufin formed was measured using the multilabel plate reader GENios at 570 nm with a reference filter at 690 nm. Data are expressed as the mean ± SD of the viability percentage relative to cells treated with the Vh (DMSO < 0.1%).

3.6 Evaluation of ROS generation

Intracellular formation of ROS was determined using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich) a non-polar and non-fluorescent molecule that, in live cells, is hydrolysed by intracellular esterases to 2',7'-dichlorodihydrofluorescein (H_2DCF). In the cytoplasm, H_2DCF, being polar, is oxidized in the presence of ROS to a fluorescent compound, 2',7'-dichlorofluorescein (DCF).[142] Briefly, after treatment with EDs, the medium was removed and 100 µL, for the 96-well plates, and 2 mL, for the 6-well plates, of H_2DCF-DA working solution were added to each well before incubating the plates in the dark at room temperature (RT) for 30 minutes. After the incubation period, the working solution was removed, and each well was washed with Dulbecco's phosphate-buffered saline without Ca2+ and Mg2+ (D'PBS, Euroclone S.p.A.). Finally, 100 µL or 2 mL of HBSS was added to each well, and the plates were incubated for 15 minutes in the dark at RT. After the incubation, intracellular ROS formation was assessed using the fluorimeter GENios, measuring fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Results were expressed as mean ± SD of the arbitrary fluorescence units (AFU) detected.

3.7 Cellular pellet collection

After 48 hours of treatment with the EDs in study, the cells were detached using a 0.02% EDTA-trypsin solution, transferred to a 15 mL tube, and centrifuged at 1,400 rpm for 8 minutes. Once the supernatant was removed, the pellet was resuspended in 1 mL of D'PBS and transferred to a 1.5 mL Eppendorf tube for further centrifugation at 6,000 rpm at 4°C for 5 minutes. The supernatant was removed again, and the pellet was stored at -80°C until use. A portion of the collected pellets was used for total RNA extraction, while the remaining part was used for protein extraction.

3.8 RNA extraction

Total RNA was extracted from the cell pellet using 'PureLink RNA Mini Kit' assay (Thermo Fisher Scientific). Specifically, the pellet was lysed using a lysis buffer containing 1% β-mercaptoethanol (Sigma-Aldrich). For each sample, 10 μL of 2-mercaptoethanol were added to 1 mL of Lysis Buffer provided in the kit. A total of 600 μL of complete Lysis Buffer was added to the cell pellet and the solution was vigorously mixed to obtain the lysate. Subsequently, an equal volume of 70% ethanol (Sigma-Aldrich) was added. The resulting solution was transferred to the provided separation column and filtered by centrifugation at 13,000 rpm for 15 seconds at RT. After centrifugation, the eluate was discarded. Once all the RNA was collected in the cartridge, the samples were washed with Wash Buffer I and II, centrifuging at 13,000 rpm for 15 seconds at RT and the eluate was discarded. The cartridge containing the bound RNA was then dried with two consecutive centrifugations at 13,000 rpm for 2 minutes each. Finally, the separation column was transferred to a clean collection tube. 20 μL of RNase-free water (Thermo Fisher Scientific) was loaded onto the cartridge, incubated for 1 minute and centrifuged for 2 minutes at 14,000 rpm at RT. The eluate containing the RNA was quantified by spectrophotometric analysis using NanoDrop™ ND-2,000 (Thermo Fisher Scientific) and finally stored at -80°C.

3.9 miRNA reverse transcription and quantification

To quantify miRNA expression, 1,000 ng of the extracted RNA was reverse transcribed into cDNA using the 'Mir-X miRNA First-Strand Synthesis and TB Green qRT-PCR' kit (Takara, Mountain View, CA, USA), following the manufacturer’s instructions. For each sample, a reaction mixture was prepared, containing the appropriate reagents provided by the kit, as listed in Table I, along with 1 μg of RNA of the sample of interest, diluted with RNase-free water.

Table I: Reverse transcription solution

Reagent	Volume (µL)
mRQ Buffer (2X)	5
RNA diluted with RNase-free water	3,75
mRQ Enzyme	1,25
Total volume	10

The samples were then reverse transcribed according to the experimental protocol described in Table II.

Table II: Reverse transcription protocol

	Temperature (°C)	Time (minutes)
Step 1	37	60
Step 2	85	5
Step 3	4	∞

At the end of the reverse transcription, the cDNA was diluted with 90 µL of RNase-free water before being stored at -20°C until further use.

The cDNA samples were then quantified by qRT-PCR exploiting once again the 'Mir-X miRNA First-Strand Synthesis and TB Green qRT-PCR' kit (Takara). For each sample, a specific solution of buffer, enzymes, and miRNA-specific primers was prepared, as described in Table III. Subsequently, 11.5 µL of the reaction mixture and 1 µL of cDNA were added to each well of a 96-well plate.

Table III: qRT-PCR solution

Reagent	Volume (µL)
ddH_2O	4,5
TB Green Advantage Premix (2X)	6,25
ROX Dye (50X)	0,25
miRNA-specific Primer (10 µM)	0,25
miRQ 3' Primer (10 µM)	0,25
cDNA	1,0
Total volume	12,5

The endogenous control U6 was selected to normalize the cDNA quantity of each sample. The reaction mixture for the endogenous control is described in Table IV.

Table IV: U6 qRT-PCR solution

Reagent	Volume (µL)
ddH_2O	4,5
TB Green Advantage Premix (2X)	6,25
ROX Dye (50X)	0,25
U6 Primer (10 µM)	0,25
miRQ 3' Primer (10 µM)	0,25
cDNA	1,0
Total volume	12,5

It was therefore possible to assess the modulation of hsa-miR-653-5p, hsa-miR-29b-3p, has miR-133b, hsa-miR-18b-5p, hsa-miR-200a-3p, and hsa-miR-146b-5p. To perform the analysis, the entire sequence of the mature miRNA was used as a specific 5' primer, while the 3' primer was the mRQ 3' primer provided by the kit. The sequences of the primers used were drawn in the genome browser Ensembl[143] and the platform for biotechnology research and development Benchling (Benchling [Biology Software] (2022)), and are listed in Table V.

Table V: Sequences of the primers used

miRNA	**Primer Sequence (5′-3′)**
Hsa-miR-18b-5p	TAAGGTGCATCTAGTGCAGTTAG
Hsa-miR-200a-3p	TAACACTGTCTGGTAACGATGT
Hsa-miR-653-5p	GTGTTGAAACAATCTCTACTG
Hsa-miR-29b-3p	TAGCACCATTTGAAATCAGTGTT
Hsa-miR-146b-5p	TGAGAACTGAATTCCATAGGCTG
Hsa-miR-133b	TTTGGTCCCCTTCAACCAGCTA

The plate was then placed in a 7900HT Fast PCR system (Applied Biosystems™, Thermo Fisher Scientific), where the appropriate thermal cycles, as reported in Table VI, were programmed.

Table VI: Thermal cycles

Step	**Temperature (°C)**	**Time (seconds)**
Denaturation	95	10
qPCR (x40 cycles)	95	5
	60	20

For each sample, an amplification curve and the corresponding Threshold Cycle (Ct) value were obtained.(Figure 7).

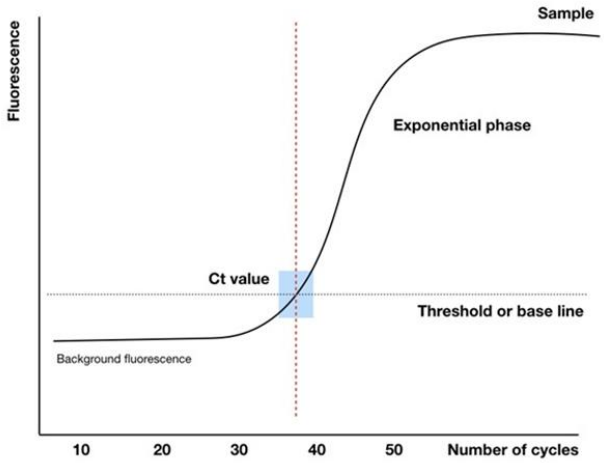

Figure 7: Schematic representation of the qRT-PCR amplification curve

The quantification of miRNAs in the samples was performed using a relative quantification method. Three experiments were run for each sample. To carry out the quantitative analysis, the cDNA value of each sample must first be normalized using the endogenous control U6, thus excluding any variations due to operator error. Subsequently, the data is compared to the value obtained for the Vh. Finally, the Fold change ($2^{-\Delta\Delta Ct}$) is calculated, a numerical value that allows for the assessment of miRNA expression levels relative to Vh. The miRNA is considered upregulated if the Fold change is greater than 2, and downregulated if the Fold change is less than 0.5.

3.10 Evaluation of gene expression levels

To quantify gene expression, the extracted RNA was reverse transcribed into cDNA using the 'High-Capacity RNA-to-cDNA' kit (Thermo Fisher Scientific). For each sample, a solution was prepared as detailed in Table VII, consisting of buffers, enzymes and 1 µg of RNA diluted with RNase-free water.

Table VII: Reverse transcription solution

Reagent	**Volume (µL)**
RT Buffer Mix (2X)	5
RT Enzyme Mix (20X)	0,5
RNA diluted with RNase-free ddH_2O	4,5
Final volume	10

The samples were then reverse transcribed by setting the thermal cycles according to the experimental protocol outlined in Table VIII.

Table VIII: Reverse transcription protocol

Setting	**Step 1**	**Step 2**	**Step 3**
Temperature (°C)	37	95	4
Time (minutes)	60	5	∞

The cDNA obtained was then diluted with RNase-free water to a final volume of 100 µL and stored at −20°C until further use.

For the quantitative analysis of gene expression by qRT-PCR, the 'PowerUp SYBR Green Master Mix' kit (Thermo Fisher Scientific) was used. For each sample, solutions with the corresponding reagents and primers were prepared as shown in Table IX. To perform the quantification, the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected to normalize the cDNA amount of each sample. The reaction mixture for the endogenous control is the same as that used for the individual samples.

Table IX: qRT-PCR solution

Reagent	**Volume (µL)**
PowerUp SYBR Green Master Mix (2X)	5
Primer Forward	0,5
Primer Reverse	0,5
cDNA diluted with RNase-free ddH_2O	4
Total volume	10

In the reaction mixture, for each gene of interest, forward primers, complementary to the initial sequence of the cDNA to be amplified, and reverse primers, complementary to the terminal section of the sequence, were used. The sequences of the primers employed are listed in Table X.

Table X: Sequences of the primers used

Gene	**Primer Sequence (5′-3′)**
ADAM12-FW	GATGTCTCCCTCGCTCGAAA
ADAM12-REV	GTCCCCTGAGACCAGAACAC
BCL2-FW	ATGTGTGTGGAGAGCGTCAA
BCL2-REV	AGTTCCACAAAGGCATCCCA
PTEN-FW	GCGGAACTTGCAATCCTCAG
PTEN-REV	AACTTGTCTTCCCGTCGTGT
BACE1-FW	GTCGGAGGGAGCATGATCAT
BACE1-REV	TTGATCTCCACCCGCACAAT
MMP2-FW	CAAGGGCATTCAGGAGCTCT
MMP2-REV	GATCTCAGGAGTGACAGGGC
HDAC4-FW	CGACGCCAAAGATGACTTCC
HDAC4-REV	CGTCTTTCGGCCACTTTCTG
CDK6-FW	TGGATCTCTGGAGTGTTGGC
CDK6-REV	GGGAGTCCAATCACGTCCAA
EGFR-FWD	CAACTGGTGTGTGCAGATCG
EGFR-REV	GACATGCTGCGGTGTTTTCA
IGF1R-FWD	ATGCTCCAAGGATGCACCAT
IGF1R-REV	CTCGATGAGCCCCATGAAGT
SH3B4P-FWD	AGCTTGTGATGGCCCTACTG
SH3B4P-REV	GGTCAGGAGCACAAAGTCCT
SOS1-FWD	CCCCTGATTCCTCCTCCTCT
SOS1-REV	CTCGGTGGAATAGCAGGAGG
GAPDH-FWD	GAAGGTGAAGTTCGGAGTCAAC
GAPDH-REV	TGGAAGATGGTGATGGGATTTC

Subsequently, 8.5 μL of the reaction mixture and 1.5 μL of cDNA were added to a 96-well plate. The plate was placed into the 7900HT Fast PCR system (Applied Biosystems™, Thermo Fisher Scientific) and the correct thermal cycles, as shown in Table XI, were set.

Table XI: Thermal cycles

Step	**Temperature (°C)**	**Time (minutes)**	**Cycles**
UDG activation	50	2	Hold
Dual-Lock DNA polymerasi	95	2	Hold
Denaturation	95	15	40
Anneal/Extend	60	1	

For each sample, an amplification curve and the corresponding Ct value are obtained. Gene quantification was performed using a relative quantification method of three replicates per sample. To conduct the quantitative analysis, the cDNA value of each sample must initially be normalized using the endogenous control GAPDH, thereby excluding any variations due to operator error. The data is then compared the Vh and finally, the Fold change ($2^{-\Delta\Delta Ct}$) is calculated. A gene is considered significantly upregulated if the Fold change is greater than 2, while it is considered significantly downregulated if the Fold change is less than 0.5.

3.11 Protein extraction and Western Blotting analysis

Initially, the cell pellet was lysed using a buffer containing protease and phosphatase inhibitors (100x), with the addition of leupeptin (2 µg/mL) and phenylmethylsulfonyl fluoride (PMSF, 100 µg/mL) (Merck Life Science S.r.L.). The cell suspension was incubated at 4°C on a plate shaker for 30 minutes. Subsequently, the cell lysate was centrifuged at 1,400 rpm at 4°C for 10 minutes. After centrifugation, the supernatant was transferred to a new Eppendorf tube. Following extraction, protein concentration was determined using the Bradford method (Bradford assay solution, Bio-Rad Laboratories S.r.L., Hercules, CA, USA) and the lysate was stored at -80°C until further use.

The samples for the protein expression analysis were obtained by adding 1 part of Complete Laemmli Sample Buffer, prepared by adding β-mercaptoethanol to Laemmli 4X (Bio-Rad Laboratories S.r.L.) to achieve a final concentration of 1X, to 3 parts of protein per sample. The mixture was then boiled at 95°C for 5 minutes and stored at -20°C.

Protein separation was performed on 4–15% TGX polyacrylamide gels (Bio-Rad Laboratories S.r.L.), running the samples for 30 minutes at 200 V in Running Buffer (25 mM Tris; 192 mM Glycine; 0,1% (w/v) SDS pH 8,3. Bio-Rad Laboratories S.r.L.). After the run, gels were photoactivated to visualize the proteins and assess the success of the separation. Subsequently, the proteins were electroblotted onto 0.45 μm nitrocellulose membranes for 1 hour at 100 V in Blotting Buffer (25 mM Tris; 192 mM Glycine; 20% (v/v) MeOH, pH 8.3. Bio-Rad Laboratories S.r.L.). The total signal of the transferred proteins on the nitrocellulose membrane was detected using UV excitation to obtain values for normalizing subsequent readings. The membranes were then incubated for 2 hours at 4°C in Block Solution (5% non-fat dry milk; TBS; 0.05% Tween 20. Bio-Rad Laboratories S.r.L.) to block all nonspecific binding sites and then at 4°C overnight with primary antibody recognizing phospho-p44/42 MAPK (pERK1/2), phospho-Akt (pAKT), phospho-mTOR (pmTOR), p53, Bax, Bcl-2, EGFR, and PTEN, provided by Cell Signaling Technologies Inc. (Danvers, MA, USA). The following day, the membranes were washed with TBS-T buffer (TBS, 0.05% Tween 20) and incubated at RT for 1 hour with a horseradish peroxidase-linked secondary antibody, either anti-rabbit or anti-mouse (1:5,000) purchased from Jackson ImmunoResearch Europe Ltd. (Cambridgeshire, UK). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) solution (Bio-Rad Laboratories S.r.L.). Subsequently, the membranes were stripped with stripping buffer (100 mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris, pH 6.7) and re-incubated with the primary antibody recognizing total Akt, mTOR, ERK1/2 (Cell Signaling Inc.) and β-actin (Merck Life Science S.r.L.), all 1:1,000, at 4°C overnight. Data analysis was performed by Quantity One software (Bio-Rad Laboratories S.r.L.), utilizing band densitometry; normalized values are expressed as the mean ± SEM of the densitometry for each experimental group.

3.12 Immunoprecipitation of Ras protein

Ras protein activation was assessed using the Active Ras Detection Kit (Cell Signaling Inc., Euroclone S.p.A.) following the manufacturer's instructions. Specifically, 200 μL of complete lysis buffer (2 μL of PMSF (100 mM) in 200 μL of 1X Lysis/Binding/Wash buffer) was used to lyse the cell pellet. Centrifugation was performed, and subsequently, the protein content in the supernatant was quantified using the Bradford assay. Afterward, spin cups were prepared

with glutathione resin and GST-Raf1-RBD, to which the cell lysate, containing 500 µg of total protein, was added. The reaction mixture was then incubated for 1 hour at 4°C with gentle agitation. After the incubation, the spin cups were washed three times with 400 µL of complete Lysis Buffer, and then 50 µL of reducing sample buffer (200 mM; 1.5 mg of DTT with 50 µL of 2X SDS Sample Buffer) were added to the resin. The eluate obtained after centrifugation was heated at 95°C for 5 minutes. From this point, the Western Blotting protocol, as previously described, was followed.

3.13 In silico target analysis

To identify potential target genes of the differentially expressed miRNAs, a comprehensive bioinformatic analysis was performed. The prediction of miRNA-target interactions was facilitated by the integration of data from multiple validated databases, including miRWalk, TargetScan, miRDB, and miRTarBase.[144] Subsequently, the predicted target genes were subjected to functional enrichment analysis using g:Profiler.[145] This bioinformatics tool maps genes to established functional pathways and identifies statistically significant overrepresented terms. The selected pathways were determined based on hypergeometric tests with a stringent p-value threshold of ≤ 0.001 and their known relevance to the mechanisms of endocrine disruption.

3.14 Statistical Analysis

Data analysis was performed using PRISM 9 software (GraphPad Software, La Jolla, CA, USA). Differences between groups were analysed using a mixed model or one-way ANOVA with Dunnet's post hoc test, and the results are expressed as the mean ± SEM of each experimental group. A p-value of less than 0.05 was considered statistically significant.

4 Results

For simplicity the results will be divided into four sections. The first one regards the selection of ED compounds and the analysis of viability and oxidative stress in the SH-SY5Y neuroblastoma cell line, to determine appropriate exposure concentrations and assess neurotoxicity. The second section focuses on epigenetic modifications and pathway analyses following exposure to ATZ, CYP, and VNZ, while the third section presents similar investigations to explore modulated pathways upon exposure to EE_2, DEP, and PFOS. Finally, the fourth section was conducted in collaboration with BioBasic Europe, for evaluating the toxicity of AVO and EHS, sunscreen filters with endocrine disrupting activities, on both SH-SY5Y and HaCaT cell lines, as well as the assessment of Ni content in commercially available cosmetic products.

4.1 Part 1: EDs selection and viability and oxidative stress analysis

4.1.1 Substances selection

Through a comprehensive review of the literature, key molecules were identified for inclusion in this study. To explore the effects of different classes of substances with endocrine-disrupting activities, compounds that are both widely utilized and commercially available on a global scale were selected. Specifically, ATZ, an herbicide, CYP, an insecticide and VNZ, a fungicide, were chosen as representative pesticides. EE_2 was selected due to its well-documented endocrine-disrupting properties and its widespread use in contraceptive pharmaceuticals by the female population. PFOS and DEP were chosen from the category of industrial chemicals, given their ubiquitous presence in a variety of consumer products. Moreover, these molecules are of particular interest due to their environmental persistence, reinforcing their classification as EPs.

4.1.2 Evaluation of cell death and oxidative stress induction in undifferentiated SH-SY5Y cells

The response to treatment with ATZ, VNZ, CYP. EE_2, DEP, and PFOS was evaluated using the continuous human neuroblastoma cell line, SH-SY5Y. Initially, cell viability was determined through the MTT assay. The cells were treated for 48 h with various

concentrations of the EDs in study (0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM) in DMEM medium without phenol red and 2% FBS. Figure 8 shows the results expressed as cell viability (%) relative to the Vh. The analysis did not show significant differences between the various concentrations of ATZ (A), VNZ (B), CYP (C), DEP (D), and PFOS (F) compared to Vh. Only the highest concentration of EE_2 (E) induced a significant mortality in the cells exposed.

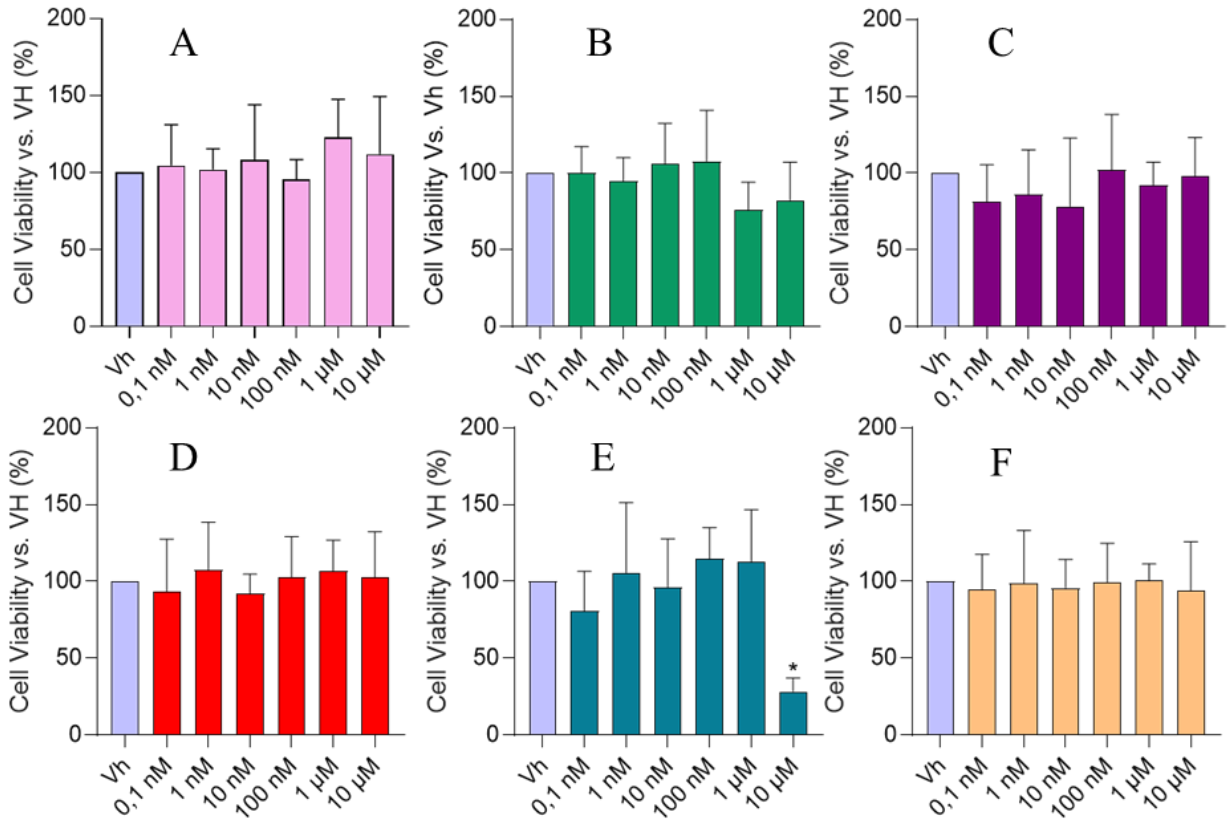

*Figure 8: Neuronal viability in undifferentiated SH-SY5Y after the treatment with ATZ (A), VNZ (B), CYP (C), DEP (D), EE_2, (E), and PFOS (F) 0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM, and 10 µM. After 48 h treatment, cell viability was evaluated using the MTT assay. Data are expressed as the mean ± SD of viability percentage relative to cells treated with the Vh of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnet).*

ROS production in SH-SY5Y cells was subsequently evaluated following exposure to ATZ, VNZ, CYP, DEP, EE_2, and PFOS at concentrations of 1 nM, 10 nM, 100 nM and 1 µM for 48 h using the fluorescent probe H_2DCF-DA. As shown in Figure 9, the treatments did not demonstrate a significant increase in ROS formation compared to the Vh for any of the concentrations investigated.

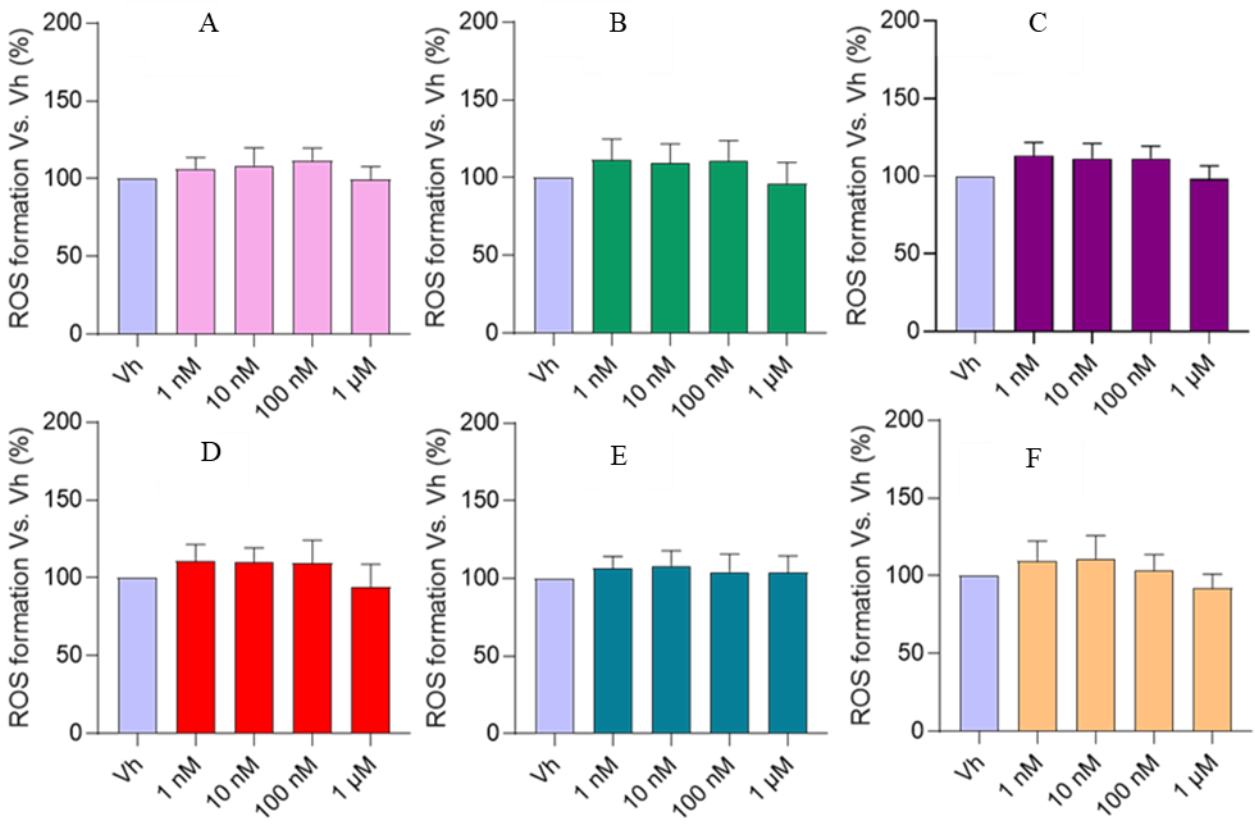

Figure 9: ROS production in undifferentiated SH-SY5Y after 48 h treatment with ATZ (A), VNZ (B), CYP (C), DEP (D), EE_2, (E), and PFOS (F) 1 nM, 10 nM, 100 nM, and 1 µM. Data are expressed as fold increases in the percentage of ROS formation versus the Vh and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

These preliminary analyses allowed the identification of subtoxic concentration ranges, where exposure to these six EDs does not induce acute cellular damage. Therefore, subsequent tests were conducted using only the 100 nM and 1 µM concentrations.

4.1.3 Evaluation of cell death and oxidative stress induction in differentiated SH-SY5Y cells

To investigate the effects of subtoxic exposure to EDs on a mature CNS model, a differentiation protocol for SH-SY5Y cells using 10 µM retinoic acid was established. After one week of treatment, the cells exhibited typical neuronal arborizations. However, this differentiation made the cells more fragile and consequently it was not feasible to assess cell viability following exposure to ATZ, VNZ, CYP, EE_2, DEP, and PFOS using the MTT assay, as it disrupts the cells and induces cell death. Instead, the AlamarBlue HSTM test was

employed on differentiated cells treated for 48 h with the six molecules at concentrations of 100 nM and 1 µM. Once again, no significant cell death was observed at any of the treatment concentrations, as it can be observed in Figure 10.

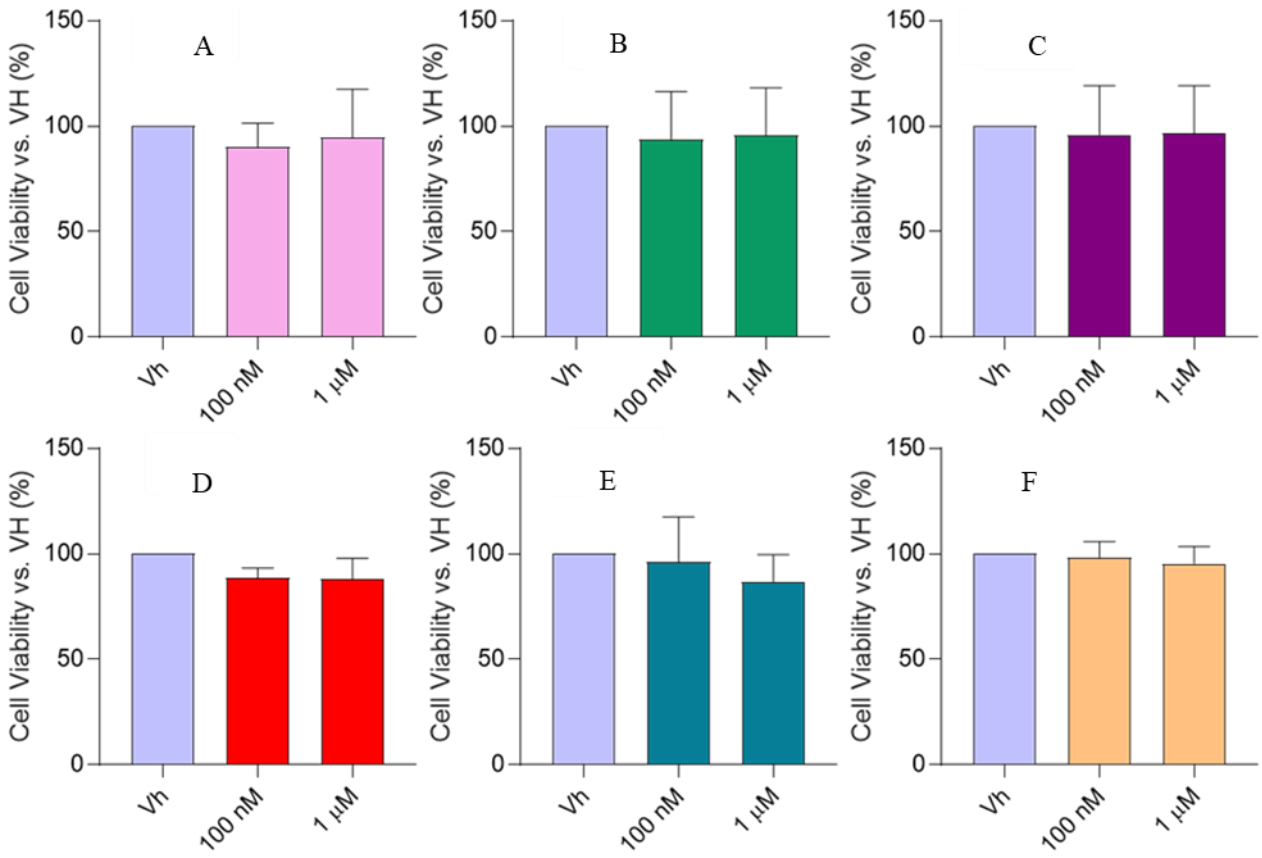

Figure 10: Neuronal viability in differentiated SH-SY5Y after the treatment with ATZ (A), VNZ (B), CYP (C), DEP (D), EE_2, (E), and PFOS (F) 100 nM and 1 µM. After 48 h treatment, cell viability was evaluated using the AlamarBlue HSTM test. Data are expressed as the mean ± SD of viability percentage relative to cells treated with the Vh of three independent experiments (One-way ANOVA, post hoc test Dunnet).

Finally, ROS production was also evaluated in differentiated SH-SY5Y cells after 48 h treatment with ATZ, VNZ, CYP, EE2, DEP, and PFOS [100 nM and 1 µM]. No significant stress was observed at the concentrations chosen (Figure 11).

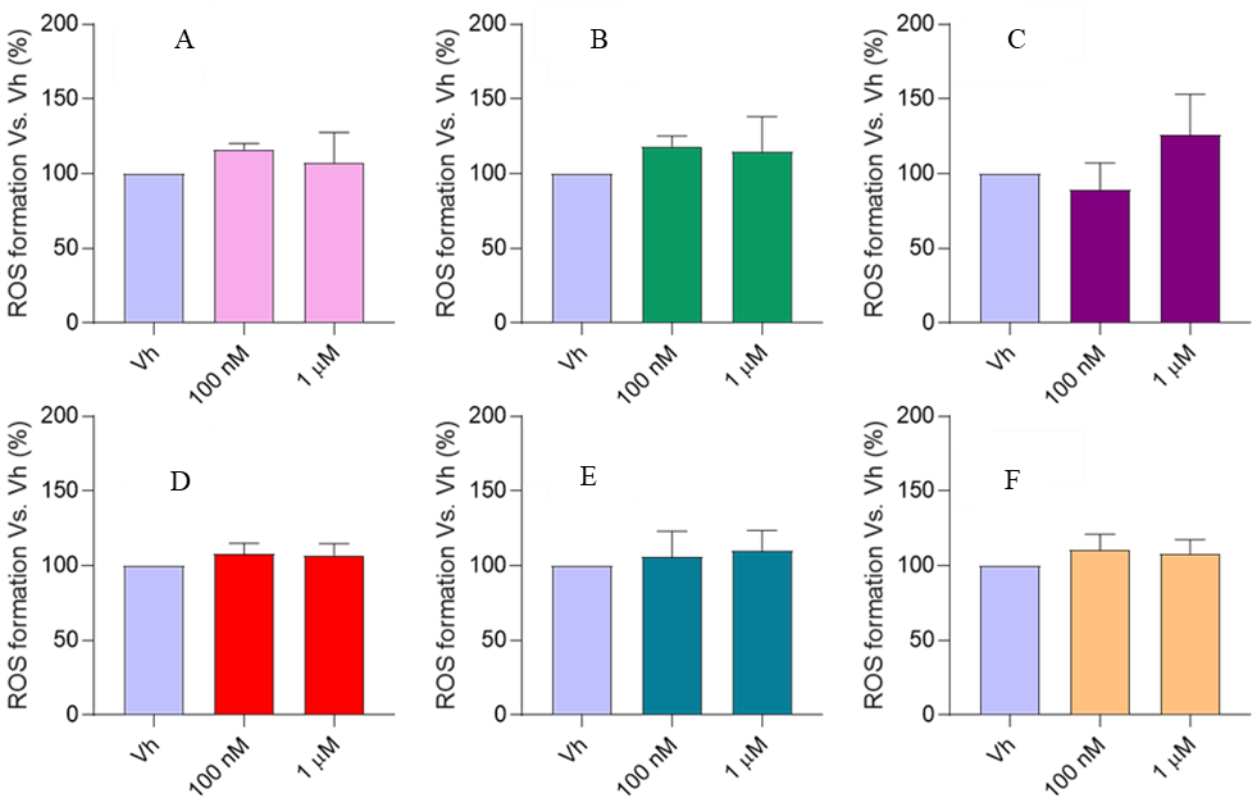

Figure 11: ROS production in differentiated SH-SY5Y after 48 h treatment with ATZ (A), VNZ (B), CYP (C), DEP (D), EE_2, (E), and PFOS (F) 100 nM and 1 µM. Data are expressed as fold increases in the percentage of ROS formation versus the Vh group and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

4.2 Part 2: ATZ, CYP, and VNZ

In the subsequent experimental phase, analyses focused on the effects of exposure to the three pesticides ATZ, VNZ, and CYP. This selection was driven by the results of miRNA modulation studies, which revealed a distinct clustering of these compounds compared to the other three EDs under investigation. These findings indicated that these pesticides are capable of modulating common pathways in cells. Furthermore, the shared classification of these molecules provided additional rationale for this division.

4.2.1 Modulation of miRNA expression profiles

In the next experimental phase, differentiated SH-SY5Y cells were treated with ATZ, VNZ, and CYP at concentrations of 100 nM and 1 µM. Epigenetic modifications were then assessed, with a specific focus on miRNA regulation. Based on a literature review, miRNAs 200a-3p,

146b-5p, and 29b-3p, were selected due to their involvement in key pathways related to neurodevelopment, neuroproliferation, neurodegeneration, and overall relevance to the CNS. qRT-PCR analysis was used to evaluate miRNA expression with single-primer detection. The table XII presents fold change values normalized to endogenous U6 miRNA, with downregulation (fold < 0.5) highlighted in blue.

miR-200a-3p exhibited the strongest modulation, being downregulated by ATZ, VNZ, and CYP [1 µM]. Significant downregulation of miR-146b-5p occurred after exposure to ATZ [100 nM]. Lastly, miR-29b-3p was significantly downregulated by exposure to ATZ and VNZ at 100 nM.

Table XII: qRT-PCR validation of miRNA expression in differentiated SH-SY5Y cells after exposure to ATZ, CYP, and VNZ

	ATZ 1	**ATZ 100**	**CYP 1**	**CYP 100**	**VNZ 1**	**VNZ 100**
miR 200a-3p	0,41	1,34	0,41	3,48	0,22	1,15
miR 146b-5p	0,56	0,49	1,32	1,06	0,97	1,51
miR 29b-3p	0,92	0,42	0,87	0,65	0,70	0,38

4.2.2 In silico target genes predictions

Following the identification of significantly modulated miRNAs, a bioinformatic analysis was conducted to determine both the target genes and the pathways in which the genes are involved. To prioritize high-confidence miRNA-target interactions, the miRWalk software was employed, leveraging a combined approach of three database (miRWalk, TargetScan, and miRDB) to identify target genes while minimizing the false positives inherent in single-tool predictions. Given the extensive number of target genes identified for the studied miRNAs, particularly miR-29b-3p, the analysis was narrowed down to those genes common to at least two miRNAs. Subsequently, the identified common target genes were visualized as a network using Cytoscape, as depicted in Figure 12.

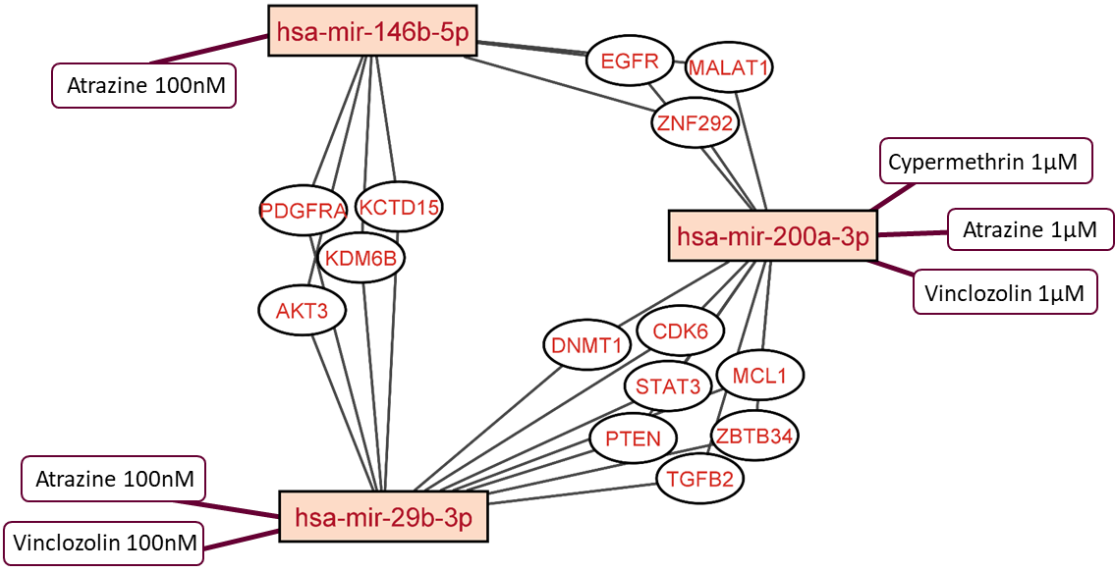

Figure 12: Descriptive network of the analyzed miRNAs, miR-146b-5p, miR-29b-3p, and miR-200a-3p (orange rectangles) and the identified common target genes (white ovals); created using Cytoscape software.

A functional enrichment analysis was conducted using g:Profiler with the Gene Ontology: Biological Process and WikiPathways databases to identify pathways potentially modulated by differentially expressed miRNAs. Once again, Cytoscape was employed to generate a visual representation of these enriched pathways (Figure 13).

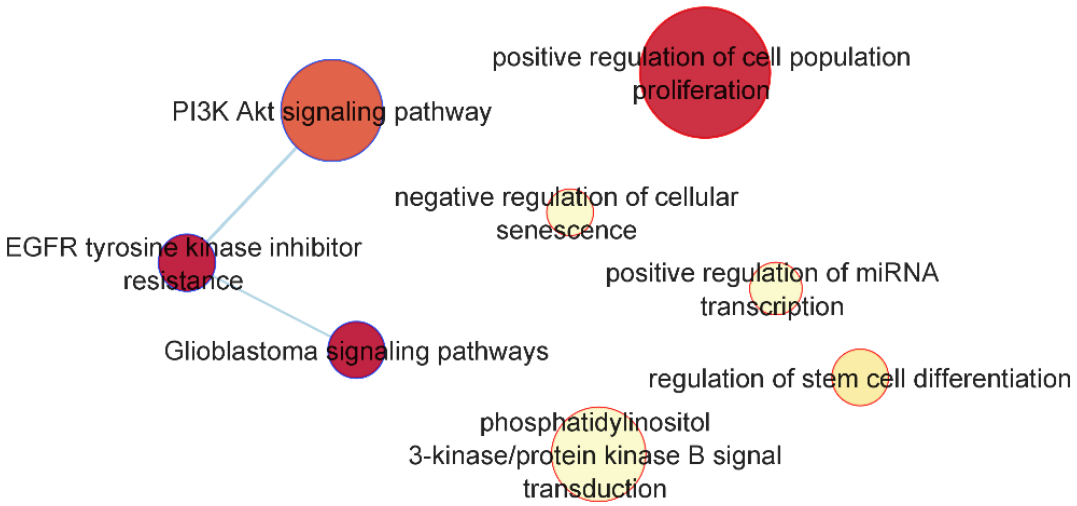

Figure 13: Enriched pathways of the target genes identified by Gene Ontology: Biological Process (red circles) and WikiPathways (blue circles) databases; created using Cytoscape software. A darker color denotes a lower p-value, signifying a greater statistical significance of the result. Moreover, the size of the circles is directly proportional to the number of genes examined within each respective pathway.

The most modulated pathways resulted to be ‘EGFR tyrosine kinase inhibitor resistance’, ‘Glioblastoma signaling pathways’, ‘positive regulation of cell population proliferation’, and ‘PI3K/Akt signaling pathway’. Notably, all these pathways are associated with cellular proliferation mechanisms and, specifically in glioblastoma, with the development of brain tumors. The PI3K/Akt/mTOR pathway, already known for its involvement in neoplastic processes, emerges as a key signaling pathway in this context.[146] Given these findings, a deeper exploration of this pathway's modulation was undertaken, involving the analysis of both gene and protein expression levels.

4.2.3 Modulation of genes expression profiles

Following the bioinformatic analysis, differentiated SH-SY5Y cells were treated for 48 h with ATZ, CYP and VNZ at concentrations of 100 nM and 1 µM. Subsequently, qRT-PCR analysis was performed to study the expression of some of the genes identified in the previous analysis. As shown in Figure 14, a significant upregulation is observed following exposure to ATZ [1 µM] for the HDAC4 (E) gene.

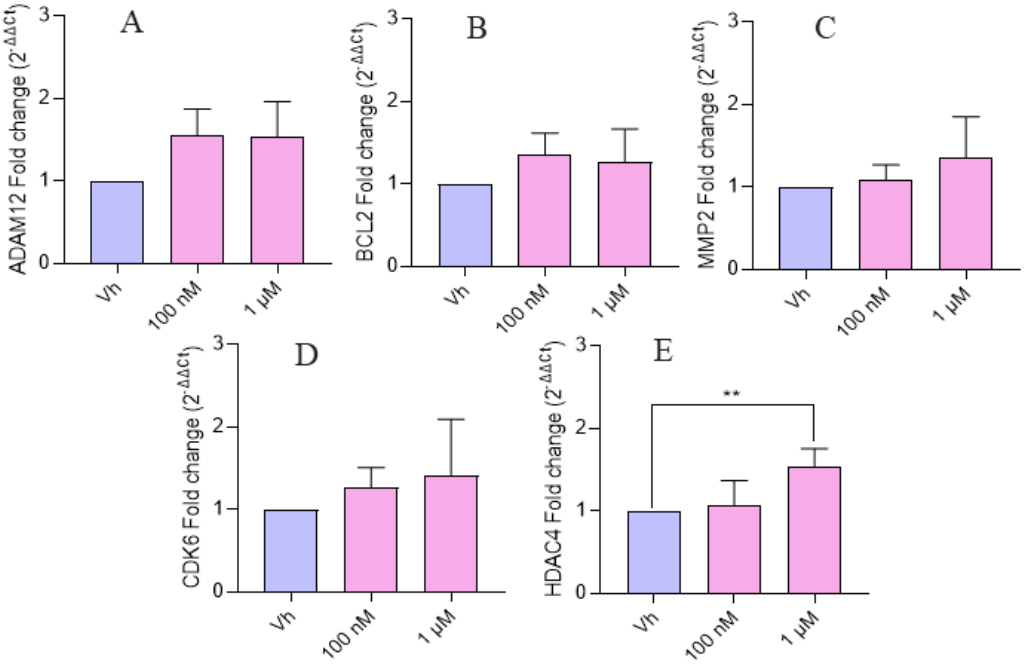

*Figure 14: Differential expression of ADAM12 (A), Bcl-2 (B), MMP2 (C), CDK6 (D), and HDAC4 (E) genes in differentiated SH-SY5Y cells treated for 48 h with ATZ [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (**p <0.01 vs. Vh. One-way ANOVA, post hoc test Dunnet).*

From Figure 15 it is possible to observe how CYP exposure can modulate the expression of the gene ADAM12 (A) at both concentrations chosen, while Bcl-2 (B) is significantly upregulated only by the lowest concentration of CYP [100 nM]. Moreover, CYP [1 µM] significantly upregulates HDAC4 (E) and MMP2 (C).

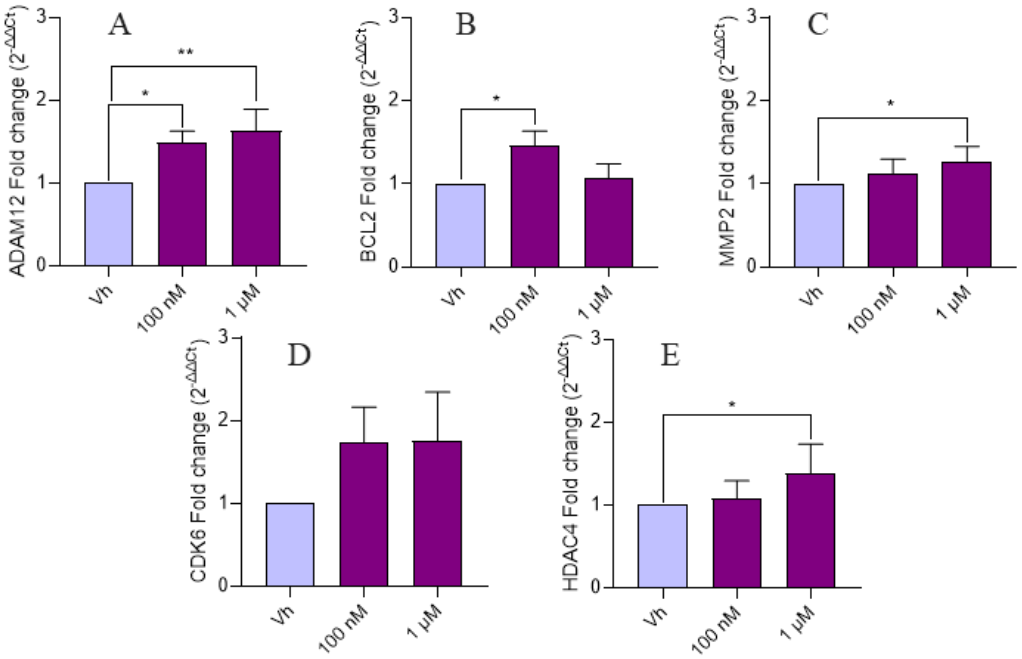

Figure 15: Differential expression of ADAM12 (A), Bcl-2 (B), MMP2 (C), CDK6 (D), and HDAC4 (E) genes in differentiated SH-SY5Y cells treated for 48 h with CYP [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (p <0.05 and **p <0.01 vs. Vh. One-way ANOVA, post hoc test Dunnet).*

Finally, ADAM12 (A) is significantly upregulated after exposure to both concentrations of VNZ, while HDAC4 (E) and MMP2 (C) are modulated only by the highest concentration of this fungicide [1 µM], as shown in Figure 16.

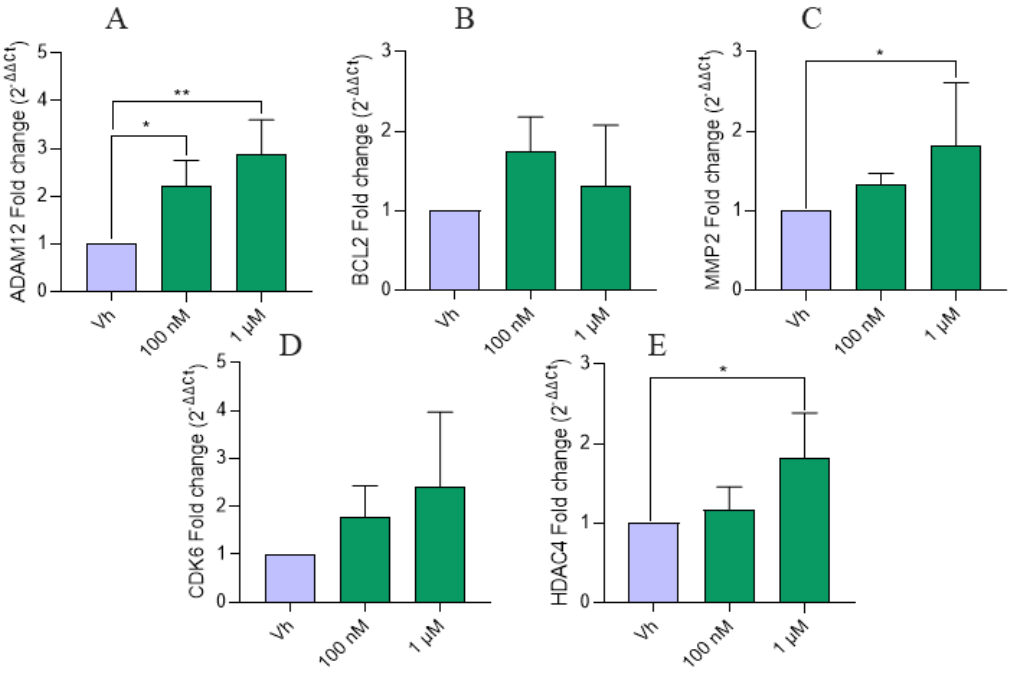

Figure 16: Differential expression of ADAM12 (A), Bcl-2 (B), MMP2 (C), CDK6 (D), and HDAC4 (E) genes in differentiated SH-SY5Y cells treated for 48 h with VNZ [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (p <0.05 and **p <0.01 vs. Vh. One-way ANOVA, post hoc test Dunnet).*

As shown in Figure 14, 15, and 16, the treatment with all three EDs led to a trend of upregulation of all the genes in study. The modulation of these genes corroborates the involvement of exposure to these pesticides in the dysregulation of the PI3K/Akt/mTOR pathway.

4.2.4 Western Blot analysis of differentiated SH-SY5Y cells

Following the results obtained through qRT-PCR, the next step of the project focused on validating the modulation of the identified pathways through protein analysis. Differentiated SH-SY5Y cells were treated for 48 h with ATZ, CYP, and VNZ at concentrations of 100 nM and 1 µM. The cell pellet was then collected, proteins were extracted, quantified using the Bradford assay and analysed by Western Blotting. Since gene modulation indicated an effect on the PI3K/Akt/mTOR pathway following exposure to these three EDs, modulation of the phosphorylation of Akt and mTOR proteins, key nodes of this pathway, was observed (Figure 17).

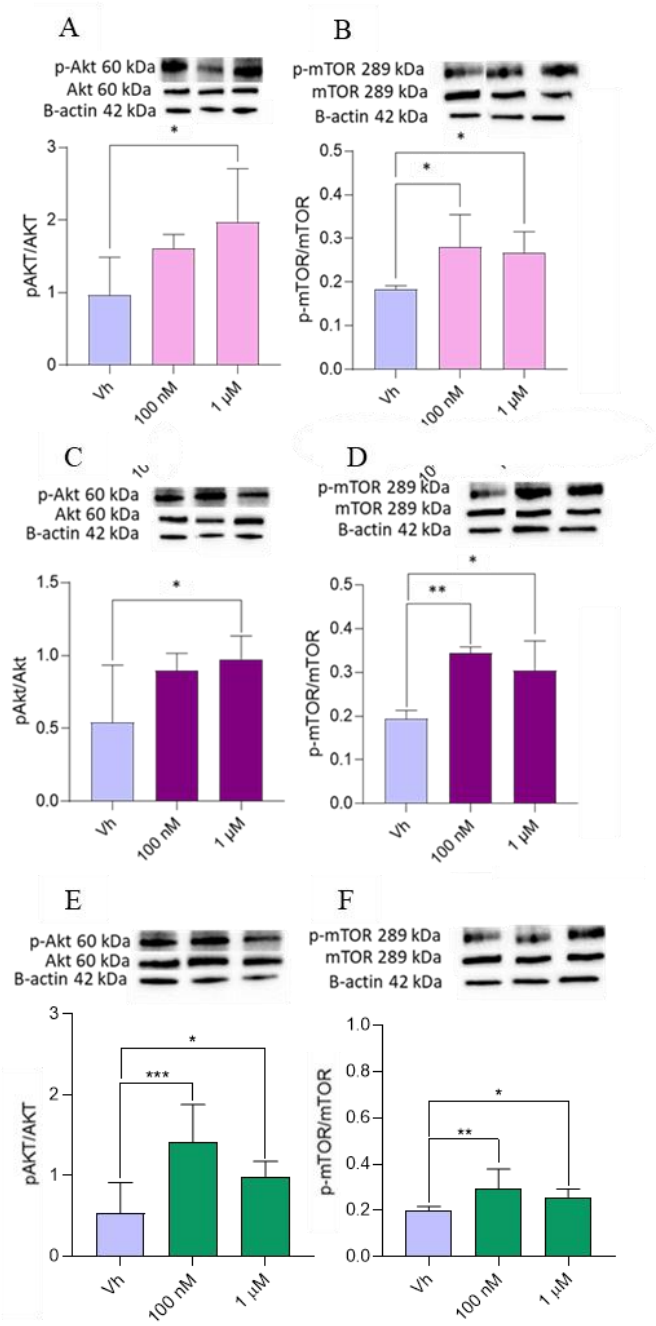

*Figure 17: Differential expression of phosphorylation of Akt (A), (C), (E), and mTOR (B), (D), (F) in differentiated SH-SY5Y cells treated for 48 h with ATZ (A, B), CYP (C, D), or VNZ (E, F) [100 nM, 1 µM]. The phosphorylation of Akt and mTOR were determined by Western Blotting at 60 and 289 kDa respectively and using total Akt or mTOR as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05, **p < 0.01, and ***p < 0.001 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Exposure to ATZ resulted in a significant upregulation of mTOR phosphorylation (B) at both concentrations, while Akt phosphorylation (A) was significantly modulated only after exposure to ATZ [1 µM]. Similarly, exposure to CYP [1 µM] upregulated Akt activation (C), while mTOR (D) was significantly phosphorylated by both concentrations of CYP. Regarding VNZ exposure, this compound significantly upregulated pAkt (E) and pmTOR (F) at both concentrations.

Given that the Akt/PI3K/mTOR pathway plays a key role in regulating cell proliferation, survival, and metabolism, the modulation of the p53 protein has also been investigated. p53, often referred to as the 'guardian of the genome,' is a crucial tumor suppressor that monitors cellular stress and DNA damage. It plays a pivotal role in maintaining genomic stability by activating DNA repair mechanisms, inducing cell cycle arrest, or triggering apoptosis when damage is irreparable.[147]

As shown in the Figure 18, exposure to ATZ [100 nM] causes a significant downregulation of this protein (A).

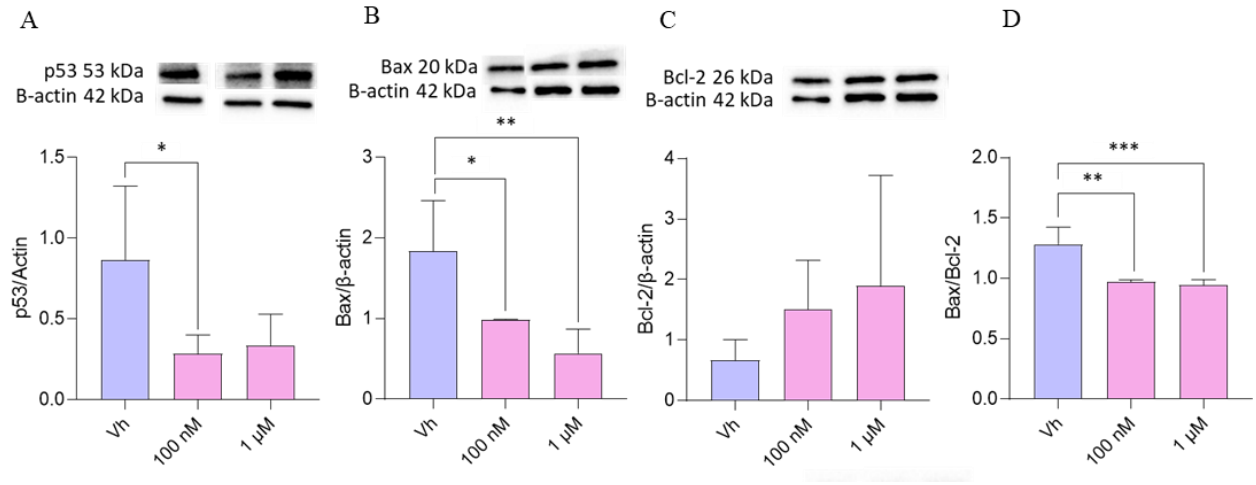

*Figure 18: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with ATZ [100 nM, 1 µM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (*p < 0.05, **p < 0.01, and *** p < 0.001 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Besides p53, the expression of Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein,[148] were also evaluated. A significant downregulation of Bax (B) can be seen after exposure to both concentrations of ATZ, as well as the ratio between Bax and Bcl-2 (D).

As shown in the Figure 19, exposure to both concentrations of CYP causes a significant downregulation of p53 protein (A), while after exposure to CYP [1 µM], Bax (B) is significantly downregulated. The ratio between Bax and Bcl-2 (D) shows a significant downregulation after exposure to both concentrations of CYP.

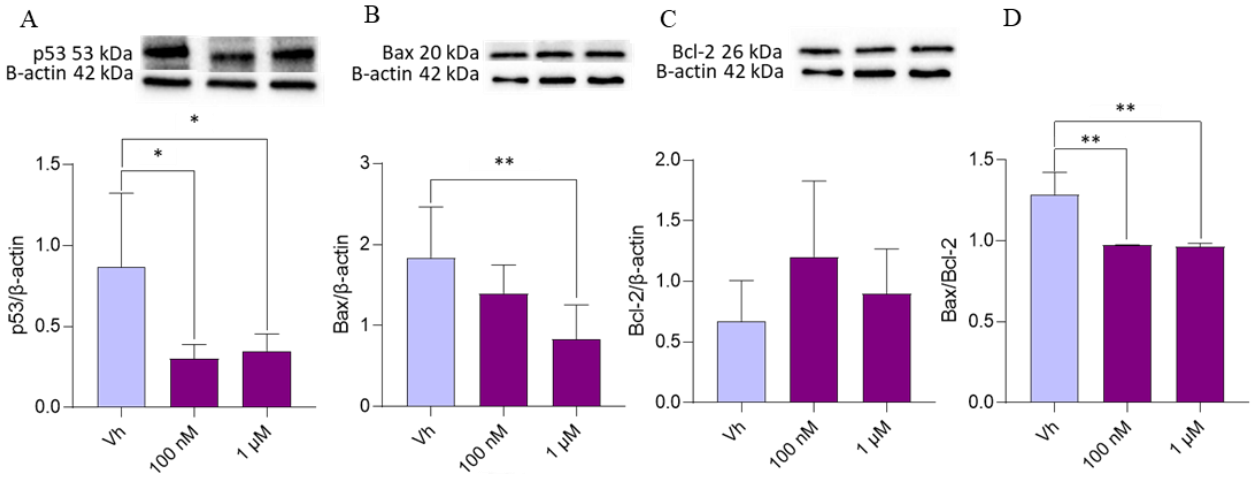

*Figure 19: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with CYP [100 nM, 1 µM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Finally, Figure 20 shows how exposure to both concentrations of VNZ causes a significant downregulation of p53 (A). Bax protein expression (B) is significantly downregulated after exposure to VNZ [1 µM], while Bcl-2 (C) is upregulated significantly after exposure to the same concentration of the fungicide. The ratio between Bax and Bcl-2 (D) shows a significant downregulation after exposure to VNZ [1 µM].

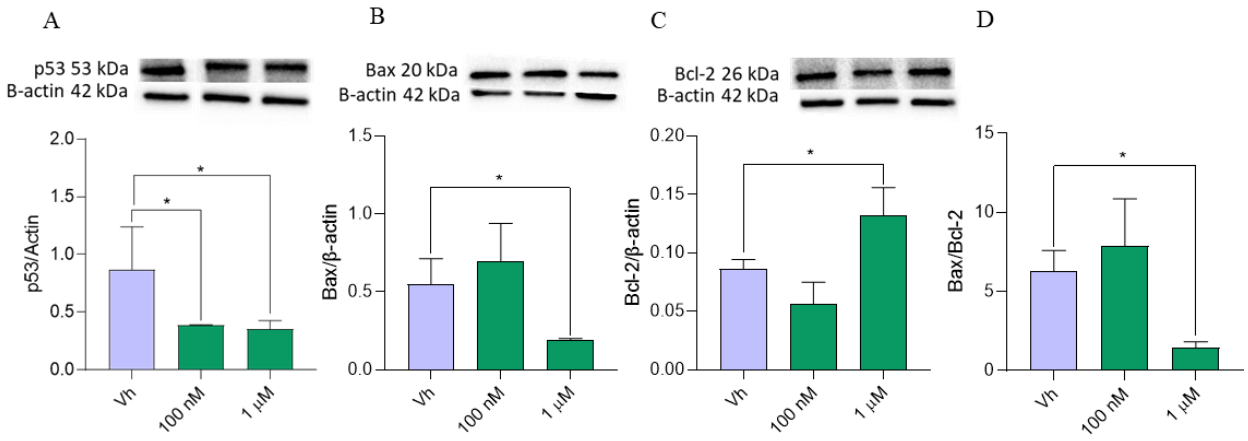

*Figure 20: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with VNZ [100 nM, 1 μM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

These initial findings revealed significant miRNA modulation following ATZ, CYP, and VNZ exposure, and confirmed alterations in target genes and proteins involved in the PI3K/Akt/mTOR and p53 pathways, which regulate cell survival and proliferation.[146,147] This modulation may underlie the neurotoxicity of these three pesticides.

4.3 Part 3: EE_2, DEP, and PFOS

4.3.1 Modulation of miRNA expression profiles

Once again, miRNAs' expression was evaluated in differentiated SH-SY5Y cells after 48 h exposure this time to EE_2, DEP, and PFOS [100 nM and 1 µM]. In this analysis, miR-200a-3p showed a significant downregulation after exposure to EE_2, DEP, and PFOS [1 µM], as well as after exposure to EE_2 and DEP [100 nM]. Exposure to the last two also induced downregulation in miR-18b-5p. miR-653-5p expression was significantly modulated after exposure to EE_2 and PFOS [100 nM] and by both concentrations of DEP. Finally, miR-133b was significantly downregulated by exposure to both concentrations of DEP and by PFOS at

100 nM. The table XIII shows fold change values normalized to endogenous U6 miRNA, with downregulation (fold < 0.5) highlighted in blue.

Table XIII: qRT-PCR validation of miRNA expression in differentiated SH-SY5Y cells after exposure to EE_2, DEP, and PFOS

	EE_2 1	**EE_2 100**	**DEP 1**	**DEP 100**	**PFOS 1**	**PFOS 100**
miR 200a-3p	0,38	0,47	0,39	0,32	0,40	1,51
miR 18b-5p	0,93	0,41	0,83	0,41	0,69	0,92
miR 653-5p	1,02	0,45	0,46	0,39	0,96	0,29
miR 133b	1,08	1,95	0,48	0,30	0,54	0,38

4.3.2 In silico target genes predictions

Using the miRWalk software, target genes were identified through three algorithms, miRWalk, TargetScan, and miRDB, with a focus on prioritizing high-confidence miRNA-target interactions. Subsequently, functional enrichment analysis was performed utilizing g:Profiler with the Gene Ontology: Biological Process database. Finally, Cytoscape software was employed to generate a network visualization (Figure 21) depicting the interactions between miRNAs, their target genes, and the enriched pathways.

Among the deregulated pathways, the 'Brain development' pathway immediately stands out, indicating that the modulation of these miRNAs is strongly implicated in diseases affecting the CNS. Some of the genes included in this pathway, EGFR, CALM2, and IGF1R are common to the 'Ras signaling pathway', also observed. As previously described, this pathway is essential for regulating cell survival, metabolism, and differentiation,[133] and it may also interfere with brain development by promoting neuronal maturation.[149] Since Ras is a GTPase,[150] this pathway can also be indirectly modulated following exposure to EE_2, DEP, and PFOS, as the miRNAs regulated by these compounds target the genes SH3BP4 and CHML, which are involved in the 'GDP-Dissociation' pathway. It is important to note that another identified pathway is the 'Activation of HIF-1 signaling', which includes the genes EGFR and IGF1R. Its activation reflects the induction of stress caused by exposure to EDs. Indeed, modulation of this pathway has been observed in several diseases, and it can lead to oxidative stress, which in turn may damage neuronal function and physiological processes.[151]

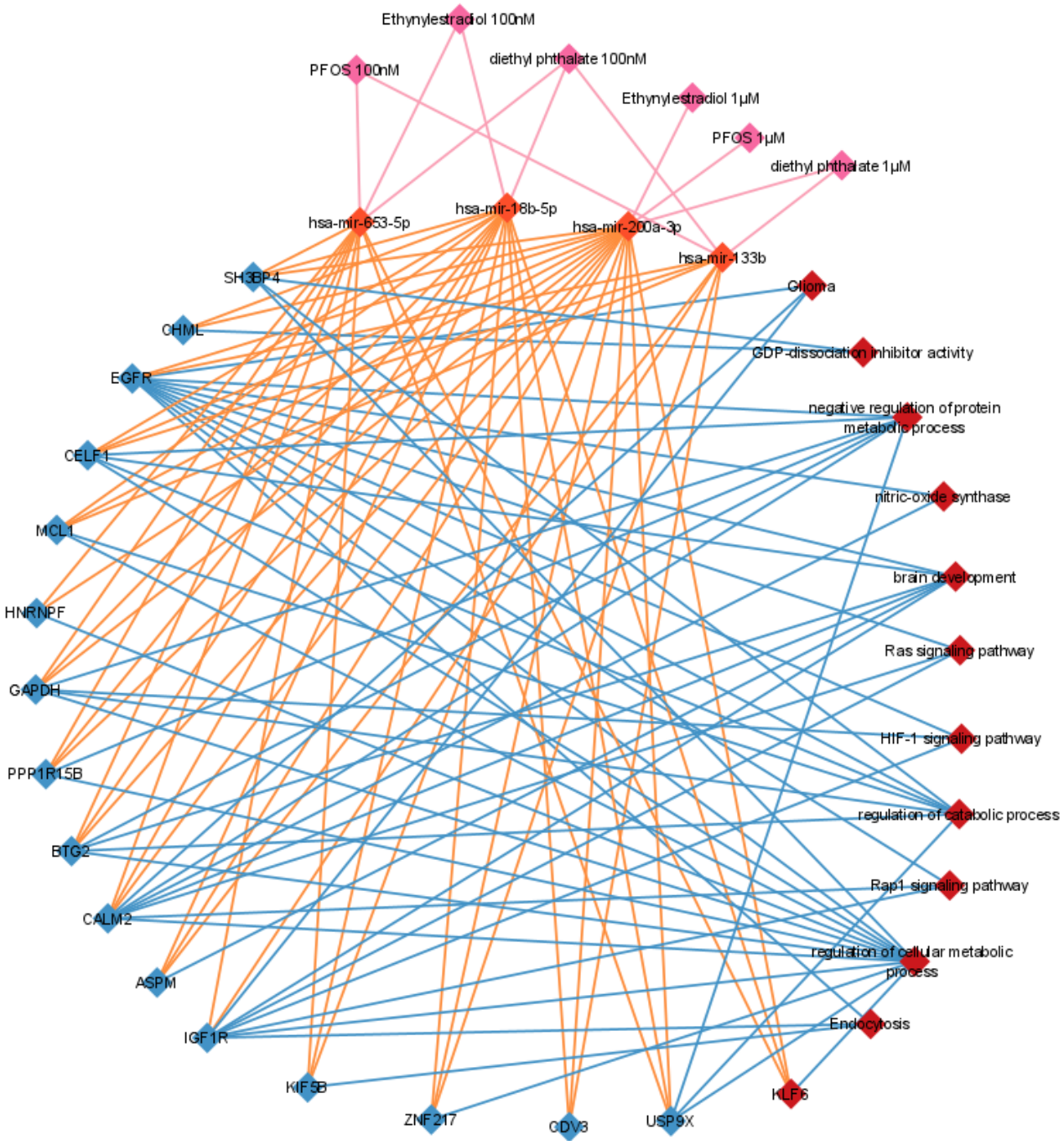

Figure 21: Descriptive network of the analyzed miRNAs, miR-18b-5p, miR-133b, miR-200a-3p, and miR-653-5p (orange diamonds), the identified target genes (blue diamonds), and the pathways in which they are involved (red diamonds), created using Cytoscape software.

4.3.3 Modulation of genes expression profiles

Since bioinformatic analysis revealed that 'Ras signaling pathway', 'brain development', 'HIF-1 signaling pathway', 'glioma development', and 'GDP dissociation inhibitor' were the pathways most implicated in neurotoxicity, the modulation of the target genes EGFR, IGF1R,

and SH3BP4 was assessed. Moreover, CDK6, SOS1, and PTEN expression was studied, being integral components of the Ras signaling pathway. Once again, differentiated SH-SY5Y cells were treated for 48 h with EE_2, DEP, and PFOS at concentrations of 100 nM and 1 µM. qRT-PCR analysis was performed to study the expression of these genes.

Significant upregulation was observed for EGFR (A) and IGF1R (B) after exposure to 1 µM EE_2 (Figure 22). Additionally, exposure to both concentrations of EE_2 led to a significant downregulation of SOS1 (F), while only EE_2 [100 nM] downregulated PTEN (C). Finally, SH3BP4 gene (D) was significantly upregulated by EE_2 [1 µM].

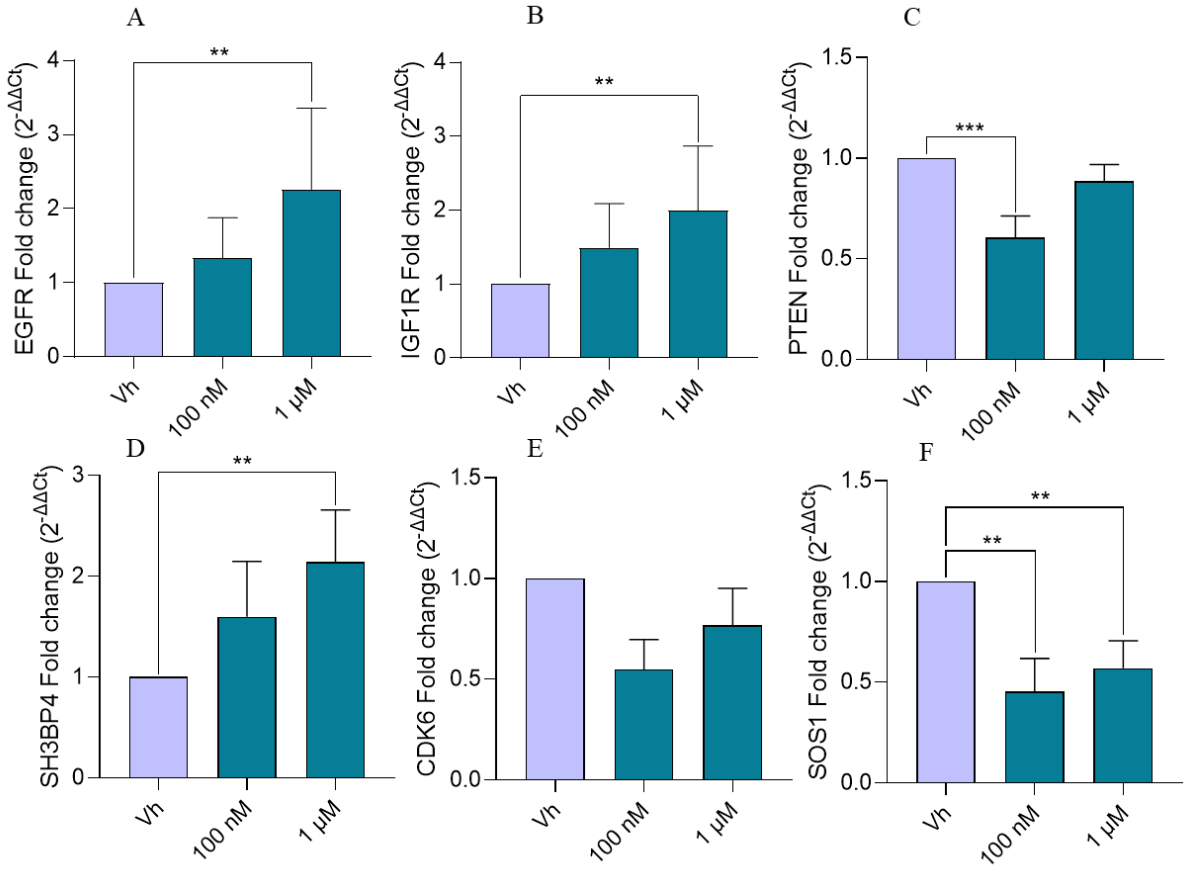

*Figure 22: Differential expression of EGFR (A), IGF1R (B), PTEN (C), SH3BP4 (D), CDK6 (E), and SOS1 (F) genes in differentiated SH-SY5Y cells treated for 48 h with EE_2 [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (**p <0.01 and ***p <0.001 vs Vh. One-way ANOVA, post hoc test Dunnet).*

As it can observed from Figure 23, both concentrations of DEP lead to a significant upregulation of EGFR (A), IGF1R (B), and SH3BP4 (D).

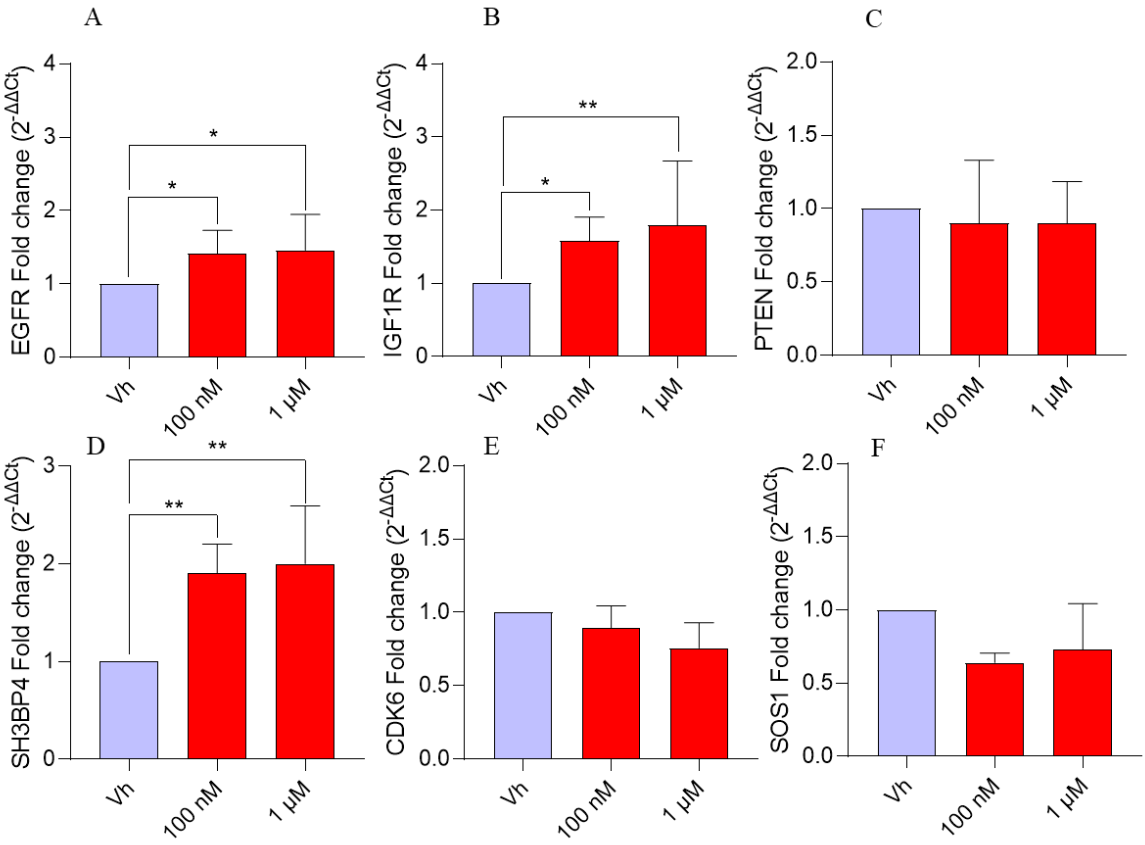

*Figure 23: Differential expression of EGFR (A), IGF1R (B), PTEN (C), SH3BP4 (D), CDK6 (E), and SOS1 (F) genes in differentiated SH-SY5Y cells treated for 48 h with DEP [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (*p < 0.05 and **p <0.01 vs Vh. One-way ANOVA, post hoc test Dunnet).*

Finally, Figure 24 shows the gene modulations caused by PFOS exposure. EGFR (A) and IGF1R (B) were significantly upregulated following exposure to PFOS [100 nM]. SH3BP4 (D), on the other hand, was significantly modulated by both the concentrations of this ED.

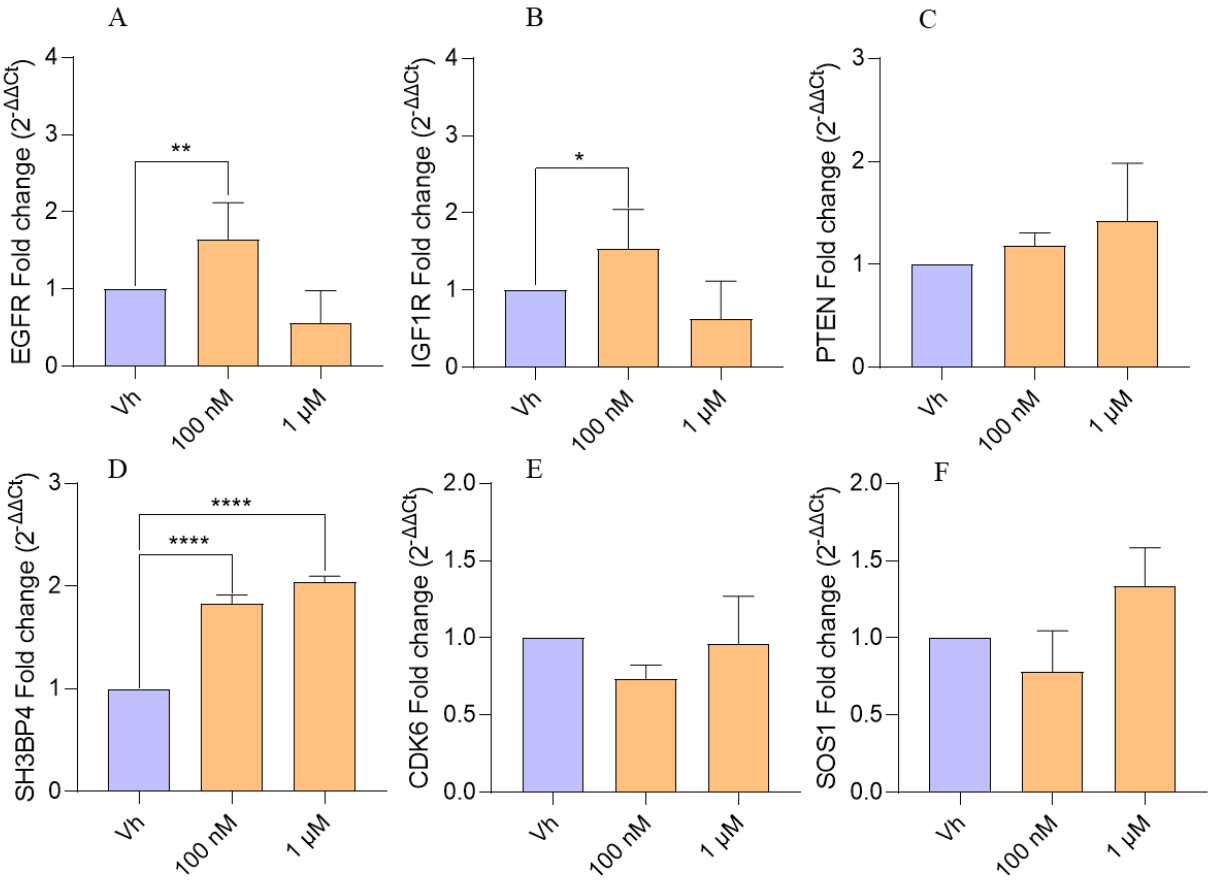

*Figure 24: Differential expression of EGFR (A), IGF1R (B), PTEN (C), SH3BP4 (D), CDK6 (E), and SOS1 (F) genes in differentiated SH-SY5Y cells treated for 48 h with PFOS [100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (*p < 0.05, **p <0.01, and **** p< 0.0001 vs Vh. One-way ANOVA, post hoc test Dunnet).*

4.3.4 Western Blot analysis of differentiated SH-SY5Y Cells

Regarding exposure to EE_2, DEP, and PFOS, Real Time analysis revealed that these substances induce modulation in the Ras pathway. For this reason, the expression of proteins involved in this pathway was analysed using Western Blotting. Differentiated SH-SY5Y cells were treated for 48 h with EE_2, DEP, and PFOS at concentrations of 100 nM and 1 µM.

A significant upregulation of the EGFR (A) protein was observed following exposure to both concentrations of EE_2 (Figure 25). Following the observed upregulation of EGFR, a receptor that regulates both the Ras pathway and the PI3K/Akt/mTOR pathway, the modulation of Akt and mTOR phosphorylation, and PTEN levels were evaluated. Indeed, pAkt (B) was

significantly upregulated by EE_2 [1 µM], while mTOR (C) displayed significant modulation of its phosphorylation after exposure to both concentrations of this chemical.

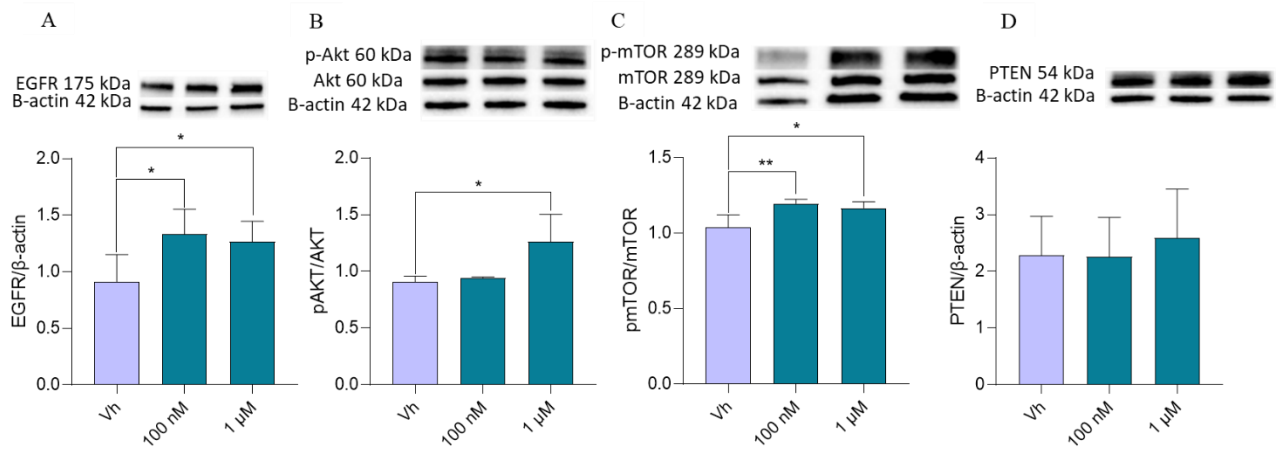

*Figure 25: Differential expression of EGFR (A), PTEN (D), ad phosphorylation of Akt (B) and mTOR (C) in differentiated SH-SY5Y cells treated for 48 h with EE_2 [100 nM, 1 µM]. The proteins levels and phosphorylation of Akt and mTOR were determined by Western Blotting at 175, 54, 60, and 289 kDa respectively and using β-actin (42 kDa) or total Akt or mTOR as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Regarding exposure to DEP (Figure 26), a significant upregulation of the EGFR protein (A) was observed following exposure to both concentrations of this ED, as well as Akt phosphorylation (B). PTEN (D) was also modulated by both DEP concentrations, but it was significantly downregulated.

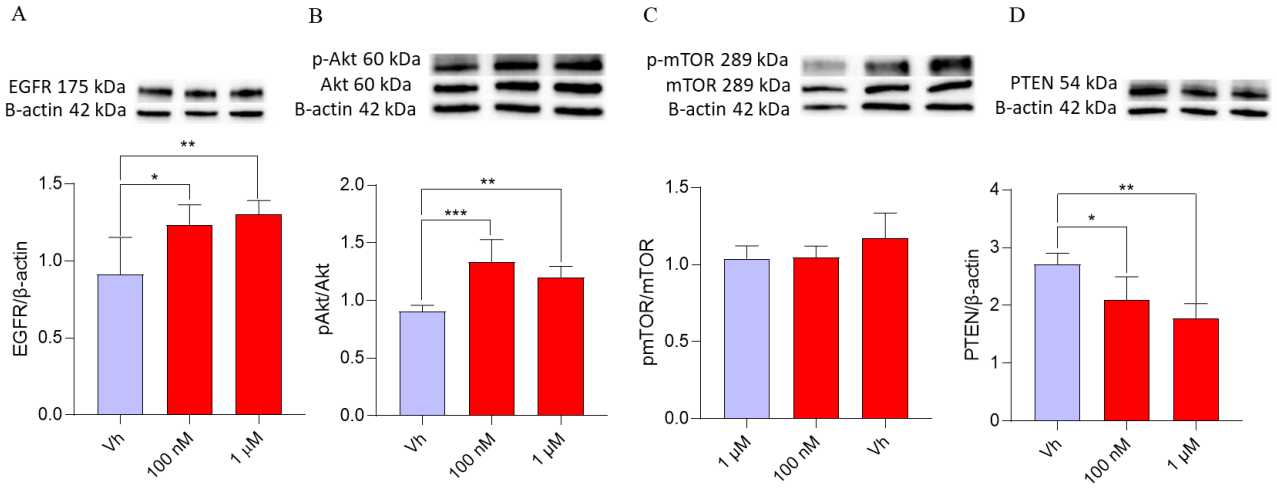

*Figure 26: Differential expression of EGFR (A), PTEN (D), ad phosphorylation of Akt (B) and mTOR (C) in differentiated SH-SY5Y cells treated for 48 h with DEP [100 nM, 1 µM]. The proteins levels and phosphorylation of Akt and mTOR were determined by Western Blotting at 175, 54, 60, and 289 kDa respectively and using β-actin (42 kDa) or total Akt or mTOR as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05, ** p < 0.01, and *** p < 0.001 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Finally, in Figure 27 it is possible to observe that PFOS significantly upregulated EGFR expression (A) and Akt phosphorylation (B) only at the highest concentration [1 µM].

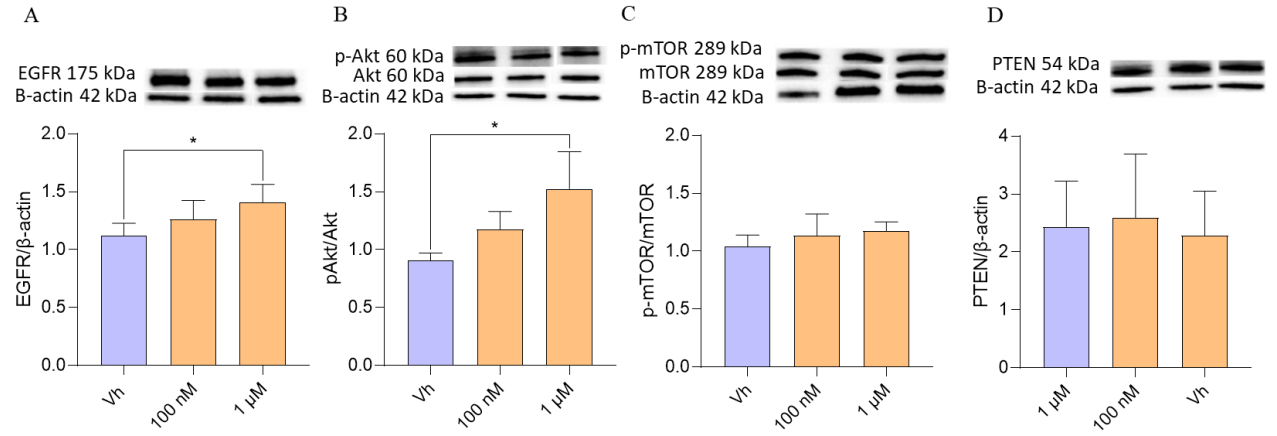

*Figure 27: Differential expression of EGFR (A), PTEN (D), ad phosphorylation of Akt (B) and mTOR (C) in differentiated SH-SY5Y cells treated for 48 h with PFOS [100 nM, 1 µM]. The proteins levels and phosphorylation of Akt and mTOR were determined by Western Blotting at 175, 54, 60, and 289 kDa respectively and using β-actin (42 kDa) or total Akt or mTOR as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Since this pathway is involved in the regulation of cell proliferation and survival, the modulation of the pro-apoptotic proteins p53 and Bax, and the anti-apoptotic protein Bcl-2, were evaluated. p53 showed a significant downregulation following exposure to both EE_2 concentrations (A). Bax (B) was significantly downregulated by both concentrations of EE_2, while Bcl-2 (C) showed an upregulation trend. The Bax/Bcl-2 ratio (D) resulted downregulated after exposure to this ED at both concentrations (Figure 28).

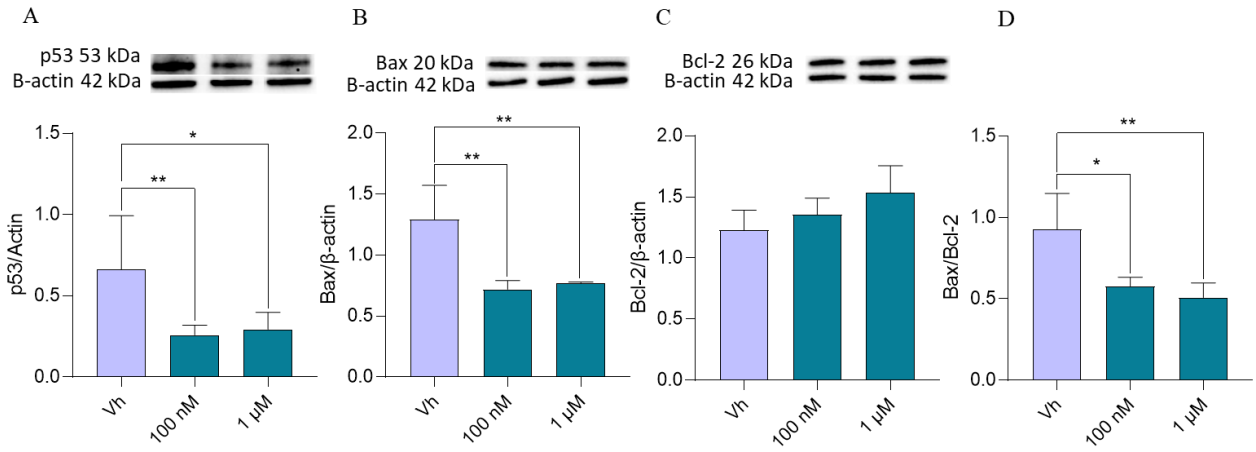

*Figure 28: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with EE_2 [100 nM, 1 µM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Exposure to DEP [100 nM] significantly upregulated Bcl-2 protein expression (C), while Bax showed only a downregulation trend (B). Bax/Bcl-2 ration, on the other hand, was significantly downregulated after exposure to DEP [100 nM] (Figure 29). Exposure to PFOS did not result in significant modulation of any of the proteins examined (Figure 30).

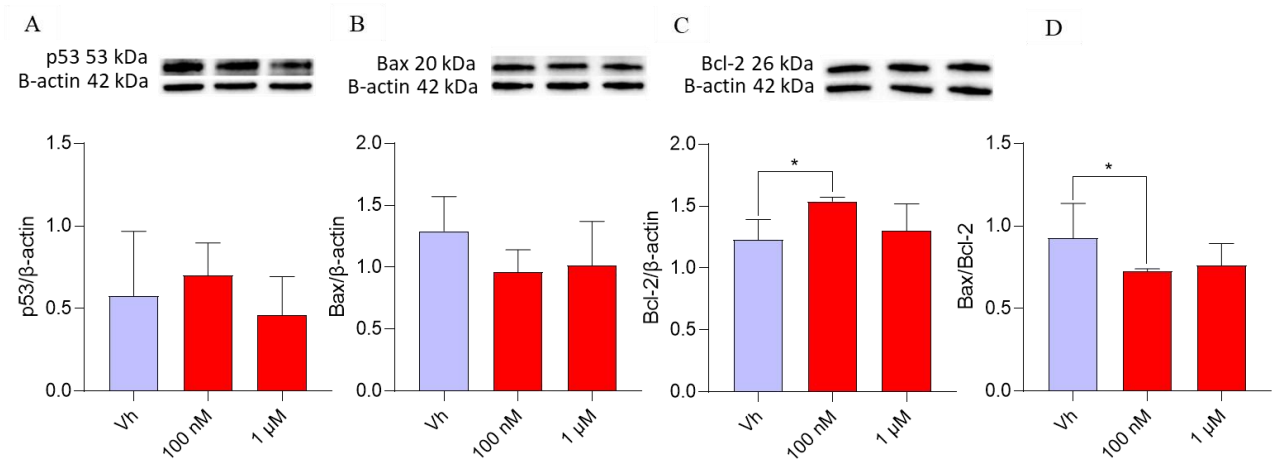

*Figure 29: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with DEP [100 nM, 1 μM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

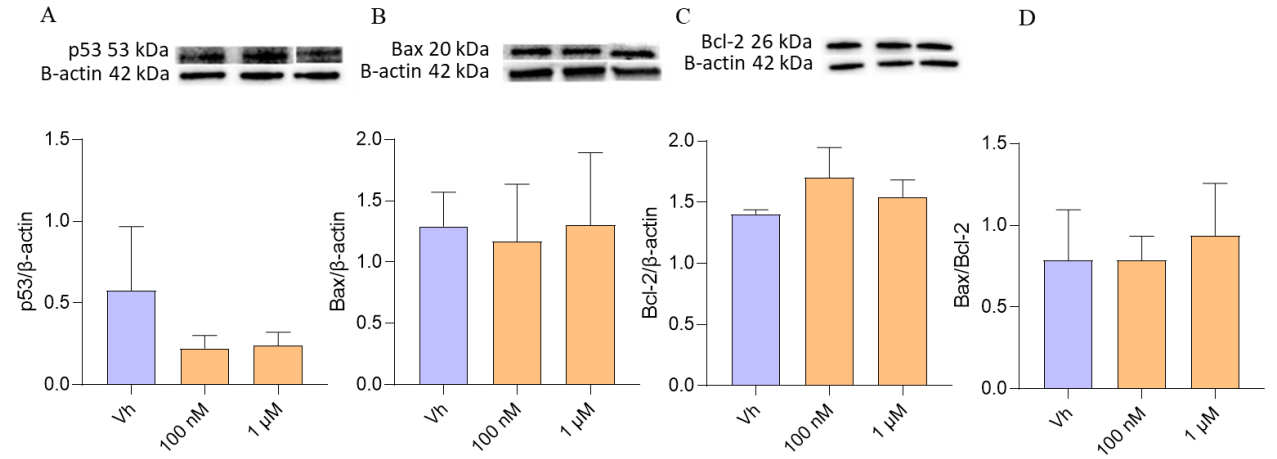

Figure 30: Differential expression of p53 (A), Bax (B), and Bcl-2 (C) in differentiated SH-SY5Y cells treated for 48 h with PFOS [100 nM, 1 μM]. The proteins levels of p53, Bax, and Bcl-2 were determined by Western Blotting at 53, 20, and 26 kDa respectively and using β-actin (42 kDa) as loading control. The ratio between Bax and Bcl-2 is also represented (D). Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and β-actin expression and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnett).

Lastly, the modulation of Ras protein itself was assessed following immunoprecipitation. As shown in the Figure 31, exposure to EE_2 [100 nM] (A) and DEP [1 µM] (B) results in a significant upregulation of this protein, confirming the modulation of this pathway.

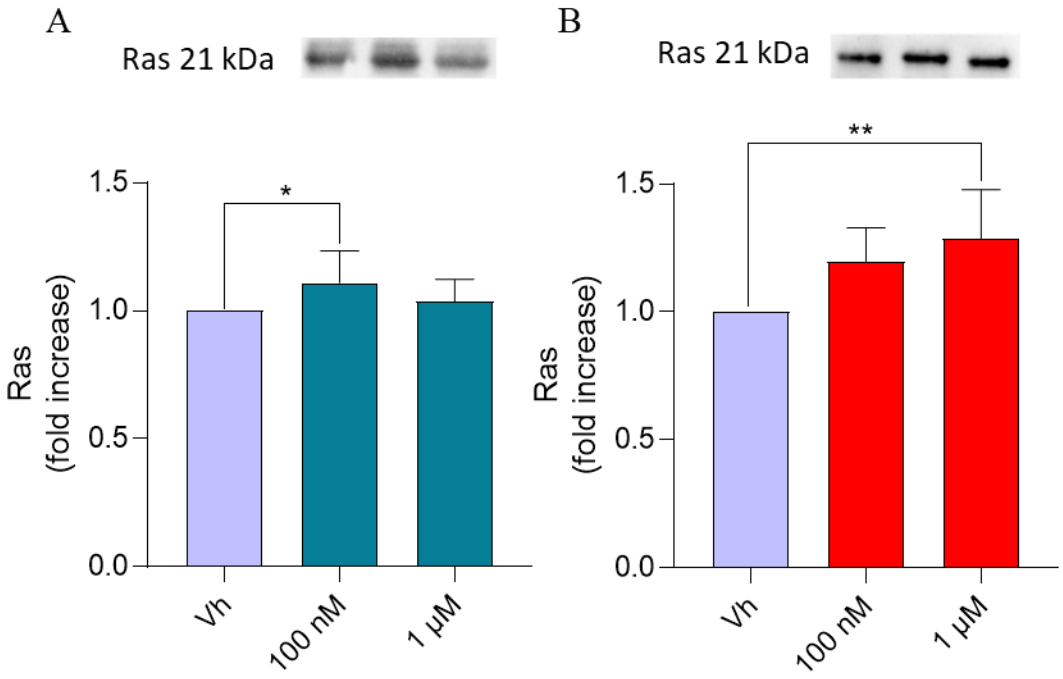

*Figure 31: Differential expression of Ras in differentiated SH-SY5Y cells treated for 48 h with EE_2 (A) and DEP (B) [100 nM, 1 µM]. Ras was determined by Western Blotting at 21 kDa and using β-actin (42 kDa) as loading control. Top: cropped representative images of Ras expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are reported as mean ± SD of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Similar to ATZ, CYP, and VNZ, exposure to EE_2, DEP, and PFOS also induced epigenetic modulations that affected the activation of the Ras, PI3K/Akt/mTOR, and p53 pathways, which are crucial for cell survival regulation. These alterations may increase the susceptibility of exposed individuals to various cellular dysfunctions, including uncontrolled cell proliferation and apoptosis.[133,146,147]

4.4 Part 4: Evaluation of sunscreens and nickel quantification

The research conducted at BioBasic Europe S.r.l. led to the identification of other two EDs, AVO and EHS, for *in vitro* studies, and allowed the quantification of Ni levels in consumer cosmetics.

4.4.1 Sunscreen molecules selection

AVO and EHS were chosen as sunscreen molecules to be investigated as the most commonly present UV filters in the products tested by BioBasics Europe between 2022 and 2023. As shown in Table XIV, which lists the UV filters with endocrine-disrupting activity identified in the various screened products, along with their percentage of occurrence and the types of formulations they were used in, EHS and AVO were identified as the two most frequently employed molecules, with a percentage of 27,14 and 19,59 respectively.

Table XIV: EDs sunscreen filters found in the products on the market

MOLECULE	N° OF PRODUCTS	%	EMULSIONS	OILS	MILKS	BIPHASICS	BUTTER & STICK	DUSTS	GELS, SPRAYS, GLOSS
4-Methylbenzylidene Camphor	0	0,00	0	0	0	0	0	0	0
Benzophenone-3 (Oxybenzone)	0	0,00	0	0	0	0	0	0	0
Benzophenone-4 (Uvinul® MS 40) (Sulisobenzone)	0	0,00	0	0	0	0	0	0	0
Butyl Methoxydibenzoylmethane (Avobenzone)	135	19,59	58	33	29	6	4	0	5
Dioxybenzone (Benzophenone- 8)	0	0,00	0	0	0	0	0	0	0
Ethylhexyl dimethyl PABA (Padymate O)	0	0,00	0	0	0	0	0	0	0
Ethylhexyl Methoxycinnamate (Octyl methoxycinnamate) (Uvinul® MC 80)	49	7,11	35	5	6	0	1	1	1
Ethylhexyl Salicylate (Octyl salicylate)	187	27,14	93	41	37	8	2	0	6
Homomenthyl Salicylate (Homosalate)	30	4,35	11	10	2	0	0	0	7
Octocrylene	106	15,38	44	28	26	1	1	0	6
PABA (Para Amino Benzoic Acid)	0	0,00	0	0	0	0	0	0	0
Phenylbenzimidazol Sulfonic Acid	119	17,27	78	5	28	8	0	0	0
Titanium Dioxide (nano)	63	9,14	48	0	2	1	3	7	2
TOTAL	689	100,00	367	122	130	24	11	8	27

4.4.2 Evaluation of cell death and oxidative stress induction in differentiated SH-SY5Y cells and HaCaT after exposure to AVO and EHS

After selecting the most frequently occurring molecules among commercial sunscreen filter products, the next step was to determine the appropriate concentrations to treat two different cellular models: HaCaT and differentiated SH-SY5Y cells. Given that the skin is the primary site of exposure to sunscreens, human keratinocytes were included in the study to investigate cutaneous toxicity alongside potential CNS effects. Both cell lines were treated for 48 h with scalar concentrations of AVO and EHS. Subsequently, the MTT assays was performed to determine cell viability and the IC50.

As shown by the graphs in Figure 32, significant HaCaT cell mortality was observed only after exposure to AVO at the highest concentration [100 µM] (A). Moreover, as shown in the curve in the same Figure, for HaCaT cells, the IC50 following exposure to AVO was identified at 57.63 µM (C).

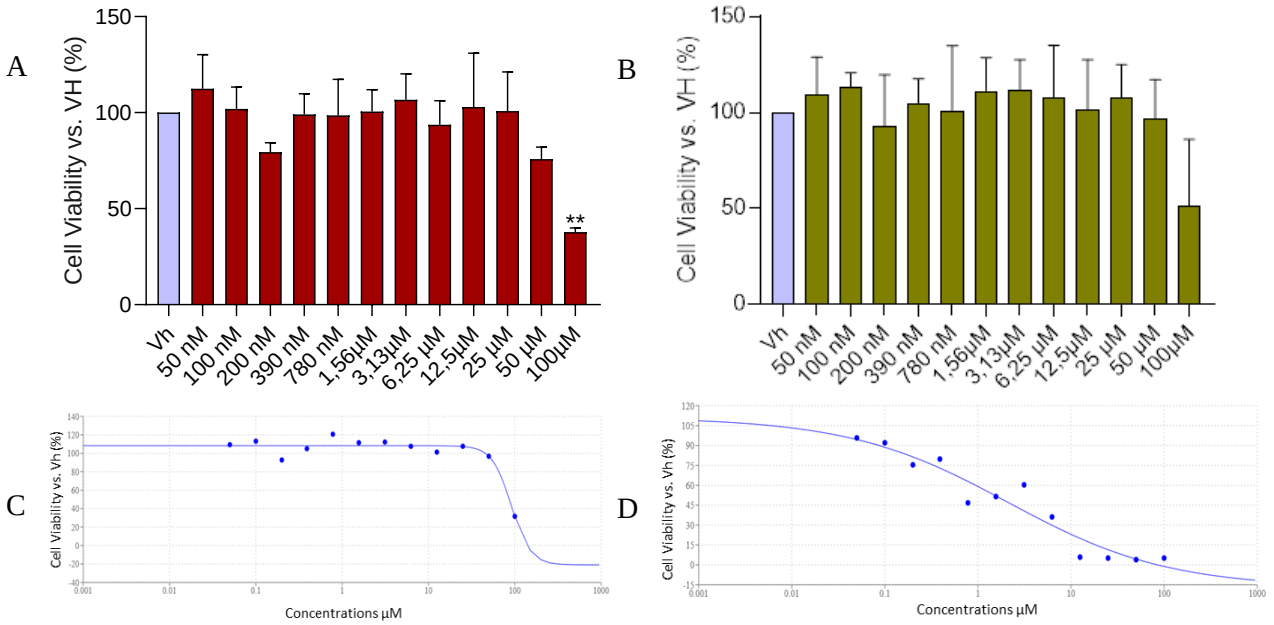

*Figure 32: Top: Neuronal viability in HaCaT cells after 48 h treatment with AVO (A) and EHS (B), evaluated using the MTT assay. Data are expressed as the mean ± SD of viability percentage relative to cells treated with the Vh of three independent experiments (**p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnet). Bottom: Curve relating cell viability with AVO (C) and EHS (D) concentrations to identify the IC50.*

Exposure to EHS (Figure 32, B) did not induce cytotoxicity in the HaCaT cellular model and resulted in an IC50 of 90.87 µM according to the MTT assay (D).

Regarding differentiated SH-SY5Y cells, exposure to increasing concentrations of AVO resulted in an IC50 at 31.75 µM based on the MTT assay (C), with significant cellular mortality (A) observed at the exposure concentration of 100 µM (Figure 33).

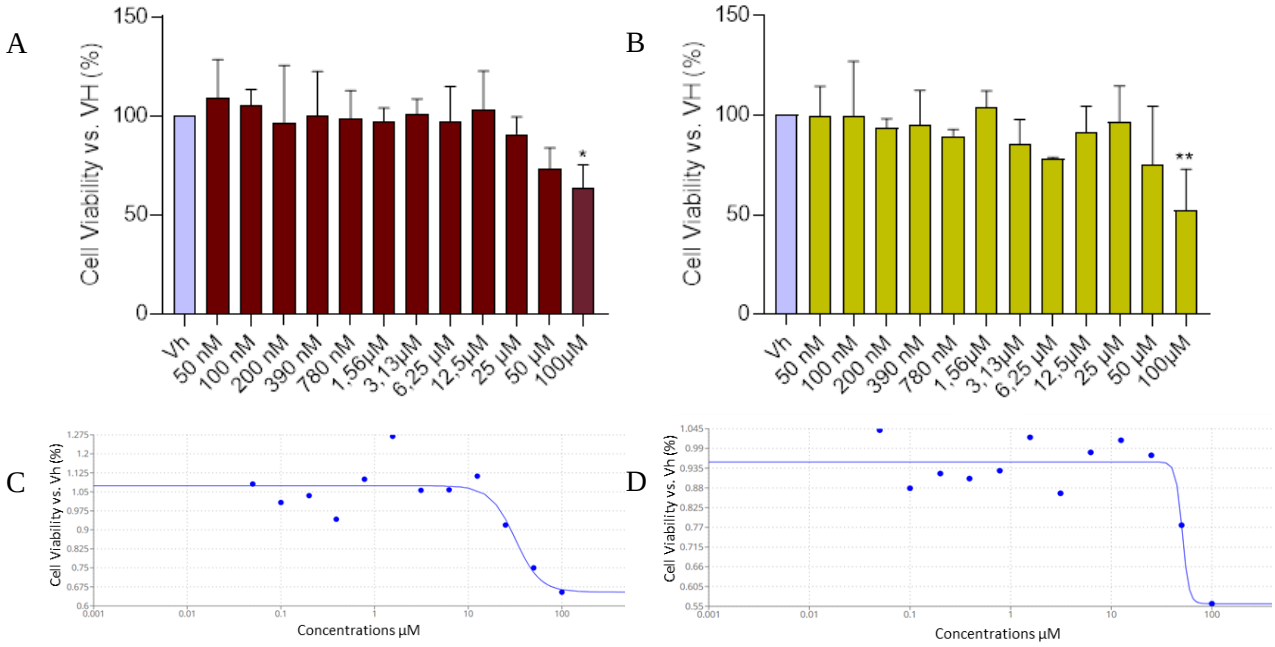

*Figure 33: Top: Neuronal viability in differentiated SH-SY5Y cells after 48 h treatment with AVO (A) and EHS (B), evaluated using the MTT assay. Data are expressed as the mean ± SD of viability percentage relative to cells treated with the Vh of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnet). Bottom: Curve relating cell viability with AVO (C) and EHS (D) concentrations to identify the IC50.*

Additionally, exposure to EHS yielded an IC50 of 50.82 µM (D) in the MTT assay, inducing significant cellular mortality (B) only at the highest concentration of exposure, 100 µM (Figure 33).

Subsequently, oxidative stress was assessed following exposure to increasing concentrations of AVO (A, C) and EHS (B, D) for 48 h. As evidenced by the graphs in Figure 34, neither of the two sunscreen agents induced significant oxidative stress at any of the concentrations tested, in either HaCaT cells (A, B) or differentiated SH-SY5Y cells (C, D).

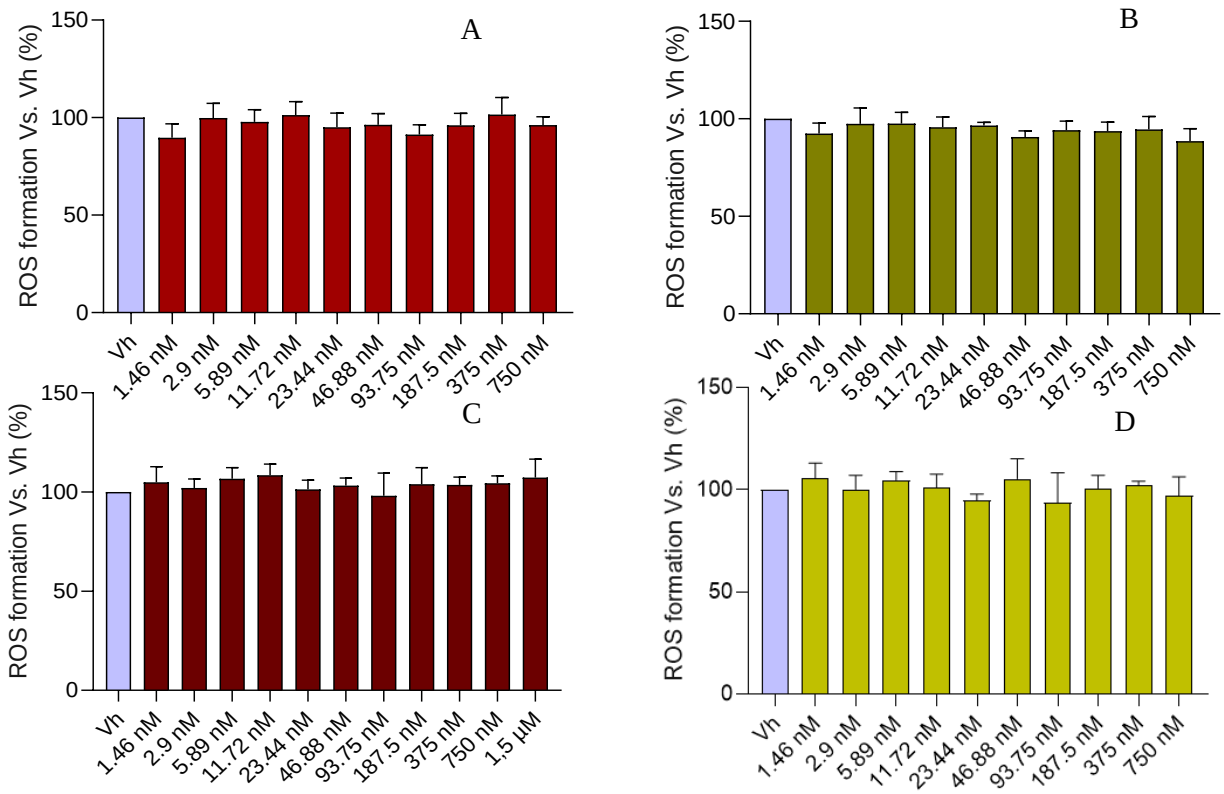

Figure 34: ROS production in HaCaT cells (A, B) and differentiated SH-SY5Y cells (C, D) after 48 h treatment with AVO (A, C) and EHS (B, D). Data are expressed as fold increases in the percentage of ROS formation versus the Vh and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

Based on these preliminary tests, it was possible to select the concentrations of 10 nM, 100 nM, and 1 µM for subsequent analyses.

4.4.3 Modulation of gene expression following exposure to AVO and EHS in HaCaT cells

Once the exposure concentrations were selected, the subsequent step was to assess whether exposure to the two sunscreens could also induce modulation of the PI3K/Akt/mTOR and Ras pathways, as previously observed following exposure to the other EDs studied. Gene expression modulation in HaCaT cells was evaluated following exposure to AVO and EHS at 10 nM, 100 nM, and 1 µM, selecting genes that belong to those two pathways. As shown in the Figure 35 and 36, these two molecules did not cause any significant modulation in the expression of BTG2, CDK6, EGFR, IGF1R, or PTEN genes.

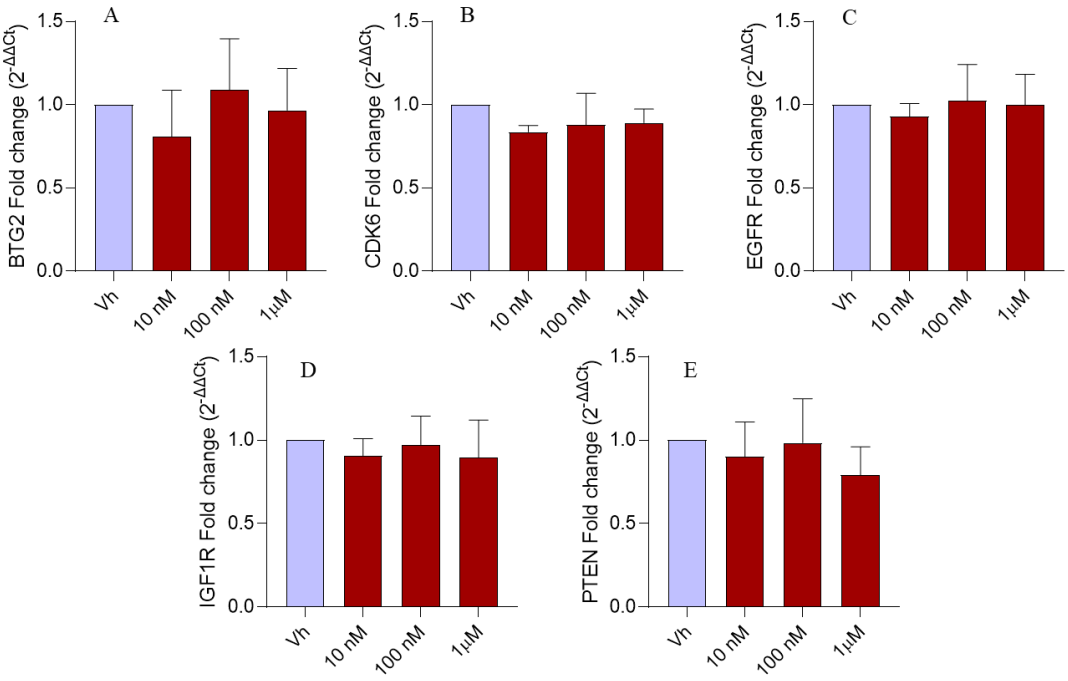

Figure 35: Differential expression of BTG2 (A), CDK6 (B), EGFR (C), IGF1R (D), and PTEN (E) genes in HaCaT cells treated for 48 h with AVO [10 nM, 100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

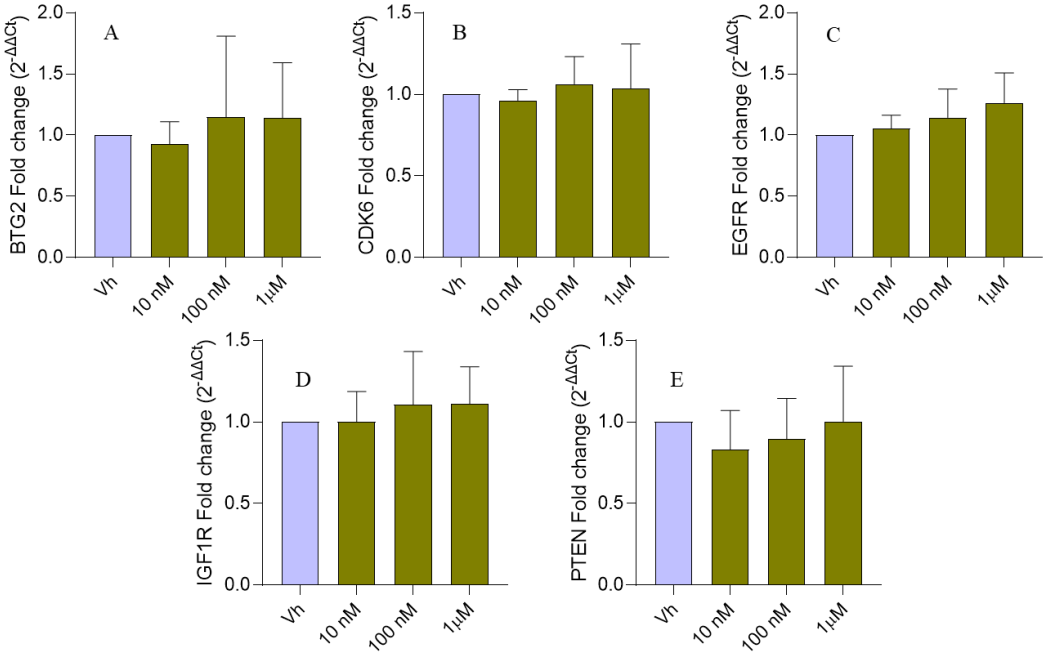

Figure 36: Differential expression of BTG2 (A), CDK6 (B), EGFR (C), IGF1R (D), and PTEN (E) genes in HaCaT cells treated for 48 h with EHS [10 nM, 100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

4.4.4 Modulation of gene expression following exposure to AVO and EHS in differentiated SH-SY5Ycells

The modulation of the same genes, EGFR, IGF1R, CDK6, BTG2, and PTEN, was also observed in differentiated SH-SY5Y cells following exposure to AVO and EHS, as shown in Figures 37 and 38. Only exposure to EHS [100 nM] resulted in a significant downregulation of the CDK6 gene. Additionally, this compound induced a non-significant trend toward upregulation of the BTG2 gene. No modulation was observed for the other genes studied.

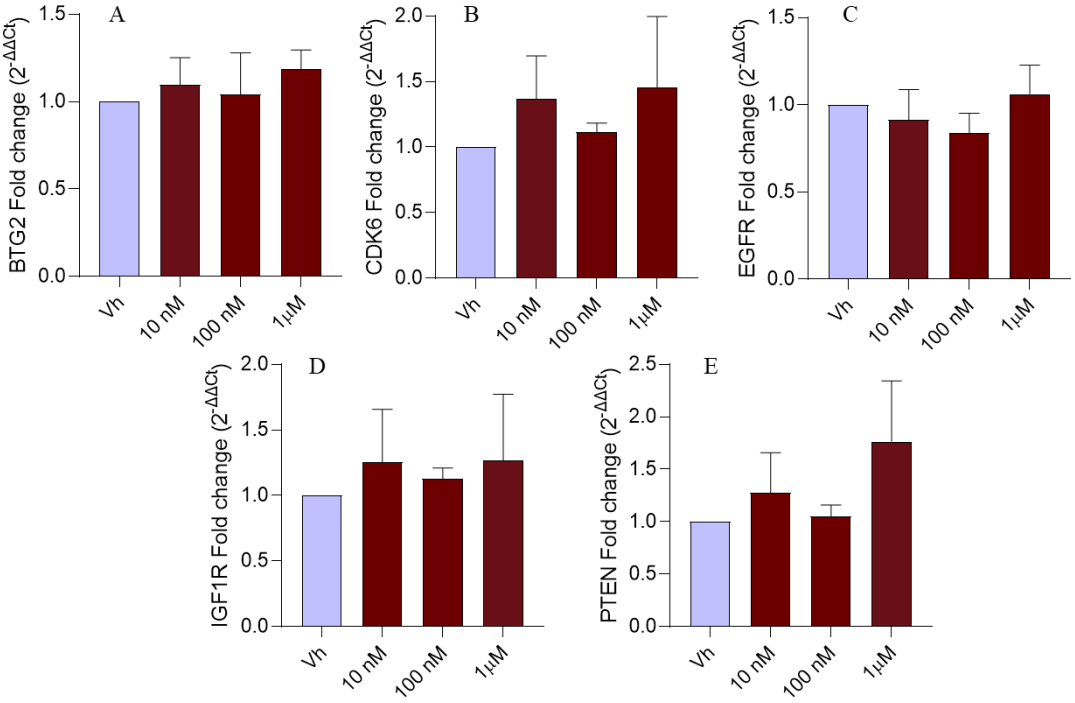

Figure 37: Differential expression BTG2 (A), CDK6 (B), EGFR (C), IGF1R (D), and PTEN (E) genes in differentiated SH-SY5Y cells treated for 48 h with AVO [10 nM, 100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (One-way ANOVA, post hoc test Dunnet).

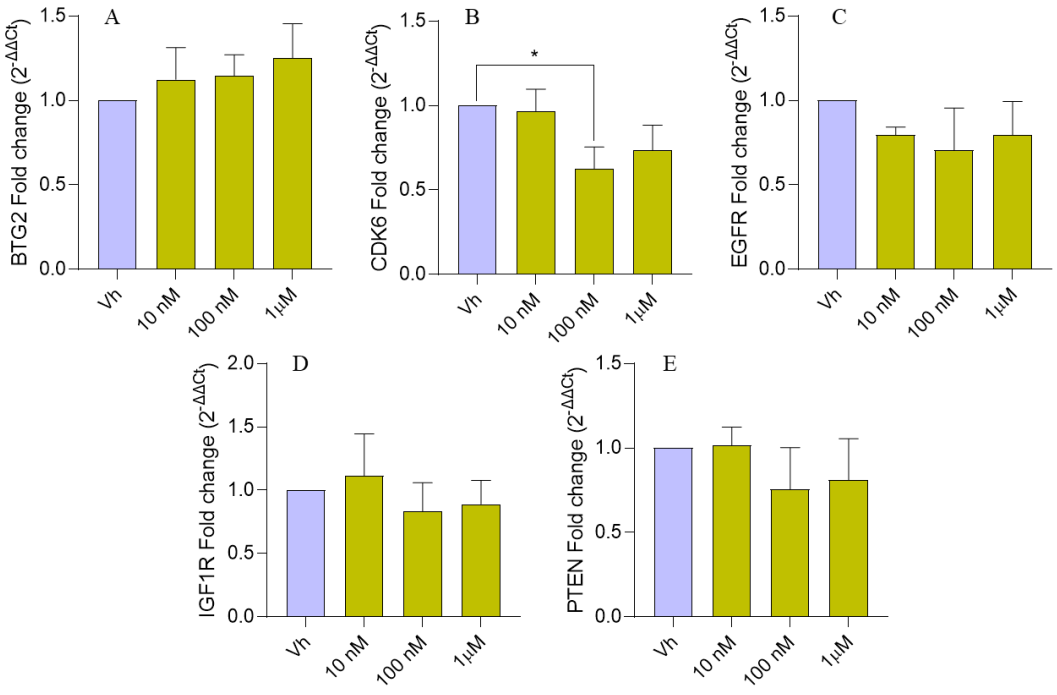

*Figure 38: Differential expression of BTG2 (A), CDK6 (B), EGFR (C), IGF1R (D), and PTEN (E) genes in differentiated SH-SY5Y cells treated for 48 h with EHS [10 nM, 100 nM, 1 µM] by single primer qRT-PCR. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method and Vh samples were considered as the calibrator of the experiment. Data are expressed as fold increases and reported as mean ± SD of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnet).*

4.4.5 Western Blotting analysis of HaCaT cells following treatment with AVO and EHS

Although gene expression analysis did not show significant changes, the protein profile modulation was still assessed in HaCaT cells treated as previously described for the qRT-PCR analysis.

As shown in Figure 39, exposure to AVO [1 µM] resulted in a significant upregulation of pmTOR (B) and p53 protein (E), the latter also observed following exposure to AVO [100 nM]. Additionally, EGFR (D) was significantly upregulated after exposure to AVO [10 nM]. No significant modulation or specific trends in the expression of the other studied proteins (pAkt, pERK 1/2, and Bax) were observed.

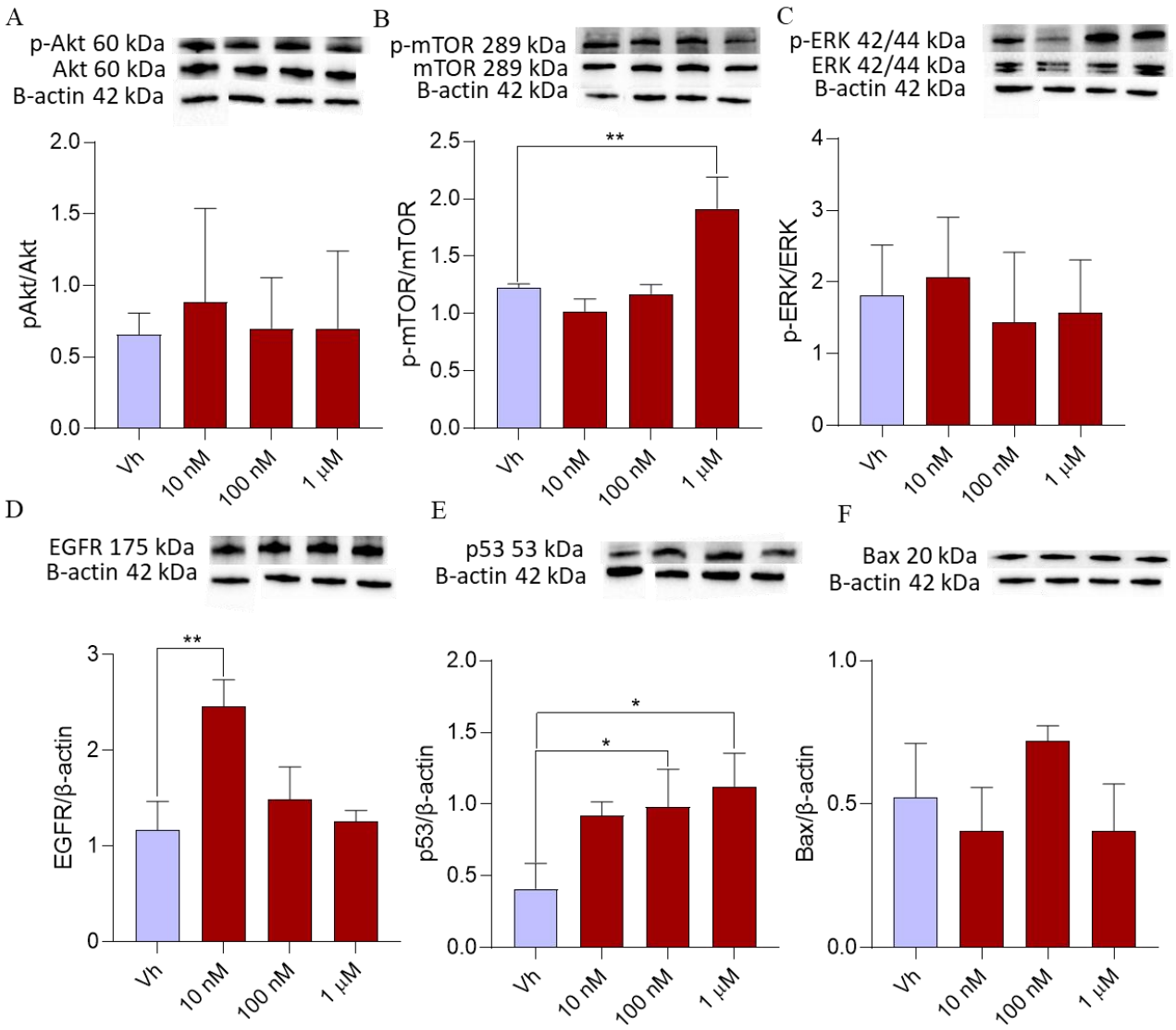

*Figure 39: Differential expression of EGFR (D), p53 (E), and Bax (F), ad phosphorylation of Akt (A), mTOR (B), and ERK ½ (C) in HaCat cells treated for 48 h with AVO [10 nM, 100 nM, 1 μM]. The proteins levels and phosphorylation of Akt, mTOR, and ERK 1/2 were determined by Western Blotting at 175, 53, 20, 60, 289, and 42/44 kDa respectively and using β-actin (42 kDa), or total Akt, mTOR, or ERK 1/2 as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05 and **p < 0.01 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

Exposure to EHS [10 nM] caused a significant upregulation of p53 in HaCaT cells, while no significant modulation was observed for the other proteins under study (Figure 40).

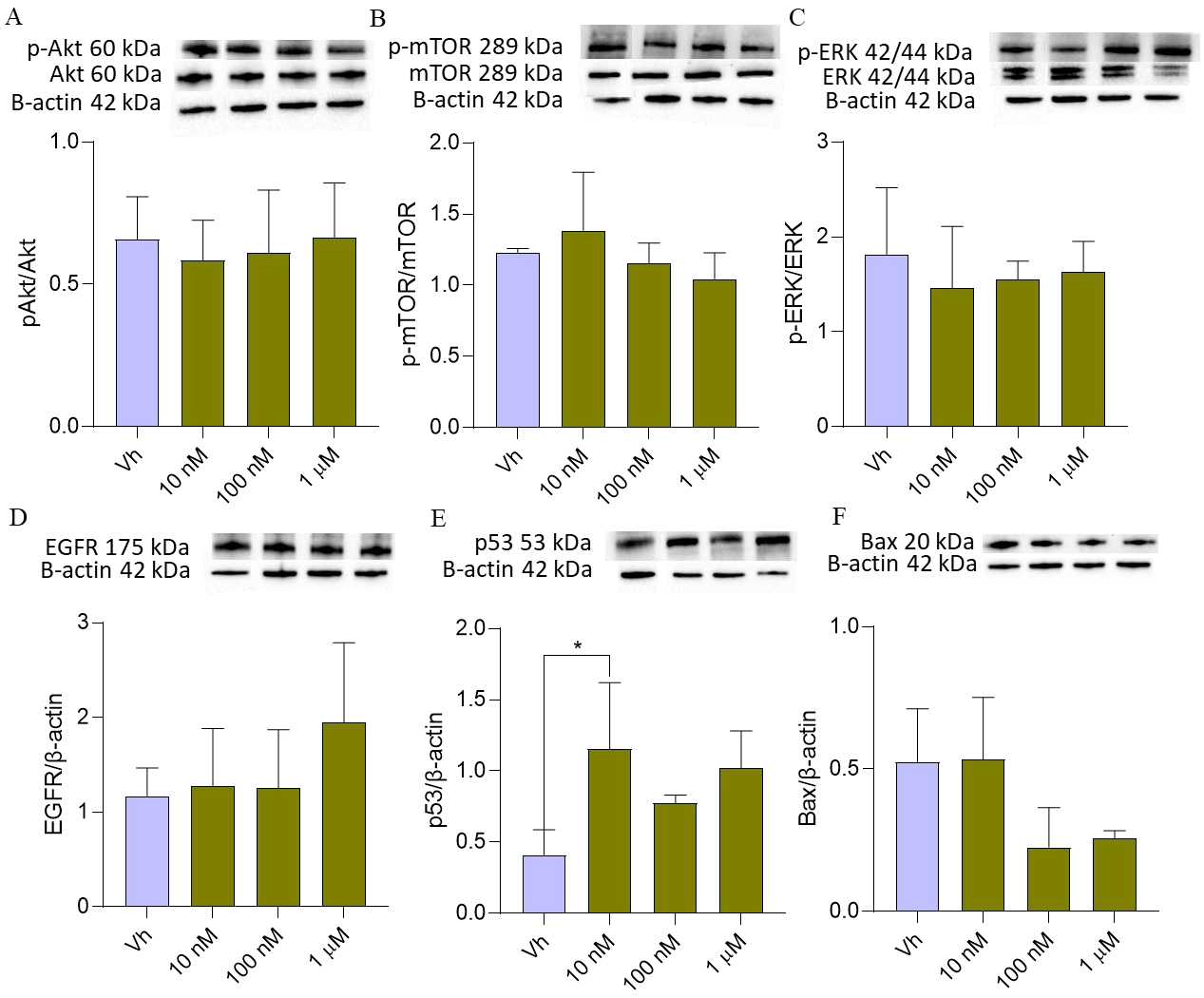

*Figure 40: Differential expression EGFR (D), p53 (E), and Bax (F), ad phosphorylation of Akt (A), mTOR (B), and ERK ½ (C) in HaCaT cells treated for 48 h with EHS [10 nM, 100 nM, 1 μM]. The proteins levels and phosphorylation of Akt, mTOR, and ERK 1/2 were determined by Western Blotting at 175, 53, 20, 60, 289, and 42/44 kDa respectively and using β-actin (42 kDa) or total Akt, mTOR or ERK 1/2 as loading control. Top: cropped representative images of the protein of interest expressions. Bottom: quantitative analysis of the Western Blotting results. The graphs show densitometry analysis of the bands appertaining to the protein of interest. Data are expressed as the ratio between the protein of interest and the corresponding loading control and reported as mean ± SD of three independent experiments (*p < 0.05 vs. Vh. One-way ANOVA, post hoc test Dunnett).*

4.4.6 Analysis of nickel levels in commercial products

Ni content in products tested by BioBasic Europe, including lotions, cleansers, and cosmetics, has also been investigated. Specifically, the formulations under study were solubilized and subsequently analysed using an Inductively Coupled Plasma spectrometer, that utilizes an argon torch to conduct a spectrophotometric analysis of the Ni concentration in the products. According to regulations, the amount of Ni in products that come into contact with the skin must be controlled. Studies indicate that a Ni concentration of 5 ppm is safe for individuals without an allergy to Ni, while a concentration of 1 ppm is considered safe for individuals with a Ni allergy. From October 2023 until April 2024, 910 products have been tested by the company. Among them, only 11, the 1.21%, showed a concentration in mg/kg of Ni higher than 1 (Table XV).

Table XV: Products analysed for Ni concentration

Total Products	Average Ni Conc.	Products with Ni >1	% Products with Ni >1
910	0,209 mg/kg	11	1,21

5 Discussion

In recent years, research has identified EDs as significant disruptors of CNS functions, with evident effects on hormone regulation, neural plasticity, and neurodevelopment. According to diverse studies, the brain is exceptionally sensitive to these compounds, which can disrupt hormonal balance, neurotransmitter activity, and cellular maintenance processes. This is particularly problematic as EDs can interfere with hormone-sensitive pathways crucial for neuron survival, signaling, and renewal.[152] miRNAs, which are essential in regulating gene expression for cellular maintenance, have shown altered expression due to ED exposure. These molecules play a crucial role in maintaining proteostasis and are increasingly studied as biomarkers of neurotoxicity caused by ED exposure. Their dysregulation may play a role in the development of cancers and neurodegenerative diseases, highlighting the need for further research in their roles within neuroendocrine disruption.[153]

Given these considerations, ATZ, CYP, VNZ, EE_2, DEP, and PFOS have been selected as representative of diverse classes of substances with established endocrine-disrupting activities. As previously outlined, following Commission Implementing Regulation (EU) 2021/2049, CYP remains an insecticide permitted at the international level, albeit with several restrictions, and is not formally recognized as an ED. Nevertheless, this substance was included in the present study due to a substantial body of research demonstrating various mechanisms of endocrine modulation.[42] Consequently, it was decided to investigate its potential to act as an ED within our experimental framework.

A comprehensive literature review enabled the identification of environmental concentration ranges for these contaminants, thereby providing insights into potential human exposure levels. The selection of treatment doses for the cells under investigation was complicated by the inherent difficulty in defining a specific exposure level to any single molecule. Indeed, the general population is exposed to subjective concentrations of individual EDs, contingent upon their occupation, residence, and lifestyle. For certain substances, such as EE_2, the analysis of direct exposure can be somewhat more straightforward due to its presence in pharmaceuticals with well-defined quantities. However, this is insufficient as the population is also exposed via numerous other routes beyond direct intake. Furthermore, while several clinical studies have assessed the presence of various classes of EDs in biological fluids such

as blood and urine, these estimations are often inefficient due to the frequent selection of population pools with similar characteristics, thus exhibiting common exposure patterns (e.g., farmers using pesticides; individuals residing in coastal areas with increased sunscreen usage, etc..). Consequently, it became necessary to base the dose selection within the concentration ranges generally identified for these molecules in the environment, commencing with a broad range to attempt to encompass both the maximum and minimum possible exposure scenarios. After selecting the concentration range to be tested, cellular mortality and oxidative stress induction were evaluated following a 48 h exposure to the six selected EDs in both non differentiated and differentiated SH-SY5Y cells. Identifying non-cytotoxic concentrations was essential to enable the following investigation of endocrine-disrupting effects at the epigenetic level. The concentrations of 100 nM and 1 µM did not alter differentiated SH-SY5Y cell viability nor ROS production. These two concentrations were therefore confirmed for subsequent analyses.

As previously mentioned, numerous studies have set the ability of EDs to exert toxic effects through epigenetic mechanisms, specifically by modulating miRNA expression.[154] It was therefore decided to investigate this epigenetic characteristic of EDs more thoroughly. An initial analysis revealed differences in the expression of the miRNAs following exposure to the three pesticides, ATZ, CYP, and VNZ, compared to the other three EDs under investigation, EE_2, PFOS, and DEP. Therefore, the study subsequently focused on these two distinct groups. Exposure to the three pesticides led to a significant downregulation of the miRNAs 200a-3p, 146b-5p, and 29b-3p. The first one, miR-200a-3p, has been extensively studied in the context of neurodegenerative diseases, with its modulation frequently associated with Alzheimer's disease pathology.[155,156] miR-200a-3p is also a key regulator of the MAPK pathway, where it inhibits the expression of this gene. Consequently, its downregulation fails to constrain this pathway, allowing it to proceed unrestricted.[157] miR-146b-5p, on the other hand, is part of a family of miRNAs that regulate immunity through a negative feedback mechanism, which prevents the overstimulation of the inflammatory response by promoting its resolution. As such, miR-146b-5p downregulation plays a crucial role in neuroinflammatory diseases, in which the inflammatory process is no longer controlled.[158] Finally, miR-29b-3p was selected for its high expression levels in the brain, particularly in neurons, astrocytes, and microglia. Deregulation of this miRNA has been

observed in various neurodegenerative diseases, while its upregulation could potentially have anti-tumor effects in the CNS.[159] It is a particularly interesting molecule due to its ability to regulate both pro-apoptotic pathways and pathways that promote cell survival.[160]

Via bioinformatic analysis, target genes were identified, and the ones that were target of at least two of the identified miRNAs were selected as the subject of further investigation. Through pathway enrichment analysis, several pathways related to cell survival and proliferation have emerged. Notably, the PI3K/Akt/mTOR pathway demonstrated one of the highest levels of modulation. Consequently, the next experimental phase focused on validating this pathway modulation upon exposure to ATZ, CYP, and VNZ by analysing gene and protein expression levels. Among the genes under study, ADAM12 stands out for its role in activating EGFR, a tyrosine kinase receptor upstream of several cell proliferation and survival pathways. Studies have identified upregulation of ADAM12 in various types of cancer,[161,162] and this finding is further confirmed by a 2020 study by Wang et al. that discovered a connection between ADAM12 and mTOR.[163] CDK6, identified by Zhang et al. as an activator of the PI3K/Akt/mTOR pathway, was further investigated.[164] The CDK family is indeed essential in the regulation of numerous cellular processes, including the cell cycle, and their imbalance is frequently observed in various types of cancer.[165] HDACs are a family of proteins essential for the regulation of gene expression, being responsible for the removal of acetyl groups from histones.[166] HDAC4 is primarily expressed in brain tissue.[167] Several studies have reported a link between inhibiting the PI3K/Akt/mTOR pathway and blocking HDAC gene expression.[168] Bcl-2 is another gene validated, since the Bcl-2 protein family includes pro-survival proteins**.**[169] Upregulation of one or more anti-apoptotic proteins is frequently observed in cancer cells, which exploit this mechanism to promote survival.[170] Lastly, MMPs are peptidases involved in numerous cellular processes; among them, MMP2 stands out for its role in the development of various cancers, where it regulates differentiation, invasiveness, and metastasis.[171] Wang et al. (2022) reported that inhibition of the PI3K/Akt/mTOR pathway resulted in decreased MMP2 expression.[172] Gene expression analysis demonstrated a marked upregulation of ADAM12, CDK6, Bcl-2, HDAC4, and MMP2 genes, with the most pronounced effects observed following exposure to CYP and VNZ. Notably, cells treated with ATZ displayed the minimal modulation, with a significant upregulation limited to HDAC4, even if this herbicide was the sole ED to significantly

modulate all three miRNAs under investigation. This effect can be attributed to the intricate nature of gene regulatory networks. The modulation of multiple miRNAs can activate numerous, often interconnected and opposing, pathways which may mitigate the effects of individual miRNAs.[127] Therefore, exposure to ATZ, by modulating multiple miRNAs, may have triggered compensatory or antagonistic mechanisms, attenuating the overall impact on gene expression.

To further validate the pathway, protein analysis using Western Blotting was performed. Significant upregulation of Akt and mTOR phosphorylation following exposure to all three pesticide molecules was observed. Simultaneously, the modulation of the p53 pathway was also individuated. p53, a transcription factor involved in the regulation of apoptosis, cell cycle arrest, autophagy, and metabolism, acts in a manner opposite to the PI3K/Akt/mTOR pathway. While the latter promotes cell survival and proliferation, p53 is a key player in initiating cellular stress responses, inducing cell cycle arrest, and activating programmed cell death when necessary.[173] Indeed, a significant downregulation of pro-apoptotic proteins and an upregulation of anti-apoptotic proteins were observed.

These findings elucidate a critical mechanism through which ATZ, CYP, and VNZ may exert their neurotoxicity. The downregulation of the three miRNAs under investigation induced alterations in the expression of both genes and proteins involved in the PI3K/Akt/mTOR pathway. The alteration in the balance between pro-apoptotic and anti-apoptotic proteins indicates a disruption in the normal regulation of cell death mechanisms, which is often associated with the early stages of cancer development, with the cells shifting toward a proliferative stage.[173]

The second part of the study focused on the analysis of epigenetic modulations induced following exposure to EE_2, DEP, and PFOS. These molecules showed significant downregulation of the miRNAs 200a-3p, 18b-5p, 653-5p, and 133b. As previously described, the deregulation of miR-200a-3p has been identified in numerous diseases related to the CNS, as well as in the development of various cancers.[155,157] Regarding miR-18b-5p, its downregulation can disrupt key cellular processes. In the CNS this results in altered neuronal functions and it can contribute to the development of neurodegenerative diseases or other CNS disorders.[174] miR-133b has been shown to inhibit the proliferation, migration, and invasion of cancer cells by directly targeting Bax and Bcl-2 genes, as well as modulating the

ERK signaling pathway.[175] Additionally, a downregulation of miR-133b has been identified in Parkinson's disease pathology, where its reduced expression may play a role in the progression of neurodegeneration.[176] miR-653-5p is also implicated in tumor pathologies. An intriguing study by Omorou et al. demonstrated that this miRNA acts as a key modulator in various types of cancer, exhibiting dual roles as either an inducer or suppressor of abnormal cell proliferation, depending on the tumor context.[177] Finally, the modulation of this miRNA can have significant effects on the CNS due to its influence on key pathways.[178]

Bioinformatic analysis revealed that miR-133b and miR-200a-3p specifically bind to and regulate the expression of the EGFR gene, while miR-133b, in conjunction with miR-653-5p, also targets IGF1R. EGFR is a tyrosine kinase receptor that plays a pivotal role in regulating intracellular signaling cascades that promote cell proliferation, survival, and differentiation.[162] IGF1R, a transmembrane glycoprotein, functions as a tyrosine kinase receptor that is involved in cellular processes such as growth, proliferation, differentiation, apoptosis, and angiogenesis, driving oncogenic progression.[179] SH3BP4 gene is directly regulated by microRNAs 200a-3p, 653-5p, and 18b-5p. SH3BP4 plays a critical role in modulating endocytosis and regulating signaling cascades downstream of EGFR, having the capacity to fine-tune the duration of this signaling.[180] Since both EGFR and IGF1R are implicated in the Ras pathway, and given the regulatory role of SH3BP4 in EGFR signaling, subsequent investigations focused on this pathway. Specifically, the study assessed the modulation of SOS1, a guanine nucleotide exchange factor that activates the Ras small GTPase, initiating the Ras signaling cascade.[181] Also PTEN, a tumor suppressor gene that acts as the primary negative regulator of the PI3K/Akt/mTOR signaling pathway,[182] and CDK6, already described for its implication in cellular proliferation and modulation of the same pathway,[164] were evaluated. Results of this study show an upregulation of EGFR, SH3BP4, and IGF1R following treatment with PFOS, DEP, and EE_2. On the other hand, upon EE_2 exposure, a downregulation of SOS1 was detected. This observation can be attributed to two potential mechanisms. Firstly, as a defence response to external stressors, the cell may have reduced SOS1 expression to mitigate the risk of aberrant Ras activation. Secondly, the pleiotropic nature of miRNAs, that can simultaneously target multiple genes within a signaling pathway, leading to both its activation and inhibition.

In light of that, the decision was made to validate Ras pathway through protein expression analysis using Western Blotting. Protein modulation confirmed that exposure to EE_2 induces significant changes in both the EGFR receptor and the Akt and mTOR proteins, supporting the hypothesis that this molecule exerts a direct effect on the deeply interconnected Ras and PI3K/Akt/mTOR pathways. Further confirmation is provided by the significant modulation of the p53 pathway, which is downregulated after exposure to this molecule. Similar results are observed following exposure to DEP, which, unlike EE_2, does not upregulate mTOR but induces a significant downregulation of PTEN while upregulating the expression of EGFR and Akt. The unchanged levels of p-mTOR suggest that DEP may specifically affect the upstream components of this pathway or be influenced by other regulatory mechanisms. This is further confirmed by the evidence that p53 pathway does not appear to be strongly impacted by DEP exposure. Both of them lead to a significant upregulation of the protein Ras. The elevated levels of EGFR and Ras indicate the induction of growth factor signalling, which triggers the downstream activation of the PI3K/Akt/mTOR pathway, as demonstrated by the increased phosphorylation of Akt. Finally, the surfactant PFOS showed an upregulation only for the EGFR proteins and Akt phosphorylation, while no modulation of the p53 pathway was observed. These results suggest that this molecule likely impacts the Ras and PI3K/Akt/mTOR pathways, as genetic analysis also confirmed this effect. It is probable that PFOS exerts its harmful action by targeting other factors within these pathways, which were not examined in this research project. For this reason, Ras expression analysis, which requires specific kits, was not performed on cells treated with PFOS.

These studies further support the hypothesis that exposure to molecules from various categories with endocrine-disrupting activity mediates their toxicity through epigenetic mechanisms that lead to the dysregulated activation of signaling pathways. Specifically, exposure to EE_2, DEP, and PFOS has been shown to modulate the Ras and PI3K/Akt/mTOR pathways, although each of these molecules targets distinct factors within these pathways. This dysregulation can increase cellular susceptibility to neoplastic development, therefore causing noxious effects in the exposed population.

The time at BioBasic Europe enabled the investigation of the actual presence of EDs in cosmetics used by the general population. Since the company performs quality test on sunscreen products, a screening of their database revealed EHS and AVO as the most

prevalent sunscreens filters with endocrine-disrupting potential present in commercially available cosmetics. Consequently, BioBasic Europe has kindly funded the purchase of these two molecules to determine whether they could induce neurotoxicity and to study their mechanism of action. Additionally, given the primary route of exposure to these substances is topical, their effects on human keratinocytes, HaCaT cells, were also investigated. Once assessed cell viability and oxidative stress, 10 nM, 100 nM, and 1 µM were selected as the concentrations of exposure. The foundation for the inclusion of an additional concentration [10 nM] beyond the two investigated for the other EDs [100 nM, and 1 µM] stems from the analysis of a study conducted by Matta et al. in 2020.[183] This research analysed the plasma levels of various sunscreen filters, including AVO and EHS, in subjects following both short and prolonged exposure via different formulations (lotion and spray). For AVO, a mean blood concentration of 4.35 ng/mL was identified, while for EHS, it was 5.2 ng/mL. Consequently, this data was leveraged to enhance the ecological validity of the study and its relevance to actual human exposure levels to these molecules. Intriguingly, the aforementioned study also analysed the quantities of product remaining on the subjects' skin post-application; these values aligned precisely within the concentration range we identified for our in vitro assays. This study also demonstrates that dermal exposure results in systemic absorption, enabling the accumulation of these molecules in the bloodstream and their subsequent transport to distal tissues and organs. This finding provides further justification for our study's investigation into the central and systemic effects of exposure to these molecules.

Gene and protein expression were therefore analysed. Once again, BTG2, EGFR, IGF1R, CDK6, and PTEN gene modulation was evaluated. These genes were chosen because they are key regulators involved in the Ras and PI3K/Akt/mTOR signaling pathways. The goal was to investigate whether exposure to these molecules could modulate the same pathways deregulated after exposure to EE_2, DEP, PFOS, ATZ, CYP, and VNZ, potentially linking the molecular mechanisms of action of these compounds. Exposure of both HaCaT cells and differentiated SH-SY5Y cells to AVO and EHS did not induce significant modulation of the analysed genes, with the only exception being the modulation of the CDK6 gene following exposure to 100 nM EHS in SH-SY5Y cells. The selective modulation of CDK6 suggests that this gene may be particularly sensitive to the effects of EDs in a neuronal context. CDK6 plays a crucial role in cell cycle regulation and neuronal differentiation, processes that are

highly relevant during neurodevelopment.[165] Unlike other genes involved in the Ras and PI3K/Akt/mTOR pathways, which may require more complex signaling interactions or thresholds of exposure to be modulated, CDK6 could be more directly influenced by changes in the cell cycle or developmental signaling processes in neurons. Additionally, the SH-SY5Y cells, being derived from a neuroblastoma, might possess a greater density of AR and ER, which can be directly targeted by EHS.[122,184] This specific modulation of CDK6 could therefore reflect a disruption in the normal neurodevelopmental signaling cascade, which may not be as readily observed in other cell types, such as HaCaT cells, that are less sensitive to these hormonal signals.

To assess a potential modulation at the protein level, Western Blot analysis of Akt, mTOR, ERK 1/2, EGFR, p53, and Bax proteins was performed following exposure to AVO and EHS in HaCaT cells only. The upregulation of mTOR and EGFR by AVO indicates that this molecule exerts its action at the Ras and PI3K/Akt/mTOR pathway levels. Interestingly, an upregulation of the p53 gene, the most crucial factor in the pro-apoptotic pathway, was also observed. This upregulation of p53 also happened following exposure to EHS in HaCaT cells. The apparent inconsistency in the findings can be explained by the fact that cells, when exposed to external factors and xenobiotics, activate protective mechanisms, including the induction of programmed cell death, to prevent the onset of diseases such as cancer. Given that the exposure to EDs was relatively brief, it is possible that multiple mechanisms were simultaneously activated, leading to opposing responses within the cell.[185]

Finally, at BioBasic Europe, tests are conducted to quantify the presence of Ni in cosmetic products, with the aim of certifying these products with the 'Nickel-Tested' label. Notably, it was observed that among more than 900 products analysed, only 1.21% contained Ni levels exceeding 1 mg/kg. This finding is particularly significant as it highlights the effectiveness of current regulations and quality control measures in the cosmetic industry.

Through the time at BioBasic Europe, it was possible to develop a more complete picture of the prevalence of EDs in cosmetics and to investigate potential mechanisms underlying their toxicity. To effectively regulate the presence of these substances in commercial products, as has been done for Ni, additional research is needed to comprehensively understand the modulations caused by this exposure.

6 Conclusions

The environmental persistence of EDs poses a global challenge, affecting human population, animals, and ecosystems alike. To safeguard public health, it is imperative to implement robust regulations governing both the production and use of EDs in consumer products. Moreover, it is crucial to assess new molecules introduced into the market for their endocrine-disrupting potential. Achieving these goals necessitates a thorough understanding of the mechanisms by which EDs exert their toxic effects across various biological systems.

This doctoral project aimed to elucidate the mechanisms underlying the neurotoxicity of six EDs, ATZ, CYP, VNZ, EE_2, DEP, and PFOS, selected based on their widespread environmental presence. Using human neuroblastoma SH-SY5Y cells, it was demonstrated that these compounds can induce, particularly at the epigenetic level, significant modulations even at subtoxic concentrations commonly found in the environment. Specifically, the Ras, PI3K/Akt/mTOR, and p53 pathways were found to be deregulated, rendering exposed cells more susceptible to uncontrolled growth and thus promoting a tumorigenic phenotype.

Furthermore, the affiliation with BioBasic Europe enabled to gather additional information on sunscreen filters with endocrine-disrupting activity, identifying EHS and AVO as the most commonly used filters in commercial products. Although analysis of the impact of these compounds on key signaling pathways did not yield conclusive evidence of significant modulation, the complexity of ED-induced toxicity suggests that additional research is required to fully understand their mechanisms of action.

While this study represents a preliminary investigation, these findings underscore the critical need for further research into the mechanisms of action of EDs. A comprehensive understanding of the diverse toxicological profiles associated with exposure to these substances is essential. Additionally, their environmental persistence and potential for synergistic interactions with other pollutants warrant further investigation. A multidisciplinary approach is necessary to address the complex challenges posed by these ubiquitous contaminants and to ensure the long-term health and sustainability of our planet.

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7 Bibliography

If the authors are 1 to 5, they are all listed. For 6 or more authors, only the first author is listed followed by "et al".

1. Yuan, Y., Jia, H., Xu, D. Y. & Wang, J. Novel method in emerging environmental contaminants detection: Fiber optic sensors based on microfluidic chips. *Sci Total Environ* **857**, (2023).
2. Gondi, R. *et al.* Algal-based system for removal of emerging pollutants from wastewater: A review. *Bioresour Technol* **344**, (2022).
3. Di Paola, D. *et al.* Chronic Exposure to Vinclozolin Induced Fibrosis, Mitochondrial Dysfunction, Oxidative Stress, and Apoptosis in Mice Kidney. *Int J Mol Sci* **23**, (2022).
4. Krishnakumar, S., Singh, D. S. H., Godson, P. S. & Thanga, S. G. Emerging pollutants: impact on environment, management, and challenges. *Environmental Science and Pollution Research* **29**, 72309–72311 (2022).
5. Feng, G., Huang, H. & Chen, Y. Effects of emerging pollutants on the occurrence and transfer of antibiotic resistance genes: A review. *J Hazard Mater* **420**, (2021).
6. Yilmaz, B., Terekeci, H., Sandal, S. & Kelestimur, F. Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. *Reviews in Endocrine and Metabolic Disorders 2019 21:1* **21**, 127–147 (2019).
7. Guarnotta, V., Amodei, R., Frasca, F., Aversa, A. & Giordano, C. Impact of Chemical Endocrine Disruptors and Hormone Modulators on the Endocrine System. *Int J Mol Sci* **23**, (2022).
8. Xiong, J. *et al.* New Signaling Kid on the Block in the Endocrine System: The Role of Extracellular Vesicles. *Endocrinology* **164**, (2023).
9. Rolfo, A. *et al.* Fetal-Maternal Exposure to Endocrine Disruptors: Correlation with Diet Intake and Pregnancy Outcomes. *Nutrients* **12**, 1–19 (2020).
10. Hassan, S. *et al.* Endocrine disruptors: Unravelling the link between chemical exposure and Women's reproductive health. *Environ Res* **241**, 117385 (2024).

11. Varticovski, L., Stavreva, D. A., McGowan, A., Raziuddin, R. & Hager, G. L. Endocrine Disruptors of Sex Hormone Activities. *Mol Cell Endocrinol* **539**, 111415 (2022).
12. Garcia-Reyero, N. The clandestine organs of the endocrine system. *Gen Comp Endocrinol* **257**, 264–271 (2018).
13. Browne, P., Van Der Wal, L. & Gourmelon, A. OECD approaches and considerations for regulatory evaluation of endocrine disruptors. *Mol Cell Endocrinol* **504**, 110675 (2020).
14. Kalofiri, P., Balias, G. & Tekos, F. The EU endocrine disruptors' regulation and the glyphosate controversy. *Toxicol Rep* **8**, 1193 (2021).
15. Liszewska, M., Czaja, K., Korcz, W., Lewiński, R. & Struciński, P. Endocrine-disrupting chemicals – pesticide regulatory issues from the EU perspective. *Regulatory Toxicology and Pharmacology* **154**, 105735 (2024).
16. Burden, N. *et al.* Resource and animal use implications of the proposed REACH information requirements for endocrine disruptor assessment. *Regulatory Toxicology and Pharmacology* **151**, 105671 (2024).
17. Natsch, A. Scientific discrepancies in European regulatory proposals on endocrine disruptors—REACH regulation quo vadis? *Arch Toxicol* **95**, 3601 (2021).
18. Schneider, M., Pons, J. L., Labesse, G. & Bourguet, W. In Silico Predictions of Endocrine Disruptors Properties. *Endocrinology* **160**, 2709–2716 (2019).
19. Presunto, M., Mariana, M., Lorigo, M. & Cairrao, E. The Effects of Bisphenol A on Human Male Infertility: A Review of Current Epidemiological Studies. *Int J Mol Sci* **24**, (2023).
20. Silva, M. C. *et al.* Combining computational tools and experimental studies towards endocrine disruptors mitigation: A review of biocatalytic and adsorptive processes. *Chemosphere* **344**, (2023).
21. Cilia, N. & Kandris, I. Training in the evaluation of pesticides (plant protection products and active substances) according to Regulation (EC) No 1107/2009. *EFSA Journal* **21**, e211007 (2023).

22. Hernandez-Jerez, A. F. *et al.* Development of adverse outcome pathways relevant for the identification of substances having endocrine disruption properties Uterine adenocarcinoma as adverse outcome. *EFSA Journal* **21**, e07744 (2023).
23. Bondareva, L. & Fedorova, N. Pesticides: Behavior in Agricultural Soil and Plants. *Molecules* **26**, (2021).
24. Tudi, M. *et al.* Agriculture Development, Pesticide Application and Its Impact on the Environment. *Int J Environ Res Public Health* **18**, 1–24 (2021).
25. Sharma, A. *et al.* Worldwide pesticide usage and its impacts on ecosystem. *SN Appl Sci* **1**, 1–16 (2019).
26. Sabarwal, A., Kumar, K. & Singh, R. P. Hazardous effects of chemical pesticides on human health-Cancer and other associated disorders. *Environ Toxicol Pharmacol* **63**, 103–114 (2018).
27. Shahid, M. & Khan, M. S. Ecotoxicological implications of residual pesticides to beneficial soil bacteria: A review. *Pestic Biochem Physiol* **188**, (2022).
28. Kim, K. H., Kabir, E. & Jahan, S. A. Exposure to pesticides and the associated human health effects. *Sci Total Environ* **575**, 525–535 (2017).
29. Hu, Y. *et al.* Impact of atrazine on soil microbial properties: A meta-analysis. *Environ Pollut* **323**, (2023).
30. Arabi, S., Heidari-Beni, M., Poursafa, P., Roshanaei, M. & Kelishadi, R. A review of the potential adverse health impacts of atrazine in humans. *Rev Environ Health* (2024) doi:10.1515/REVEH-2024-0094.
31. Zhang, C. *et al.* Atrazine induced oxidative stress and mitochondrial dysfunction in quail (Coturnix C. coturnix) kidney via modulating Nrf2 signaling pathway. *Chemosphere* **212**, 974–982 (2018).
32. Liu, J. *et al.* Transcriptome analysis reveals the mechanisms of hepatic injury caused by long-term environmental exposure to atrazine in juvenile common carp (Cyprinus carpio L.). *Environ Sci Pollut Res Int* **30**, 36545–36556 (2023).
33. Deng, S. *et al.* Advances in understanding and mitigating Atrazine's environmental and health impact: A comprehensive review. *J Environ Manage* **365**, (2024).

34. Zhao, H. *et al.* Endocrine toxicity of atrazine and its underlying mechanisms. *Toxicology* **505**, (2024).
35. Wu, K. *et al.* Gestational vinclozolin exposure suppresses fetal testis development in rats. *Ecotoxicol Environ Saf* **203**, (2020).
36. Abarikwu, S. O., Ezim, O. E., Ikeji, C. N. & Farombi, E. O. Atrazine: cytotoxicity, oxidative stress, apoptosis, testicular effects and chemopreventive Interventions. *Frontiers in toxicology* **5**, (2023).
37. D'Amico, R. *et al.* Chronic Exposure to Endocrine Disruptor Vinclozolin Leads to Lung Damage via Nrf2-Nf-kb Pathway Alterations. *Int J Mol Sci* **23**, (2022).
38. Marcoccia, D., Smeriglio, A., Mantovani, A., Trombetta, D. & Lorenzetti, S. Intracellular distribution of vinclozolin and its metabolites differently affects 5α-dihydrotestosterone (DHT)-induced PSA secretion in LNCaP cells. *Reprod Toxicol* **111**, 83–91 (2022).
39. Kang, C. Q., Meng, Q. Y., Dang, W., Shao, Y. J. & Lu, H. L. Effects of chronic exposure to the fungicide vinclozolin on gut microbiota community in an aquatic turtle. *Ecotoxicol Environ Saf* **239**, (2022).
40. Ham, J., Jang, H., Song, G. & Lim, W. Cypermethrin induces endoplasmic reticulum stress and autophagy, leads to testicular dysfunction. *Sci Total Environ* **902**, (2023).
41. Rebuzzini, P. *et al.* Chronic cypermethrin exposure alters mouse embryonic stem cell growth kinetics, induces Phase II detoxification response and affects pluripotency and differentiation gene expression. *Eur J Histochem* **64**, (2020).
42. Ganguly, S. *et al.* Endocrine disruptive toxicity of cypermethrin in Labeo catla: Involvement of genes and proteins related to the HPG axis. *Science of The Total Environment* **901**, 165958 (2023).
43. Filardi, T., Panimolle, F., Lenzi, A. & Morano, S. Bisphenol A and Phthalates in Diet: An Emerging Link with Pregnancy Complications. *Nutrients* **12**, (2020).
44. Filardi, T., Panimolle, F., Lenzi, A. & Morano, S. Bisphenol A and Phthalates in Diet: An Emerging Link with Pregnancy Complications. *Nutrients* **12**, (2020).
45. Das, M. T. *et al.* Remediation strategies for mitigation of phthalate pollution: Challenges and future perspectives. *J Hazard Mater* **409**, (2021).

46. Pallotti, F. *et al.* Mechanisms of Testicular Disruption from Exposure to Bisphenol A and Phtalates. *J Clin Med* **9**, (2020).
47. Prasad, B. Phthalate pollution: environmental fate and cumulative human exposure index using the multivariate analysis approach. *Environ Sci Process Impacts* **23**, 389–399 (2021).
48. Tuan Tran, H. *et al.* Phthalates in the environment: characteristics, fate and transport, and advanced wastewater treatment technologies. *Bioresour Technol* **344**, (2022).
49. Kim, S., Thapar, I. & Brooks, B. W. Epigenetic changes by per- and polyfluoroalkyl substances (PFAS). *Environ Pollut* **279**, (2021).
50. Zhang, M. *et al.* A new mechanistic insight into the association between environmental perfluorooctane sulfonic acid (PFOS) exposure and attention deficit and hyperactivity disorder (ADHD)-like behavior. *Neurotoxicology* **99**, 254–263 (2023).
51. Radetić, L., Plantak, L., Loborec, J. & Grčić, I. Super-resistance of PFOS in water: Is it beatable? *Chemosphere* **362**, (2024).
52. Rumph, J. T., Stephens, V. R., Archibong, A. E., Osteen, K. G. & Bruner-Tran, K. L. Environmental Endocrine Disruptors and Endometriosis. *Adv Anat Embryol Cell Biol* **232**, 57 (2020).
53. Rogers, R. E., Chai, S., Pask, A. J. & Mattiske, D. M. Prenatal exposure to diethylstilbestrol has long-lasting, transgenerational impacts on fertility and reproductive development. *Toxicol Sci* **195**, 53–60 (2023).
54. Saaristo, M., Johnstone, C. P., Xu, K., Allinson, M. & Wong, B. B. M. The endocrine disruptor, 17α-ethinyl estradiol, alters male mate choice in a freshwater fish. *Aquat Toxicol* **208**, 118–125 (2019).
55. Torres, N. H. *et al.* Environmental aspects of hormones estriol, 17β-estradiol and 17α-ethinylestradiol: Electrochemical processes as next-generation technologies for their removal in water matrices. *Chemosphere* **267**, (2021).
56. Yien Fang, T., Praveena, S. M., Aris, A. Z., Syed Ismail, S. N. & Rasdi, I. Quantification of selected steroid hormones (17β-Estradiol and 17α-

Ethynylestradiol) in wastewater treatment plants in Klang Valley (Malaysia). *Chemosphere* **215**, 153–162 (2019).

57. Adeel, M., Song, X., Wang, Y., Francis, D. & Yang, Y. Environmental impact of estrogens on human, animal and plant life: A critical review. *Environ Int* **99**, 107–119 (2017).
58. Voisin, A. S., Kültz, D. & Silvestre, F. Early-life exposure to the endocrine disruptor 17-α-ethinylestradiol induces delayed effects in adult brain, liver and ovotestis proteomes of a self-fertilizing fish. *J Proteomics* **194**, 112–124 (2019).
59. Lee, S. *et al.* Single and mixture toxicity evaluation of avobenzone and homosalate to male zebrafish and H295R cells. *Chemosphere* **343**, (2023).
60. Serpone, N. Sunscreens and their usefulness: have we made any progress in the last two decades? *Photochem Photobiol Sci* **20**, 189–244 (2021).
61. The SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation, 11th revision, 30-31 March 2021, SCCS/1628/21. *Regul Toxicol Pharmacol* **127**, 105052 (2021).
62. Suh, S., Pham, C., Smith, J. & Mesinkovska, N. A. The banned sunscreen ingredients and their impact on human health: a systematic review. *Int J Dermatol* **59**, 1033–1042 (2020).
63. Shetty, N., Schalka, S., Lim, H. W. & Mohammad, T. F. The effects of UV filters on health and the environment. *Photochem Photobiol Sci* **22**, 2463–2471 (2023).
64. Yuan, S. *et al.* Environmental Fate and Toxicity of Sunscreen-Derived Inorganic Ultraviolet Filters in Aquatic Environments: A Review. *Nanomaterials (Basel)* **12**, (2022).
65. Kim, H., Kim, D. & An, Y. J. Microplastics enhance the toxicity and phototoxicity of UV filter avobenzone on Daphnia magna. *J Hazard Mater* **445**, (2023).
66. Ka, Y. & Ji, K. Waterborne exposure to avobenzone and octinoxate induces thyroid endocrine disruption in wild-type and thrαa-/- zebrafish larvae. *Ecotoxicology* **31**, 948–955 (2022).

67. Liu, Y., Wang, Y., Li, N. & Jiang, S. Avobenzone and nanoplastics affect the development of zebrafish nervous system and retinal system and inhibit their locomotor behavior. *Sci Total Environ* **806**, (2022).
68. Ka, Y. & Ji, K. Waterborne exposure to avobenzone and octinoxate induces thyroid endocrine disruption in wild-type and thrαa-/- zebrafish larvae. *Ecotoxicology* **31**, 948–955 (2022).
69. Zhao, G. *et al.* UV filter ethylhexyl salicylate affects cardiovascular development by disrupting lipid metabolism in zebrafish embryos. *Sci Total Environ* **888**, (2023).
70. Hiller, J. *et al.* Toxicokinetics of urinary 2-ethylhexyl salicylate and its metabolite 2-ethyl-hydroxyhexyl salicylate in humans after simulating real-life dermal sunscreen exposure. *Arch Toxicol* **93**, 2565–2574 (2019).
71. Aprile, A. & De Bellis, L. Editorial for Special Issue "Heavy Metals Accumulation, Toxicity, and Detoxification in Plants". *Int J Mol Sci* **21**, 1–5 (2020).
72. Fasae, K. D. & Abolaji, A. O. Interactions and toxicity of non-essential heavy metals (Cd, Pb and Hg): lessons from Drosophila melanogaster. *Curr Opin Insect Sci* **51**, (2022).
73. Renu, K. *et al.* Molecular mechanism of heavy metals (Lead, Chromium, Arsenic, Mercury, Nickel and Cadmium) - induced hepatotoxicity - A review. *Chemosphere* **271**, (2021).
74. Paschoalini, A. L., Savassi, L. A., Arantes, F. P., Rizzo, E. & Bazzoli, N. Heavy metals accumulation and endocrine disruption in Prochilodus argenteus from a polluted neotropical river. *Ecotoxicol Environ Saf* **169**, 539–550 (2019).
75. Liu, D., Shi, Q., Liu, C., Sun, Q. & Zeng, X. Effects of Endocrine-Disrupting Heavy Metals on Human Health. *Toxics* **11**, (2023).
76. Swathi Krishna, S., Kuriakose, B. B. & Lakshmi, P. K. Effects of phytoestrogens on reproductive organ health. *Arch Pharm Res* **45**, 849–864 (2022).
77. Canivenc-Lavier, M. C. & Bennetau-Pelissero, C. Phytoestrogens and Health Effects. *Nutrients* **15**, (2023).

78. Domínguez-López, I., Yago-Aragón, M., Salas-Huetos, A., Tresserra-Rimbau, A. & Hurtado-Barroso, S. Effects of Dietary Phytoestrogens on Hormones throughout a Human Lifespan: A Review. *Nutrients* **12**, 1–25 (2020).
79. Juraschek, L. M., Kappenberg, A. & Amelung, W. Mycotoxins in soil and environment. *Science of The Total Environment* **814**, 152425 (2022).
80. Caserta, D., De Marco, M. P., Besharat, A. R. & Costanzi, F. Endocrine Disruptors and Endometrial Cancer: Molecular Mechanisms of Action and Clinical Implications, a Systematic Review. *Int J Mol Sci* **23**, (2022).
81. Huang, J. *et al.* Occurrence, removal, and risk assessment of emerging contaminants in aquatic products processing sewage treatment plants. *Environ Sci Pollut Res Int* **30**, 117772–117784 (2023).
82. Stafoggia, M. *et al.* Joint effect of heat and air pollution on mortality in 620 cities of 36 countries. *Environ Int* **181**, (2023).
83. Ottinger, M. A., Brugger, K. E. & Touart, L. Endocrine-disrupting chemicals. *Best Pract Res Clin Endocrinol Metab* **35**, 101566 (2021).
84. Domingo, J. L. & Nadal, M. Human exposure to per- and polyfluoroalkyl substances (PFAS) through drinking water: A review of the recent scientific literature. *Environ Res* **177**, (2019).
85. Córdoba-Tovar, L., Marrugo-Negrete, J., Barón, P. R. & Díez, S. Drivers of biomagnification of Hg, As and Se in aquatic food webs: A review. *Environ Res* **204**, (2022).
86. Wan, M. L. Y., Co, V. A. & El-Nezami, H. Endocrine disrupting chemicals and breast cancer: a systematic review of epidemiological studies. *Crit Rev Food Sci Nutr* **62**, 6549–6576 (2022).
87. Khairul Hasni, N. A. *et al.* Occurrence of endocrine disruptors in Malaysia's water systems: A scoping review. *Environ Pollut* **324**, (2023).
88. Teixeira, L. C. G. M. *et al.* Occurrence and removal of drugs and endocrine disruptors in the Bolonha Water Treatment Plant in Belém/PA (Brazil). *Environ Monit Assess* **193**, (2021).
89. Silva, A. B. P., Carreiró, F., Ramos, F. & Sanches-Silva, A. The role of endocrine disruptors in female infertility. *Mol Biol Rep* **50**, 7069–7088 (2023).

90. Yang, H., Du, Z., Lv, W. J., Zhang, X. Y. & Zhai, H. L. In silico toxicity evaluation of dioxins using structure-activity relationship (SAR) and two-dimensional quantitative structure-activity relationship (2D-QSAR). *Arch Toxicol* **93**, 3207–3218 (2019).
91. Guan, L. L., Lim, H. W. & Mohammad, T. F. Sunscreens and Photoaging: A Review of Current Literature. *Am J Clin Dermatol* **22**, 819 (2021).
92. Kahn, L. G., Philippat, C., Nakayama, S. F., Slama, R. & Trasande, L. Endocrine-disrupting chemicals: implications for human health. *Lancet Diabetes Endocrinol* **8**, 703–718 (2020).
93. Rattan, S. & Flaws, J. A. The epigenetic impacts of endocrine disruptors on female reproduction across generations†. *Biol Reprod* **101**, 635–644 (2019).
94. Predieri, B., Iughetti, L., Bernasconi, S. & Street, M. E. Endocrine Disrupting Chemicals' Effects in Children: What We Know and What We Need to Learn? *Int J Mol Sci* **23**, (2022).
95. de Paula, L. C. P. & Alves, C. Food packaging and endocrine disruptors. *J Pediatr (Rio J)* **100 Suppl 1**, S40–S47 (2024).
96. Predieri, B., Iughetti, L., Bernasconi, S. & Street, M. E. Endocrine Disrupting Chemicals' Effects in Children: What We Know and What We Need to Learn? *Int J Mol Sci* **23**, (2022).
97. Predieri, B., Iughetti, L., Bernasconi, S. & Street, M. E. Endocrine Disrupting Chemicals' Effects in Children: What We Know and What We Need to Learn? *Int J Mol Sci* **23**, (2022).
98. Galbiati, V. *et al.* Immune and Nervous Systems Interaction in Endocrine Disruptors Toxicity: The Case of Atrazine. *Frontiers in toxicology* **3**, (2021).
99. Hilz, E. N. & Gore, A. C. Sex-specific Effects of Endocrine-disrupting Chemicals on Brain Monoamines and Cognitive Behavior. *Endocrinology* **163**, bqac128 (2022).
100. Richardson, J. R., Fitsanakis, V., Westerink, R. & Kanthasamy, A. G. Neurotoxicity of Pesticides. *Acta Neuropathol* **138**, 343 (2019).
101. De Caroli Vizioli, B., Silva da Silva, G., Ferreira de Medeiros, J. & Montagner, C. C. Atrazine and its degradation products in drinking water source and supply:

Risk assessment for environmental and human health in Campinas, Brazil. *Chemosphere* **336**, (2023).

102. Peritore, A. F. *et al.* Effect of Pesticide Vinclozolin Toxicity Exposure on Cardiac Oxidative Stress and Myocardial Damage. *Toxics* **11**, (2023).
103. Thacharodi, A. *et al.* Endocrine disrupting chemicals and their effects on the reproductive health in men. *Environ Res* **236**, (2023).
104. Jane L Espartero, L. *et al.* Health-related toxicity of emerging per- and polyfluoroalkyl substances: Comparison to legacy PFOS and PFOA. *Environ Res* **212**, (2022).
105. Hu, M. *et al.* Physiologically-based toxicokinetic modeling of human dermal exposure to diethyl phthalate: Application to health risk assessment. *Chemosphere* **307**, (2022).
106. Suh, S., Pham, C., Smith, J. & Mesinkovska, N. A. The banned sunscreen ingredients and their impact on human health: a systematic review. *Int J Dermatol* **59**, 1033–1042 (2020).
107. Schatzer, C. A. de F. *et al.* Microencapsulation of sunscreen reduces toxicity of its components to A. salina: Biochemical, behavioral and morphological studies. *Ecotoxicol Environ Saf* **281**, (2024).
108. Chatzigianni, M. *et al.* Environmental impacts due to the use of sunscreen products: a mini-review. *Ecotoxicology* **31**, 1331 (2022).
109. Combarnous, Y. & Nguyen, T. M. D. Comparative Overview of the Mechanisms of Action of Hormones and Endocrine Disruptor Compounds. *Toxics* **7**, (2019).
110. Gaggi, G., Barbagallo, F., De Toni, L. & Bucci, I. Editorial: Endocrine disruptors: mechanism of action and implications for human health. *Front Cell Dev Biol* **12**, (2024).
111. La Merrill, M. A. *et al.* Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat Rev Endocrinol* **16**, 45–57 (2020).
112. Combarnous, Y. & Diep Nguyen, T. M. Comparative Overview of the Mechanisms of Action of Hormones and Endocrine Disruptor Compounds. *Toxics* **7**, (2019).

113. Qu, K., Song, J., Zhu, Y., Liu, Y. & Zhao, C. Perfluorinated compounds binding to estrogen receptor of different species: a molecular dynamic modeling. *J Mol Model* **25**, (2018).
114. Corti, M., Lorenzetti, S., Ubaldi, A., Zilli, R. & Marcoccia, D. Endocrine Disruptors and Prostate Cancer. *Int J Mol Sci* **23**, (2022).
115. Zhao, H. *et al.* Endocrine toxicity of atrazine and its underlying mechanisms. *Toxicology* **505**, (2024).
116. Warner, G. R., Mourikes, V. E., Neff, A. M., Brehm, E. & Flaws, J. A. Mechanisms of action of agrochemicals acting as endocrine disrupting chemicals. *Mol Cell Endocrinol* **502**, (2020).
117. Hong, H. *et al.* Human sex hormone-binding globulin binding affinities of 125 structurally diverse chemicals and comparison with their binding to androgen receptor, estrogen receptor, and α-fetoprotein. *Toxicol Sci* **143**, 333–348 (2015).
118. Küblbeck, J. *et al.* The EDCMET Project: Metabolic Effects of Endocrine Disruptors. *Int J Mol Sci* **21**, (2020).
119. Nicolella, H. D. & de Assis, S. Epigenetic Inheritance: Intergenerational Effects of Pesticides and Other Endocrine Disruptors on Cancer Development. *Int J Mol Sci* **23**, (2022).
120. Nicolella, H. D. & de Assis, S. Epigenetic Inheritance: Intergenerational Effects of Pesticides and Other Endocrine Disruptors on Cancer Development. *Int J Mol Sci* **23**, (2022).
121. Voisin, A. S., Suarez Ulloa, V., Stockwell, P., Chatterjee, A. & Silvestre, F. Genome-wide DNA methylation of the liver reveals delayed effects of early-life exposure to 17-α-ethinylestradiol in the self-fertilizing mangrove rivulus. *Epigenetics* **17**, 473–497 (2022).
122. Zhou, R. *et al.* Epigenetic mechanisms of DNA methylation in the transgenerational effect of ethylhexyl salicylate on zebrafish. *Chemosphere* **295**, (2022).
123. Budakoti, M. *et al.* Micro-RNA: The darkhorse of cancer. *Cell Signal* **83**, (2021).
124. Ho, P. T. B., Clark, I. M. & Le, L. T. T. MicroRNA-Based Diagnosis and Therapy. *Int J Mol Sci* **23**, (2022).

125. Wang, N., Chen, C., Ren, J. & Dai, D. MicroRNA delivery based on nanoparticles of cardiovascular diseases. *Mol Cell Biochem* **479**, 1909–1923 (2024).
126. Condrat, C. E. *et al.* miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells* **9**, (2020).
127. Crisafulli, L. & Ficara, F. Micro-RNAs: A safety net to protect hematopoietic stem cell self-renewal. *Wiley Interdiscip Rev RNA* **13**, (2022).
128. Xiong, S. *et al.* An miR-200 Cluster on Chromosome 23 Regulates Sperm Motility in Zebrafish. *Endocrinology* **159**, 1982–1991 (2018).
129. Omorou, M. *et al.* The emerging role of miR-653 in human cancer. *Cancer Epidemiol* **79**, (2022).
130. Kim, K. Y. *et al.* Downregulated miR-18b-5p triggers apoptosis by inhibition of calcium signaling and neuronal cell differentiation in transgenic SOD1 (G93A) mice and SOD1 (G17S and G86S) ALS patients. *Transl Neurodegener* **9**, (2020).
131. Jahangard, Y. *et al.* Therapeutic Effects of Transplanted Exosomes Containing miR-29b to a Rat Model of Alzheimer's Disease. *Front Neurosci* **14**, (2020).
132. Bokobza, C. *et al.* miR-146b Protects the Perinatal Brain against Microglia-Induced Hypomyelination. *Ann Neurol* **91**, 48–65 (2022).
133. Song, Y. *et al.* Targeting RAS-RAF-MEK-ERK signaling pathway in human cancer: Current status in clinical trials. *Genes Dis* **10**, 76–88 (2022).
134. Guo, Y. *et al.* ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med* **19**, (2020).
135. Yu, L., Wei, J. & Liu, P. Attacking the PI3K/Akt/mTOR signaling pathway for targeted therapeutic treatment in human cancer. *Semin Cancer Biol* **85**, 69–94 (2022).
136. Tewari, D., Patni, P., Bishayee, A., Sah, A. N. & Bishayee, A. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: A novel therapeutic strategy. *Semin Cancer Biol* **80**, 1–17 (2022).
137. Li, Q., Li, Z., Luo, T. & Shi, H. Targeting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways for cancer therapy. *Molecular biomedicine* **3**, (2022).

138. Li, Q., Li, Z., Luo, T. & Shi, H. Targeting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways for cancer therapy. *Molecular biomedicine* **3**, (2022).
139. Jachter, S. L., Simmons, W. P., Estill, C., Xu, J. & Bishop, C. V. Matrix-free three-dimensional culture of bovine secondary follicles to antral stage: Impact of media formulation and epidermal growth factor (EGF). *Theriogenology* **181**, 89–94 (2022).
140. Pruccoli, L., Morroni, F., Sita, G., Hrelia, P. & Tarozzi, A. Esculetin as a Bifunctional Antioxidant Prevents and Counteracts the Oxidative Stress and Neuronal Death Induced by Amyloid Protein in SH-SY5Y Cells. *Antioxidants (Basel)* **9**, 1–16 (2020).
141. Lavogina, D. *et al.* Revisiting the Resazurin-Based Sensing of Cellular Viability: Widening the Application Horizon. *Biosensors (Basel)* **12**, (2022).
142. D'agostino, A., Di Palma, T., Cecchini Gualandi, S. & Boni, R. Fluorescence Spectroscopy for the Diagnosis of Endometritis in the Mare. *Animals (Basel)* **12**, (2022).
143. Cunningham, F. *et al.* Ensembl 2022. *Nucleic Acids Res* **50**, D988–D995 (2022).
144. Sticht, C., De La Torre, C., Parveen, A. & Gretz, N. miRWalk: An online resource for prediction of microRNA binding sites. *PLoS One* **13**, (2018).
145. Kolberg, L. *et al.* g:Profiler-interoperable web service for functional enrichment analysis and gene identifier mapping (2023 update). *Nucleic Acids Res* **51**, W207–W212 (2023).
146. Yu, L., Wei, J. & Liu, P. Attacking the PI3K/Akt/mTOR signaling pathway for targeted therapeutic treatment in human cancer. *Semin Cancer Biol* **85**, 69–94 (2022).
147. Liu, Y., Su, Z., Tavana, O. & Gu, W. Understanding the complexity of p53 in a new era of tumor suppression. *Cancer Cell* **42**, 946–967 (2024).
148. Maarouf, R. E., Azab, K. S., El Fatih, N. M., Helal, H. & Rashed, L. Withania somnifera Alter BCL2/Bax signaling and trigger apoptosis of MCF-7 and MDA-MB231 breast cancer cells exposed to γ-radiation. *Hum Exp Toxicol* **42**, (2023).
149. Schmidt, S. I., Blaabjerg, M., Freude, K. & Meyer, M. RhoA Signaling in Neurodegenerative Diseases. *Cells* **11**, (2022).

150. Fernández-Pisonero, I. *et al.* A hotspot mutation targeting the R-RAS2 GTPase acts as a potent oncogenic driver in a wide spectrum of tumors. *Cell Rep* **38**, (2022).
151. Korbecki, J. *et al.* Chronic and Cycling Hypoxia: Drivers of Cancer Chronic Inflammation through HIF-1 and NF-κB Activation: A Review of the Molecular Mechanisms. *Int J Mol Sci* **22**, (2021).
152. Street, M. E., Audouze, K., Legler, J., Sone, H. & Palanza, P. Endocrine Disrupting Chemicals: Current Understanding, New Testing Strategies and Future Research Needs. *Int J Mol Sci* **22**, 1–5 (2021).
153. Ramírez, V. *et al.* Role of endocrine disrupting chemicals in children's neurodevelopment. *Environ Res* **203**, (2022).
154. Kalinina, T., Kononchuk, V., Klyushova, L. & Gulyaeva, L. Effects of Endocrine Disruptors o, p'-Dichlorodiphenyltrichloroethane, p, p'-Dichlorodiphenyltrichloroethane, and Endosulfan on the Expression of Estradiol-, Progesterone-, and Testosterone-Responsive MicroRNAs and Their Target Genes in MCF-7 Cells. *Toxics* **10**, (2022).
155. Li, H. *et al.* miR-200a-3p Regulates PRKACB and Participates in Aluminium-Induced Tau Phosphorylation in PC12 Cells. *Neurotox Res* **40**, 1963–1978 (2022).
156. Majumdar, A., Venkatesh, I. P. S., Swarup, V. & Basu, A. Short-chain fatty acids abrogate Japanese encephalitis virus-induced inflammation in microglial cells via miR-200a-3p/ZBTB20/IKβα axis. *mBio* **15**, (2024).
157. Pham, T. T. *et al.* MicroRNA gga-miR-200a-3p modulates immune response via MAPK signaling pathway in chicken afflicted with necrotic enteritis. *Vet Res* **51**, (2020).
158. Bokobza, C. *et al.* miR-146b Protects the Perinatal Brain against Microglia-Induced Hypomyelination. *Ann Neurol* **91**, 48–65 (2022).
159. Wang, H., Li, Z., Gao, J. & Liao, Q. Circular RNA circPTK2 regulates oxygen-glucose deprivation-activated microglia-induced hippocampal neuronal apoptosis via miR-29b-SOCS-1-JAK2/STAT3-IL-1β signaling. *Int J Biol Macromol* **129**, 488–496 (2019).

160. Klatt, C. L., Theis, V., Hahn, S., Theiss, C. & Matschke, V. Deregulated miR-29b-3p Correlates with Tissue-Specific Activation of Intrinsic Apoptosis in An Animal Model of Amyotrophic Lateral Sclerosis. *Cells* **8**, (2019).
161. Xu, J., Wang, Y., Jiang, J., Yin, C. & Shi, B. ADAM12 promotes clear cell renal cell carcinoma progression and triggers EMT via EGFR/ERK signaling pathway. *J Transl Med* **21**, (2023).
162. Levantini, E., Maroni, G., Del Re, M. & Tenen, D. G. EGFR signaling pathway as therapeutic target in human cancers. *Semin Cancer Biol* **85**, 253–275 (2022).
163. Wang, L. *et al.* ADAM12 silencing promotes cellular apoptosis by activating autophagy in choriocarcinoma cells. *Int J Oncol* **56**, 1162–1174 (2020).
164. Zhang, L., Zhang, L. & Guo, F. MiRNA-494-3p Regulates Bupivacaine-Induced Neurotoxicity by the CDK6-PI3K/AKT Signaling. *Neurotox Res* **39**, 2007–2017 (2021).
165. Nebenfuehr, S., Kollmann, K. & Sexl, V. The role of CDK6 in cancer. *Int J Cancer* **147**, 2988–2995 (2020).
166. Sild, M. & Booij, L. Histone deacetylase 4 (HDAC4): a new player in anorexia nervosa? *Mol Psychiatry* **24**, 1425–1434 (2019).
167. Wang, L. *et al.* Histone Deacetylase 4 Inhibition Reduces Rotenone-Induced Alpha-Synuclein Accumulation via Autophagy in SH-SY5Y Cells. *Brain Sci* **13**, (2023).
168. Xu, Z. *et al.* METTL14-regulated PI3K/Akt signaling pathway via PTEN affects HDAC5-mediated epithelial-mesenchymal transition of renal tubular cells in diabetic kidney disease. *Cell Death Dis* **12**, (2021).
169. Kaloni, D., Diepstraten, S. T., Strasser, A. & Kelly, G. L. BCL-2 protein family: attractive targets for cancer therapy. *Apoptosis* **28**, 20–38 (2023).
170. Gupta, V. A., Ackley, J., Kaufman, J. L. & Boise, L. H. BCL2 Family Inhibitors in the Biology and Treatment of Multiple Myeloma. *Blood Lymphat Cancer* **11**, 11–24 (2021).
171. Peng, K., Zhang, Y., Liu, D. & Chen, J. MMP2 is a immunotherapy related biomarker and correlated with cancer-associated fibroblasts infiltrate in melanoma. *Cancer Cell Int* **23**, (2023).

172. Deng, Q. *et al.* Nickel nanoparticles affect the migration and invasion of HTR-8/SVneo cells by downregulating MMP2 through the PI3K/AKT pathway. *Toxicol In Vitro* **80**, (2022).
173. Wang, H., Guo, M., Wei, H. & Chen, Y. Targeting p53 pathways: mechanisms, structures, and advances in therapy. *Signal Transduct Target Ther* **8**, (2023).
174. Kim, K. Y. *et al.* Downregulated miR-18b-5p triggers apoptosis by inhibition of calcium signaling and neuronal cell differentiation in transgenic SOD1 (G93A) mice and SOD1 (G17S and G86S) ALS patients. *Transl Neurodegener* **9**, (2020).
175. Xu, Y., Ma, Y., Liu, X. L. & Gao, S. L. miR-133b affects cell proliferation, invasion and chemosensitivity in renal cell carcinoma by inhibiting the ERK signaling pathway. *Mol Med Rep* **22**, 67–76 (2020).
176. Dong, L. G. *et al.* MiR-133b inhibits MPP+-induced apoptosis in Parkinson's disease model by inhibiting the ERK1/2 signaling pathway. *Eur Rev Med Pharmacol Sci* **24**, 11192–11198 (2020).
177. Omorou, M. *et al.* The emerging role of miR-653 in human cancer. *Cancer Epidemiol* **79**, (2022).
178. Guo, J. B. *et al.* Comparative Transcriptome Profiling Reveals Changes of microRNAs Response to Exercise in Rats with Neuropathic Pain. *Neural Plast* **2021**, (2021).
179. Stefani, C. *et al.* Growth Factors, PI3K/AKT/mTOR and MAPK Signaling Pathways in Colorectal Cancer Pathogenesis: Where Are We Now? *Int J Mol Sci* **22**, (2021).
180. Imbastari, F. *et al.* MACC1 regulates clathrin-mediated endocytosis and receptor recycling of transferrin receptor and EGFR in colorectal cancer. *Cell Mol Life Sci* **78**, 3525–3542 (2021).
181. Daley, B. R. *et al.* SOS1 and KSR1 modulate MEK inhibitor responsiveness to target resistant cell populations based on PI3K and KRAS mutation status. *Proc Natl Acad Sci U S A* **120**, (2023).
182. Rademacher, S. & Eickholt, B. J. PTEN in Autism and Neurodevelopmental Disorders. *Cold Spring Harb Perspect Med* **9**, (2019).

183. Matta, M. K. *et al.* Effect of Sunscreen Application on Plasma Concentration of Sunscreen Active Ingredients: A Randomized Clinical Trial. *JAMA* **323**, 256 (2020).
184. Maioli, S., Leander, K., Nilsson, P. & Nalvarte, I. Estrogen receptors and the aging brain. *Essays Biochem* **65**, 913–925 (2021).
185. Zhou, Y. *et al.* Expanding Roles of the E2F-RB-p53 Pathway in Tumor Suppression. *Biology (Basel)* **12**, (2023).