Effect of exposure to persistent toxic pollutants (PTPs) on the incidence of cancer disease in the Canarian population: a review.

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1. Abstract

Organochlorine pesticides (OCs) have been associated with some chronic diseases, such as breast cancer, diabetes and obesity. In the present study have investigated the effects of some OCs, individually and in combination, which had been previously detected in the human serum of the Canarian population, on normal human mammary epithelial cells (HMEC) at concentrations close to those found in human beings. The OCs studied were: aldrin, dieldrin, o,p'-DDE, p,p'-DDE, p,p'-DDD, o,p'-DDT, p,p'-DDT.

Organochlorines have similar properties to estrogen, and these chemicals have been associated with estrogen-dependent breast cancer. When studying the effect exerted by 17β-estradiol on HMEC, it was found that the cell viability profile exposed to estradiol was similar to that obtained in the HMEC exposed to the OC-DDTs mixture. Emphasizing therefore that the effects exerted by one of the most important mixtures of organochlorines could be related to the known estrogenic propierties of these chemicals (PF Valerón et al., 2009).

The effect exerted for the H-mixture and BC-mixture environmentally relevant on the gene expression profile, found in healthy women and patients with breast cancer in mammary epithelial cells was clearly different, while the H-mixture decreased the expression of tumour supressor genes (EPHA4 and EPHB2), the BC-mixture increased the expression of oncogenes associated with breast cancer (GFRA1, BHLHB8) (Javier Rivero et al., 2016).

2. Introduction

The introduction of insecticides has greatly improved diet and health of the population by increasing the agricultural productivity and controlling the infectious diseases transmitted by plagues. Therefore, is not surprising that the use of insecticides is increasing (Sparks et al., 2013). However, the wide use of insecticides has also sparked growing concern over their health impact. Insecticides are one of the main causes for environmental pollution, and growing body of evidence is suggesting that exposure to insecticides can also potentiate the risk of chronic diseases, among which are included: cancer, obesity and diabetes (Hectores et al., 2011, Karami-Mohajeri y Abdollahi, 2011, Kuo et al., 2013; Lee et al., 2014a; Rezg et al., 2010b). This review has focused in the knowledges which have been acquired in the relation between the exposure to insecticides and the development of cancer or other chronic diseases in the Canarian population.

3. Insecticide classification and action

Persistent organic pollutants (POPs) are chemical compounds from industrial waste and agricultural pesticides, which are very spread in the environment in all the regions all over the world. Its use was banned in 1970, but these chemicals are still being detected in the environment, food, biota and human being due to its great persistence and degradation resistance. As endocrine disruptors, they interfere with the tissue functions which react hormonally through the deregulation in the hormonal signal and the cellular function.

The main features of the POPs are:

1. They are organic compounds, which don't decompose easily, because their molecular structure is based on carbon.
2. They have lipophilic properties; resist physical, chemical and biological degradation. Thus, once a POP has come into the environment, it will remain.
3. They are bioaccumulative. In fact, their persistence in the environment leads to bioaccumulation in animals and also to biomagnification in the

food chain, resulting in the bioaccumulation of these chemicals in the human body, including adipose tissue, fatty tissue, breast milk, or serum.

4. They can be transported over long distances and cause a dangerous pollution. This can be verified by currents of air, water or migratory species.
5. They cause negative effects in human health and/or the ecosystems, considering that they are chemical pollutants.

When humans consume food polluted by POPs, these are accumulated in the fat tissues. The mothers transmit the POPs to their babies, both in humans and in animals through the breastmilk. Likewise, in non-mammalian animals, POPs are transmitted from the mother to her babies through the eggs.

There is a lot of literature that associate the following diseases and human disabilities with POPs:

1. Cancer and tumours, including breast cancer (BC), testicular cancer, pancreas cancer, lymphoma non-Hodgkin, multiple myeloma and the appearance of leukaemia in the adult age among others.
2. Neurological disorders, as problems in learning and memory decline.
3. Immunologic abolition.
4. Reproductive disorders, decrease in spermatozoa, spontaneous abortion and birth defects.
5. Other diseases, with a higher incidence of diabetes type 2, endometriosis, hepatitis and cirrhosis.

Among the most distinguished chemicals are the organochlorine pesticides (OCPs), such as DDT and its major metabolites (DDD and DDE), aldrin, dieldrin, endrin and lindane. All of them have been individually related with breast cancer because they exert estrogenic effects on mammary epithelial cells. Nevertheless, human beings are exposed to mixes of theses organochlorine compounds in the environment, which biological effects can be significantly different from those exerted by any organochlorine taken individually.

In the same way, more studies have demonstrated the implication of these compounds in the obesity, which are specially adquired through the diet. It is proved that diet rich in animals' fat have more organochlorine contaminants, so not surprising that these diets are the greatest entry way for these contaminants into the organism.

Due to their effect in the increase of the fat and obesity these chemical compounds been called "Obesogens", and they are also toxics and they are linked with two disorders very related with the diabetes: the metabolic syndrome and the insulin resistance.

Different governments have developed many projects and agreements in order to control the exposure and emission of POPs. Most of these programs had ambitious goals that were not achieved. On May 23, 2001, more than 90 countries signed the Stockholm Convention on Persistent Organic Pollutants. The Convention entered into force on May 17, 2004 with a main goal: "Protecting human health and the environment of persistent organic pollutants, reducing or eliminating their emissions into the environment". The list of POPs that is currently considered is the following:

- **Annex A** (substances that must be eliminated): pesticides (aldrin, chlordane, dieldrin, endrin, heptachlor, CHC, mirex, hexachlorobenzene or toxaphene) and industrial chemicals (i.e. polychlorinated biphenyls (PCBs)).
- **Annex B** (restricted substances): pesticides (DDT) and other industrial chemicals included.
- **Annex C** (reduction of emissions of unintentionally produced substances): industrial sub-products included.

The following is a brief description of the pesticides (prevalent in the Canary population), as published in the official website of the Stockholm Convention (www.pops.int):

- **Aldrin**: a pesticide applied to soils to kill termites, grasshoppers, corn rootworm, and other insect pests, aldrin can also kill birds, fish, and humans. In humans, the fatal dose for an adult male is estimated to be about five grams. Humans are mostly exposed to aldrin through dairy products and animal meats. Studies in India indicate that the average daily intake of aldrin and its byproduct dieldrin is about 19 micrograms per person.

- DDT: it was widely used during World War II to protect soldiers and civilians from malaria, typhus, and other diseases spread by insects. DDT continued to be used to control disease, and it was sprayed on a variety of agricultural crops, especially cotton. DDT continues to be applied against mosquitoes in several countries to control malaria. Its stability, its persistence (as much as 50% can remain in the soil 10-15 years after application), and its widespread use have meant that DDT residues can be found everywhere; residual DDT has even been detected in the Arctic. The short-term acute effects of DDT on humans are limited, but long-term exposures have been associated with chronic health effects. DDT has been detected in breast milk, raising serious concerns about infant health.

- Dieldrin: used principally to control termites and textile pests, dieldrin has also been used to control insect-borne diseases and insects living in agricultural soils. The pesticide aldrin rapidly converts to dieldrin, so concentrations of dieldrin in the environment are higher than dieldrin use alone would indicate. Dieldrin is highly toxic to fish and other aquatic animals, particularly frogs, whose embryos can develop spinal deformities after exposure to low levels. Dieldrin residues have been found in air, water, soil, fish, birds, and mammals, including humans. Food represents the primary source of exposure to the general population, being the second most common pesticide detected in a US survey of pasteurized milk.

- Endrin: this insecticide is sprayed on the leaves of crops such as cotton and grains. It is also used to control mice, voles and other rodents.

- Endosulfan: it is a synthetic organochlorine compound commonly used as an agricultural insecticide. It has been sold from the mid 1950s but it is now banned in at least 60 countries with former uses replaced and its production is decreasing. It has the potential for long-range transport of endosulfan residues. Endosulfan is in the Arctic at increasing levels in water, air and biota.It is highly acutely toxic via oral, dermal and inhalation routes of exposure and it is associated to human poisoning. Contradictory opinions on the potential for endocrine disruption have been presented. a benchmark approach has been performed with lindane having similar toxicity than endosulfan.

- Heptachloro: primarily used to kill soil insects and termites, heptachlor has also been used more widely to kill cotton insects, grasshoppers, other crop

pests, and malaria- carrying mosquitoes. Laboratory tests have shown high doses of heptachlor to be fatal to mink, rats, and rabbits, with lower doses causing adverse behavioral changes and reduced reproductive success.Heptachlor is classified as a possible human carcinogen. Food is the major source of exposure for humans, and residues have been detected in the blood of cattle from the US and from Australia.

- Hexachlorobenzene: first introduced in 1945 to treat seeds, HCB kills fungi that affect food crops. It was widely used to control wheat bunt. It is also a byproduct of the manufacture of certain industrial chemicals and exists as an impurity in several pesticide formulations. Mothers passed HCB to their infants through the placenta and through breast milk. In high doses, HCB is lethal to some animals and, at lower levels, adversely affects their reproductive success. HCB has been found in food of all types. A study of Spanish meat found HCB present in all samples. In India, the estimated average daily intake of HCB is 0.13 micrograms per kilogram of body weight.

- Alpha and beta hexachlorocyclohexane: although the intentional use of alpha-HCH as an insecticide was phased out years ago, this chemical is still produced as unintentional by-product of lindane. It is highly persistent in water in colder regions and may bioaccumulate and biomagnify in biota and arctic food webs. This chemical is subject to long-range transport, is classified as potentially carcinogenic to humans and adversely affects wildlife and human health in contaminated regions.

- **Lindane:** it has been used as a broad-spectrum insecticide for seed and soil treatment, foliar applications, tree and wood treatment and against ectoparasites in veterinary and human applications. The production of lindane has decreased rapidly in the last years. Lindane is persistent, bioaccumulates easily in the food chain and bioconcentrates rapidly. There is evidence for long-range transport and toxic effects in laboratory animals and aquatic organisms.

- Mirex: this insecticide is used mainly to combat ants and termites. Direct exposure to mirex does not appear to cause injury to humans, but studies on laboratory animals have caused it to be classified as a possible human carcinogen. It is considered to be one of the most stable and persistent pesticides. The main route of human exposure to mirex is through food, particularly meat, fish, and wild game.

3. Effects of the insecticides on the risk of chronic diseases

There is a huge body of evidence on the relation between exposure to pesticides and elevated rate of chronic diseases such as different types of cancers, diabetes, neurodegenerative disorders like Parkinson, Alzheimer, cardiovascular disease and reproductive disorders among others. The common feature of chronic disorders is a disturbance in cellular homeostasis, which can be induced via pesticides' primary action like perturbation of ion channels, enzymes, receptors or mediated via pathways. (Sara Mostafalou et al., 2013).

3.1. Breast cancer

A wide variety of organic synthetic chemicals, such as the organochlorines pesticides (OCs) have been released into the environment in the last decades. The organochlorines are persistent and have toxic characteristics for the wild life and human beings.

Human exposure to OCs is essentially through the environment or food (D.J. Ecobichon et al., 1995; S.A. Lee et al., 2007). Over the years, human exposure to a wide variety of OCs has been evaluated throughout the world (S.A. Lee et al., 2007; O.P. Luzardo et al., 2006).

The results showed that the worldwide population had detectable residues of more than one organochlorine. On the other hand, it could be said that people are currently exposed to countless combinations of organochlorines. Therefore, the effects exerted by mixtures of OCs should be examined (N. Rajapakse et al., 2002; L.D. Boada et al., 2007).

Many OCs and some of their metabolites have been found to induce estrogen-like effects in exposed humans, so they may be involved in endocrine pathologies related to estrogenic effects, such as breast cancer (S.M. Snedeker et al., 2001; R.J. Gellert et al., 1972; M. López-Cervantes et al., 2004).

In European Union, BC is currently the most significant cause of death from malignancies in women, with around 90,000 deaths in 2013 (Maruthappu et al., 2015). Whereas BC mortality and incidence are lower in mainland Spain than in other European countries, there are alarming rates of mortality because of this type of cancer in the archipelago of the Canary Islands, specifically in Gran Canaria Island. (Cabanes et al., 2009; Lopez-Abente et al., 2004).

The etiology of BC is complex, with genetic, epigenetic and environmental factors contributing to the development of the disease. BC risk is significantly influenced by genetics, but over 70% of the women that are diagnosed have sporadic cancer or tumours not associated with inheritance of any major identified high risk genes. It is thought that the risk of BC can be modified by life- style and environment. Besides the genetic influence, the most established factors contributing to BC are related to cumulative exposure of the breast tissue to endogenous estrogens.

The well-known association between breast cancer and prolonged exposure to estrogens suggests that the environmental estrogens, such as OCs, could play an important role in the cellular and molecular changes that occur during breast carcinogenesis. These changes convert normal cells into latent tumour cells by altering the genetic material making easier their proliferation (M.C. Pike et al., 1993). Currently, genetic studies on the expression level of key genes in tumor development may offer additional information about the first steps of carcinogenesis.

Moreover, a number of organochlorine pesticides such as DDT, aldrin, dieldrin, endrin and their metabolites are considered xeno-estrogens (Colborn et al., 1993; Snedeker, 2001), which have been related with environmentally induced breast cancer (Jaga and Dharmani, 2003; Snedeker, 2001; Wolff et al., 2000; Zumbado et al., 2005).

3.2. Diabetes type 2

Insuline-like growth factor I (IGF-I) and dioxin-like polychlorobiphenyls (DL-PCBs) have been associated with the pathogenesis of several diseases like cancer, diabetes and growth disorders. Because it has been suggested that organohalogenated contaminants could influence IGF-I levels in adults.

IGF-I is an amino acid peptide synthesized in the liver in response to growth hormone (GH). Serum levels of IGF-I are influenced by gender, age, body mass index (BMI) and dietary lifestyle factors (Gómez JM et al., 2003). The influence of BMI on serum IGF-I levels is well known (Gomez JM et al., 2003; Succurro E et al., 2010; Crowe FL et al., 2011). The Canary Islands population has a rather high proportion of over-weighted and obese people (more than 60%), and

subjects with higher BMIs have higher levels of DL-PCBs. The results reinforce the importance of BMI on both, IGF-1 and DL-PCBs serum.

The median free circulating IGF-1 values did not differ significantly between genders, while evidencing the physiological profile of serum IGF-1 levels, which significantly decrease with increasing age. Subjects with non-detectable levels of DL-PCBs were younger than those showing detectable levels of DL-PCBs. As a consequence, subjects showing undetectable DL-PCBs serum levels had higher IGF-1 median values.

The serum levels of IGF-1 and DL-PCBs showed a trend of inverse association in the whole population. It has to be highlighted that this clear inverse relationship was evidenced in the youngest subgroup of women (18-45 years old), with BMI below the population mean (27 kg/m^2) showed an evident inverse association between DL-PCBs and IGF-I serum levels.

There is a crosslink between the IGF-system and other hormonal systems. IGF-I and insulin have complementary roles in the regulation of blood glucose (Lewitt et al., 1994). Animal and cell studies suggest that diverse PCBs and dioxins alter glucose and insulin metabolism. Moreover, PCB 156 and other DL-PCBs have been associated with higher risk of developing type 2 diabetes mellitus (Philibert A et al., 2009; Everett CJ et al., 2007; Lee DH et al., 2007; Uemura H et al., 2008). It can be speculated that the negative association between DL-PCBs and the IGF system activity might be related with the low IGF-I serum levels described for diabetic patients.

3.3. Obesity

In the last years, the obesity's prevalence, defined as BMI (Body Mass Index) >30kg/m^2, has reached alarming proportions with 30-80% of the adult European population being overweight (BMI >25kg/m^2) and 1/3 of the population with obesity. Earlier the increased of obesity was attributed to caloric intake and exercise reduction, but has appeared some evidences that environmental factors could be involved in the increased of obesity, called endocrine disrupting chemicals.

A study was carried out to evaluate the association between serum levels of POPs and the prevalence of obesity, in 98 obese and 47 lean individuals, all of

them adult women and men, over 18 years old, with an average age of 40 years old.

	Obese subjects	Lean subjects
Diabetes	12 of the 98	1 of the 46
BMI	40kg/m²	23kg/m²
Abdominal fat	Waist 122 cm WHR 0.99	Waist 79 cm WHR 0.81
Fat Mass %	49	24
TAT, VAT, SAT (cm²)	830, 189, 641	234, 60, 174
Glucose 120 (mg/dl)	135	88
Insulin 120 (mg/dl)	94	25

*Total adipose tissue (TAT), visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT).

There was studied the correlation between the waist, the percentage of fat mass and the serum levels of POPs, since the percentage of fat mass and the corporal distribution of this one differs in men and women.

In the total group, it was found a positive correlation between waist and βHCH, and between the FM% and βHCH. However, a negative correlation between the waist and PCB 153, 180, 170, sumPCB, and between FM% and the POP levels in the total group.

According to gender:

- Male subgroup: βHCH correlated significantly positive, and PCB 180 and 170 correlated negative with waist and FM%.
- Female subgroup: FM% correlated significantly inverse with the four PCBs and their sum, but not with the βHCH. The waist in the women correlated significantly with βHCH, and inversely with PCB 153, 180, 170 and sumPCB.

Analyzing the distribution of abdominal fat in more detail, it should be point out that the significant negative correlation between PCBs and TAT was almost exclusively due to SAT. In contrast, βHCH correlated significantly positive with

both, SAT and VAT. A significant correlation between fasting glucose and none of the POPs levels could not be established. There is a six-fold increase in the sum of PCB serum concentration between the youngest and the oldest in our population. In the group with individuals aged under 25 years old, the mean sum of PCB was 48,5 ng/g lipids, which increased to 315,8 ng/g lipids for individuals aged more than 50 years old.

In the Spanish population, (Agudo et al. 2009) have founded that the obese subjects (BMI > 30kg/m²) had concentrations in the serum PCB lower compared with the lean group. In contrast, the group with a BMI between 25 y 30 kg/m² shows higher concentrations. A possible explanation may be found in the dilution capabilities of the PCBs: because these lipophilic contaminants are preferably stored in the adipose tissue, a higher percentage of fat mass will lead to a fast and efficent storage, with lower serum levels as a consequence. Indeed, a significant negative correlation between serum levels of PCB 153, 170, 180 and sumPCB and percentage of fat mass were detected, which supports this hypothesis. A significant negative association between serum PCB levels and the amount of abdominal fat, particularly subcutaneous abdominal fat, seems to suggest that PCBs stored in subcutaneous fat are diluted less easily in the bloodstream. A confused factor may be the fact that the time to remove PCBs in obese invididuals can be different from that observed in lean inviduals. (Flesh-Janys et al. 1996) as a matter of fact it showed that the subjects with higher BMI had reduced the elimination of dioxins, although it hadn't been clearly demonstrated for PCBs.

βHCH, it was observed a positive relation with the BMI. (Jakszyn et al. 2009) also found a positive relation between BMI and serum βHCH concentration in the Spanish population, which wasn't confirmed by others. Because βHCH is the most hydrophilic substance among the ones which have been analized in this study, it is expected to be the most easily to detect in the serum. (Jung et al. 1997) established that the βHCH is eliminated more slowly in subjects with a high percentage of body fat. A significantly positive correlation was observed between FM% and βHCH in this study. In the current population, no difference was detected in BMI according serum levels of pp-DDE. Other authors found a significant difference in BMI, which could be due to the more advanced age of participants (Hue et al. 2007). Recently, Karmaus et al. 2009 Reported a

possible positive relationship between maternal levels of DDE and BMI in the chilfren of adult women.

In this study wasn't collected data from from other obesogenic factors, such as sedentary lifestyle, diet, history of obesity in the family, or obesogenic medication. Moreover, the design of this study is a representative section. Therefore, a causal relationship between POPs in the serum and obesity is difficult to determine.

The data from this study confirm the influence of POPs on glucose metabolism, with a statiscally significant higher insulin resistance (as measured by HOMAIR) with higher levels or serum βHCH. PCB 180, 170 and sumPCB are negatively correlated with HOMAIR. Higher levels of PCB in the serum were found in the group with a lower BMI, indicating that the endocrine disrupting effect of PCBs could involve a different pathway thatn the classic insulin resistence inducing obesity effects. No statiscally difference was found in fasting glucose or in HOMAIR for pp-DDE. In the study sample, 13 participants were diagnosed with diabetes, one of them was a participant of the lean group. It couldn't be detected a difference in serum POP levels between obese participants with and without diabetes. Because the small number of diabetics, it was estimated that the group was too small to detect that difference.

The study data confirm the lifetime accumulation of POP in the human body. Previous studies have clearly demonstrated the ability of POPs to accumulate in the human body throughout life. This positive relationship between age and serum levels of POPs was previously observed in lean, obese and severely obese patients.

Exposure to POPs has declined in recent decades, due to the cessation of production and/or use in many products investigated. Together with the short duration of exposure, this can also contribute to the significant difference in the burden of endocrine disrupters in young adults versus obese and lean subjects.

There are some limitations or deficiencies associated with this study. Data were collected over a long time of period, covering almost ten years. The environmental PCB burden is known to have decreased substantially every decade. It would be really interesting to investigate the relationship between POPs concentration in serum and fat. Taking into account the information of the literature (Mckinney et al. 1994; Arsenescu V. et al. 2008; Staels B. et al. 2007;

Mullerova D. et al. 2008), in particular the date on the influence via peroxisome proliferator-activated receptor ɣ, an effect on the signalling of adipose cells by POPs seems indeed possible. In the present population, however, no samples were collected. Therefore, it is impossible to make an assumption on the concentration of POPs in fat and its effect on the energetic homeostasis of the adipose cell.

In conclusion, a positive relation was shown between the less lipophilic disruptor βHCH and BMI, while a negative relation was found between the serum level of the more lipophilic PCBs and BMI. It couldn't be found a statiscally significant relation between serum levels of pp-DDE and BMI. This study is in concordance with other previous reports describing a positive relation between βHCH levels and insulin resistance, while additionally a negative relationship was found between serum levels of PCBs and insulin resistance. Combined, these results suggest that the diabetogenic effect of exposure to low doses of POPs may be more complex than a simple obesogenic effect. The exact mechanisms of influence of POPs on body energy homeostasis remain largely unkown.

4. Cellular response: cytotoxicity and proliferative effect

We studied the effect of some organochlorines, individually and in combination, which had been previously detected in the human serum of the Canarian population, in primary mammary epithelial cells (HMEC) at concentrations close to those found in this population. The organochlorines studied were: aldrin, dieldrin, o,p'-DDE, p,p'-DDE, p,p'-DDD, o,p'-DDT, p,p'-DDT.

Previous studies have shown that the exposure to DMSO (Dimetilsulfoxide), which is used as an organic dissolvent, could induce ultrastructural changes in cells. To clarify the possible cytotoxic and proliferative effects exerted by DMSO, the HMEC (human mammary epithelial cells) were exposed to DMSO at the same concentration used throughout this work, obtaining the concentrations set forth in **Table 1**. The results showed that there were no differences between the proportion or the proliferative ratio between the control and cells treated with DMSO (PF Valerón et al., 2009).

Table 1. Final concentration (1x) of OC compounds added to the clonetics mammary epithelial cell basal medium (MEGM). These concentrations were calculated to correspond to OC serum concentrations found in people living in the Canary Islands.

Compound	Concentration (μM)
Aldrin	0.2
Dieldrin	0.035
o,p′-DDE	1.43
p,p′-DDE	1.08
p,p′-DDD	1.33
o,p′-DDT	0.31
p,p′-DDT	0.46

4.1. Effect at individual level

To test the possibility that the effect exerted by individual organochlorines on human mammary epithelial cells might be different from that exerted by the combination of these, HMEC were individually exposed to different increasing concentrations of these organochlorines. There were no differences in cell viability between control and HMEC exposed to low concentrations (x1 and x100) for any pesticide. However, the survival curves obtained were very different at high concentrations (x500 and x1000). Therefore, p,p'-DDD seems to be the most cytotoxic for HMEC, with an evident reduction of cell growth at 24-48 hours of treatment in x500 and x1000. Similarly, o,p'-DDE exerted a remarkable cytotoxic effect at high concentrations. In contrast, p,p'-DDT and the major metabolite of DDT, p,p'-DDE, didn't appear to be as cytotoxic as high concentrations as p,p'-DDD and o,p'-DDE. On the other hand, comparing OCs non-DDTs (aldrin and dieldrin) does not appear to affect cell viability at any concentration (PF Valerón et al., 2009).

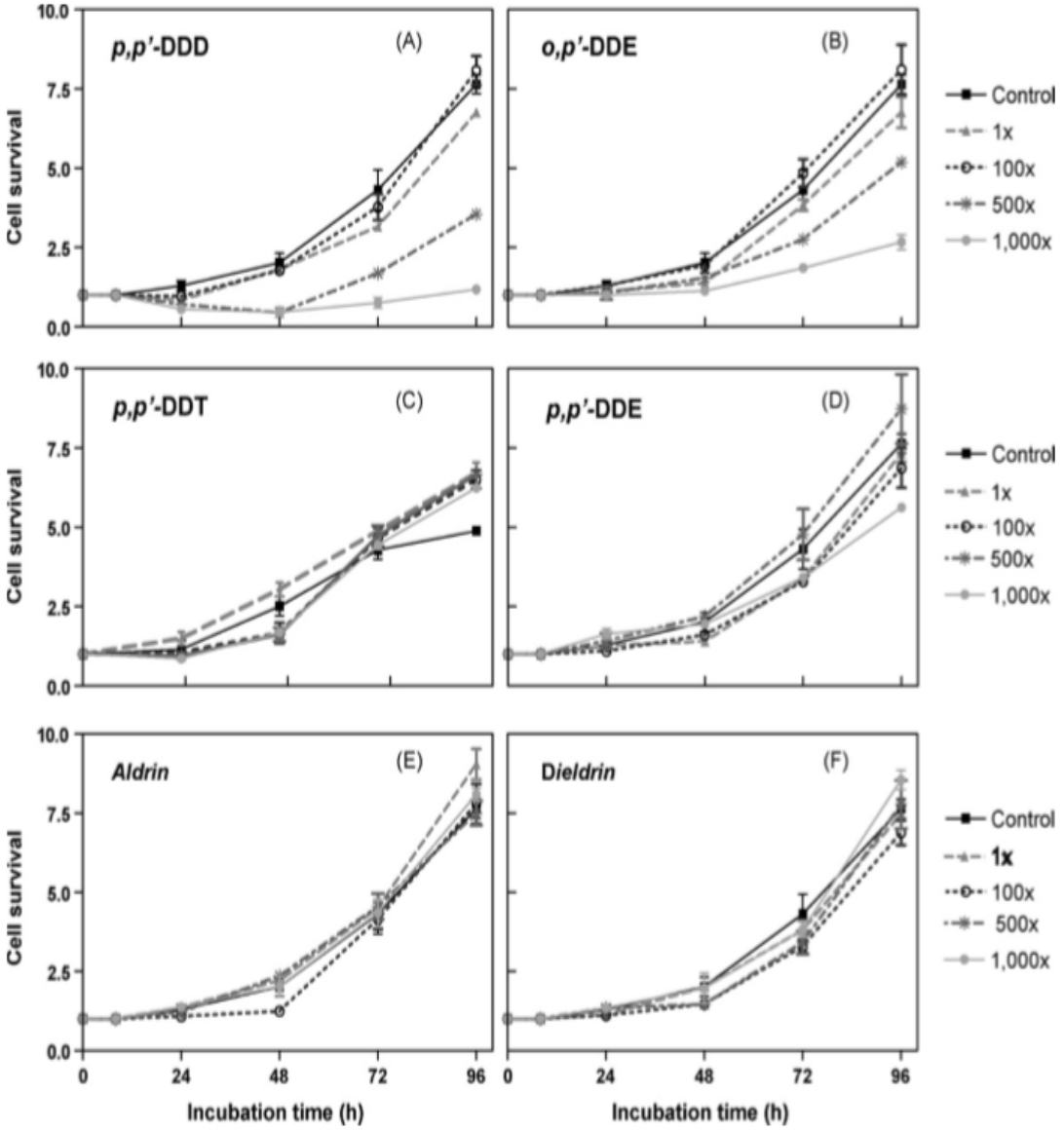

Figure 1. Dose- and time-dependent cytotoxic effects of OCs individually on the viability of HMEC. HMEC were exposed to increasing concentrations of o,p'-DDD (A), o,p'-DDE (B), p,p'-DDT (C), p,p'- DDE (D), aldrin (E), and dieldrin (F). Each experiment was performed at least theree times. In the figure, each data point represents the mean ± SD of three replicates in one representative experiment. See Table 1 for details of concentrations.

4.2 Effect in combination

The first was to analyzed the effect of the most prevalent OCs-mix in the population of the Canary Islands (p,p'-DDD + p,p'-DDE + o,p'-DDE + aldrin + dieldrin) (M. Zumbado et al. 2005; O.P. Luzardo et al. 2006). It was observed that the OCs-mix exert a cytotoxic effect at the highest concentrations (x500 and x1000), whereas at concentrations of 1x and 100x the cell viability was similar to the control group, as shown in the graph (Figure 2).

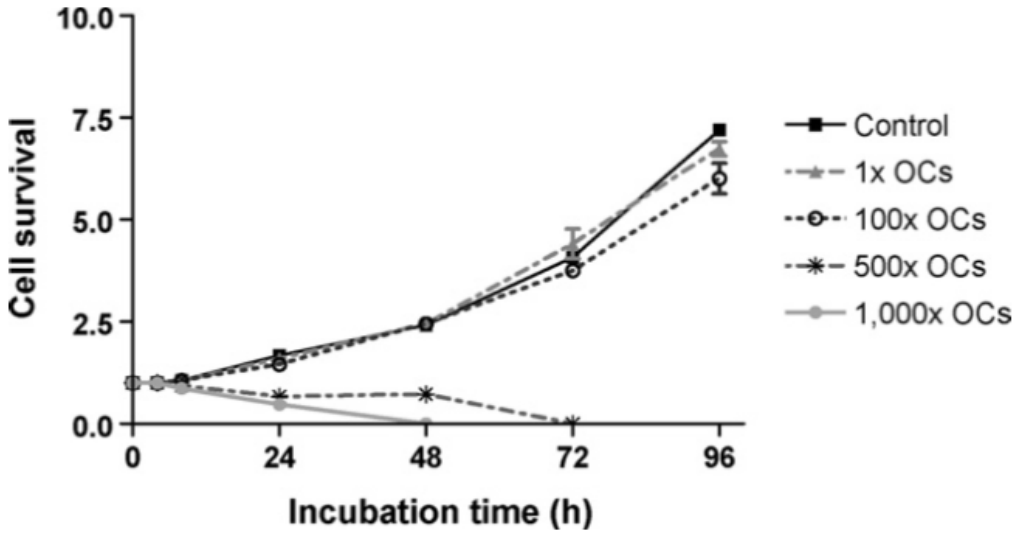

Figure 2. Dose- and time-dependent cytotoxic effects of a mixture of OCs (p,p'-DDD + p,p'-DDE + o,p'-DDE + aldrin + dieldrin) on the viability of HMEC. HMEC were exposed to an OCs mixture present in the Canary Islands' population at 1x, 100x, 500x, and 1000x concentrations, for 0, 8, 24, 48, 72, and 96 h. Each experiment was performed at least three times. In the figure, each data point represents the mean ± SD of three replicates in a single representative experiment. See Table 1 for details of concentrations.

The OCs-DDTs-metabolites-mixture (p,p'-DDD + p,p'-DDE + o,p'-DDE) and OCs-cyclodienes-mixture (aldrin + dieldrin), induced a loss in cell viability at the lowest concentration evaluated (1x). In contrast, the OCs-DDTs-mixture (p,p'-DDT + p,p'-DDE + p,p'-DDD) didn't exert any cytotoxic effect on the proliferative capacity of HMEC at the lowest concentration (x1), but at concentrations of 1x and 100x appeared to exert a proliferative effect on HMEC.

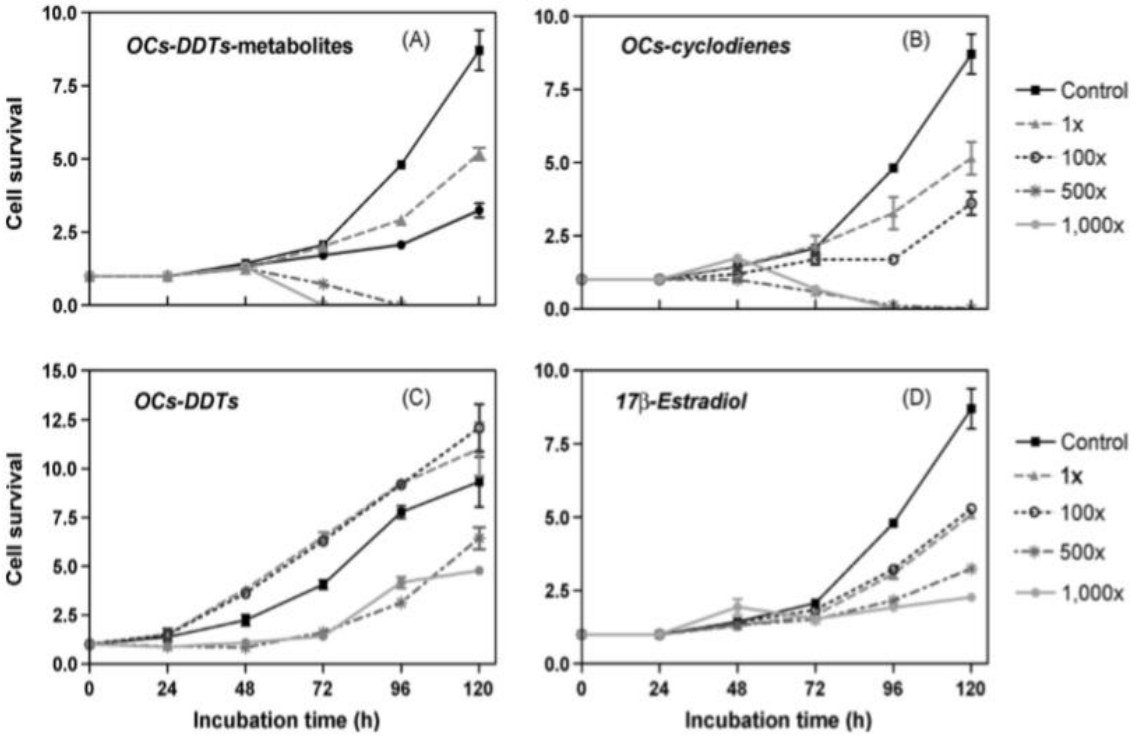

Figure 3. Dose- and time-dependent cytotoxic and/or proliferative effects exerted by others OCs mixture on HMEC. HMEC were exposed to three different OCs mixtures described in the Canary Islands' population at 1×, 100×, 500×, and 1000× concentrations, for 0, 8, 24, 48, 72, and 96 h: (A) OCs-DDTs-metabolites mixture (p,p'-DDD plus p,p'-DDEplus o,p'-DDE). (B) OCs-cyclodienes mixture (aldrin plus dieldrin). (C) OCs-DDTs mixture (p,p'-DDT plus p,p'-DDE plus p,p'-DDD). (D) The effect exerted by 17-estradiol exposure on HMEC viability at 1× concentration (0.18nM), 100× concentration (0.18 µM), 500× concentration (0.9 µM), and 1000× concentration (1.8 µM). Each experiment was performed at least three times. In the figure, each data point represents the mean ± SD of three replicates in a single representative experiment. See Table 1 for details of concentrations.

However, the result was different at greater concentrations than 100x, where the three OCs-mixtures evaluated seemd to exert a clear cytotoxic effect after 48 hours of treatment. Nevertheless, the cytotoxic effect exerted by OCs-DDT-mixture at the highest concentrations (500x and 1000x) was lower than the effects exerted by OCs-DDT-metabolites-mixture or OCs-cyclodienes-mixture.

Taking into account that the pesticides evaluated are considered estrogenic chemicals, it was decided to compare their effect with that produced by 17β-estradiol. The results indicated that estrogen exerted a detrimental effect on

HMEC at all concentrations, although it must be pointed out that estradiol was not able to completely prevent cell proliferation at the highest concentrations (500x and 1000x), being similar to the effect caused by OCs-DDTs mixture, whereas at the same concentrations (500x and 1000x) OCs-DDTs-metabolites or OCs-cyclodienes exerted a dramatic detrimental effect on HMEC.

4.3 The most prevalent OCPs in the Canary population

The level of contamination by OCPs showed by the general population of the Canary Islands has been extensively studied. Despite the fact that most OCPs pesticides were banned in Spain in the late 1970's, our results have already shown that the people living in the Canary Islands presented a relatively high degree of contamination by OCPs (including DDT and its derivatives, aldrin dieldrin, endrin, and lindane). Furthermore, such results seemed to indicate the existence of chronic exposure to OCPs that persisted in the late 1990's [Zumbado M. et al., 2005; Longnecker MP et al., 1997].

Specifically, exposure to organohalogenated contaminants has been linked to breast cancer (BC) etiology. Among environmental contaminants linked to BC, most studies have focused in organochlorine pesticides (OCPs) due to their endocrine-disrupting properties (estrogenic or antian-drogenic effects) observed both *in vivo* and *in vitro* studies and to their biochemical features (high lipophilic and resistant to biotransformation) that result in their accumulation in body fat.

It has to highlighted that most studies focused in the study of only one compound, and they did not take into account that human beings are exposed simultaneously to multiple OCPs, and that the biological effects exerted by the mixture of OCPs vary considerably from those exerted by any OCP individuallly.

Bearing in mind the alarming rates of mortality by BC among women from the Canary Islands (Spain) and more specifically in women from Gran Canaria Island, the aim of our study was to compare the profile of mixtures of OCPs detected in women diagnosed with BC living in Gran Canaria Island with that detected in healthy women selected among participants from our previous population-based studies, and secondarily to evaluate whether OCPs´ exposure could be considered as a risk factor for BC in such population.

Thus, adult healthy women from Gran Canaria Island (n = 103) were selected among the representative population-sample obtained in the Canary

Islands Nutrition Survey (ENCA 1998) previously evaluated regarding their OCPs serum levels. On the other hand, women with a histological confirmed first diagnosis of breast cancer (n = 121) were recruited between April 1999 and June 2001 from the two University Hospitals of Gran Canaria Island. Cases and controls were selected according to place of residence (Gran Canaria Island). Our result showed that most prevalent mixture of organochlorines among healthy women was the combination of lindane and endrin, and this mixture was not detected in any affected women. Breast cancer patients presented more frequently a combination of aldrin, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), and this mixture was not found in any healthy woman. After adjusting for covariables, the risk of breast cancer was moderately associated with DDD (OR = 1.008, confidence interval 95% 1.001-1.015, p = 0.024).

Table 2. Median levels of organochlorine compounds (ng/g lipid) previously described in the serum of the studied women (Boada et al., 2012) and their corresponding final concentrations assayed.

	Healthy women			Breast cancer patients			
Compound	pM	Median (p5th–p95th)	1× (nM)	100× (μM)	Median (p5th–p95th)	1× (nM)	100× (μM)
Aldrin	364.90	0.0 (0.0–100.1)	2.8	0.28	75.8 (0.0–116.4)	3.2	0.32
Dieldrin	380.90	0.0 (0.0–46.2)	1.2	0.12	0.0 (0.0–72.0)	1.9	0.19
Endrin	380.90	29.1 (0.0–1279.0)	33.6	3.36	-	0	0
Lindane	290.80	0.0 (0.0–111.4)	3.8	0.38	0.0 (0.0–220.0)	7.6	0.76
p,p'-DDE	318.04	167.7 (45.0–706.0)	22	2.2	300.1 (106.1–653.3)	20	2.0
p,p'-DDD	320.04	0.0 (0.0–129.2)	4	0.4	551.1 (0.0–1108.2)	34.6	3.46
p,p'-DDT	354.50	217.0 (0.0–1428.6)	40	4.0	153.0 (0.0–327.9)	9.2	0.92

Abbreviations: p5th–p95th, percentiles 5 and 95 of the distribution.

This study indicates that healthy women show a very different profile of organochlorine pesticide mixtures than breast cancer patients, suggesting that organochlorine pesticide mixtures could play a relevant role in breast cancer risk. In consequence, we assessed the cytotoxic response induced by these two OC mixtures in primary human breast epithelial cells (HMEC). The two OC mixtures showed similar cytotoxic effects on HMEC. At concentrations of 50X and 100X, survival of cells exposed to the H-mixture was 46.2 and 15.0%, respectively, referred to the control (ANOVA test, p = 0.024 and p = 0.009,

respectively). For the BC-mixture, cell survival at 50X and 100X was 39.9 and 19.1%, respectively, referred to the control (ANOVA test, p = 0.017 and p = 0.010, respectively). We did not observe statistically significant differences between concentrations at 1X and 10X compared with the control. Nevertheless, it has to be highlighted that the present results are different to those previously reported for individual OCs on HMEC (Valeron et al., 2009).

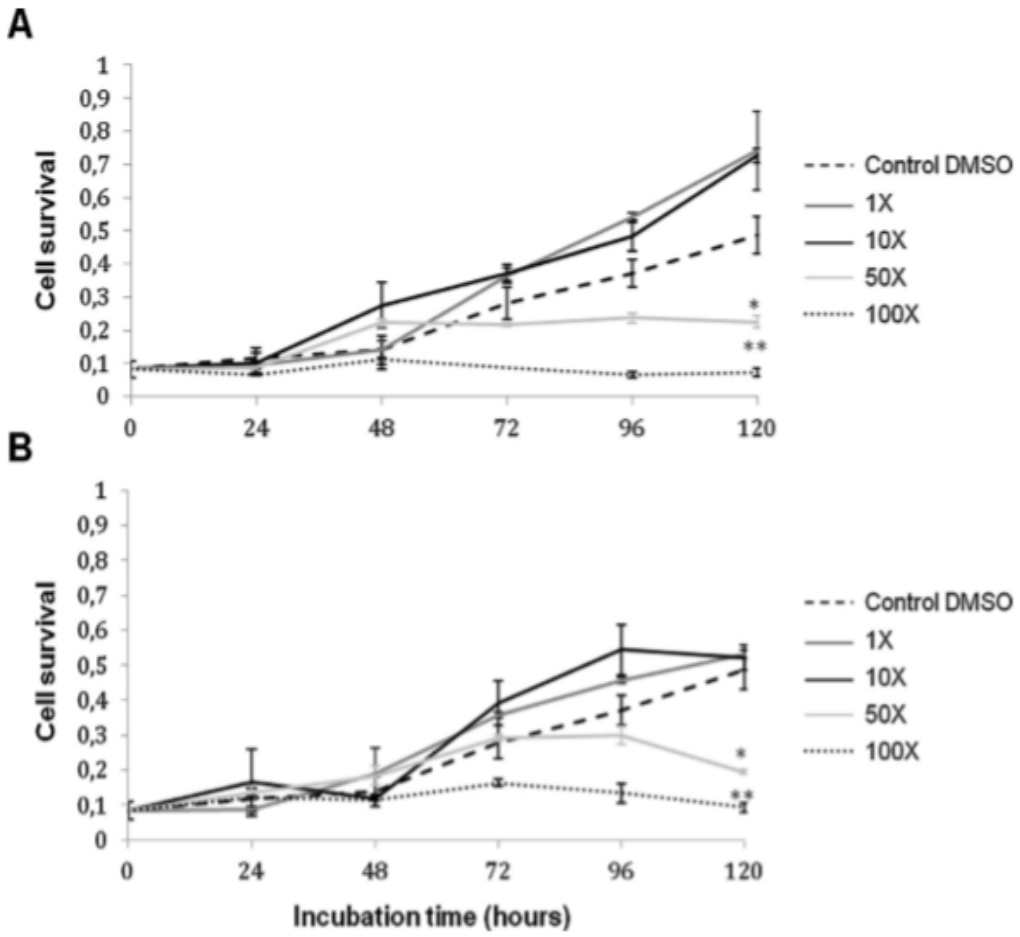

Figure 4. Dose and time-dependent proliferative effects of OCs mixtures on the viability of HMEC. HMEC were exposed to increasing concentrations of H-mixture (A), and BC-mixture (B). Each experiment was performed at least three times. In the figure, each data point represents the mean ± SD of three replicates in one representative experiment. *p < 0.05; **p < 0.01.

5. Endocrine disruption

Before evaluating the effect on both mixtures (H and BC) of the estrogen/androgen balance, we evaluated the cytotoxic/proliferative response in MDA-MB-231 cells, which is a cell line in which the estrogen receptor (ER) is deficient. This experiment allowed us to evaluate the effect of the mixtures, irrespective of their estrogenic action potential. We evaluated the proliferative effect of the two OC mixtures compared to cells treated with 17β-estradiol (control) in the cell culture medium over a 120 hours period.

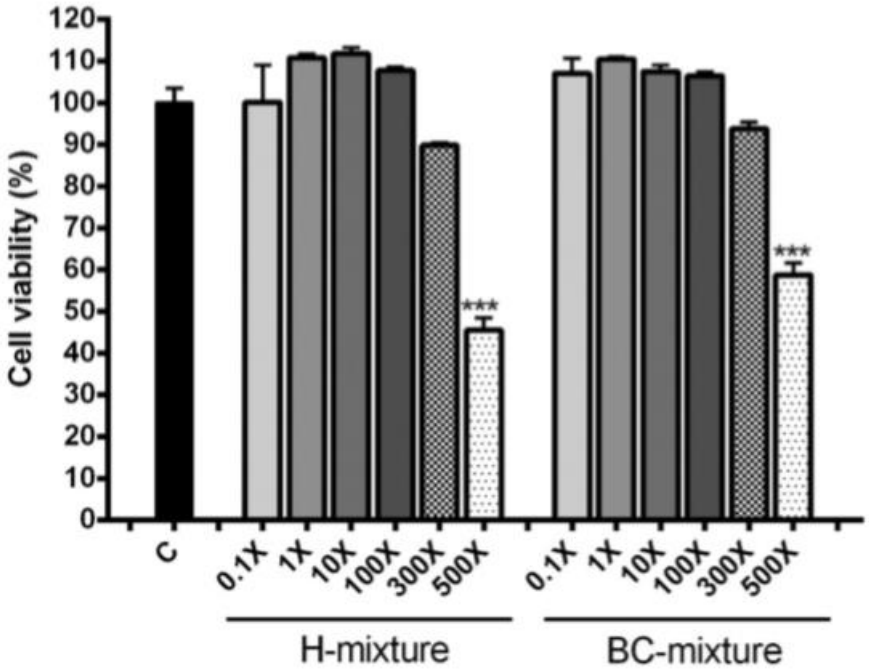

Figure 5. The cell growth inhibition ofoestrogen-receptor negative (MDA-MB-231) human mammary carcinoma cells was tested after 120 h of incubation. Cells were treated with 17β-Oestradiol (Control, [E2] = 100 pM) or each OC mixture in phenol red-free DMEM supplemented 10% charcoal-dextran (CD) foetal bovine serum (FBS). After 5 days, the number of viablecells wasmeasuredusing theSRBassay (mean ± SD, n = 3).

Both mixtures showed a similar viability (Fig 5). Thus, for H-mixture the proliferative responses at 300X and 500X were of 89,75% and 45,49% respectively compared with the control. obtained a proliferative response % at concentrations of 300x and 500x respectively. BC-mixture elicited a proliferative response of 93,76% and 58,64% at concentrations of 300x and 500x respectively. These differences were only significant in the 500x concentration

case for both mixtures, suggesting that the evaluated mixtures are cytotoxic at concentrations of 500-fold or higher.

Based on this result, the subsequent experiments aimed to evaluate the potential endocrine disrupting capacity endocrine disrupting potencial of these two mixtures were performed at non-cytotoxic concentrations (ranging between 0,1x and 300x).

5.1. Estrogenicity of organochlorine mixtures

To determine the estrogen-like effects of OC mixtures, the ability of these mixtures to promote dose-dependent growth of MCF-7 BUS cells was determined. We evaluated by the E-Screen the proliferation of these cells in abscence of estrogen (negative control), in presence of 17β-estradiol (positive control), and in presence of a range of concentrations of H and BC-mixtures.

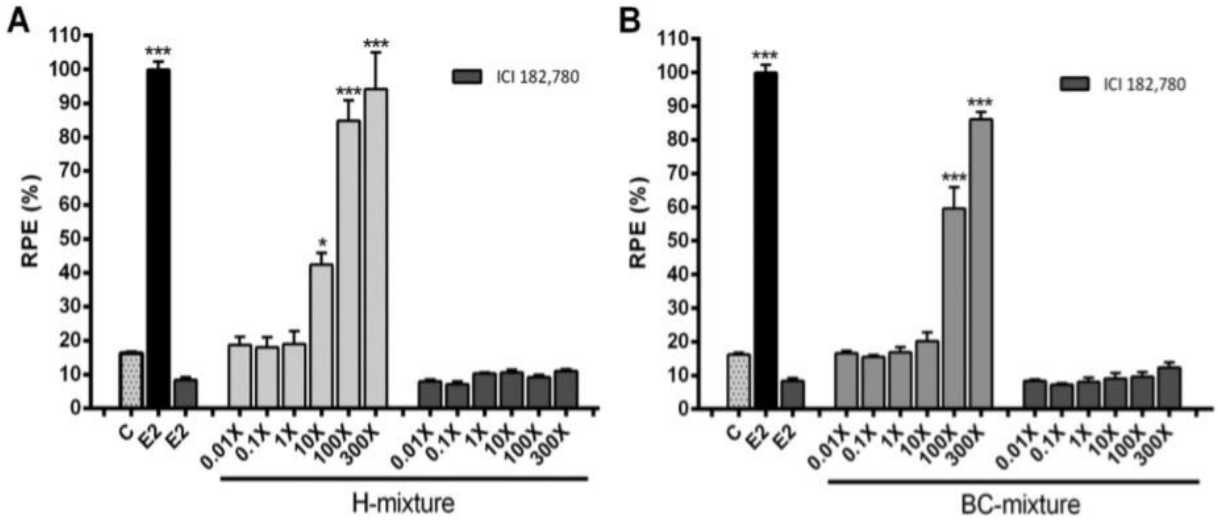

Figure 6. Relative proliferative effect percentage (RPE%) induced by the different concentrations of the mixtures tested with and without co-incubation with 1 μM ICI 182,780 in MCF-7BUS. A) Increasing concentrations of the H-mixture, and B) Increasing concentrations of the BC-mixture. Cell proliferation was assessed after 5 days of treatment. Each bar represents the mean ± SE of three independent experiments. *pb0.05 ,**pb0.01 ,***pb0.001, versus the negative control (untreatedcells). Differences were assessed by ananalysis of variance followed byone-tailed Bonferroni post hoc test.

The estrogen receptor (ER) is expressed in MCF-7 BUS cells, making this cell line appropiate to determine the estrogenic effects of the chemicals. As shown in Figure 6, in both mixtures the highest *relative proliferative effect percentage* (RPEs, %) were corresponded at 300x (94,2% H-mix, 86,2% BC-mix) compared to the maximum response E_2 (RPE = 100%). Despite, at two non-cytotoxic concentrations (10X and 100X), the H-mixture exerted a stronger proliferative effect than the BC-mixture. Thus, the RPE values obtained for the H-mixture were 42,4% and 84,9% at 10x and 100x respectively, and in the BC-mixture were 20,2% and 59,6% at 10x and 100x respectively. The results suggest that the H-mix may be more estrogenic than the BC-mix.

Taking into account these results, MCF-7 BUS cells were treated with the anti-estrogen ICI 182.780 to determine if the observed cell proliferation depends on the activation of the estrogen receptor (ER). This anti-estrogen is a well-known pure ER antagonist that can bind to ERα or ERβ to affect the dimerization of this receptor and accelerate its degradation, thus leading to the supression of estrogen-dependent gen (Nuttall et al., 2001; Robertson, 2001).

As expected, the results showed that the maximum proliferative effect of E2 (100%) was drastically reduced when MCF-7 BUS cells were treated with 1µm of ICI 182.780 (RPE = 8,4%). Similarly, the induced cell growth was completely abolished by co-treatment with OC mixtures and the anti-oestrogen ICI 182,780. These findings indicated that both OC mixtures evaluated exhibit oestrogenic activity related to ER activation.

5.2. Androgenicity of organochlorine mixtures

The signalling pathways of estrogen and androgen are well known to exert opposite influence on mammary cells. However, it has been reported that some pesticides may exert anti-androgenic effects (Wilson et al., 2008), the possibility that OC mixtures act as agonists or antagonists of the AR has not been explored in depth. Therefore evaluating the possible androgenic/anti-androgenic action of these two OC-mixtures by the A-Screen, which measures the androgen-dependent inhibition in the MCF7-AR1 cell line. These cells overexpress (AR), and contains five times more AR than wild-type MCF-7 cells.

Thus, these cells:

- retain their ability to proliferate when they are exposed to estrogens.
- do not proliferate when they are treated with androgens.
- are prevented from proliferating in response to oestrogens by natural and synthetic androgens
- proliferate in response to androgen antagonists in the presence of oestrogens and androgens because the anti-androgens antagonise the androgen-induced proliferative shut off in MCF7-AR1 cells (Szelei et al., 1997).

The A-Screen assay showed that both mixtures (H and BC-mixture) induced a dose-dependent increase in MCF7-AR1 proliferation in presence of 100pM E_2 or 17β-estradiol and 100 pM de DHT (Dihydrotestosterone). Figure 7A.

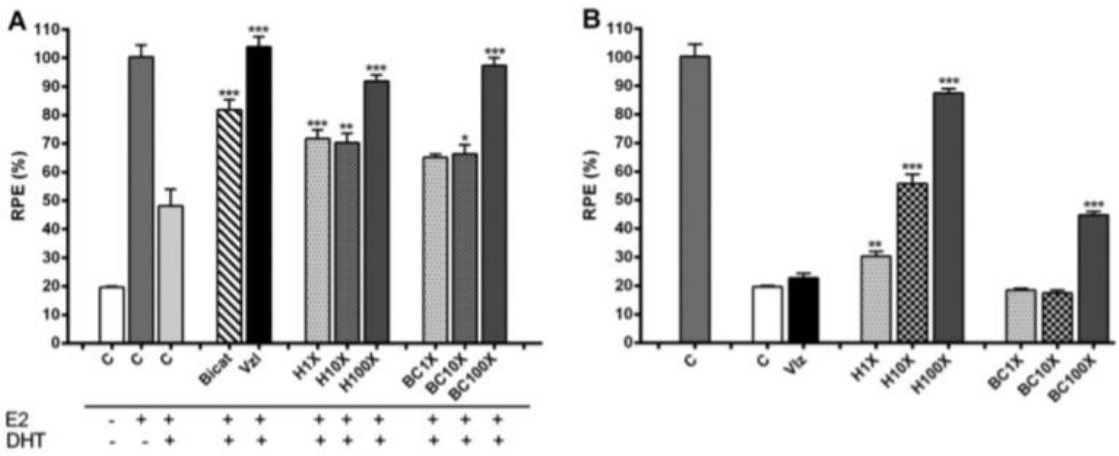

Figure 7. .Relative proliferative effect percentage (RPE%)induced by the different concentrations of the H- and BC-mixtures in MCF7-AR1. Cell proliferation was assessed after 5 days of treatment. Each bar represents the mean ± SE of three independent experiments. A) Proliferative effects of the mixtures co-incubated with 17β-Oestradiol (E2; 100pM) and dihydrotestosterone (DHT;100pM). B) Proliferative effects of the mixtures versus the negative control (untreated cells). Final concentration of vinclozolin = 10μM.*p b 0.05,**p b 0.01,***p b 0.001, Differences were assessed byananalysisof variancefollowed by a one-tailed Bonferronipost hoc test.

As expected, DHT inhibited the cell proliferation which was induced by E_2. Both OC-mixtures increased their cell proliferation to 100x concentration compared to the E2 + DHT treatment, suggesting that the mixtures may exert an anti-androgenic effect on MCF7-AR1 cells. To clarify whether the reversal of the proliferation inhibited by DHT can be due to de antagonistic action of the androgen receptor (AR, which allows to exhibit androgenic and anti-androgenic actions) or due to estrogenic activation, we evaluated the effect of MCF7-AR1 cells on OC-mixtures in absence of E2 y DHT. Obtaining that the H-mixture showed a significant proliferative response at all concentrations evaluated, Figure 7B, in comparison with BC-mixture that only showed a significant proliferative response at the highest concentration (100x), whereas at lower concentrations showed an effect similar to that found in the MCF7-AR1 cells treated with the anti-androgenic drug Vinclozolin.

The results seems to indicate that both OC-mixtures exert a proliferative effect on mammary cancer cells, but taking into account that in BC-mixture it was not induced the proliferation of MCF7-AR1 cells in abscence of sex hormones unlike in H-mixture, it suggests that in BC-mixture there is a greater affinity for the androgen receptor (AR) than for the estrogen receptor (ER). The fact that p,p'-DDD is a major constituent of BC-mixture could explain the anti-androgenic effect, because this metabolite of DDT shows a greater affinity for AR than for the ER (Maness et al., 1998). Consequently, the additional anti-androgenic effect of BC-mixture might be due to the high levels of p,p'-DDD in this mixture.

6. Transcriptional regulation of protein kinases

6.1. Pesticides and/or combinations

In order to expand the scope of this study, it was evaluated the expression of 94 kinase and non-kinase genes, paying special attention to TGF-beta superfamily receptors (transforming growth factor beta), ACVRL-1 (activin A receptor type II), and ACVR1C (activin A receptor type 1C). There is increasing evidence that activin and the activin receptor in the normal human breast and in breast cancer cell lines are actively involved in mammary cell growth and morphogenesis (J.E. Burdette et al., 2005). In addition to the members of

PDGFRA (platelet-derived growth factors receptors alpha) and PDGFRB (beta), and its glycoprotein tyrosine kinase receptors on structurally related surface (KIT). It has been shown that a high level of c-kit expression occurs frequently in invasive breast cancer, and its expression is associated with estrogen receptor (A. Eroglu et al., 2007). Moreover, it was evaluated the regulation of the members of erbB/HER type I tyrosine kinase receptor family (ERBB3) and anaplastic lymphoma kinase ki-1 (ALK-1). The overexpression of the ErbB family of tyrosine kinase receptors is viewed as an important sign in the development of many breast tumours. It occurred also the 30-fold increase in the expression of anaplastic lymphoma kinase (ALK), which is expressed in different subtypes of human breast cancers.

As shown in Figure 8, using 18S gen expression as housekeeping gen. Exposure to OCs-mixture (p,p'-DDD + p,p'-DDE + o,p'-DDE + aldrin + dieldrin) at a serum concentrationn of 100x for five days, markedly increased the expression of a number of protein kinases. Therefore, the increase in the levels of mRNA in ACVRL1, ALK-1 and KIT in HMEC were evident, Figure 8A. Besides, mRNA levels in ERRB3 and ACVR1C were higher in cells treated with OCs-mixture than in the control or in cells treated with estradiol, Figure 8B.

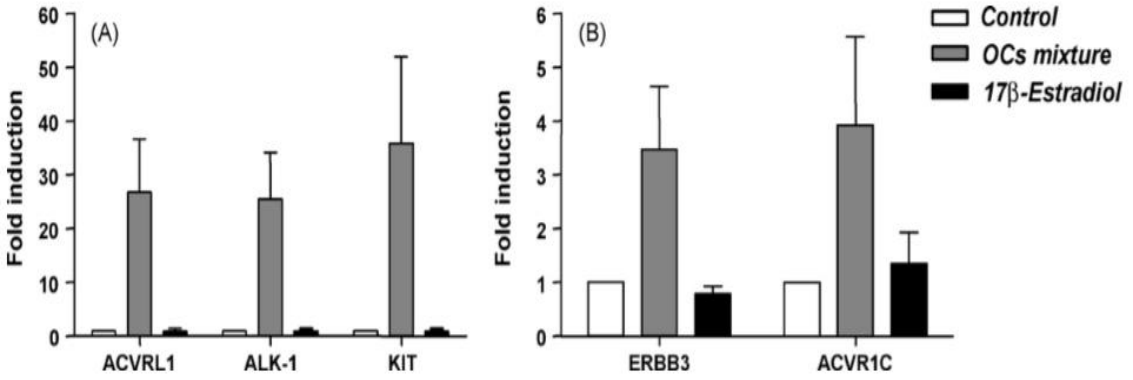

Figure 8. Profile of transcriptional induction of kinases and non-kinases genes in HMEC exposed to OCs-mixture (p,p'-DDD plus p,p'-DDE plus o,p'-DDE plus aldrin plus dieldrin) or 17β-estradiol for 5 days to an estimation of human fatty tissue concentration (100-fold higher than serum concentratio ndescribed in human beings).HMEC cultures were exposed to DMSO (control), estimation to fatty tissue concentration (100-fold higher than serum concentration) of OCs mixture and 17β-estradiol for 5 days. (A) Effects exerted on ACVRL1, ALK-1, and KIT mRNA levels. (B) Effects exerted on ERBB3 and ACVR1C. 18S expression was performed for each sample as housekeeping genes.The results shown represent the fold changes of each sample relative to the reference sample (mean±SD of three independent experiments).

As well as, the expression of PDG gene receptors was also studied. Bearing in mind that the PDGF pathway is essential for tumour progression, it was evaluated the expression of PDGFRA and PDGFRB in HEMC exposed to OCs-mixture. Autocrine PDGFR signalling promotes mammary cancer metastasis (M.Jechlinger et al., 2006), and the PDGFRA expressed in invasive breast carcinomas is associated with biological aggresiveness (A. Carvalho et al., 2005). Interestingly, estradiol-treated HMEC only induces the expression of PDGFRA, eight more times, but not of PDGFRB, which was only raised by exposure of the OCs-mixture, Figure 9.

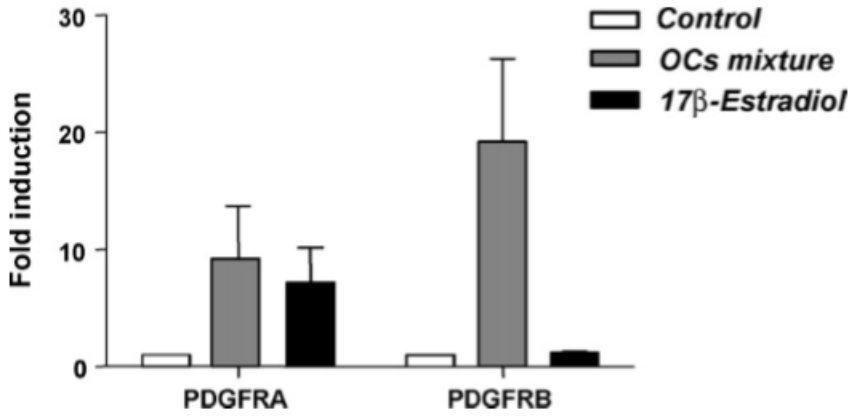

Figure 9. Profile of transcriptional induction of PDGFRA and PDGFRB genes in HMEC exposed to OCs-mixture (p,p'-DDD plus p,p'-DDE plus o,p'-DDE plus aldrin plus dieldrin) or 17β-estradiol for 5 days to an estimation of human fatty tissue concentration (100-fold higher than serum concentration described in human beings). 18S expression was performed for each sample as housekeeping genes. The results shown represent the fold changes of each sample relative to the reference sample (mean±SD of three independent experiments).

6.2 Organochlorides mixtures in healthy and cancer woman

Tyrosine kinase are regulatory proteins that play an important role in cell growth and differentation of normal cells. TKs represent the major class of proto-oncogenes, and many of them have been induced or inhibited in breast cancer, being the candidates to play an important role in carcinogenesis (Meric et al., 2002). It was studied the gene expression profile of 94 protein kinases and non-kinases after the exposition of HMEC to both OC-mixtures, including the genes which were regulated by the OC-mixtures after the normalization by the 18S gen, determining a total of 80 genes. The 18S gen or 18S ribosomal

RNA gen is widely used for qRT-PCR due to its invariant expression through tissues, cells and experimental treatments.

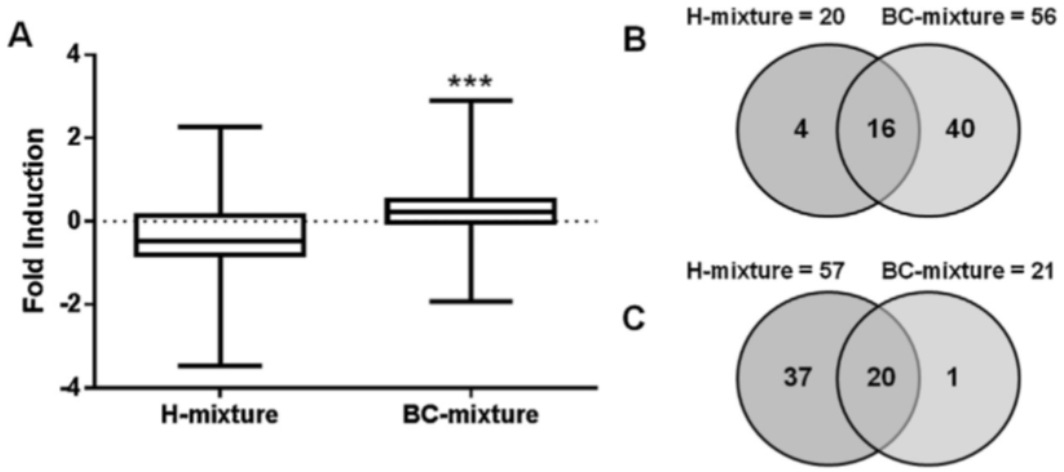

Figure 10. Effects of healthy mixture (H-mixture) and breast cancer mixture (BC-mixture) on gene expression profiling of primary human mammary epithelial cells. A: Box plot showing a statistical evaluation of the differences in the mean expression changes induced by H-mixture and BC-mixture. The lines inside the boxes represent the medians, the boxes cover the 25–75th percentiles, and the minimal and maximal values are shown by the ends of the bars. Median value for H-mixture = -0.47, median value for BCmixture = 0.24. ***p < 0.0001. B: Venn diagram showing the number of genes up-regulated by H-mixture and BC-mixture. The overlapping area shows genes for which expression was altered by both pesticide mixtures. Value of universal (U), defined as the total number of genes represented in the diagram was 76 genes. C: Venn diagram showing the number of genes down-regulated by H-mixture and BC-mixture. The overlapping area shows genes for which expression was altered by both pesticide mixtures.U = 78 genes.

In Figure 10 it is shown how the pattern of gene expression was different in HMECs exposed to H/mixture and BC/mixture respectively. In Figure 10B a total of 20 and 56 genes were up-regulated by the action of H-mixture and BC-mixture. In Figure 10C a total of 57 and 21 genes were down-regulated by the action of H-mixture and BC-mixture respectively. The overlapping areas of the graphs B and C represent genes whose expression has been altered by both pesticide mixtures, a total of 36 genes, of which 16 genes were up-regulated by the action of both mixtures, and a total of 20 genes were down-regulated by the action of both mixtures. These results indicate us that the expression of the genes depends on each mixture of organochlorines, supporting the hypothesis

that the effect on genes and by extension the effect in the cell, depends on the chemical mixtures.

It was calculated the absolute distance of expression for the differentially regulated genes, and the five most differentially regulated genes were shown in Figure 11. Glial cell line derived neurotrophic factor family receptor alpha 1 (GFRA1) was down-regulated by the exposure of cells to the H-mixture, but up-regulated by the exposure to the BC-mixture (fold induction = -1.20 and 0.83, respectively; absolute distance = 2.03). GRFA1, together with other proteins, binds artemin (ARTN), which has been implicated in promoting oncogenicity, tumor growth and invasiveness in diverse human malignancies including breast cancer (Kang et al., 2009). It has been published that the expression of GFRA1, especially when combined with ARTN expression, may be useful predictors of disease progression and outcome in specific subtypes of mammary carcinoma (Esseghir et al., 2007; Wu et al., 2013). Moreover, mRNA levels of GFRA1 are higher in patients younger than 35 years old and in advanced stages of the disease (Wu et al., 2013). The fact that the BC-mixture exerted a clear up-regulation of that gene is of interest, and offers a novel role of environment on gene regulation that deserves attention, especially in a region with high levels of mortality due to the disease (Cabanes et al., 2009). It has to be highlighted that early age of diagnosis and advanced stages (III-IV) are well-known bad prognosis factors for BC.

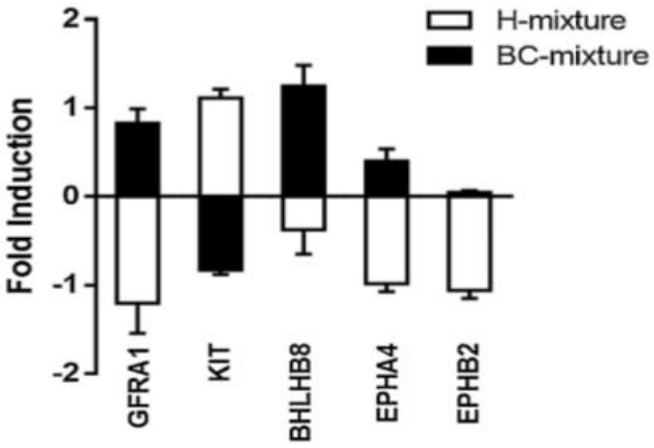

Figure 11. Effects exerted by H-mixture and BC-mixture of OCs on the five genes that showed the most different regulation: GFRA1, KIT, BHLH8, EPHA4 and EPHB2 mRNA levels. HMEC were exposed to the 10x OC mixtures for 5 days.18S expression was performed for each sample as housekeeping gene. The results shown representthe fold changes of each sample relative to the reference sample.

The fold induction of v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) was 1.11 and -0.83 for HMEC exposed to 10x of H-mixture and BC-mixture during five days, respectively (absolute distance = 1.94). KIT is a proto-oncogene that encodes a transmembrane receptor tyrosine kinase named c-kit (Bose et al., 2010). The expression and function of c-kit in breast cancer is a quiet controversial subject, but several studies have proposed that the loss of c-kit expression is associated with tumor progression even for BC (Roussidis et al., 2007). As reported, it was observed a down-regulation of KIT in HMEC exposed to the BC-mixture, reinforcing an environmental regulation of that gene, as it was previously reported (Valeron et al., 2009).

BC-mixture caused 1.25-fold increase in the expression of basic helix-loop-helix family, member a15 (BHLHB8) (fold induction = -0.37 for H-mixture; absolute distance = 1.62). It was reported in this study for the first time the expression of BHLHB8 in HMEC and its regulation by different mixtures of OCs. This gene is a tissue-restricted Class II basic helix-loop-helix (bHLH) transcription factor expressed in lactating mammary glands which is essential for the maintenance of the fully differentiated alveolar state (Zhao et al., 2006). It is also expressed by human neoplastic and non-neoplastic plasma cells (Yeung et al., 2012) and is down-regulated in gastric chief cells undergoing experimentally induced metaplasia (Lennerz et al., 2010). The consequences of this regulation for cell's fate are unknown and deserve deep research.

Finally, EPHA4 and EPHB2 genes were down-regulated in HMEC exposed to 10x of the H-mixture during five days (fold induction = -0.98 and -1.06, respectively; absolute distance with BC-mixture = 1.38 and 1.11 respectively). The role of Eph receptors in cancer is extremely complex and remains controversial, with evidence suggesting both tumor promoting and tumor suppressive functions (Miguelena Bobadilla et al., 2015; Ruiz-Suarez et al., 2015). However, several pre-clinical and laboratory studies support the function of Eph receptor tyrosine kinases in growth, metastasis, and neovascularization of breast cancer (Miguelena Bobadilla et al., 2015). It has to be taken into account that there are 14 receptors and 8 ligands present in the human genome, with expression patterns that often overlap and promiscuous interaction betweenligands and receptors that include bi-directional signaling

and pleiotropic functions which makes highly difficult to discriminate the real role of specific genes of this family (Ruiz-Suarez et al., 2015). In general terms, expression of many of the Eph receptors is often elevated in a wide variety of tumors, including breast cancer (Miguelena Bobadilla et al., 2015). We hav addressed the expression of these genes in HMEC, which is a non-carcinogenic primary cell line. It has been reported that ephrin-induced Eph receptor forward signalling in nontransformed mammary epithelial cells appears to transduce an inhibitory signal that may keep cells quiescent and noninvasive (Rivero et al., 2015; Boada et al., 2015). It was observed that the H-mixture down-regulated the expression of some members of the family, which were slightly up-regulated by the exposure of cells to the BC-mixture (fold induction = 0.4 and 0.05 for EPHA4 and EPHB2, respectively). Taken together, the results reinforce the role of EPHA4 and EPHB2 in the breast tissue and suggest a novel environmental regulation of these genes which could be relevant for breast carcinogenesis.

7. Conclusion

- None of the pesticides have an effect on cell proliferation at existing doses in humans.
- All the combinations had more proliferative effects than estradiol at the existing doses in humans (1x and 100x), although this did not happen at high concentrations.
- Specific mixtures were found for women with breast cancer and healthy women, which composition were different for H-mixture (Aldrin, Dieldrin, Endrin, Lindane, p,p'-DDE, p,p'-DDD and p,p'-DDT) and BC-mixture (Aldrin, Dieldrin, Lindane, p,p'-DDE, p,p'-DDD and p,p'-DDT), and also their concentration.
- The specific mixtures did not show significant differences in their proliferation.
- The specific mixtures did not show differences to estrogenic effects.
- The breast cancer mixture was clearly anti-androgenic at doses closed to those found in humans.
- The expression pattern in human mammary epithelial cells (HMEC) induced by realistic organochlorine mixtures described in healthy women and in women diagnosed with breast cancer was significatly different, which explain, at least

partially and for the fist time, why some mixtures seem to be more carcinogenic than others

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