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Degree of Doctor of Philosophy in Pharmacology and Toxicology

Pesticides exposure and premature thelarche: experimental and epidemiological tools for toxicological assessment

PhD Candidate XXXIV Cycle

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Summary

SUMMARY

Pesticides, widely used in agriculture to protect plants and produce a high quality crop, cause serious human health concerns due to a high number of associated comorbidities such as cancer, neurological, cardiovascular and reproductive diseases including premature thelarche and early puberty.

In addition, several pesticides are identified as endocrine disruptors because they may interfere with the functions of the hormonal system. Children due to their developmental stage, peculiar lifestyle and eating habits are more susceptible to the adverse effects of endocrine disruptors.

The present study is within the framework of the project "Integrated approach to evaluate children agricultural pesticide exposure and health outcome" (PEACH project) and deals with two aspects converging in the toxicological risk assessment, i.e., the hazard identification and the exposure assessment.

Specifically, the study is focused on:

(a) the evaluation of the potential toxicological effects of three pesticides widely used in agriculture, such as chlorpyrifos (CPF), imidacloprid (IMI) and glyphosate (GLY), through an *in vitro* study comparing the response of two mammary cell lines;

(b) the setting up of a questionnaire to be used within the epidemiological study of PEACH project, to design a sampling plan of food locally produced and consumed by the enrolled girls for pesticide residues determination and dietary exposure assessment.

In vitro toxicological study

The possible toxicological effects of CPF, IMI and GLY were evaluated by an *in vitro* study using two human mammary gland cell lines: MCF-7 a breast cancer cell line, commonly used, and MCF-12A a non-tumorigenic cell line, that, although barely used in toxicological studies, could be a more appropriate model simulating the "effective cellular condition" of healthy people; therefore

it may be more suitable to investigate the effects of pesticide exposure and the possible association with the idiopathic premature thelarche (IPT), as clue of pubertal disorders. In this respect, the ultimate goal of the study was to set up a three-dimensional (3D) cell culture system reproducing the *in vivo* physiology of the mammary gland, by using the selected cell lines as preparatory step for further studies.

To evaluate the effects on cell viability and metabolism and endocrine activity, both cell lines were treated with the three pesticides at concentrations derived from real exposure values in children as reported by epidemiological data.

The cytotoxic potential of the substances was measured by CyQUANT® and MTS assays able to detect cell proliferation and cell viability, respectively; cells were treated with five ten-fold serial dilutions of CPF (120 pM- 1.2 μM), IMI (160 pM–1.6 μM) and GLY (230 pM- 2.3 μM).

In order to evaluate if the three pesticides might induce oxidative stress, altered the intracellular ATP production as well as apoptosis or necrosis, appropriate assays were performed in both cell line i.e., Reactive Oxygen Species (ROS) and Adenosine triphosphate (ATP) detection and Annexin V assays were performed in both cell lines.

Furthermore, the endocrine activity of CPF, IMI and GLY was elucidated by the evaluation of their ability to interfere with estradiol production as well as the gene expression of specific nuclear receptors involved in the development of the mammary gland including estrogen receptors α and β (ERα ERβ), androgen receptor (AR), progesterone receptor (PgR) and Aryl hydrocarbon receptor (AhR). Specifically, estradiol concentration and gene expression were determined by Estradiol ELISA assay and real-time PCR, respectively, by using three ten-fold serial dilutions of CPF (1.2, 12 or 120 nM), IMI (1.6, 16 or 160 nM), GLY (2.3, 23 or 230 nM).

Cell proliferation and cell viability were differently affected in MCF-7 and MCF-12A cell lines. In MCF-7 cells, only two pesticides exerted an effect on cell

proliferation at different concentrations: CPF at the highest concentration tested induced an increase while GLY at the lowest induced a decrease. In MCF-12A, all three pesticides induced a dose-dependent decrease in cell proliferation. The results showed that IMI and GLY are able to affect cell proliferation differently depending on the cell model postulating MCF-12A as a more sensitive model. The effects on cell proliferation in MCF-12A are supported by an increased apoptotic signal for GLY indicating activation of the apoptotic mechanism in the cell death process, whereas in MCF-7 cells no significant effect in apoptotic or necrotic signals was observed.

In MCF-7 cells, all three pesticides induced a dose-dependent decrease in cell viability although in a different pattern, while in MCF-12A cells, CPF induced a dose-dependent decrease in cell viability, IMI at the lowest concentration tested induced an increase in cell viability and GLY had no effect. The decrease in cell viability observed in MCF-7 cells was supported by the decrease in ATP level induced by all three pesticides. No effect in ATP production was observed in MCF-12A.

In MCF-7 cells, CPF at the two highest concentrations induced a decrease in ROS production, in contrast, IMI at the lowest concentration induced an increase in ROS production, whereas no effect was recorded for GLY.

In MF-12A cells, intracellular ROS production was decreased by all three pesticides.

In both cell lines, all three pesticides increased estradiol (E2) secretion although in different patterns.

Furthermore, in MCF-7 cells, ERα gene expression was induced by CPF and IMI, whereas it was downregulated by GLY; all three pesticides downregulated ERβ and PgR gene expression and upregulated AR although with different patterns; moreover, IMI e GLY upregulated also AhR although with different pattern.

In MCF-12A cells, ERα and ERβ gene expression was upregulated by IMI and GLY; the same pesticides downregulated AR, PgR and AhR although with different pattern; only CPF downregulated ERβ with no effect on the other receptors.

The final step of the *in vitro* study was to set up a 3D cell culturing that closely mimic the *in vivo* mammary gland. For this purpose, a hydrogel system and a magnetic system were set up and compared by using MCF-7 and MCF-12A cell lines, respectively. The magnetic system aggregating cells under magnetic forces appeared to be as a more suitable 3D cell culturing to study the effects of chemicals providing more reproducible experimental methodology and conditions.

Epidemiological study: Food Frequency Questionnaire

The aim was to develop a food frequency questionnaire to be filled in by the girls (or their parents) enrolled in the case-control study performed in the PEACH project. The food frequency questionnaire is divided into food groups (i.e., fruit, vegetables, meat, fish, cereals, eggs, oil) and food commodities. For each commodity, the quantity consumed, the frequency of consumption and the place of purchase are required. Data from the structured questionnaire were elaborated to sample food locally produced and consumed by girls, for pesticide residues determination. Food samples were collected in 17 local farms and 25 private gardens. At the end of PEACH project, data from questionnaires will be also used to estimate pesticide dietary intake.

Overall, the results of this study, including both the *in vitro* toxicological study and the epidemiological study, provide evidence on pesticides toxicology and suitable tools for chemical risk assessment.

1 Introduction

1.1 Toxicological Risk Assessment

Humans or, more generally, living organisms may be exposed to chemicals through water and food ingestion, air inhalation, or skin contact. Chemicals include both synthetic and natural compounds, such as mycotoxins, plant and animal toxins, for which it is important to establish the potential hazard for human health [1].

Toxicology is the discipline dealing with the assessment of the effects of chemicals on living organisms, formerly based on the paradigm of Philippus Aureolus Theophrastus Bombastus von Hohenheim (1493–1541), also known as Paracelsus and considered the "father" of this discipline: ‘‘In all things there is a poison, and there is nothing without a poison. It depends only upon the dose whether a poison is poison or not”. According to this paradigm, toxicology evaluates the adverse effects of the substances qualitatively and quantitatively in relationship to their amount [2].

Specifically, the chemical risk assessment (RA) is a multi-step process required to determine the safe dose of a chemical and the possible adverse effects on human health.

RA process consists of four stages (Fig.1):

1. Hazard identification (HI) - which substances should be tested and what are the possible adverse effects?

2. Dose (concentration) - response (effect) relation - what concentration of the substance causes harm to humans?

3. Exposure assessment - what concentration of a substance are people really exposed to and what is the intensity and duration?

4. Risk characterization - what is the risk caused by this substance?

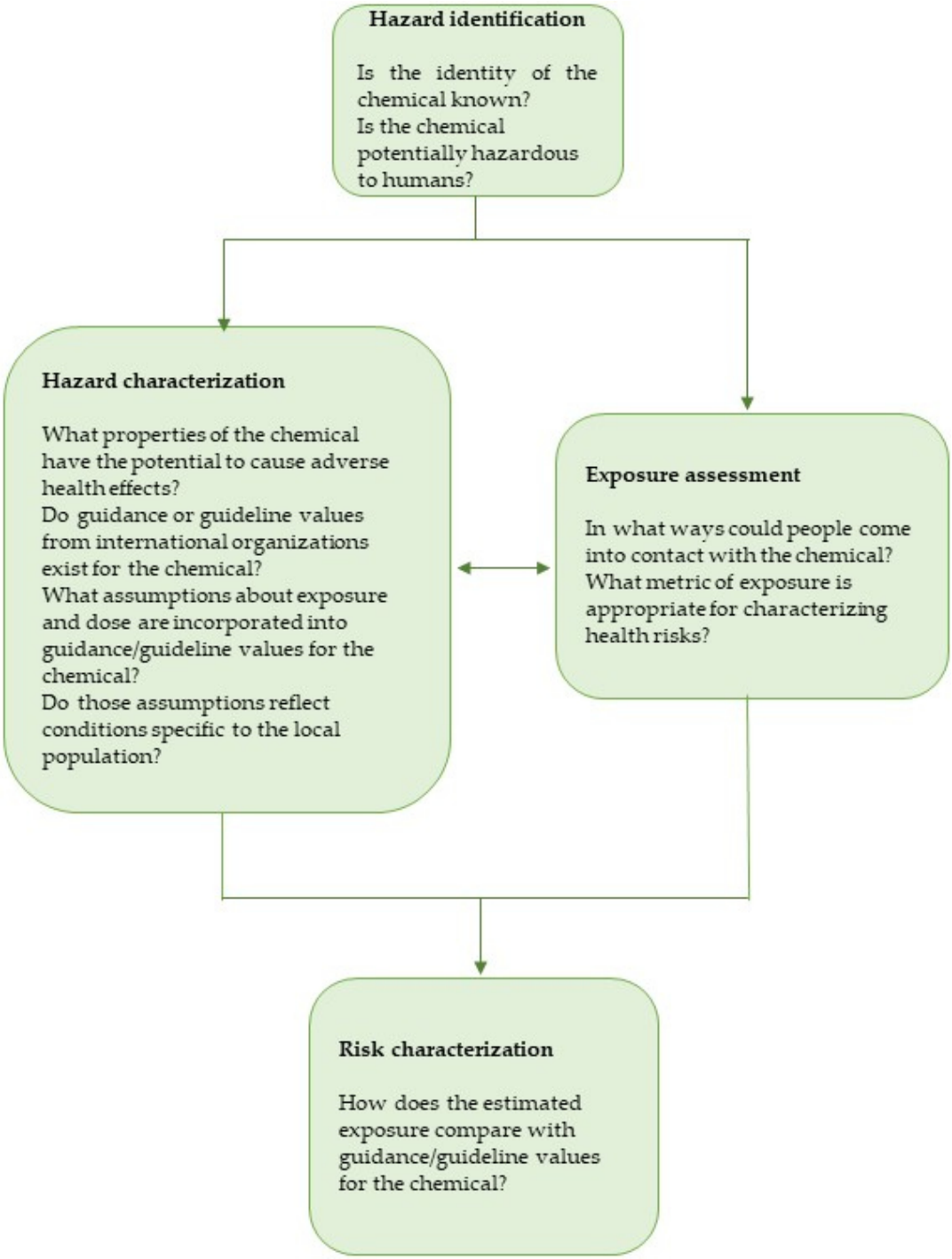


Figure 1. The 4 steps of the Risk Assessment.

In the RA process it is important to define the terms "hazard" and "risk" often mistakenly used as synonyms: "hazard" refers to a qualitative description of adverse effects caused by a substance due to its physical, chemical or biochemical properties, whereas the term "risk" refers to the quantitative measure of the likelihood of an adverse effect as a result of exposure [3].

1.1.1 Hazard Identification

The first RA stage is the hazard identification (HI) through the study of physicochemical properties (e.g., solubility, pH and chemical structure) and toxicological characteristics of a chemical. In particular, HI aims at characterizing the chemical behaviour within the body (i.e., adsorption, distribution, metabolism and excretion), which are the most affected organs or tissues, by which mechanisms the compound exert its effect and the exposure conditions under which harm may occur [4]. Such information is obtained by performing toxicological and epidemiological studies.

Toxicological studies consist of *in vitro*, *in silico*, and *in vivo* approaches which may be performed by validated or not validated methods. Internationally accepted guidelines for chemical test and assessment are provided by the Organization for Economic Co-operation and Development (OECD); obtained data could be used for regulatory purposes.

In vivo studies may be performed using different animal models (e.g., rodents and non-rodents), administering the compound under study under controlled conditions and taking into account the different routes of exposure (oral, dermal, inhalation exposure), the duration of exposure (acute, subacute, subchronic, and chronic studies) as well as the more relevant doses of exposure for human health. The physiological systems target of the chemical under study may be then analysed to evaluate the adverse effects. As regards the exposure duration, acute toxicity generally refers to a short time of exposure (single exposure or dose received over a period of 24h), whereas subacute, subchronic and chronic exposures refer to repeated doses for a period of about 14-28 days, 13 weeks and more than 52 weeks up to the whole life, respectively considering the rodent life span.

The use of animals is still a gold standard in RA, however some aspects have to be considered including ethical issues, high costs and possible relevance for

humans. For this reason, over the years, international organizations such as ECVAM (European Centre for the Validation of Alternative Methods), ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) and OECD released regulations that increasingly limited the use of animals and promoted the use of alternative methods to animal studies through the validation of *in vitro* toxicity tests. The development and increased use of *in vitro* assays were strength by William M.S. Russell and Rex L. Burch in "The Principles of Humane Experimental Technique" [5], by providing a set of recommendations known as the "Principle of the 3Rs" aimed at encouraging a more responsible and careful animal experimentation.

Specifically, the 3Rs correspond to:

REDUCTION: reduction in the number of animals used for a specific study

REFINEMENT: improvement of experimental design to reduce stress and suffering to animals

REPLACEMENT: replacement (even partial) of animal testing with alternative methods of comparable validity.

On the basis of the Principle of the 3Rs, the European Union published the Directive 2010/63/EU in 2010 (implemented in Italy by the Legislative Decree 4 March 2014, n. 26.) on the protection of animals used for scientific purposes, with the ultimate goal of increasingly moving towards a replacement of animal testing.

This has led to a greater development and use of alternative methods based on *in vitro* and *in silico* approaches, which, however, currently do not allow to completely replace animal testing for all end-points of interest. *In silico* methods use computational models to simulate and predict toxicological outcomes upon chemical exposure on the basis of *in vivo* and *in vitro* data [6]. In *in vitro* studies, by the use of cell cultures, subcellular organelles and tissue preparations from laboratory animals or humans, it's possible to identify the mechanism and mode of action of a chemical substance at cellular, molecular, and biochemical level.

However, the limitation in these approaches is that observations are limited to the cellular model implied not considering the feedback mechanisms with other surrounding organs, thus providing information on a single target tissue that cannot be translated to the whole organism. Moreover, *in vitro* cellular models have a limited ability to metabolize chemical compounds and their derivatives compared to same organ in a living organism. In this respect, the use of 3D cultures mimicking *in vivo* cell microenvironments represents a new challenge for toxicity testing leading to a more robust assessment [7].

As regards epidemiological studies, they may be differently carried out depending on whether exposure is predetermined or not (experimental and observational studies, respectively) and when the assessment is performed (prospective or retrospective studies).

Observational studies may be descriptive or analytical; a descriptive study merely describes the frequency of a disease or the background exposure levels in a population and it is often the first step in an epidemiological investigation. Otherwise, an analytical study examines the relationships among health status and other variables, for example the association between exposure to some chemical compounds and the incidence of a pathology. Cohort studies and case-control studies belong to this group; in particular, case-control studies investigate the relationship between disease and exposure by comparing people with a particular disease (called cases) and an appropriate matched control group of people not affected by the disease (comparison or reference group). Conversely, in cohort studies all the population is observed for exposure and disease outcomes with the aim to provide the best information on causality by calculating the risk of developing the disease. Although conceptually simple, cohort studies are the most challenging requiring also long follow-up periods because disease can occur long after exposure. These types of studies are called prospective, but they can also be conducted retrospectively if the exposure data have been collected before the start of the evaluation.

Experimental studies are other types of epidemiological studies including randomized controlled trials, in which patients affected by the same disease are assigned to different groups and subjected to different treatments or interventions then comparing the outcomes, and field trials or community trials in which the participants are healthy people or communities, respectively, who are presumed to be at risk [7].

Overall, all the different toxicological studies provide relevant information on the mechanism and adverse effects exerted by chemical substances. The integration of data from different approaches support a more robust RA.

1.1.2 Dose (concentration) - response (effect) relationship

Dose (concentration) - response (effect) relationship, also referred to as risk estimation, is the second stage of RA representing the quantitative evaluation of the adverse effects.

This process identifies both probability and potency of the exposure effects of a chemical by quantifying dose-response and dose-effect relationships on the basis of animal, cell or human data. The obtained dose-response curves allow to identify at which exposure level of a given substance corresponds a response in an exposed population.

Dose-effect curve identifies the relationship between the dose and the magnitude of change produced on an individual or animal. It applies to measurable changes that give a gradual response to increasing doses of a substance [8].

By this approach is possible to determine the LO(A)EL (Lowest Observed Adverse Effect Level), which is the lowest dose at which a toxic effect occurs, and the NOAEL (No Observed Effect Level) which represents the highest experimental dose level of a compound at which no significant effect is observed in a group of exposed individuals compared to a suitable group of control individuals. Such parameters are then used to derive guidance values

below which exposure to a chemical do not represents an appreciable risk for human health [1].

1.1.3 Exposure assessment

People are exposed to a variety of chemicals through different routes including inhalation, dermal contacts and/or ingestion. The evaluation of actual exposure to toxic substances is a key element in RA to program and adopt appropriate measures to protect human health, taking into account the established guidance values as well as the possible association of exposure with adverse effects [9]. The American National Research Council (NRC) in the report "Exposure Science in the 21st Century: A Vision and a Strategy", defines exposure as "the collection and analysis of quantitative and qualitative information needed to understand the nature of contact between receptors and physical, chemical, or biologic stressors" [10].

In particular, the exposure assessment to a chemicals takes into consideration [1]:

- the route of exposure (air, water, food, skin contact);
- the concentration to which an individual is exposed;
- the intensity, frequency and duration of exposure;
- the identification of the exposed population (age, sex, weight, etc.).

Sex and age represent critical factors influencing exposure to chemicals. Indeed, several population studies have shown different patterns of exposure in men and women which thus may represent different risk factors for some diseases onset such as those related to the reproductive and metabolic spheres [11]. Another relevant issue is the lifestage at which humans are exposed to chemicals since different effects may occur due to different physiological, metabolic and hormonal status [12-14].

In this respect, pregnancy, childhood and elderly deserve more attention as critical lifestages. Among them, children are particularly susceptible and

vulnerable to the adverse effects of chemicals due to their peculiar lifestyle, dietary habits and developmental stage [15].

Human exposure to chemicals is evaluated by performing epidemiological studies and measuring the concentration of the chemical parent compound and/or its metabolites as biomarkers of exposure in biological matrices (i.e., urine, blood, hair, nails). The obtained data represents the combined exposure from all the possible different routes (oral, inhalation or skin contact), each contributing according to the rate of absorption, intake, metabolism, storage, excretion, and abundance of target macromolecules at the cellular level.

Alternatively, an indirect approach to evaluate human exposure is to measure chemical concentrations in the environment or in food then estimating human intake over time (air inhalation, food intake, duplicate diet studies) [16]. Often two or more methods are combined to obtain all possible information on the extent of exposure to a compound.

1.1.4 Risk characterization

The final stage in the RA process is the risk characterization which is carried out by regulatory bodies such as the European Food Safety Authorithy (EFSA) combining and integrating the information obtained from the first three steps to provide an estimate of the probable incidence of adverse effects on human health; by this process, the quantitative and qualitative definition of the risk levels associated with exposure to a chemical substance are established. Specifically, EFSA provides the Acceptable Daily Intake (ADI) or the Tolerable Daily Intake (TDI), which estimate the amount of a substance present in food or drinking water that can be consumed throughout life without presenting an appreciable health risk. In particular, ADI applies to chemicals such as food additives, residues of pesticides and veterinary drugs, whereas TDI applies to substances not deliberately added to foods (e.g., contaminants) [17].

The calculation for TDI or ADI values is given by the ratio between the NOAEL obtained in an animal study and an uncertainty factor (UF) which varies according to the severity of the measured effects. Generally, UF assumes a value of 100 including a factor 10 due to data extrapolation from an animal model to humans and a further factor 10 for inter-individual differences related to metabolism within the human population. However, if serious adverse effects are found, additional UFs are considered from the available scientific data. On the contrary, if the assessment is based on human data UFs are lower [7].

Overall, by the establishment and application of TDI and ADI values, the general population is aware of the possible risks related to the voluntary/involuntary assumption of chemical substances during their lifetime.

1.2 Pesticides

As reported by the European Union: “a 'pesticide' is something that prevents, destroys, or controls a harmful organism ('pest') or disease, or protects plants or plant products during production, storage and transport. The term includes, amongst others: herbicides, fungicides, insecticides, acaricides, nematicides, molluscicides, growth regulators, repellents, rodenticides and biocides” [18].

Pesticides are biologically active against target organism through different modes of action; for example, organophosphates and carbamates act on the nervous system as acetylcholinesterase inhibitors [19,20], pyrethroids interact with the nicotinic receptor as sodium channel agonists (nAChR) [21] and herbicides control development of weeds by inhibiting synthesis of some amino-acids or antagonizing the action of natural growth regulators [22]. Although pesticides have been designed to act selectively against a target pest, they may be toxic to non-target organisms, including humans, due to biological similarities between organisms [23].

Similarly to other chemicals, humans are exposed to pesticide residues through the consumption of food or water, by inhalation or skin contact. Manifold effects associated with pesticides exposure have been observed, including cancer, reproductive disorders, neurodegenerative, cardiovascular and respiratory diseases and developmental disorders [24]. Exposure to organophosphates, a more studied group of pesticides, has been associated to effects on cholinesterase enzymes [25], mitochondrial function [26] and neurodegeneration [27] leading to Alzheimer’s and Parkinson’s diseases, recently associated to glyphosate exposure [28]. Exposure to chlorpyrifos, a widely used organophosphate, may also result in obesity, hyperlipidemia, and Type 2 diabetes mellitus [29].

Carbamate, organochlorine and organophosphate pesticide exposure has been associated to metabolic disorders, including obesity, hyperlipidemia and Type

2 diabetes mellitus through a mechanism affecting mitochondrial function with a consequent derangement of lipid and carbohydrate metabolism [26,27].

In order to protect consumers' health and to reduce the impact of pesticides exposure, their production and presence of residues in food are regulated by the European Commission and regulatory agencies which have promoted and implemented measures for the sustainable use of pesticides since 2009 [30]. In particular, the Regulation (EC) no. 396/2005 [31], established the maximum residue levels (MRLs) of pesticides allowed in feed and food of plant and animal origin; each Member State has to carry out official controls (e.g., Piano Nazionale Residui in Italy) [32] to ensure that foods placed on the market comply with such legal limits. MRLs are established by the European Commission on the basis of the scientific opinions provided by EFSA on the possible risks associated with the presence of pesticide residues in food.

1.3 Endocrine system and nuclear receptors

The endocrine system is constituted by several endocrine glands and organs (Fig. 2) secreting a number of hormones which are then transported by the bloodstream to reach target compartments and is extremely sensitive and able to respond to low doses of hormones. Through hormones, little molecules mainly represented by peptides and acting as 'messengers', the endocrine system plays a crucial role in maintaining the physiological homeostasis of the human body, in regulating body growth, metabolism, sexual development as well as reproduction [33].

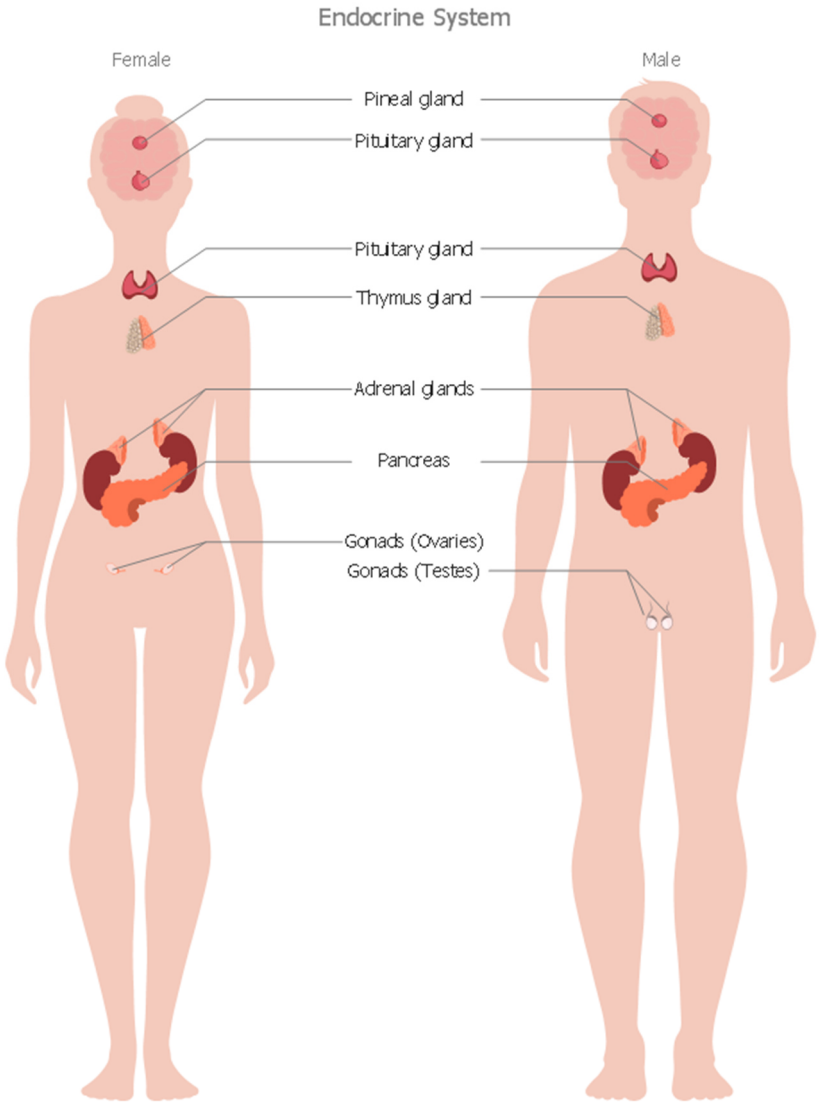


Figure 2. The endocrine system and glands.

The biological actions of endogenous hormones are mediated by the binding with high-affinity receptors present in the cell nucleus and called nuclear receptors (NRs) [34]. The binding of an hormone with its receptor initiates a cascade of events starting with the dimerization of the NR and leading to multiple effects depending on the hormone-receptor pair. Hormones may also trigger other ligand-independent and more rapid responses by differently interacting with hormone receptors expressed on the plasma membrane rather than in the nucleus [35].

Nuclear receptor

NRs are a superfamily of transcription factors regulating several physiological processes (e.g., metabolism, development and reproduction) [36].

NRs structure (Fig. 3) is composed of an N-terminal domain (AB), a DNA-binding domain (DBD) (C), a hinge region (D), a ligand-binding domain (LBD) (E), and a C -terminal domain (F).

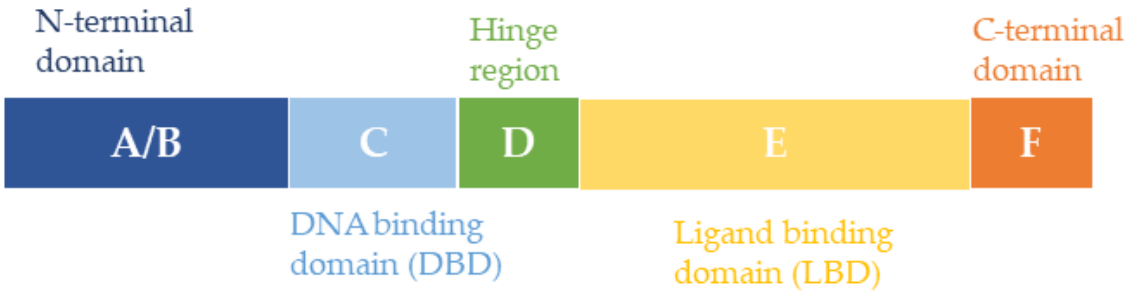


Figure 3. Schematic illustration of the nuclear receptor.

In particular:

- the N-terminal domain has a highly variable sequence among NRs also containing a ligand-independent transcription activation function (AF-1);
- the DBD domain contains zinc finger motifs important for the binding to a DNA consensus sequence called hormone response elements (HRE); this region is highly conserved among receptors;

- the Hinge region connects the DBD and LDB domains;
- the LBD domain is responsible for binding to the hormone and together with the DBD contributes to the receptor dimerization interface. This domain contains a ligand-regulated transcriptional activation function (AF-2) for the recruitment of transcriptional co-activators. LDB is moderately conserved in sequence and highly conserved in the structure between the various NRs;
- the C-terminal domain has a flexible conformation and is highly variable among NRs [37,38].

Among the many NRs expressed in humans, Estrogen receptors α and β, Androgen Receptor, Progesterone receptor, and Aryl hydrocarbon receptor are particularly relevant in the context of our study, so they are described more in detail.

1.3.1 Estrogen receptors α and β

The Estrogen receptors (ERs) belong to the steroid/thyroid hormone superfamily of NRs [39], and their main ligands are estrogens, 17β-estradiol in particular. There are two main forms of ERs, α and β, encoded by separate genes (ESR1, estrogen receptor 1 (ER-alpha); ESR2, estrogen receptor 2 (ER-beta), respectively) (Fig. 4). Both ERs are widely expressed in different tissue types, but with some different expression patterns; for example, ERα is more expressed in the liver, while in the intestine and the lungs only ERβ is expressed [40,41]. The binding of estrogen to ERs in the cytoplasm triggers a series of conformational changes in the receptor structure which dimerizes and translocates to the nucleus where it binds to specific DNA promoter sequences in target genes; the receptor-DNA binding recruits a series of co-activators and other transcription factors forming the preinitiation complex and activating the transcription of estrogen-regulated genes. A small pool of ERs is also localized in the plasma membrane and their activation determines non-genomic response and signals mainly through direct or indirect coupling with G proteins [42].

Therefore, estrogen signaling may involve both transcriptional and non-transcriptional events with sustained and rapid consequences, respectively [43]. In addition to these mechanisms, ERs can also be involved in regulation of estrogen-sensitive genes by binding with some specific transcription factors as NFκB and Sp1 or by cross-talk with other NRs [44].

ERα and ERβ are differently responsive to estrogens, and, as a consequence differently regulate functions within cells and tissue. Generally, ERα promotes cell proliferation and survival whereas ERβ inhibits cell proliferation and gene expression inducing pro-apoptotic effects, and thus, overall, antagonizing ERα action, as demonstrated in several *in vitro* studies on breast, ovarian and prostate cancer cell lines [45-47].

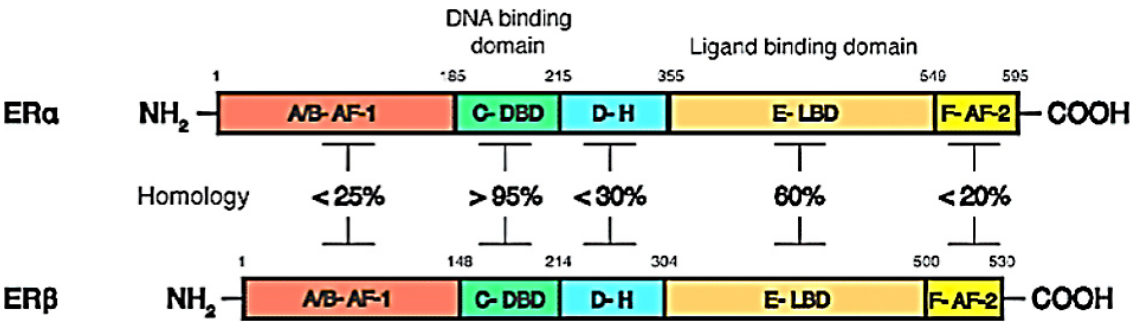


Figure 4. Structure and homology between Erα and Erβ. The image was extracted from Kwakowsky et al. [48].

Estrogens are the main regulators of female reproductive system, but they control many other physiological processes in mammals, including cardiovascular system, bone integrity, mammary gland development, cognition and behavior [49]. Dysregulation of estrogen signaling may perturb all these vital functions leading, for example, to deranged reproductive development, cognitive disorders, including schizophrenia, bipolar disorder, major depressive disorder [50]), cardiovascular diseases, as well as several types of cancer including breast, ovarian, prostate, colon [51]. Any compound mimicking

estrogen action may thus interfere with these fundamental functions within the body determining adverse effects with possible severe consequences.

1.3.2 Androgen Receptor

The Androgen receptor (Fig. 5) is a member of the NRs superfamily, thus similarly to ERs is a ligand-inducible transcription factor. The main steroidal androgens are testosterone (T) and its metabolite 5-dihydrotestosterone (DHT). Their binding to AR induces receptor dimerization, translocation into the nucleus, binding to its cognate response elements and recruitment of co-regulators to promote the expression of target genes [52-54]. Also, androgens can employ non-genomic effects interacting with AR on the plasma membrane [55] or inducing second messenger signal transduction cascades, such as free intracellular calcium increase, and activation of protein kinase A, protein kinase C, and MAPK. The non-genomic action of androgens has been implicated in a number of cellular effects, including gap junction communication, aortic relaxation, and neuronal plasticity [56-59].

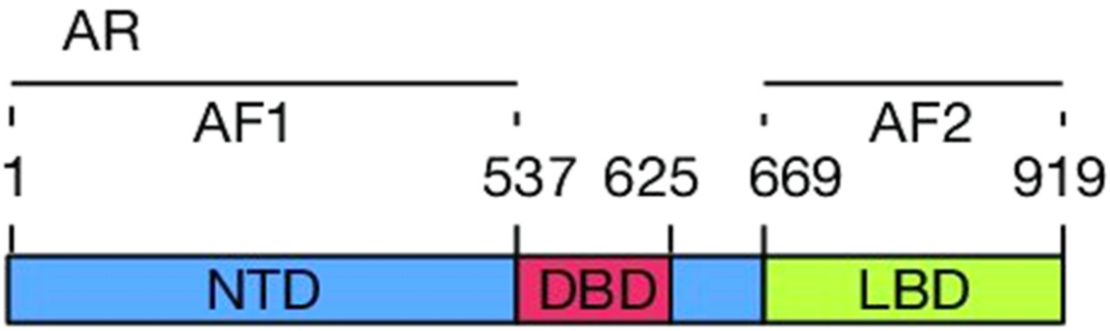


Figure 5. Androgen receptor structure. The image was extracted from Takayama [60].

AR is mainly expressed in male reproductive organs (prostate, testes) but it's present also in the hypothalamus, pituitary and liver [61].

The appropriate regulation of androgen activity is necessary for a range of developmental and physiological processes, particularly male sexual

development and maturation, as well as maintenance of male reproductive organs and spermatogenesis [62-64]. Dysregulation of androgen signaling perturbs normal reproductive development and accounts for a wide range of pathological conditions such as androgen-insensitive syndrome, prostate cancer, and spinal bulbar muscular atrophy [65]. Among compounds antagonizing AR action, flutamide is one of the more studied with a number of documented adverse effects including induction of proliferation in mammary epithelial cells in administered female mice [66].

1.3.3 Progesterone receptor

The Progesterone receptor (Fig. 6) belongs to the NR subfamily 3 group C (NR3C), which has two isoforms, PgRA and PgRB, differing in 164 amino acids at the N-terminal of PgRB. The isoform A is a repressor of transcriptional activity including the isoform B, while isoform B is a positive regulator. Cytoplasmic PgR is transcribed and assembled into an inactive multiprotein chaperone complex. When Cytoplasmic PgR binds the progestin, the natural ligand, a conformational change takes place leading to the receptor dimerization and the binding to the promoter sequences on the target genes into the nucleus which involves the recruitment of specific co-activators. Apart progestins, PgR expression levels are regulated also by 17β-estradiol or estrogen bound to estrogen receptors (ER) [67].

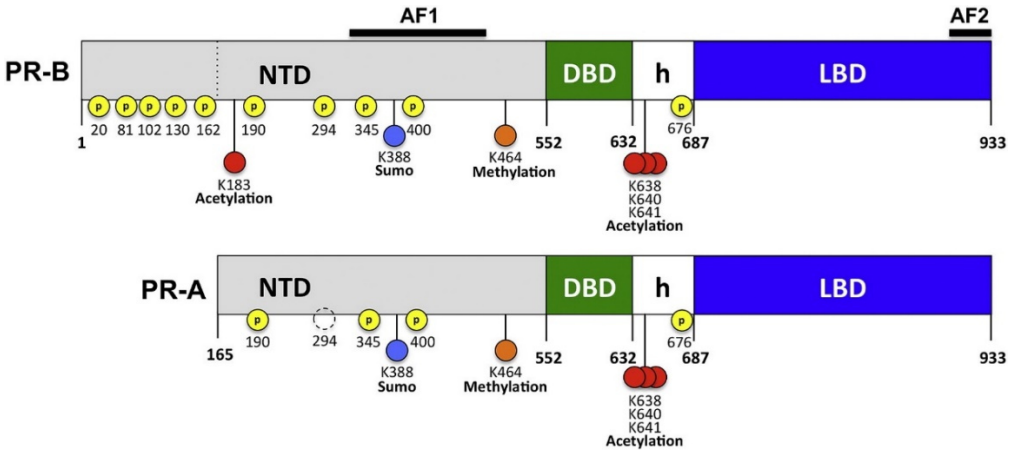


Figure 6. Structure of the two isoforms of PgR: PgRA and PgRB. The image was extracted from Grimm et al. [68].

In humans PgR is expressed in several types of tissue such as mammary gland, utero, brain, bone, ovary and testes [69]. In particular, PgR is involved in mammary gland ductal morphogenesis, ovulation, and lobuloalveolar differentiation [70].

1.3.4 Aryl hydrocarbon receptor

The Aryl hydrocarbon receptor (AhR) (Fig. 7), also known as the dioxin-receptor, is a member of the basic helix-loop helix/Per-ARNT-SIM (bHLH-PAS) gene family of transcription factors and it is the only ligand-activated one.

After ligand binding, AhR migrates in the cell nucleus forming an heterodimeric complex with the AhR nuclear translocator ARNT [71] which binds to specific genomic enhancer sequences (up to 59) termed Dioxin- or Xenobiotic-Responsive Elements (DREs or XREs); this interaction leads to transcriptional activation of genes involved in complex cellular responses such as cell cycle progression and apoptosis, phase I drug-metabolizing enzymes like Cytochromes P-450 1A1 (CYP1A1), CYP1A2, and CYP1B1 and phase II enzymes including glutathione-S-transferase Ya subunit, UDP glutathione transferase, aldehyde dehydrogenase, and NAD(P)H quinone oxidoreductase (NQOR) [72]. In addition, a number of Estradiol (E2)- regulated genes are controlled by AhR at either the transcriptional or post-transcriptional level [73].

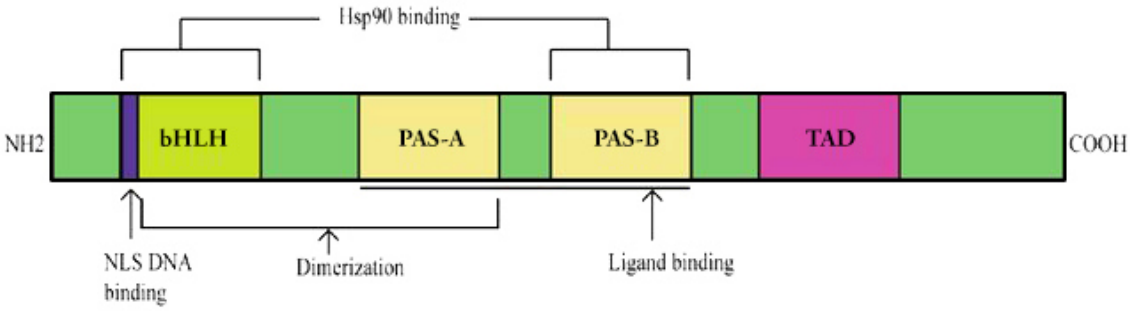


Figure 7. Aryl hydrocarbon receptor structure. The image was extracted from Zhu et al. [74].

AhR influences several physiological processes, including cell proliferation, differentiation, and inflammation. AhR dysregulations have been associated with decreased fertility, oxidative stress and damage to the reproductive tract [75].

The majority of AhR ligands are environmental contaminants such as polycyclic aromatic hydrocarbons, TCDD [76], related polychlorinated dibenzo-pdioxins and dibenzofurans [77], and dietary indole carbinols present in cruciferous vegetables and pesticide [78].

1.4 Endocrine disruptors

Endocrine disruptors (EDs) are a diversified class of chemicals (EDCs) that interfere with the hormonal system and defined by the World Health Organization as: "*… an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"* [79].

Specifically, EDs are compounds capable of mimicking the action of natural hormones altering the normal endocrine functions within the body. EDs can act through several mechanisms including:

- direct interactions with hormone receptors, both by evoking an unexpected cellular response at the wrong time or in an excessive degree (agonist effects), and by hindering the binding of the natural hormone (antagonistic effects);
- bindings with transport proteins in the blood by altering the amount of circulating hormones and, therefore, their bioavailability;
- interactions with enzymes involved in steroid and/or metabolic biosynthesis, thus affecting the speed and capacity of hormone synthesis [80].

Initially, EDs were observed to exert actions through steroid hormone receptors, such as ERα and ERβ, AR, PgR, and the thyroid hormone receptors (THRα and THRβ). Recent evidence indicates that EDs are also capable of interacting with other receptors, such as retinoid receptors (RAR and RXR), non-nuclear steroid hormone receptors (e.g., membrane ERs), nonsteroidal receptors (e.g., AhR), and peroxisome proliferator-activated receptors [81].

Furthermore, it is increasingly evident that many EDs can act as agonists in some tissues and as antagonists in others. Indeed, each tissue expresses a characteristic set of co-activators and co-repressors that could differently modulate the action of ligands in a tissue-specific way. This implies that EDs may derange same pathways in different ways.

Many substances released into the environment are able to interfere with the endocrine system: i.e., persistent organic pollutants (POPs) such as dioxins, polychlorinated biphenyls (PCBs), pesticides, or compounds used as additives to plastics, such as phthalates or bisphenol A. There are also natural substances such as phytoestrogens, including isoflavones and lignans, mainly contained in soy and cereals, but also in cosmetics as active ingredients [82] (Fig. 8).

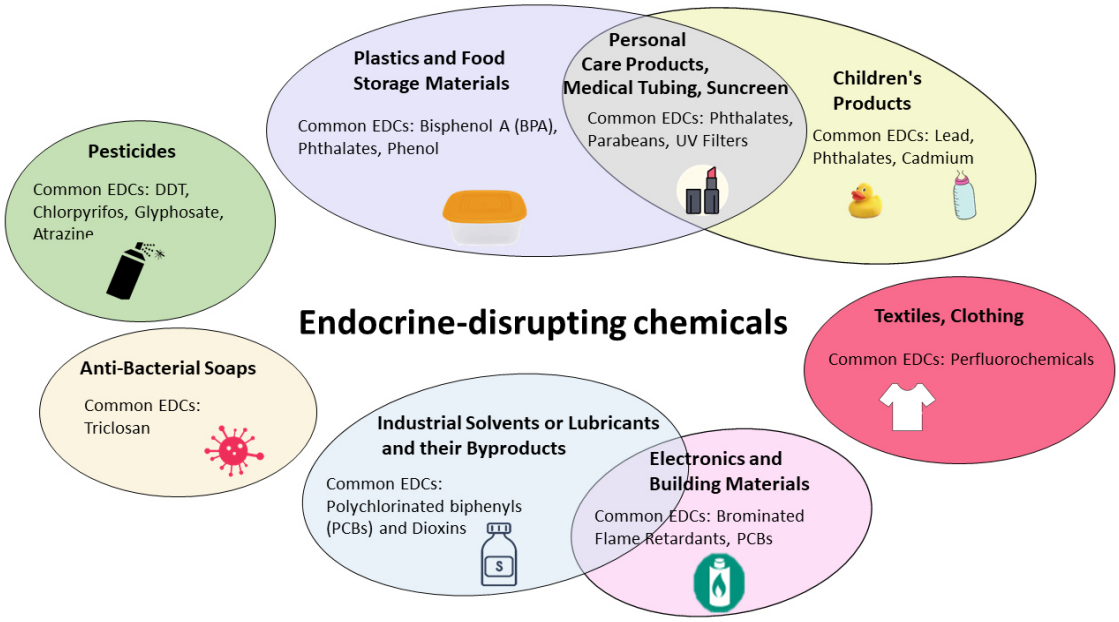


Figure 8. Examples of Common Endocrine disruptors.

Many pesticides can act as endocrine-disrupting chemicals (EDCs). For example, chlorpyrifos-methyl, propiconazole, carbaryl, and methiocarb can act as agonists or anti-agonists of ERα and ERβ, while cyproconazole and prochloraz interact with aromatase functions, enzyme responsible for estrogen biosynthesis from androgens, and with steroid hormone metabolism [83]. Within the same class of chemicals, different compounds may exert diverging effects. For example, the carbamate aldicarb acts as an inhibitor of 17 beta-estradiol and progesterone activity [84], whereas carbofuran increased the levels of progesterone, cortisol and estradiol and decreased the testosterone levels in adult male rats [85].

Other pesticides like mancozeb, zineb, dimethoate, trichlorfon and malathion mainly affect thyroid homeostasis and signaling with consequences on the hypothalamus-pituitary-thyroid axis [83,86].

Since effects induced by pesticides may derive from different mechanisms, EFSA and the European Chemicals Agency (ECHA) have developed a strategy to be considered when evaluating the endocrine disrupting properties of pesticides based on evidence that the adverse effects are a consequence of the endocrine mode of action, i.e., it alters the functions of the endocrine system [87]. Furthermore, the European Commission recently approved the regulation (EU) 2018/605 which establishes the criteria to determine EDs properties; when a chemical is identified as ED, it can no longer be placed on the market [88].

1.5 Pesticides and mammary gland

The regulation of mammary gland development starts since the first months of life, when the hypothalamic-pituitary-gonadal axis (HPG axis) is transiently activated in the newborn and it's then inactivated until the onset of puberty, beginning in girls between 8 and 13 years of age. During puberty, the re-activation of HPG axis and the release of the gonadotropin-releasing hormone (GnRH) by the hypothalamus induces the secretion of the gonadotropin luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) from the anterior pituitary gland, that in turn regulate the hormonal production by the gonads [83,89].

Since the onset of puberty is activated by a set of cross-communicating hormones and neurotransmitters along the HPG axis, any imbalance perturbing this phase may detrimentally affect sexual development.

In the last decades, a progressive shortening of developmental time in girls and a consequent increased incidence of precocious puberty and premature thelarche were observed worldwide. In girls, precocious puberty is defined as the development of secondary sexual changes before the age of 8 years. The onset of precocious puberty in girls has an estimated incidence of between 1/5,000 and 1/10,000 [90]. The term premature thelarche, describes isolated mammary development in the absence of other signs of pubertal maturation in girls before the age of 8 with a prevalence ranging from 2.2% to 4.7% among girls aged 0 to 48 months [91]. Thelarche can be defined as progressive if accompanied by a gradual increase in breast size and not associated with other signs of puberty, cyclical if at least one regression occurs followed by new breast augmentation, persistent if no change in breast size occurs, and regressive if regression of breast tissue occurs [92].

The pubertal breast development is mainly controlled by ovarian estrogen and progesterone hormones, regulating the ductal morphogenesis and the epithelial

growth of the mammary gland through their respective estrogen receptors (ERα, ERβ), and the PgR [93].

However, other nuclear receptors are also involved such as the AR, which has a compensatory role in mammary gland development during puberty, counteracting the extent of proliferation, ductal extension and buds morphology [94], and the AhR, which is not strictly required for breast development [95], but due to its cross-talk with ERs, is relevant as regulator of proliferation [96].

An increasing number of studies suggest that pesticides exposure can affect children's health [97]. One of the effects that pesticides may exert as EDs is to derange the hypothalamic–pituitary–gonadal axis unbalancing proper puberty timing [24,98,99]. Indeed, children exposure to pesticides has been associated with premature development of the reproductive system, including premature thelarche [100,101], as reviewed in Coppola et al., [83].

The mechanisms may involve agonistic and anti-agonistic activities toward both ERs (ERα and ERβ) and AR (i.e., chlorpyrifos methyl, propiconazole, carbaryl, methiocarb), as well as interaction with aromatase functions and steroid hormone metabolism (i.e., cyproconazole, prochloraz) [84]. A transactivation ER assay using a human breast cancer cell line (MVLN) showed the estrogenic activities of terbuthylazine, propiconazole, prothioconazole, cypermethrin and malathion while also demonstrated that bitertanol, propiconazole and mancozeb have anti-androgenic activity and terbuthylazine, propiconazole and prothioconazole can act as aromatase activity inducers [102,103]. Also relevant is the interaction with the pregnane X receptor that entails the implication of pesticides on cellular metabolism and the detoxification process by regulating the transcription of genes involved in the microsomal cytochrome P450 and conjugation enzymes (i.e., fenbuconazole, tebuconazole), thus possibly affecting steroid hormones homeostasis. The thyroid homeostasis and signalling are also affected by mancozeb, zineb,

dimethoate, trichlorfon and malathion with adverse impact on hypothalamus-pituitary-thyroid axis [86].

Exposure of female rats to chlorpyrifos, increased LH, FSH and E2 serum levels, possibly due to an effect on anterior pituitary function [104]. Similarly, imidacloprid increased FSH and decreased LH and progesterone serum levels in treated female rats, probably due to an alteration in GnRH release, with consequences on ovary morphology [105]. In an *in vitro* study on swine granulosa cells, glyphosate decreased E2 and increased progesterone secretion [106].

Effects evident at puberty may have had an origin in utero since many pesticides may cross the placental barrier, as observed in girls whose mothers worked in greenhouses, displaying an early breast development with concomitant higher serum levels of androstenedione, a precursor of both androgens and estrogens, and lower levels of the anti-Mullerian hormone [107], which generally increases during the pre-pubertal period.

In utero and lactational exposure to the anti-androgenic vinclozolin altered rat mammary gland development evident at puberty, with increased cell proliferation and excessive ductal branching [108].

Chlorpyrifos was demonstrated to affect the mammary gland in female adult rats by increasing cell proliferation and the number of ducts as well as PgR expression involved in promoting ductal development [109]. A similar effect was also observed *in vitro* model using a human breast cancer cell line (MCF-7) by a mechanism possibly involving the phosphorylation of ERα [110].

1.6 PEACH project presentation

Several pesticides, including acaricides, insecticides, herbicides and fungicides, are recognized as endocrine disruptors (EDs) since they can interfere with the dysregulation of sexual, thyroid and neuro-endocrine hormones. General population is exposed to pesticides mainly via food intake, considering the presence of residues in food as detected by the yearly monitoring plan control, even if the majority of them is under the maximum residue level permitted. Although pesticides residue levels are yearly monitored thus assuring consumers that their presence is under the established MRLs, a relevant issue is related to the risk posed by multiple residues found in all food products. Since children are particularly vulnerable to ED adverse effects due to their developmental stage, peculiar life style and dietary habits, the exposure to pesticides may represent an important risk factor associated with the onset of puberty [15].

In this frame, the project "Integrated approach to evaluate children agricultural pesticide exposure and health outcome" (PEACH project, funded by the Italian Ministry of Health, RF-2016-02364628) deals with the increased incidence of idiopathic premature thelarche in girls observed by pediatricians of the National Health System in a specific area of Central Italy (Fermo, Macerata), characterized by an intensive agriculture practice.

In order to evaluate the possible association between the exposure to pesticides and the premature thelarche in girls, an integrated approach was set up, including: i) a prospective case-control study; ii) girls' exposure assessment to pesticides by internal level determination and dietary exposure evaluation; iii) a toxicological study by using an *in vitro* model [83].

The case control study is performed by enrolling 60 girls with an idiopathic premature thelarche (cases) and a corresponding number of healthy subjects (controls), matched by age and ethnicity, living in the selected area. For each

subject, 45 different pesticides and their metabolites are measured in urine samples. With respect to dietary exposure, different food groups with potential pesticide contamination, including vegetables, fruit, cereals, fish, meat, dairy products, eggs, honey, oil are collected from farms and private gardens in the selected area and pesticides levels are evaluated in each food category. Toxicological effects of three widely used pesticides (two organophosphates, glyphosate and chlorpyrifos, and a neonicotinoid, imidacloprid) are evaluated by using a human breast cell line as representative of the target organ of thelarche, treated at real pesticide exposure concentrations occurring in children and assessing some endpoints related to vitality, mitochondrial activity and endocrine functionality.

Data integration will provide a contribution to risk assessment of pesticides to children health, by correlating pathology with individual and environmental factors (dietary habits, agricultural practices, related use of pesticides, land use) and evaluating potential effects in children health, strictly related to real levels of exposure.

2 Aim of the study

Pesticides are compounds widely used in agriculture to protect the plants and produce a high quality crop. Despite the beneficial aspects on plant production, exposure to pesticides residues through contaminated foods causes serious health concerns due to high number of associated comorbidities such as cancer, neurological, cardiovascular and reproductive diseases [111,112].

In addition, several pesticides are identified as endocrine disruptors since they can affect the hormonal system [113] and therefore, are more susceptible to the toxic effects of pesticides, due to their developmental stage, peculiar life style and dietary habits [15]. Several studies suggest that pesticides exposure can affect children's health, including problems with neurological development, birth defects and premature development of the reproductive system such as premature thelarche [100,101,114].

In the frame of the PEACH project, the present study deals with two aspects of the toxicological risk assessment:

a) to study the potential toxicological effects of three relevant pesticides widely used in agriculture such as chlorpyrifos (CPF), imidacloprid (IMI) and glyphosate (GLY) as potential risk factors of premature development of mammary gland, a main target organ of premature thelarche, by an *in vitro* approach performed using both tumorigenic (MCF-7) and non-tumorigenic (MCF-12A) mammary gland cell lines;

b) to set up and validate a questionnaire on food habits as a tool of the epidemiological study to design a food sampling plan and assess the dietary exposure of girls to pesticides.

In vitro toxicological study

The toxicological assessment of pesticide effects was carried out by an *in vitro* study implying a battery of tests on two human mammary gland cell lines, namely: MCF-7, a breast cancer cell line commonly used in cancer and

toxicological studies, and MCF-12A, a non-tumorigenic cell line, less frequently used in toxicological studies. The rational for their choice is that MCF-7 cells are easily cultivated and results obtained on this cell line are comparable with a large literature. On the contrary, MCF-12A cells, represent a more appropriate model simulating the "effective cellular condition" in healthy people therefore more suitable to reflect real effects of pesticide exposure and the possible association with the idiopathic premature development of mammary gland, as clue of pubertal disorders. The results obtained are used to compare the response of the two cell lines in order to define a more suitable model of study and to better characterize the toxicological impact of exposure to selected pesticides.

In addition, to better recapitulate *in vivo* physiology of mammary gland, both cell lines were used to set up a 3Dcell culture system for further studies.

MCF-7 and MCF-12A cell lines were treated with the three pesticides at concentrations derived from real exposure values in children as reported by epidemiological data [115-119].

The *in vitro* test battery included general toxicological assays as well as approaches investigating endocrinological derangements induced by CPF, IMI and GLY in MCF-7 and MCF12-A. In the general toxicological assessment, we evaluated the cytotoxic potential of the substances by determining cell proliferation and cell viability by CyQuant and MTS assay, respectively. To better identify cell death mechanisms, induction of apoptosis and necrosis were time-course assessed by the Annexin V assay. As regards mitochondrial functionality, induction of oxidative stress was assessed by measuring ROS; besides also intracellular ATP production was performed by a luminescent assay.

To assess the three pesticides in relation to their endocrine disruption action [120-122], we examined their ability to interfere with estradiol secretion in mammary gland cell lines by an Estradiol ELISA assay. Further, the gene

expression of specific nuclear receptors involved in the development of the mammary gland, including ERα, ERβ, AR, PgR and AhR, was evaluated by real-time PCR. The final step of the study, was to set up a 3D *in vitro* model that would better represent the physiological situation *in vivo* by using MCF-7 and MCF-12A cell lines. For this purpose, a hydrogel system and a magnetic system were set up and compared.

Epidemiological study: Food Frequency Questionnaire

In the epidemiological study, a food frequency questionnaire (FFQ) was developed to be filled in by enrolled girls (or their parents) in the case-control study performed in the PEACH project. The FFQ is divided into sections according to food groups (i.e., fruit, vegetables, meat, fish, cereals, eggs) and food commodities. For each commodity, the quantity consumed, the frequency of consumption and the place of purchase are required. The questionnaire thereby ensures the sampling of locally produced foods consumed by girls and finally, the estimate of pesticide exposure of girls from dietary intake.

3 *In vitro* study

3.1 Background

3.1.1 *In vitro* models and cell cultures

In vitro studies involve the use of cell cultures or tissue preparations from laboratory animals or humans. The use of human cells for *in vitro* studies represent a valid, reliable and informative alternative to experimentation in humans which is, for obvious ethical reasons, regulated and restricted.

Cell cultures may consist of primary cells, i.e., cells derived from explanted organisms grown in an appropriate culture environment where they are able to replicate a limited number of times, or immortalized cell lines, mainly derived from tissues of tumorigenic origin or from non-tumorigenic samples properly engineered, able to proliferate indefinitely. Further models are represented by stem cells which are undifferentiated and capable of self-renewal and differentiation into one or more cell types depending on the received stimulus [123].

Compared to *in vivo* studies, *in vitro* cellular models allow to perform a higher number of experiments using a large number of different experimental conditions, especially when applying high throughput approaches. By *in vitro* testing, it's possible to explore adverse effects at molecular level thus characterizing modes of action and promising biomarkers of effects. Further, and more importantly, their imply eliminates the problems related to the interspecific extrapolation as occurring for animal data.

The most commonly used method for *in vitro* testing is a two-dimensional (2D) model in which cells grow in monolayer adherent, when to a surface of a flask or dish, or in suspension. The 2D model is well established and internationally accepted system for toxicological study, however it partially reflects the *in vivo* cellular microenvironment lacking of the complex interactions with the

neighbouring cells and the extracellular matrix (ECM), composed by proteoglycans and fibrous protein such as collagens, elastins, fibronectins and laminins, thus not allowing to simulate the functionality of an organ or tissue [124]. Cell-ECM interactions are important because they are involved in several cell growth processes and functions, including: a) structural support in cellular migration, proliferation and survival; b) release of growth factors; c) regulation of cell-cell signaling; d) defining properties of rigidity or elasticity; e) enabling neovascularization and tissue remodelling. To overcome 2D model limitations, efforts have been made to develop more complex *in vitro* models. One of these is the three-dimensional (3D) cell culture in which cells are surrounded by ECM, reproducing *in vitro* the microarchitecture found *in vivo* [125]. 3D models can be obtained through the formation of spheroids, organoids, or engineered models. Spheroids consist in groups of aggregated cells together, organoids are aggregates of different cell types that assemble into a 3D structure, and engineered models use porous scaffolds made from biomaterials that attempt to replicate the architecture of a tissue *in vitro* [126].

A 3D cellular system can be obtained by growing cells in suspension in non-adherent plates or by using gel-like substances designed to control the physical, mechanical, and biological properties of cell cultures. Among the gel-like substances, the Matrigel, obtained from sarcoma samples of mice called Engelbreth-Holm-Swarm, is used for its content of ECM components and growth factors. However, its variability in the composition makes difficult to keep under control the culture environment and to reproduce the experiments [127].

Therefore, alternative scaffold of a synthetic nature has been designed, such as the hydrogel [128] formed of poly vinyl alcohol (PVA), poly-2-hydroxyethyl methacrylate (pHEMA), or polyethylene glycol (PEG). The composition of the hydrogel is reproducible and enhance nutrient exchange and improve cell proliferation. However, being composed of synthetic molecules, hydrogels does

not inherently contain the signal molecules necessary to recreate the cellular microenvironment associated with natural gels [129].

Recently, a magnetic 3D technology has been developed to recreate the *in vivo* environment without the use of artificial scaffolds. This technology allows to obtain the formation of spheroids or organoids through the use of inert and biocompatible nanoparticles (nanoshuttle) that adhere to the cell membrane and make the cells magnetic. In this way, through the use of a magnet, the cells are rapidly 3D printed. Although the magnetic 3D bioprinting technology is more expensive than the above methods, it allows cells to synthetize the endogenous ECM, without any artificial substrate, as well as to obtain a reproducible model in a very short time [130].

3.1.2 Chlorpyrifos

Chlorpyrifos (CPF, IUPAC nomenclature: diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-λ^5-phosphane, molecular formula $C_9H_{11}NO_3PS$ (Fig. 9); molecular weight 350.6 g/mol) is an organophosphate pesticide used as insecticide, acaricide and nematicide [131].

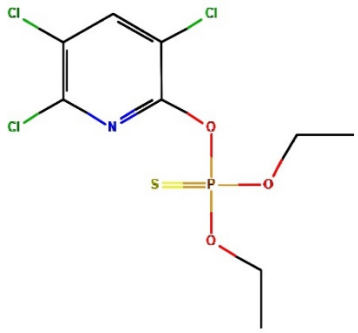


Figure 9. CPF chemical structure. (modified from PubChem).

Chlorpyrifos affects the nervous system by interacting with the active site of acetylcholinesterase (AChE) which has the function of degrading the neurotransmitter acetylcholine involved in the activation of cholinergic neurons and nerve cells controlling signals in the peripheral nervous system. Inactivation of AChE induces over-stimulation of neurons resulting in sensory

and behavioural disturbances, coordination loss, decreased motor function, respiratory depression, tremors, and death [132,133].

CPF is metabolized in the liver by the cytochrome P450 (CYP450) system to chlorpyrifos oxon and subsequently to 3,5,6-trichloro-2-pyridinol (TCP). These metabolites result equally toxic and more persistent in the organism than their parent compound [134-136].

Chlorpyrifos is also considered an EDC interacting as antagonist of progesterone receptor and as both agonist and antagonist of the AhR and ERα; in addition, it is able to induce anti-androgenic effects [81,137,138]. *In vivo* studies performed at Istituto Superiore di Sanità demonstrated for the first time that developmental exposure to CPF affected the expression of neuroedocrine peptides as oxitocin and vasopressin [139] as well as of ERβ [140] in mice hypothalamus. Further, effects on mice thyroid function were also identified [141]. Several evidence report CPF effects also on the reproductive system, like alterations in the reproductive cycle, enlargement of mammary glands, a significant increase in the thickness of the epithelium of the ovarian and uterine surface, an increase in the follicular diameter and atresia [142]. The observed enlargement of the mammary gland may be due to an activation of ERα, as observed *in vitro* in CPF-treated MCF-7 and MDA-MB-23 breast cell lines displaying increased cell proliferation [109,110,143].

Administration of CPF to adult female rats at 0.01mg/kg body weight (bw)/day increased the number of mammary gland ducts, cell proliferation as well as PgR expression but decreased serum estradiol and progesterone levels at higher concentration (1 mg/kg bw/day) [109]. Further, CPF exposure of adult female rats to 0.1 and 2.5 mg/kg bw/day altered mammary gland morphology increasing ductal thickness and branching as well as bud diameter and number [142]. Promotion of breast tubulogenesis through the activation of the AhR pathway was also observed in MCF-7 treated with CPF [144].

3.1.3 Imidacloprid

Imidacloprid (IMI, IUPAC nomenclature: (*NE*)-*N*-[1-[(6-chloropyridin-3-yl)methyl] 42midazolidine-2-ylidene]nitramide, molecular formula $C_9H_{10}ClN_5O_2$ (Fig. 10); molecular weight 255.66 g/mol) is a neonicotinoid insecticide used to protect crops, soil and seems [145].

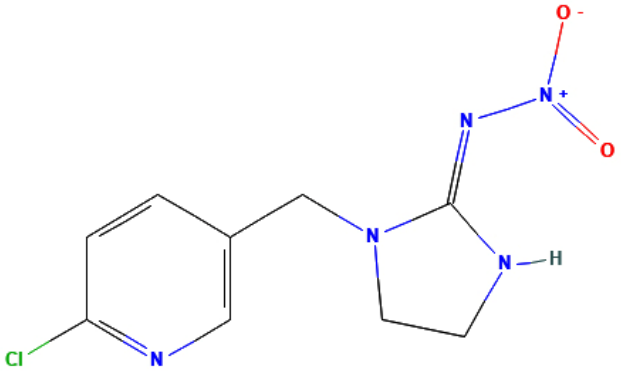


Figure 10. IMI chemical structure. (modified from PubChem).

Similarly to CPF, IMI acts on the nervous system by interacting with AChE inducing over-stimulation of neurons. In the organisms, IMI is metabolized in liver in two major metabolites: 6-chloronicotinic acid (6-CNA) and imidazolidine [146]. IMI exposure has been associated with neurological diseases, developmental and reproductive disorders, genotoxic and mutagenic alterations and hepatotoxic effects [147,148]. *In vivo* study showed that exposure of female rats to IMI induced pathomorphological changes of the reproductive organs and alteration of LH, FSH and PgR levels [149], whereas in male mice IMI affected testicular morphology by down-regulating AR expression and reducing serum Testosterone levels [150]. *In vitro* study in MCF-7 transfected cells showed the capability of IMI to trigger ER-mediated estrogenic activity [151], however, no *in vivo* studies investigated IMI adverse effects on mammary gland development. Despite the limited data available, there is evidence that IMI can act as an EDC.

3.1.4 Glyphosate

Glyphosate (GLY, IUPAC nomenclature: 2-(phosphonomethylamino) acetic acid, molecular formula $C_3H_8NO_5P$ (Fig. 11); molecular weight 169.07 g/mol) is a broad spectrum and systemic organophosphate herbicide [152].

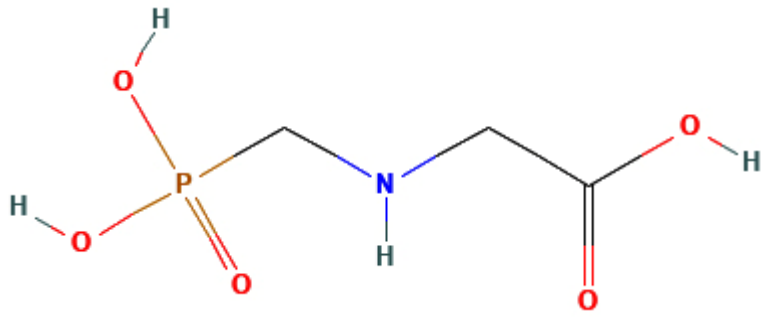


Figure 11. GLY chemical structure. (modified from PubChem).

It inhibits the enzyme 5-enolpiruvilshikimato-3-phosphate synthase (EPSPS) in plants which is involved in the biosynthesis of essential aromatic amino acids [153] . The International Agency for Research on Cancer (IARC) classified GLY as probably carcinogenic; however, available toxicity data on GLY and its metabolite aminomethylphosphonic acid (AMPA) are no sufficient to limit its use. Currently, the ECHA has initiated a new safety evaluation.

Recent evidence demonstrated human exposure to GLY as detectable levels were found in urines being possibly associated to toxicological effects on the immune system [117,154]. *In vivo* study showed that GLY and its commercial products induced toxic effect in reproductive organs, including uterus and testis; in addition, GLY interfere with hypothalamic-pituitary-gonadal axis [155-157].

Data from *in vitro* studies have reported conflicting results on possible endocrine disrupting effects of GLY.

In one study, GLY did not induce any ER-mediated estrogenic activity and produced no antiestrogenic effect when tested in combination with E2 in hepatic and breast cell lines (HepG2 and MDA-MB453-kb2) [158]. However,

other *in vitro* experiments showed that GLY may induce an estrogenic activity mediated by ER activation, similar to 17β-estradiol (E2) in a transfected epithelial mammary gland cell line (T47D-KBluc cells) [159], also promoting proliferation in MCF-7 cells increasing ER*α* gene expression via a ligand-independent mechanism, but only at high concentrations [98].

In addition, GLY inhibited AR and ER*α* expression in HepG2 cells moreover modifying aromatase enzyme activity in placental JEG3 cells and human HEK293 cells [151]. At mammary gland level, the early post-natal administration of a GLY-based herbicide formulation (GLY at 2 mg/kg bw) increased the hyperplasia of ducts and altered the stroma morphology; further, it induced ER*α* and PgR gene expression [160].

3.2 Materials and Methods

3.2.1 Cell culture

MCF-7 (HTB-22) (Fig. 12A) cell line was purchased from ECACC (Salisbury, UK) and grown in Dulbecco's Modified Eagle Medium (DMEM medium) (Gibco, Grand Island, NY), supplemented with 10% Fetal Bovine Serum (Gibco), 100 U/mL penicillin/streptomycin and 2 mM L-glutamine (Gibco).

MCF-12A (CRL-10782) cells (Fig. 12B) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in DMEM/F12 medium containing 5% equine serum (ES), 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 0.1 μg/ mL cholera toxin, and 10 μg/mL insulin.

Both cell culture media were phenol red-free to avoid any unintentional estrogenic effect. Both cell lines were maintained in a humidified Steri-Cult 200 Incubator (Forma Scientific, Marietta, OH, USA) at 37 °C, 5% CO_2 and 95% air.

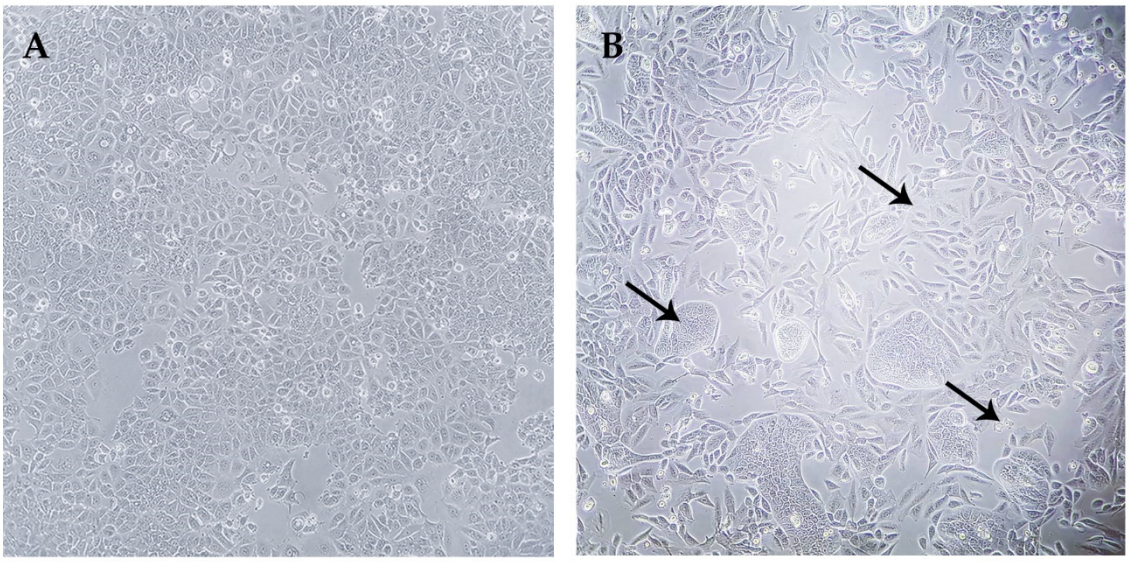


Figure 12. Cell morphology of MCF-7 (A) and MCF-12A (B) cell lines. MCF-12A cells grow as a heterogeneous population; arrows indicate epithelial cells, single fibroblast-like cells, and spherical cells.

3.2.2 Chemicals

CPF (CAS no. 2921-88-2, purity ≥ 98.0 %), IMI (CAS no. 138261–41-3, purity ≥ 98.0 %) and GLY (CAS no. 1071-83-6, purity ≥ 98.0 %) were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). Stock solutions were prepared in dimethyl sulfoxide (DMSO) for CPF (12 mM) and IMI (16 mM), whereas GLY was dissolved in water (23 mM).

3.3 Assessment of effects on cell viability and metabolism

3.3.1 Cell viability and proliferation assay

In order to detect the amount of metabolically active cells and the amount of DNA in the cells, which not necessarily correlates with cellular metabolic activity, MTS assay and fluorimetric CyQuant® assay were performed.

The colorimetric MTS assay (Cell Titer 96 Aqueous One Solution reagent; Promega, Madison, WI, USA) is based on the cell capacity to reduce tetrazolium salts to formazan by dehydrogenases enzymes [161] (Fig. 13).

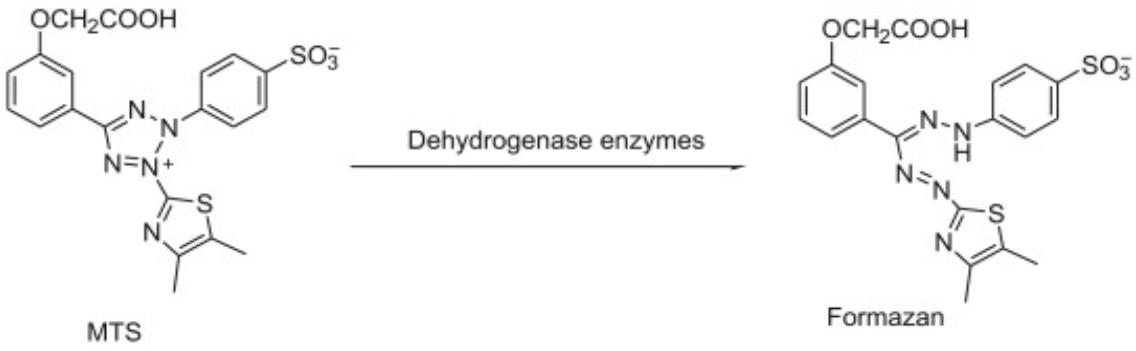


Figure 13. Enzymatic conversion to formazan. The image was extracted from [162].

The fluorimetric CyQuant® assay (CyQuant® Direct Cell proliferation Assay; Life Technologies, Paisley, UK) [163] is based on a cell-permeant DNA-binding dye in combination with a background suppression reagent. The masking dye blocks staining of dead cells with compromised cell membranes, inducing only DNA staining of healthy cells (Fig. 14).

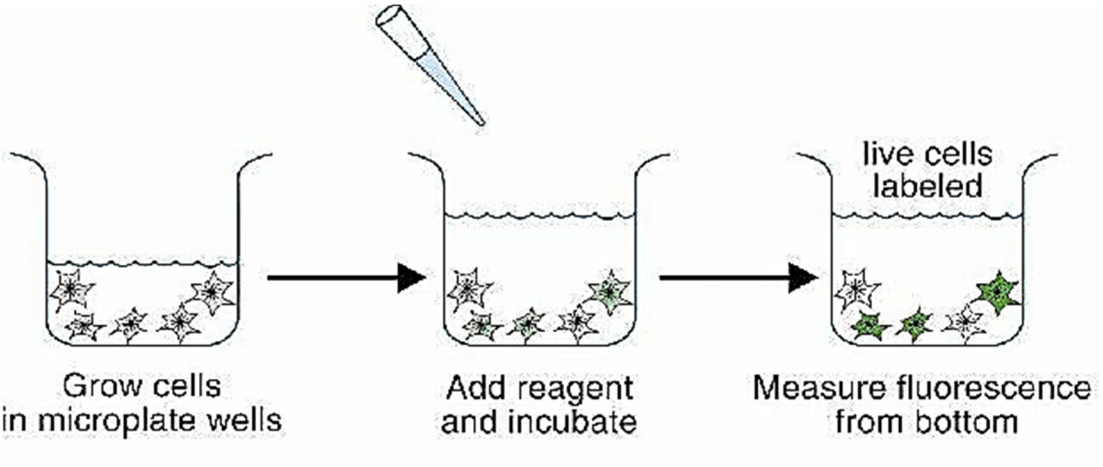


Figure 14. CyQuant® Direct Cell proliferation Assay. The assay uses a DNA-binding dye in combination with a masking reagent inducing only DNA staining of healthy cells. The image was extracted from www.thermofisher.com.

According to manufacturers' protocols, for each assay 10,000 cells/well were plated in 96 flat-bottomed multiwells and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion. Medium was then replaced with fresh medium added with five ten-fold serial dilutions of CPF (120 pM - 1.2 nM - 12nM – 120 nM - 1.2 μM), IMI (160 pM – 1.6 nM – 16 nM – 160 nM –1.6 μM), GLY (230 pM – 2.3 nM -23 nM – 230 nM - 2.3 μM) or medium alone as control, in triplicated wells, incubating cells for 72h at 37 °C. Pesticide stock solutions were diluted with culture medium immediately before use; since DMSO final concentration not exceeded 0.01% (v/v) being not toxic, we did not include solvent control cells in the design [164]. For each pesticide, the range of concentrations used was established starting from the mean exposure levels detected in urine samples of children and reported in biomonitoring studies, i.e., CPF 12 nM, IMI 16 nM and GLY 23 nM [115-119]. After incubation, 20 μl of MTS reagent or 100 μl (equal volume as culture media) of 2X CyQuant Detection Reagent were added to each well, incubating 60 min at 37 °C.

The Victor 3 Multilabel Reader (PerkinElmer, MA, USA) was used to read absorbance at 490 nm (MTS assay) or fluorescence from bottom with a green filter (485nm excitation - 535 nm emission) (CyQuant assay), setting control cells as 100% viable. Three independent experiments were performed for each assay

and the GraphPad Prism v 5.01 software (GraphPad Software Inc., La Jolla, CA, USA) was used to derive and visualize the best curve fit for each assay.

3.3.2 Apoptosis-Necrosis assay

Apoptosis and necrosis are biological processes leading to cell death. Apoptosis, also called programmed cell death, is an active process involving specific metabolic pathways, leading to the activation of caspases (proteases) which digest the proteins of the cytoskeleton causing loss of contact between the cells, chromatin condensation and degradation, and formation of cell apoptotic fragments. Necrosis defines a process of involuntary death after a traumatic "insult" such as treatment with chemical agents or pathogens resulting with a release of inflammatory factors and morphological changes and organelles disruption [165].

In our study the apoptosis and necrosis processes were evaluated by using the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay kit (Promega). This assay is a real-time live-cell (non-lytic) assay that measures the exposure of phosphatidylserine (PS) on the outer leaflet of the cell membrane during the apoptotic process. When the flip occurs, PS binds to the detection reagent containing two annexin V fusion proteins with complementary units of a luciferase emitting a luminescence signal when in close proximity. If apoptosis progresses toward necrosis, the cell membrane gets damaged and the cell-impermeant profluorescent DNA dye may enter the cells and emit a fluorescence signal (Fig. 15).

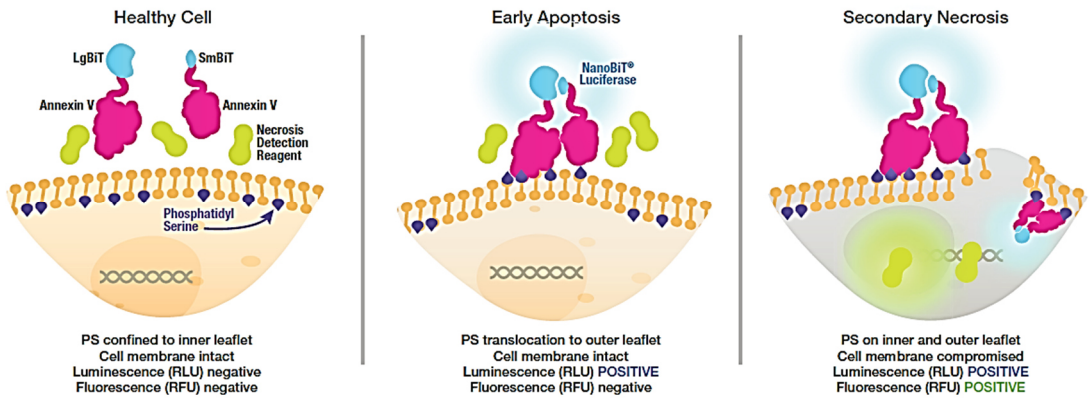


Figure 15. Annexin V Apoptosis and Necrosis Assay. This assay allows real-time bioluminescence detection of PS exposure on apoptotic cells. Time-dependent increases in luminescence that occur before increases in fluorescence reflect the apoptotic process. Increases in both luminescence and fluorescence signals indicate secondary necrosis following apoptosis or other non-apoptotic mechanisms. The image was extracted from www.promega.com.

The assay was used following the manufacturer's protocol. About 10,000 MCF-7 and MCF-12A cells/well, diluted in 100 µl/well, were seeded on 96-well solid white bottom microplates. The next day, medium was removed and cells were treated with five ten-fold serial dilutions of CPF (120 pM- 1.2 µM), IMI (160 pM–1.6 µM), GLY (230 pM- 2.3 µM) or medium alone as control, also adding 100 µl of kit's reagents mix solution. Plates were read for both luminescence and fluorescence (485-535 nm) signals by Victor 3 Multilabel Reader each hour for the first 8h and then at 24h of exposure normalizing values to control cells readings for each incubation time. The assay was performed in triplicate.

3.3.3 ATP levels assessment

Adenosine 5'-triphosphate (ATP) represents the major energy unit of the cell and is involved in multiple cellular processes. Intracellular ATP concentrations can change in response to different types of stimuli, such as nutrients, hormones, and cytotoxic agents [166].

In order to investigate whether CPF, IMI and GLY were able to influence intracellular ATP levels, the ToxGlo™ mitochondrial assay (Promega) was performed (Fig. 16).

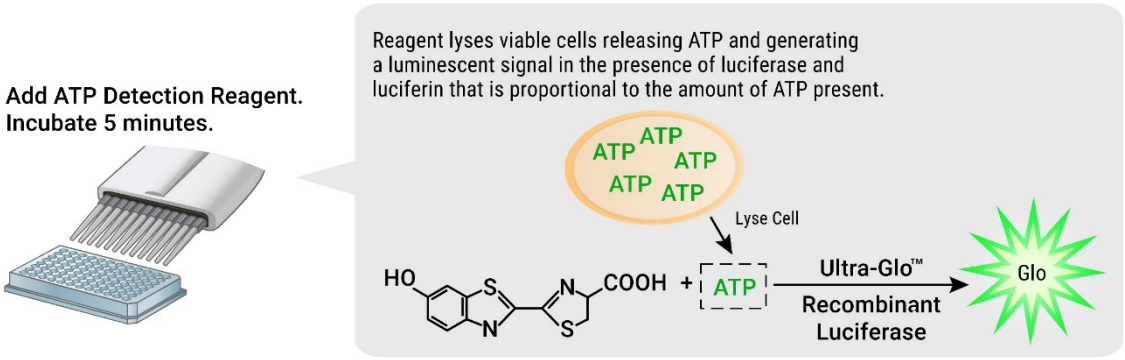


Figure 16. ToxGlo™ mitochondrial assay. ATP is quantified by using an ATP-sensing reagent, leading to cell lysis and generation of a luminescent signal proportional to the amount of ATP present.

According to manufacturer's protocol, about 10,000 MCF-7 or MCF-12A cells/well were plated in 96 flat-bottomed multiwells and incubated overnight at 37 °C to permit cell adhesion. The experimental design (i.e., cell treatment and pesticide concentrations) was the same as for cell viability/proliferation assessment (see paragraph 3.3.1). After 72h incubation, 100µl of ATP Detection Reagent was added to each well stirring at 500 rpm for 5 minutes. Luminescence was then read on a Victor 3 Multilabel Reader (Perkin Elmer), normalizing readings with respect to control cells set at 100%. Three independent experiments were performed and GraphPad Prism v 5.01 (GraphPad Software Inc.) was used to derive and visualize the best curve fit.

3.3.4 Reactive Oxygen Species assay

ROS are principally produced by mitochondria during physiological cellular respiration. Under normal conditions, the cell produces free radicals through various processes, the most important being oxidative phosphorylation, which is crucial for energy production in the form of ATP. As a consequence of this metabolic cycle, superoxide anion (O_2^-) is constantly formed as a natural product of mitochondrial respiration. Other examples of ROS are hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen (O_2). Free radicals easily react with several types of molecules, such as carbohydrates, lipids, proteins, nucleic acids, damaging them and often compromising their function. Generally, the lifetime of ROS in biological systems is only a few seconds, so methods developed to detect mitochondrial and cellular ROS require probes that react very rapidly with them producing stable products.

To evaluate the intracellular ROS levels, a fluorometric 2',7'-dichlorodihydrofluorescein diacetate assay (ROS Detection Assay Kit, BioVision, Milpitas, CA) was performed according to manufacturer's protocol. The assay allows the assessment of intracellular H_2O_2 formation through a dichlorofuorescein probe. In living cells, the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is modified by cellular esterases to form a non-fluorescent H_2DCF. After oxidation by intracellular ROS, H_2DCF will produce the highly fluorescent product DCF (Ex/Em 495/529 nm) (Fig. 17).

About 10,000 MCF-7 or MCF-12A cells/well were plated in 96 flat-bottom microplates and incubated overnight allowing adhesion. The next day, medium was removed and 100µl/well of ROS Assay Buffer were added to wash the cells; after buffer removal, 100µl/well of cell permeant ROS Assay Label 1X were added, incubating 45 min at 37 °C. Excess ROS label solution was then removed and cells were treated for 24h with CPF (at 1.2-12-120 nM), IMI (at 1.6-16-160 nM), GLY (at 2.3-23-230 nM) or medium alone as control. H_2O_2 100 µM was used as a positive control. At the end of treatment, plates were read by the Victor 3

Multilabel Reader (PerkinElmer) measuring fluorescence from bottom at 485 nm of excitation - 535 nm of emission, setting control cells as 100%.

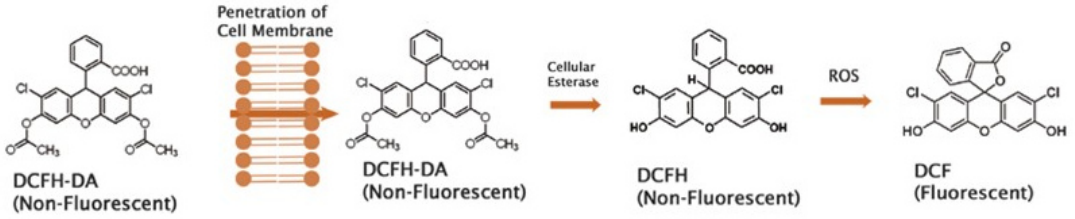


Figure 17. Mechanism of conversion of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) to fluorescent DCF in the presence of ROS.

3.4 Assessment of endocrine activity

3.4.1 Cellular treatment

MCF-7 and MCF-12A cells were plated in culture flasks and incubated overnight at 37 °C, 5% CO_2 and 95% humidity to permit cell adhesion. Then, cells were treated with CPF (1.2, 12 or 120 nM), IMI (1.6, 16 or 160 nM), GLY (2.3, 23 or 230 nM), or medium alone as control for 72h at 37 °C. Three independent experiments were performed for each pesticide, at different replication passages from thawing. At the end of incubation, cell monolayers and supernatants were collected and stored at -80 °C until use.

3.4.2 Estradiol ELISA assay

17β-estradiol (E2) is synthesized in several tissues or organs, such as ovaries, placenta, breast, brain and adipose tissue [167,168]. E2 is essential for the development of the female reproductive organs and secondary sex characteristics, including mammary gland.

MCF-7 and MCF-12A culture supernatants were assessed for 17β-estradiol (E2) secretion by the Estradiol parameter assay kit (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. By the provided E2 standard solution, six three-fold serial dilutions (3000 pg/ml - 12.3 pg/ml) were prepared and assessed along with the samples in duplicated wells for both cell lines. Absorbance was

read at 450 nm by Victor 3 Multilabel Reader (PerkinElmer); unknown E2 concentrations in samples were derived from the standard curve by the Graph Pad Prism v 5.01 software (GraphPad Software Inc.).

3.4.3 Real-time PCR

The effect of CPF, IMI and GLY on gene expression of specific nuclear receptors involved in mammary gland development (ERα, ERβ, AR, PgR and AhR), was assessed by real time-PCR analysis.

Total RNA content was extracted by control and treated MCF-7 and MCF-12A cell pellets using the Norgen RNA kit (Norgen, Thorold, Canada) according to manufacturer's protocol. Nabi Nano Spectrophotometer (MicroDigital Co., Ltd, Korea) was used to determine RNA quantity.

An aliquot of total RNA (1 μg) from each sample was reverse transcribed to cDNA using the SensiFAST cDNA Synthesis Kit (Bioline Reagents Ltd., London, UK). Specific primers for the selected panel of genes (ERα, ERβ, AR, AhR, and PgR) as well as for GAPDH and ACTB as reference genes, were designed through the web application Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences are listed in Table 1. Reactions were run in duplicate on 96-well PCR plates using the SensiMix SYBR kit (Bioline). The thermal program was as follows: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min; 1 dissociation cycle from 55 to 95 °C (30 s/°C) to verify amplification products. Threshold cycles (Ct) were obtained by the LineGene 9600 PCR V.1.0 software (Bioer) and $\Delta\Delta$Ct values were calculated using control cells as calibrators and the geometric mean of housekeeping genes Ct values as normalizer.

Gene	*RefSeq accession*		*Sequence 5′ to 3′*
GAPDH	NM_002046.4	fw	ACTCCTCCACCTTTGACGCT
		rev	CTTCAAGGGGTCTACATGGC
ACTB	NM_001101.5	fw	CAGCAAGCAGGAGTATGACG
		rev	GTGAACTTTGGGGGATGCTC
ERα	NM_000125.3	fw	ACTGCGGGCTCTACTTCATC
		rev	GGCTGTTCCCAACAGAAGAC
ERβ	NM_001040275.1	fw	CTCTTTTGCCTGAAGCAACG
		rev	CTGGGCAGTTAAGGAGACCA
AR	NM_000044.3	fw	CCCATCTATTTCCACACCCA
		rev	GCAAAGTCTGAAGGTGCCAT
AhR	NM_001621.4	fw	TTCCACCTCAGTTGGCTTTG
		rev	GGACTCGGCACAATAAAGCA
PgR	NM_000926.4	fw	AGGTCTACCCGCCCTATCTC
		rev	AGTAGTTGTGCTGCCCTTCC

Table 1. Sequences of the specific primers used in Real time PCR analysis with accession numbers of the corresponding genes.

3.5 3D *in vitro* model

3.5.1 Hydrogel cell culturing

In the set-up of bioactive materials that best represent the ECM, one of the factors to consider is the ability of the cells to bind the scaffold. For this purpose, one of the most widely used peptides is an arginine-glycine-aspartate amino acid sequence, or "RGD", capable of promoting cell adhesion. Cell adhesion proteins present on the cell membrane, as well as integrins, recognize this sequence and bind to it.

The set-up of 3D cell cultures was achieved with VitroGel® RGD High Concentration hydrogel (TheWell Bioscience Inc. North Brunswick, NJ). This system is a permeable, tunable, xeno-free modified with cell adhesive peptide RGD to promote cell attachment with a neutral pH.

The MCF-7 cells were harvested using trypsin and suspended in a complete DMEM medium; cells were distributed in 96 plates (Greiner Bio-One) at different concentrations, including 5×10^3, 7.5×10^3 and 1.0×10^4 to optimize the number of cells needed to achieve the formation of the spheroid.

To perform the 3D cell culture in 96-well plate, the hydrogel was firstly allowed to warm up to 37°C. In the meantime, a dilution solution of PBS and Millipore distilled water was prepared at 1: 1 (v / v) ratio and placed at 37 ° C. Then, hydrogel and dilution solution were mixed in the ratio 1: 3 (v / v). A cell suspension containing 40% FBS was prepared to have 10% FBS cells in the final hydrogel solution. The hydrogel cell suspension solutions were then mixed in the ratio 3:1 (v/v), adding 50 μL of the hydrogel-cell mixture to each well of a 96 well plate. The plate was incubated 30 minutes at room temperature for hydrogel formation, without tilting. 50 μL DMEM + 10% FBS were then carefully added on top of the hydrogel incubating at 37 ° C overnight The next day 25 μL of complete DMEM was added to each well. On the third day of incubation, approximately 80% DMEM was removed and 75 μL of complete

DMEM was added to each well (Fig. 18). Cells were incubated for spheroid formation and subsequently observed.

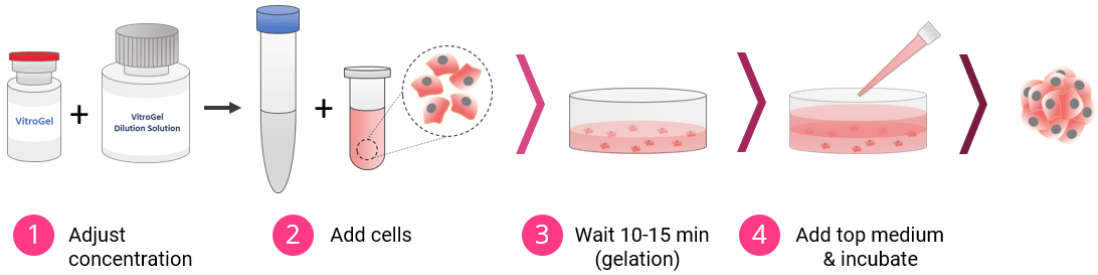


Figure 18. Schematic illustration of 3D culture preparation with hydrogel system. The image was extracted from www.thewellbio.com.

3.5.2 Magnetic cell culturing

The magnetic 3D cell culture was obtained by using 96 well Bioprinting Kit and 6 well Bio-Assembler Kit, Levitating Drive (Greiner Bio-One S.r.l, Italia). This technology is based on the use of 50 nm diameter nanoshuttles made of gold, iron oxide and poly-L-lysine. These nanoparticles magnetize cells by electrostatically binding to cell membranes during an overnight incubation. Both nanoparticles and magnetic forces have been shown to have no effect on cellular activity including proliferation, viability, metabolism and oxidative stress.

Specifically, with the magnetic 3D bioprinting system, cells are printed into spheroids by placing a microplate containing magnetized cells on top of a magnetic unit which induces cell aggregation and spheroid printing on the bottom of each well, in a time ranging from about 15 minutes to several hours. After, the magnet is removed and the spheroid is cultured normally. By magnetic levitation, cells are levitated from the bottom of the plate using a magnet placed on top. When cells levitate, the magnetic forces act as an invisible scaffold that rapidly aggregates the cells to harden cell-cell interactions and ECM synthesis [169-173].

This method was applied only to MCF-12A by firstly plating cells in T-25 culture flasks and make them grow till 80% confluence. Then, following manufacturer's protocol, 1 μL Nanoshuttle TM-PL was added for each 10^4 cells, incubating overnight at 37 °C, 5% CO_2 and 95% humidity to permit Nanoshuttle TM-PL adhesion to cell membrane. The next day, MCF-12A cells were harvested using trypsin and resuspended in complete DMEM/F12 medium. Then, cells were distributed in CELLSTAR® Cell-Repellent 96 or 6-Well Plates (Greiner Bio-One) at different concentrations, including 5×10^3, 1.0×10^4, 1.5×10^4 and 2.8×10^5, 3.2×10^5 and 3.6×10^5 cells in 100 μL and 2 mL respectively, to optimize the number of cells needed to achieve the formation of the spheroid. The levitating drive is then placed on the top of the closed plate and transferred into the incubator (Fig.19) for spheroid formation.

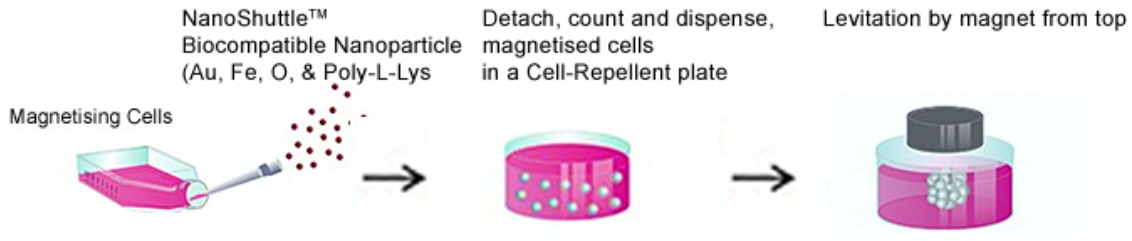


Figure 19. Schematic spheroid formation with magnetic levitation.

3.6 Statistical analysis

Data were analysed by JMP 10 Software (SAS Institute Inc., Cary, NC, USA). Statistical differences among treated and control groups were evaluated by one-way analysis of the variance (ANOVA) followed by post-hoc Dunnett's test where appropriate. The significance threshold was set at $P<0.05$ for all the tests.

3.7 Results

3.7.1 Cell viability and cell proliferation

Cell viability of MCF-7 cells treated with CPF, IMI and GLY was dose-dependently decreased with similar patterns by the three pesticides (Fig. 20). Specifically, CPF and IMI significantly decreased cell viability at all concentrations tested except for CPF 120 pM and IMI 160 pM, whereas GLY induced a significant decrease in cell viability at 2.3 nM, 230 nM and 2.3 μM concentrations. The pesticides did not significantly affect cell proliferation except for CPF 1.2 μM and GLY 230 pM.

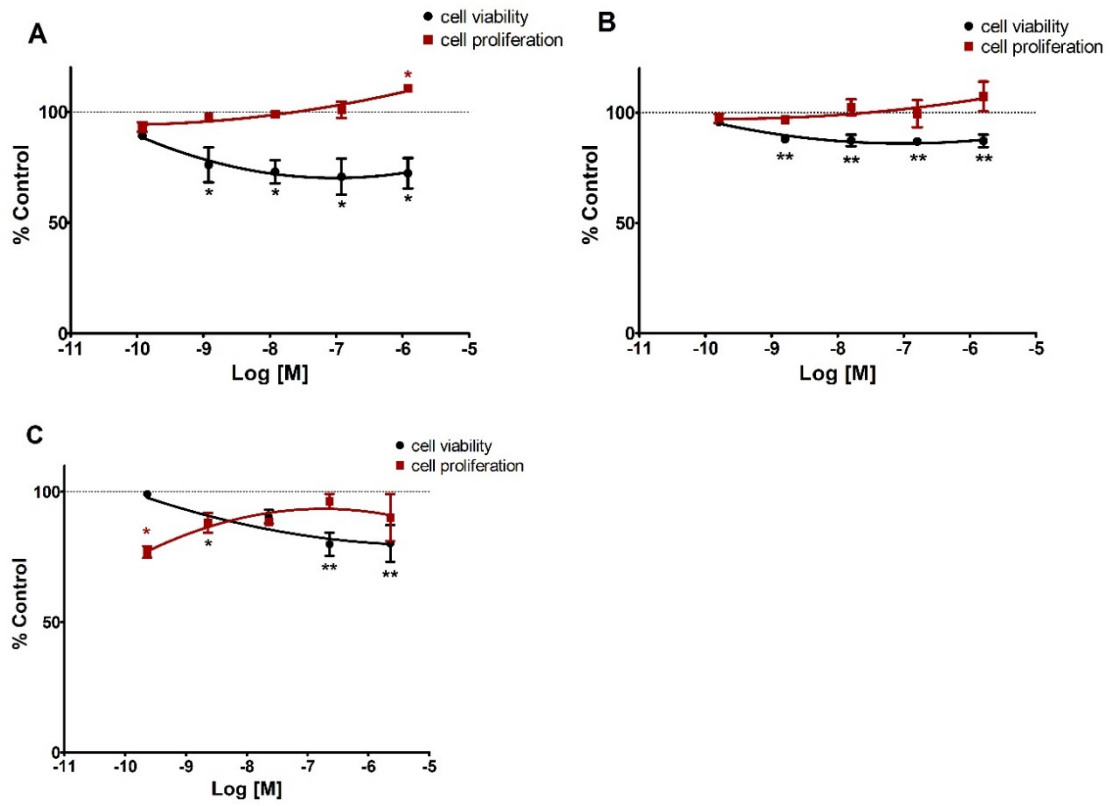


Figure 20. Dose-response curves for cell viability (black) and cell proliferation (red) assays in MCF-7 cells treated for 72h with CPF (A), IMI (B) or GLY (C). Values are means ± SEM of three independent experiments with the control set at 100%. Log [M] -8 value corresponds to 12 nM CPF, 16 nM IMI and 23 nM GLY. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$.

Similarly to MCF-7, also in MCF-12A cell viability was dose-dependently decreased, with similar patterns, by the three pesticides, being significant in cells treated with CPF at the two highest doses of (120 nM and 1.2 μM), and IMI at 160 pM. Cell proliferation was significantly reduced by CPF at all tested concentrations, by IMI at the highest dose (1.6 μM) and by GLY at the higher doses (23 nM, 230 nM and 2.3 μM) (Fig. 21).

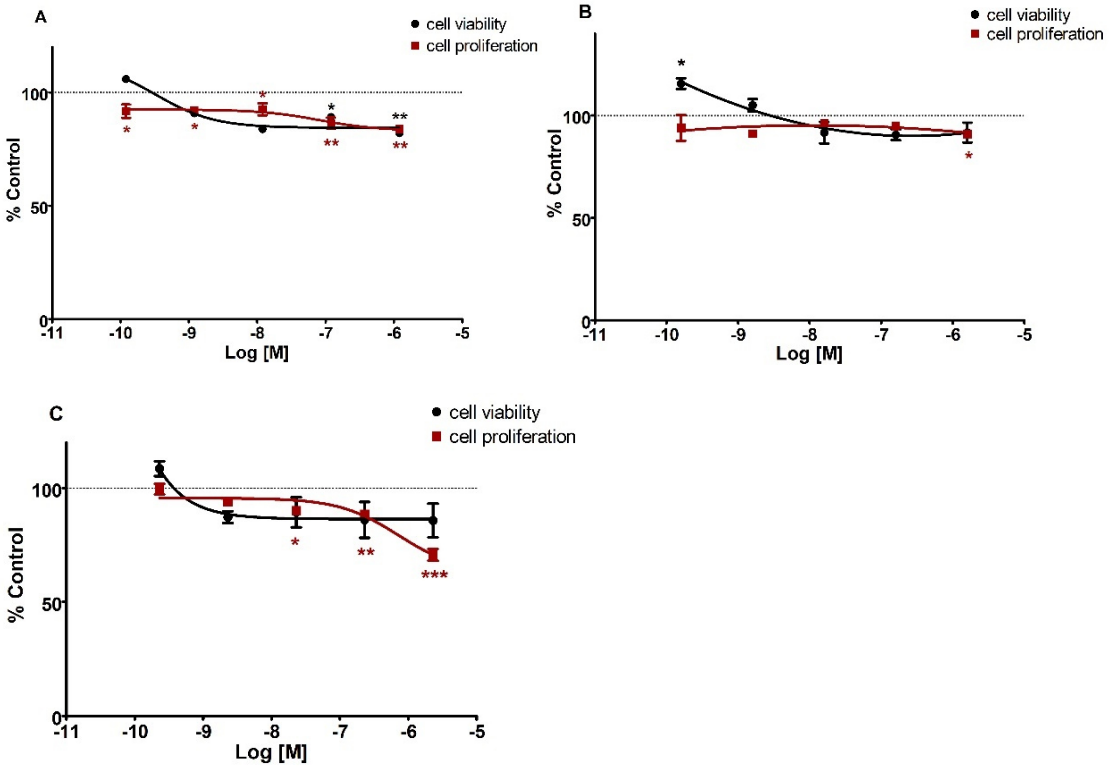


Figure 21. Dose response curves for cell viability (black) and cell proliferation (red) assays in MCF-12A cells treated for 72h with CPF (A), IMI (B) or GLY (C). Values are means ± SEM of three independent experiments with the control set at 100%. Log [M] -8 value corresponds to 12 nM CPF, 16 nM IMI and 23 nM GLY. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7.2 Apoptosis and necrosis time course

Treatment with CPF, IMI and GLY did not induce statistically significant differences in apoptotic and necrotic signals in MCF-7 cells, at all tested concentrations (Fig. 22).

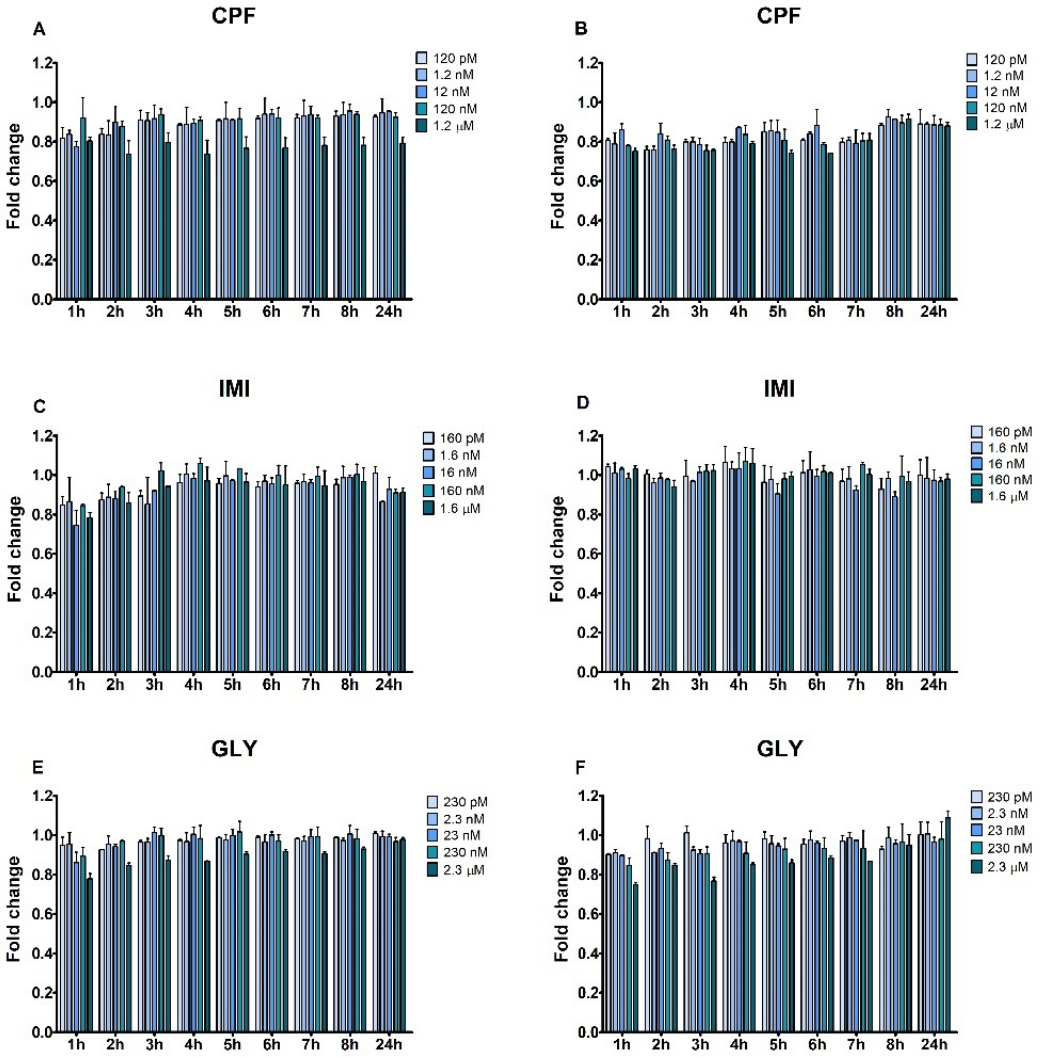


Figure 22. Apoptotic and necrotic signals evaluated by Annexin V assay in MCF-7 cells treated with CPF (A, B), IMI (C, D) and GLY (E, F) or medium alone as control for 24h. Values are fold change of three independent experiments.

MCF-12A cells treated with CPF showed a statistically significant decrease in apoptotic signal after 6h at 120 nM, 8h at 12 nM, and 24h at 12 nM and 120 nM. Also IMI induced a decrease in apoptotic signal after 8h at 16 nM and 1.6 μM.

On the contrary, GLY treatment determined an increase in apoptotic signal at all concentrations except 2.3 μM, but with a statistical significance only at 7 and 8h at 2.3 nM. No significant necrotic effect was observed in MCF-12A cells (Fig. 23).

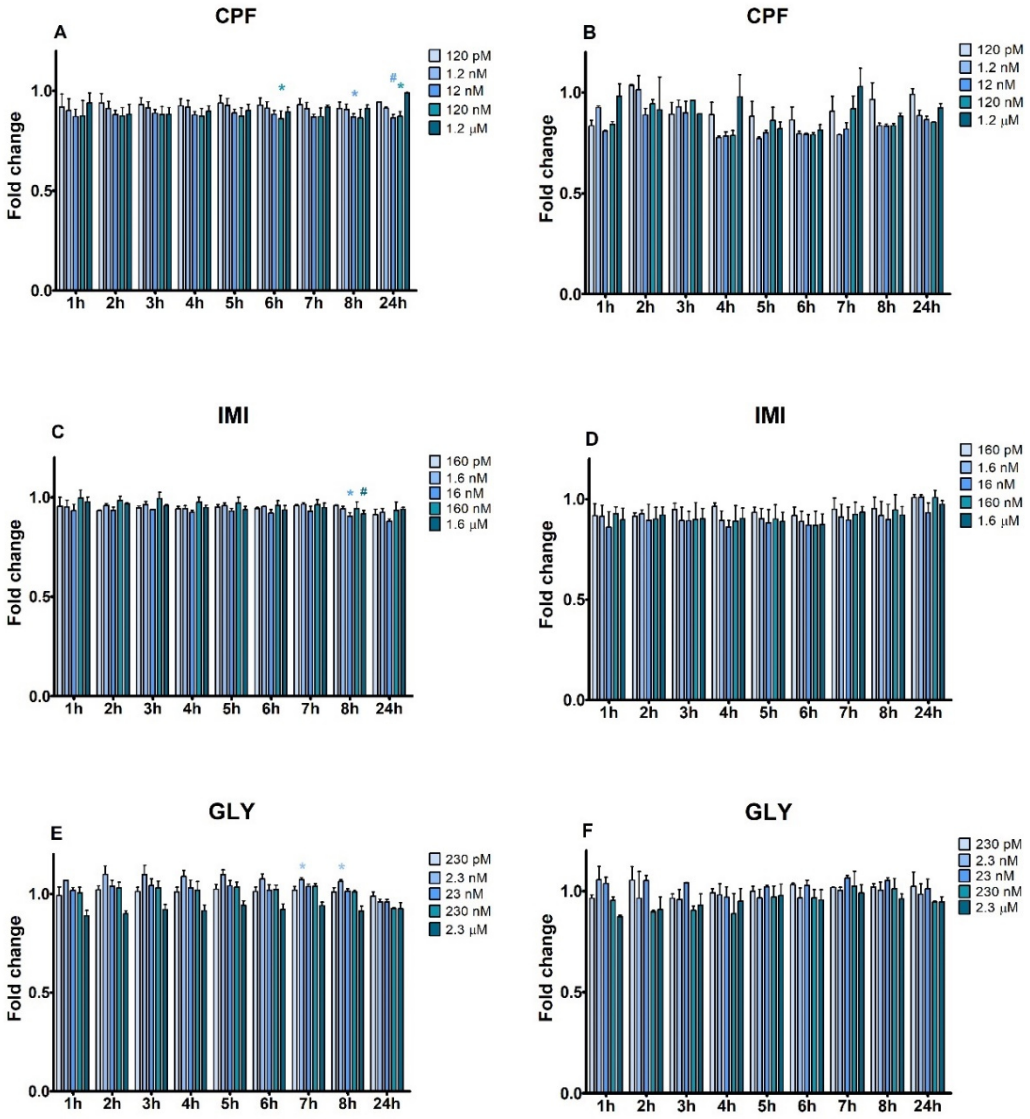


Figure 23. Apoptotic and necrotic signals evaluated by Annexin V assay in MCF-12A cells treated with CPF (A, B), IMI (C, D) and GLY (E, F) or medium alone as control for 24h. Values are fold change of three independent experiments. Asterisks and hashtags indicate statistically significant differences with respect to control cells: * $p < 0.05$; # $p < 0.01$.

3.7.3 ATP levels

The intracellular ATP levels were evaluated in MCF-7 and MCF-12A after 72h of exposure to three pesticides. In MCF-7 cells, all three pesticides induced a

decrease in intracellular ATP levels but with different patterns: a comparable decrease at all concentrations for IMI, concentration-dependent decrease for GLY, and inverse dose-related effect for CPF. Specifically, CPF and IMI determined a significant decrease in intracellular ATP levels at all tested concentrations, whereas GLY at the two highest concentrations (230 nM and 2.3 μM) (Fig. 24).

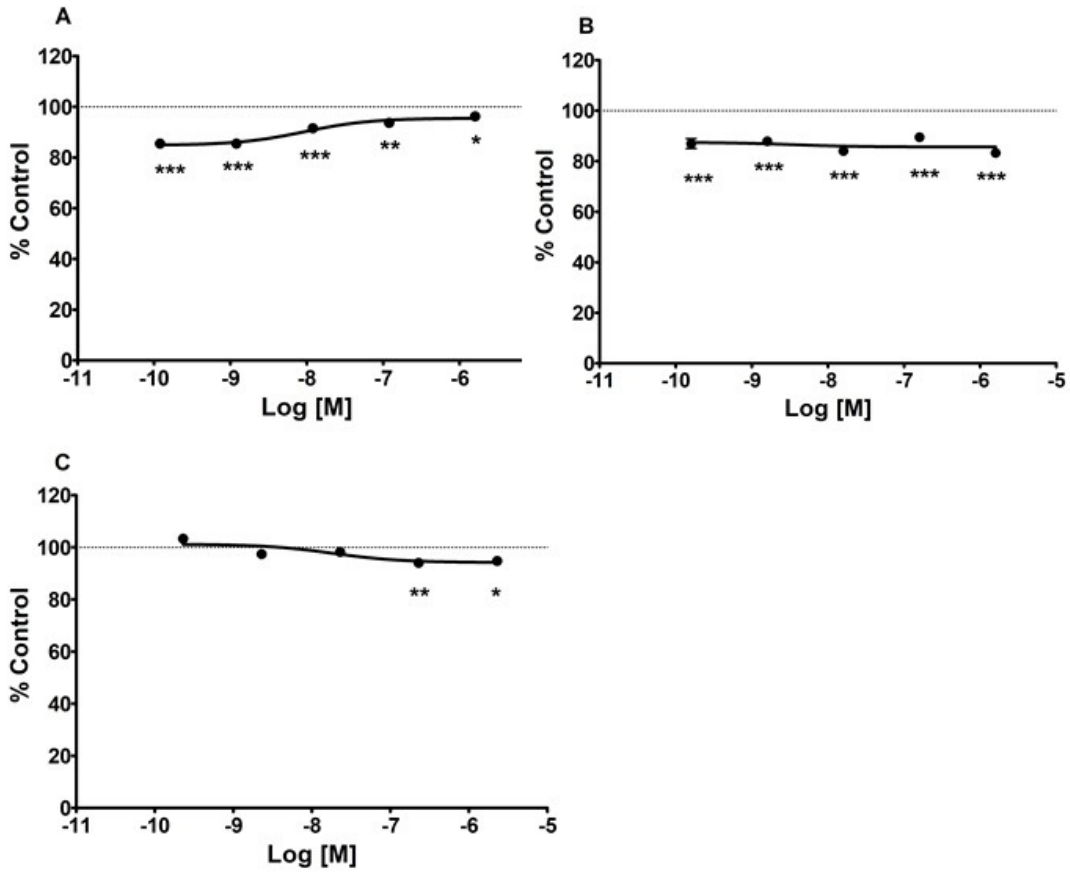


Figure 24. ATP levels in MCF-7 cells treated for 72h with CPF(A) IMI (B) or GLY (C). Values are means ± SEM of three independent experiments with control cells set at 100%. Log [M] -8 value corresponds to 12 nM CPF, 16 nM IMI and 23 nM GLY. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In MCF-12A cells (Fig. 25) the pesticides did not affect intracellular ATP levels.

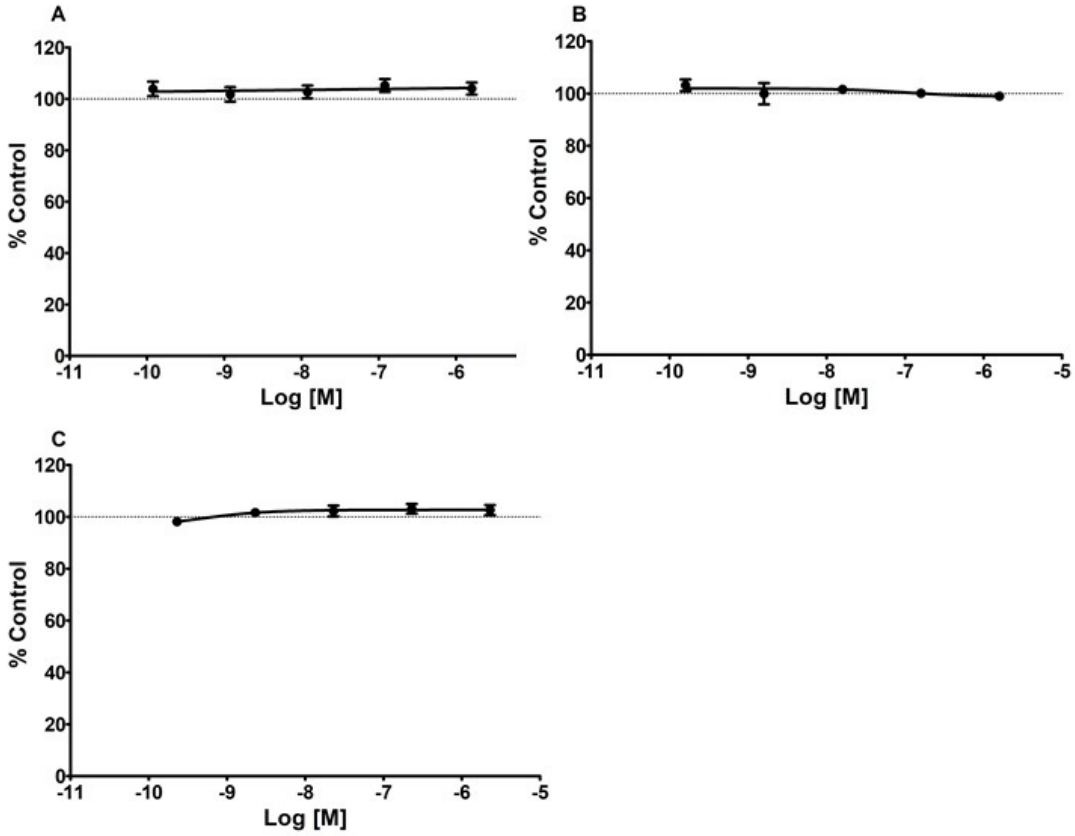


Figure 25. ATP levels in MCF-12A cells treated for 72h with CPF(A) IMI (B) or GLY (C). Values are means ± SEM of three independent experiments with control cells set at 100%. Log [M] -8 value corresponds to 12 nM CPF, 16 nM IMI and 23 nM GLY. Asterisks indicate statistical significance: * $p < 0.05$.

3.7.4 Reactive Oxygen Species

CPF, IMI and GLY differently affected intracellular ROS production after 24h of exposure in MCF-7 and MCF-12A cell lines, as shown in Figure 26 A and B, respectively. Treatment of MCF-7 cells with CPF at 12 and 120 nM induced a significant decrease of ROS production, whereas IMI at 1.6 nM determined a slight significant increase in intracellular ROS production. GLY induced no effects at any tested concentration. In MCF-12A cells, the three pesticides induced a statistically significant reduction in intracellular ROS species compared to control cells, at all concentrations following treatment with CPF and GLY, and at the two highest concentrations of IMI. As expected, the positive

control H_2O_2 100 μM, determined a strong significant increase of intracellular ROS levels in both cell lines.

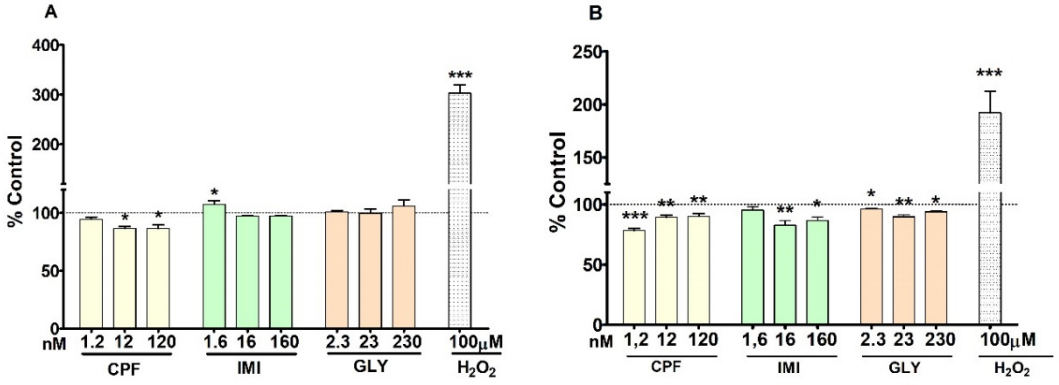


Figure 26. Intracellular ROS production in MCF-7 cells (A) and MCF-12A cells (B) treated with CPF, IMI, GLY or H_2O_2 as positive control. Values are means ± SEM of three independent experiments with control cells set at 100%. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7.5 Estradiol secretion

E2 secretion was evaluated in MCF-7 and MCF-12A cell lines after 72h of exposure to three pesticides, at three concentrations.

E2 secretion was statistically increased in MCF-7 cells by CPF at all tested concentrations, by IMI at 1.6 and 16 nM and by GLY at 2.3 nM.

In MCF-12A cells, E2 secretion was increased by CPF only at the lowest concentration and by IMI and GLY only at the highest concentration (Fig. 27).

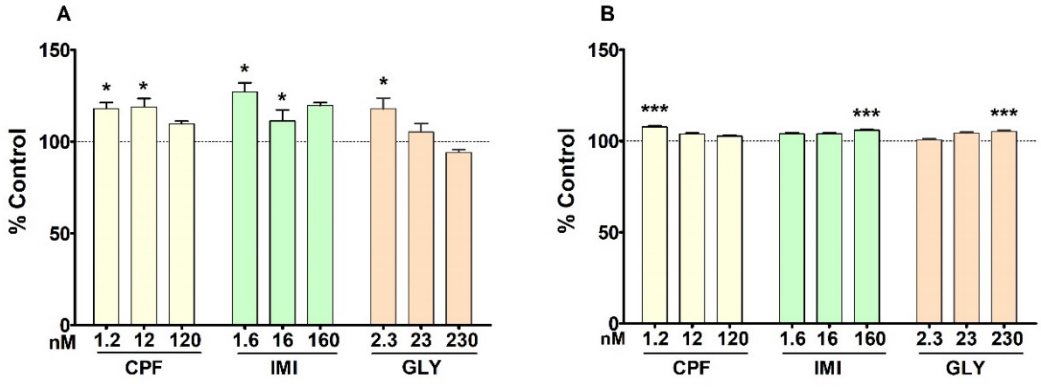


Figure 27. E2 secretion in MCF-7 cells (A) and MCF-12A (B) following treatment with CPF, IMI and GLY or medium alone as control, for 72h. Values are means ± SEM of three independent experiments with control cells set at 100%. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7.6 Nuclear receptors gene expression

The gene expression levels of the selected nuclear receptors (i.e., ERα, ERβ, AR, AhR and PgR) in MCF-7 are shown in Figure 28. ERα gene expression was significantly up-regulated by CPF and IMI at all tested concentrations and by GLY at the intermediate concentration (23 nM). Conversely, GLY at 2.3 nM significantly down-regulated ERα expression. ERβ gene expression was repressed by CPF at the lowest and highest doses (1.2 and 120 nM) and by IMI and GLY at the lowest doses (1.6 nM and 2.3 nM, respectively). The expression of AR was dose-dependently increased by GLY, being significant at the two highest doses (23 and 230 nM), by IMI at the two highest doses (16 and 160 nM), and by CPF at 120 nM. PgR gene expression was significantly down-regulated by CPF and IMI at all tested concentrations, and by GLY at the intermediate dose only (23 nM). AhR gene expression was induced following treatment with IMI at the low and intermediate concentrations (1.6 nM and 16 nM) and with GLY at the intermediate concentration (23 nM); no effect was exerted by CPF.

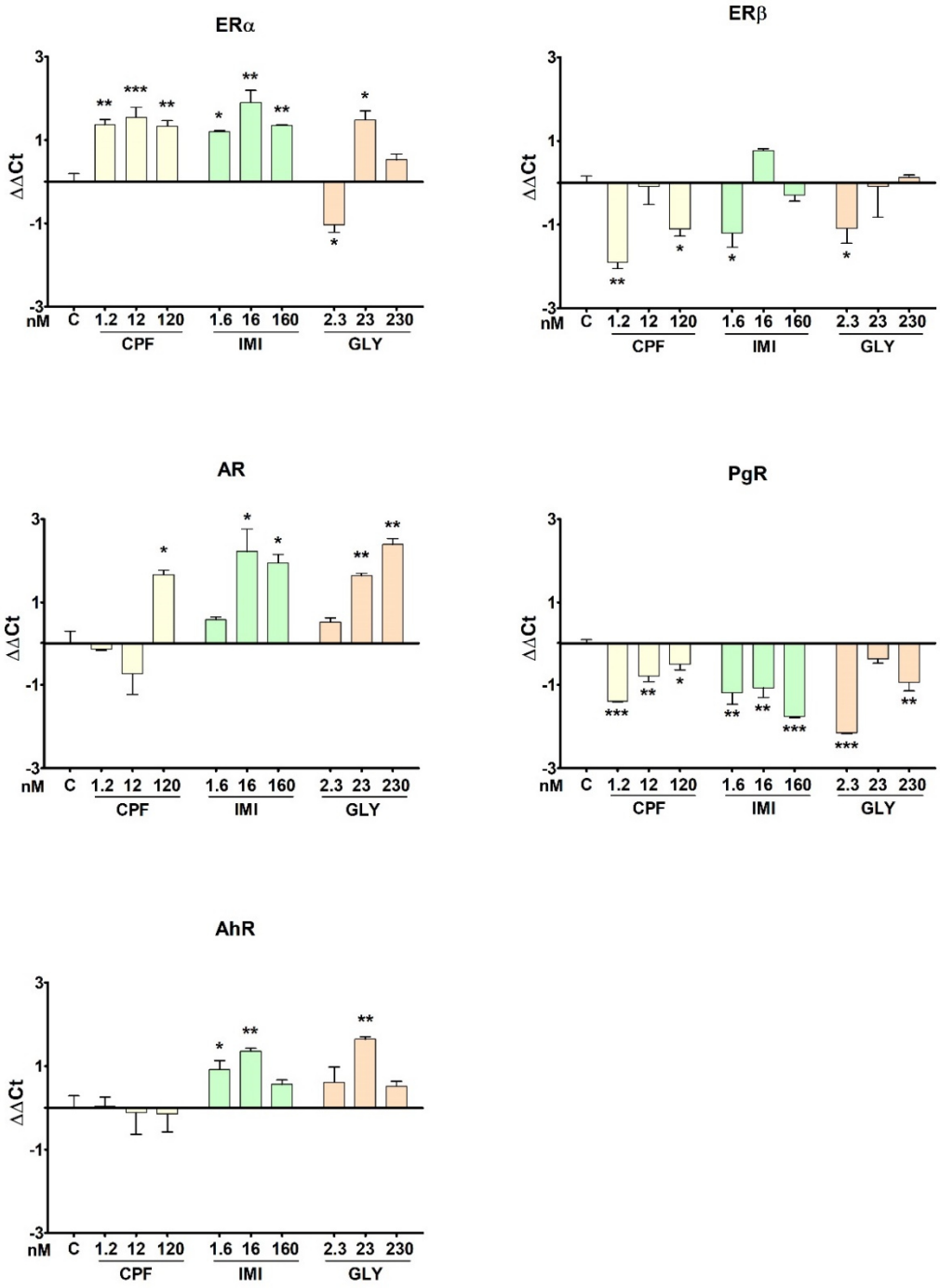


Figure 28. Gene expression levels of nuclear receptors assessed in MCF-7 cells following treatment with CPF, IMI and GLY for 72h. Data are mean ΔΔCt values ± SEM of three independent experiments, with control samples as calibrators and the geometric mean of GAPDH and ACTB as reference. Asterisks indicate the statistical significance: * $p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The effects of the three pesticides on the gene expression levels of the nuclear receptors ERα, ERβ, AR, AhR and PgR in MCF-12A are shown in Figure 29. ERα gene expression was significantly up-regulated by IMI at 16 and 160 nM and by GLY at all tested concentrations, whereas no effect was observed for CPF. ERβ gene expression was dow-regulated by CPF although being significant only at the intermediate concentration (16 nM). Conversely, ERβ gene expression was significantly up-regulated by IMI and GLY at all tested concentrations. AR gene expression was repressed by IMI at the lowest dose (1.6 nM) and by GLY at the two highest doses (23 and 230 nM). No significant effect was observed upon CPF treatment.

The expression of PgR was down-regulated by all three pesticides, although significance was observed only after treatment with IMI and GLY at all concentrations tested. AhR gene expression was decreased by GLY in a dose-dependent manner with statistical significance at lower and intermediate concentrations (2.3 and 23 nM) and by IMI at the highest dose (160 nM), while no significant effect was exerted by CPF.

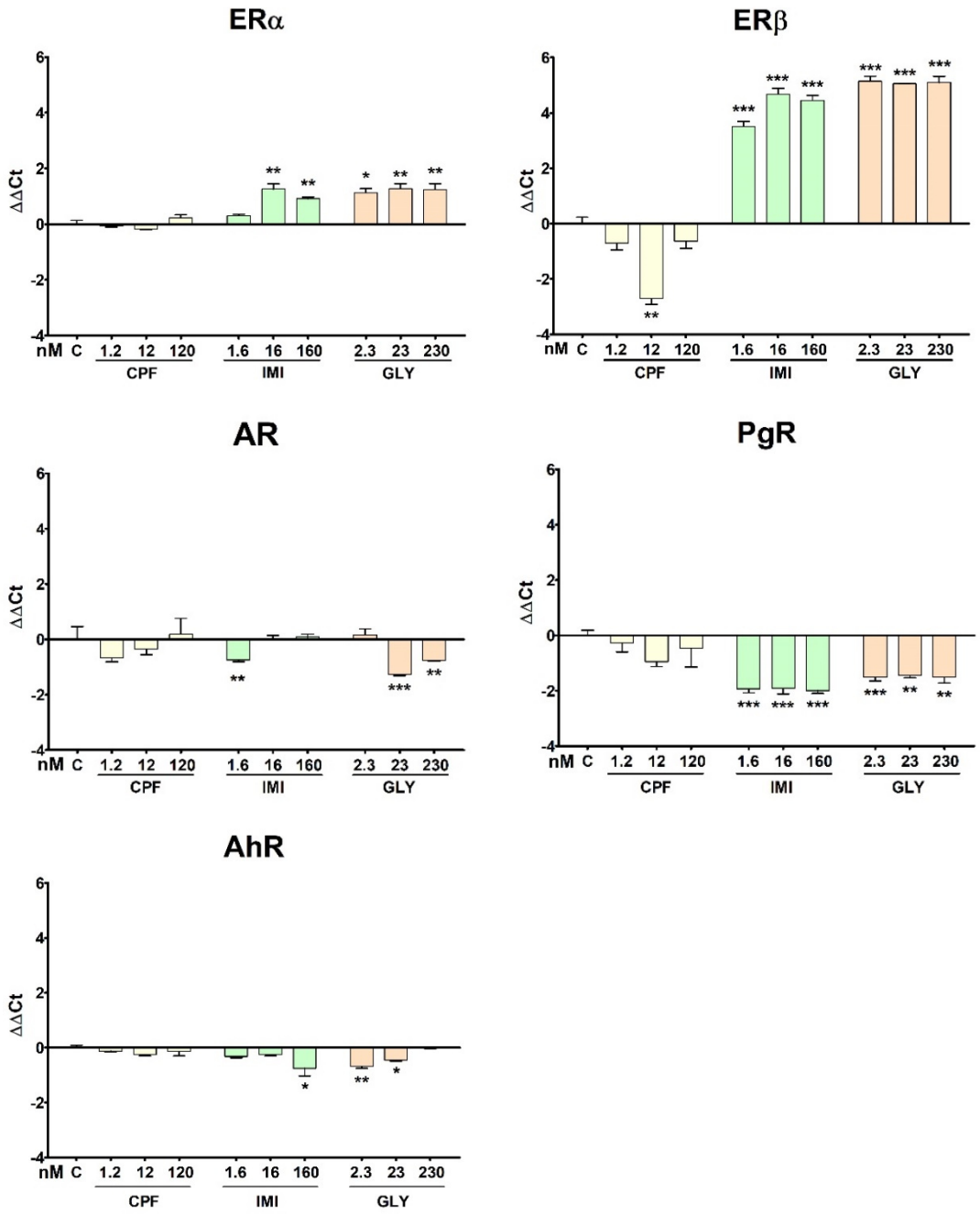


Figure 29. Gene expression levels of nuclear receptors assessed in MCF-12A cells following treatment with CPF, IMI and GLY for 72h. Data are mean ΔΔCt values ± SEM of three independent experiments, with control samples as calibrators and the geometric mean of GA GAPDH and ACTB as reference. Asterisks indicate the statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7.7 3D *in vitro* model- Hydrogel and magnetic levitation cell culturing

3D spheroids were obtained using two different methods. Hydrogel was used with MCF-7 cells and magnetic levitation with MCF-12A cells.

We observed that the optimal cell concentration for spheroid fomation using MCF-7 in hydrogel is 7.5×10^3 cells. Specifically, MCF-7 cells encapsulated in the hydrogel matrix formed spheroids of different sizes, as shown in Figure 30, after 7 days of culture.

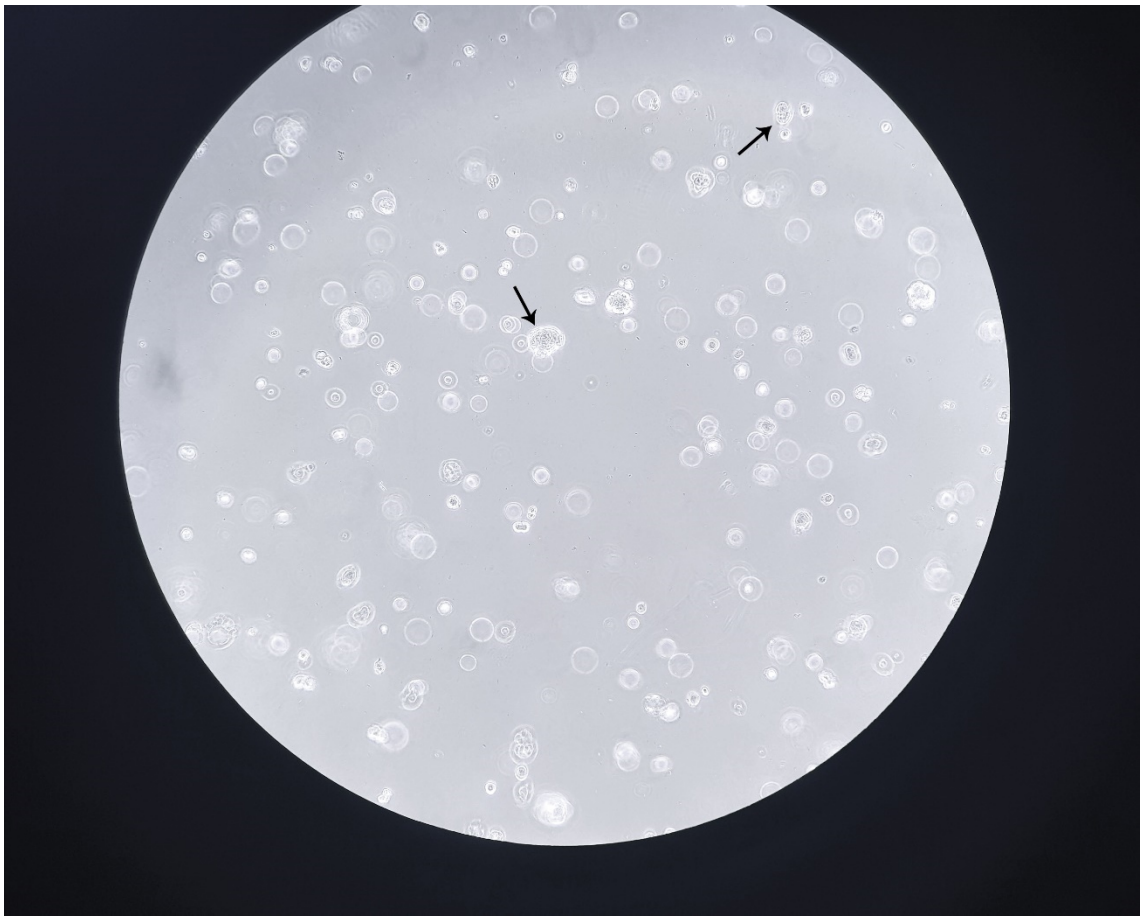


Figure 30. 3D spheroids of MCF-7 cells after 7 days of culture. The arrows indicate spheroids of different sizes.

We observed that the number of cells needed to obtain a compact, regular-shaped spheroid using MCF-12A in magnetic levitation is 3.2×10^5 cells.

MCF-12A cells cultured in magnetic levitation started to move and aggregate after about 15 min of culturing (Fig. 31 A). After about 2h (Fig 31 B), the cells were aggregated to form a spheroid and after 24h when the magnet was removed the cells formed a compact spheroid (Fig 31 C).

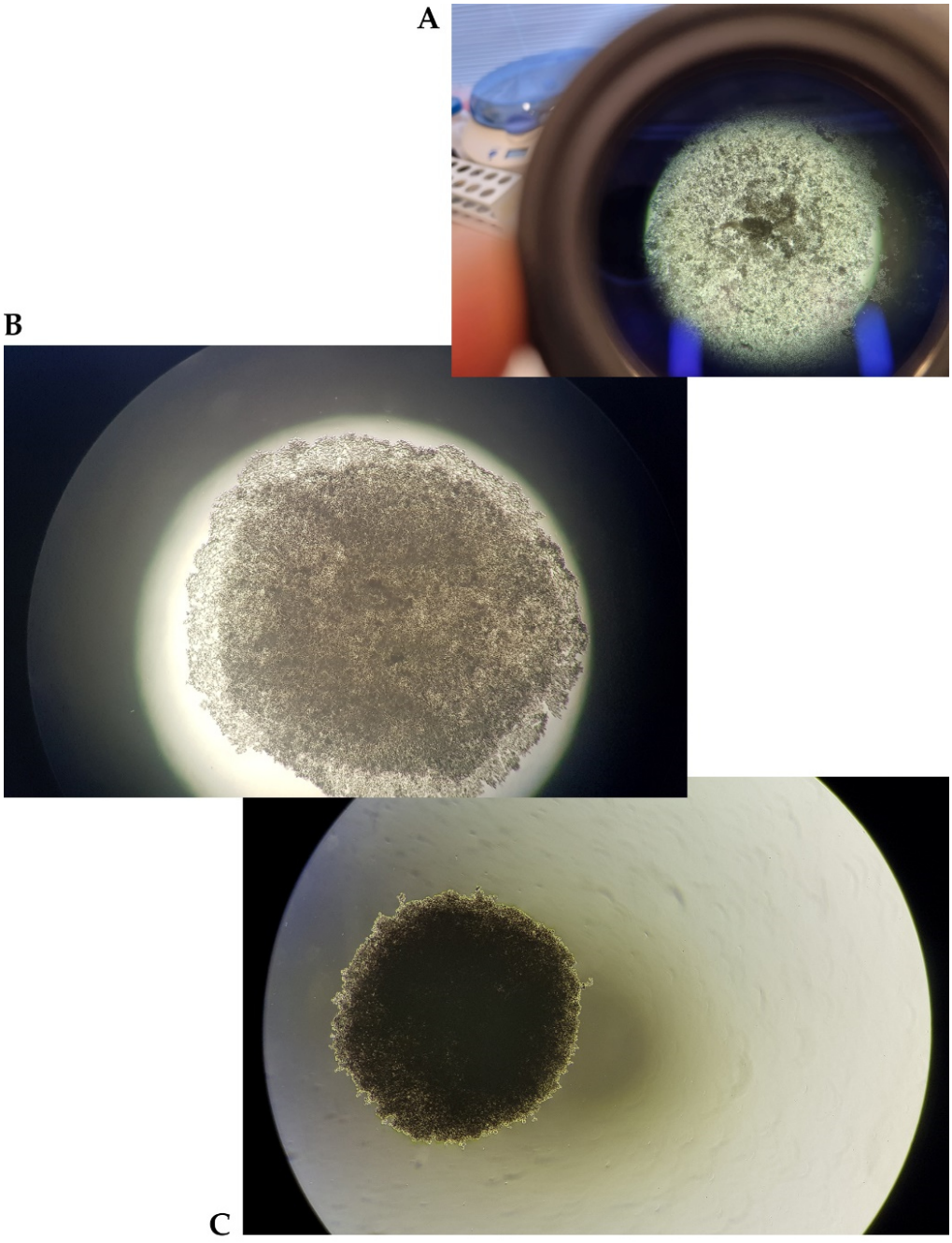


Figure 31. 3D spheroids of MCF-12A cells after 15 min in A, 2h in B and 24h in C.

3.8 Discussion

This *in vitro* study evaluated the toxicological effects of CPF, IMI and GLY exposure by comparing the response of two human breast cell lines, i.e., the MCF-7 cancer cell line and the MCF-12A non-tumorigenic cell line, as demonstrative of the effective cell condition of healthy people, to better characterize molecular alterations related to several toxicological endpoints, possibly involved in mammary gland development, which is pathologically deranged in girls affected by premature thelarche.

Among the available human non-tumorigenic breast cell lines, we selected the MCF-12A because of its capability to express ERs [174], thus being a more suitable model for the purpose of our study. Our study confirmed such expression, moreover demonstrating that MCF-12A cells also express AR, PgR and AhR. Moreover, we also verified the ability of the MCF-12A cells to grow in monolayer as a heterogeneous population of cells characterized by epithelial cells surrounded by fibroblast-like and spherical cells (Fig. 12 B), forming in a 3D model acini and ducts with an organisation comparable to those in the mammary gland, as reported in a previous study [175].

Interestingly, to our knowledge, these are the first data concerning CPF, IMI and GLY effects in MCF-12A cells.

To evaluate toxicological effects of CPF, IMI and GLY we selected exposure concentrations in the range of those really occurring in children [115-119], coincident with the nanomolar concentrations used in our experiments (i.e., CPF 12 nM; IMI 16 nM; GLY 23 nM).

Cell proliferation was differently affected in MCF-7 and MCF-12A cell lines. In MCF-7 cells, only two pesticides exerted an effect on cell proliferation at different concentrations, specifically CPF at 1.2 μM induced an increase whereas GLY at 230 pM induced a decrease in proliferation. In MCF-12A, all three pesticides induced a dependent-dose decrease in cell proliferation,

although with different patterns: CPF at all concentrations tested, IMI at the highest concentration, and GLY at the three highest concentrations tested. Our results confirmed the ability of CPF to affect cell proliferation, as observed in previous studies reporting MCF-7 cell proliferation by CPF at 50 nM for long (10 days) but not for short exposure times (24h to 96h) [110]. Moreover, our data showed for the first time that CPF exerts this effect at low concentrations, for short time of exposure (72h) and regardless of the cell model used.

IMI decreased cell proliferation in MCF-12A cells but not in MCF-7 cells. Since the same effect has been shown by an E-screen test using MCF-7 treated with higher dose, equal to 50 mg/l corresponding to about 195 μM [98], it is possible that IMI is able to affect cell proliferation differently according to the dose and cell model: at higher doses in MCF-7 but at lower doses in MCF-12A cells, postulating the latter as a more responsive model.

Similarly, GLY has been demonstrated to induce cell proliferation in MCF-7 cells at higher concentrations (> 500 μM) [98] than those tested in this study; however, our results showed no effect in MCF-7 cells and a decrease in MCF-12A cells, demonstrating also in this case a greater sensitivity of the latter model. The observed decrease of cell proliferation in MCF-12A was supported by an increase in apoptotic signal for GLY at 2.3 nM at 7 and 8h indicating the activation of the apoptotic mechanism. Conversely, the observed slight decrease in apoptotic signal for CPF (after 6h at 120 nM, 8h at 12 nM and 24h at 120 and 12 nM) and IMI (8h at 16 nM and 1.6 μM) suggests different cell death processes could be involved in the decrease of observed cell proliferation. In MCF-7 cells, none of the three pesticides induced significant changes in apoptotic or necrotic signals. To our knowledge, there are no data in human breast cell lines investigating apoptotic or necrotic mechanisms, therefore, these are the first available data providing information on the effects of CPF, IMI, and GLY on human mammary cell lines, both tumorigenic (MCF-7) and non-tumorigenic (MCF-12A).

A dose-dependent decrease in cell viability was observed in MCF-7 cells treated with all three pesticides although with different patterns. Dissimilar effect is observed in MCF-12A cells according to each pesticide: CPF induced a dose-dependent decrease in cell viability, IMI induced an increase in cell viability at 160 pM and GLY had no effect. The effects on cell viability in MCF-7 cells were supported by the decrease in ATP level induced by all three pesticides, whereas no effect in ATP production was observed in MCF-12A.

In MCF-7 cells no effect on intracellular ROS production was recorded for GLY, whereas CPF at 12 and 120 nM induced a decrease of ROS production; in contrast, IMI at 1.6 nM induced an increase in ROS production. Differently, in MF-12A cells intracellular ROS production was decreased by all three pesticides, although with different patterns. Several evidence reported the ability of the three pesticides to deplete ATP synthesis, concomitant or not with a ROS increase. In particular, in dopaminergic neural cells and induced pluripotent stem cells, micromolar CPF impaired mitochondrial membrane potential with a consequent reduction in ATP; in the same experimental condition a ROS increase was also observed [176,177]. In A549, CPF induced intracellular ROS at 10 and 100 nM, decreasing cell vitality only at micromolar concentrations [178]. In the same range of concentrations, CPF induced morphological alterations of mitochondria in HeLa cells as well as ATP depletion in SH-SY5Y cells [179].

Decreased ATP production was also observed upon IMI treatment of isolated rat liver mitochondria in the micromolar range, without affecting mitochondrial membrane potential [180]. No increase in ROS production was observed in lymphoblastoid cells treated with IMI up to 391 nM [181].

GLY at 0.036 g/L increased intracellular Ca^{2+} concentrations in rat Sertoli cells [182] due to its ability to enhance mitochondrial membrane permeability, which finally leads to a decreased ATP synthesis [183]. Inhibition of ATP synthesis following GLY exposure was observed also in human peripheral blood

mononuclear cells, although in the millimolar range [184] and in swine granulosa cells in the micromolar range [106]. In female adult mice administered with 250 or 500 mg/kg bw GLY, ATP decrease was observed in ovary, with concurrent increase in ROS levels and decrease of the mitochondrial membrane potential [185].

Thus, although the evidence indicated that the three pesticides differently affect the mitochondrial membrane potential/permeability, in MCF-7 all of them impair ATP synthesis; at high exposure concentrations this effect may translate into an increase of ROS production, not observed in our experiment performed at human exposure concentrations. How this could affect mammary gland development has to be further investigated.

In MCF-7 and MCF-12A cells, the three pesticides under study differently affected E2 secretion and gene expression of NRs involved in mammary gland development, representing also relevant clues for endocrine disruption action. To our knowledge, this is the first time that CPF, IMI and GLY effects are simultaneously assessed on this panel of nuclear receptors in both MCF-7 and non-tumorigenic MCF-12A cell lines.

E2 secretion is affected by all three pesticides in both cell lines although to a different extent. This effect was generally supported by an up-regulation of ERα gene expression in both cell lines but with some exceptions; indeed, in MCF-7 cells, ERα was induced by CPF and IMI, whereas GLY downregulated its expression at the lowest dose and induced it at the intermediate dose. In MCF-12A cells, ERα was up-regulated by IMI and GLY whereas CPF did not affect its expression. Noteworthily, all three pesticides downregulated ERβ in MCF-7 cells whereas a strong expression induction was observed in MCF-12A cells upon IMI and GLY treatment at all doses; on the contrary, CPF repressed ERβ also in MCF-12A cells. Thus, a different balance of ERα and ERβ receptors may occur following treatment with these pesticides, even at these human relevant

concentrations, with possible consequences on the proper mammary gland development.

PgR gene expression was repressed by all pesticides in MCF-7 and by IMI and GLY in MCF-12A. In MCF-7, all three pesticides up-regulated AR whereas only IMI and GLY upregulated AhR. These same pesticides downregulated AR and AhR also in MCF-12A although to a lesser extent. No effect was induced by CPF on these two receptors in MCF-12A cells. These results support dissimilar interacting mechanism underlying the action of each pesticide in relationship to the tumorigenic or non- tumorigenic cell line considered, which may differ in co-activators'/co-repressors' abundances. Of note, CPF almost did not affect NRs gene expression in MCF-12A, except for the downregulation of ERβ, at least in the range of concentrations tested.

A recent study on MCF-7 treated with CPF reported decreased ERα gene expression after short (24h) and long (14 days) exposure times at 10 and 100 μM, with corresponding protein increasing at same times but at lower exposure concentrations (0.1- 10 μM). A parallel dose-related increase in AhR gene expression was observed after 14 days of exposure but not at 24h [143]. Thus, AhR activation appeared delayed compared to ERα, which may explain the lack of effect observed in our study. In addition, tested concentrations were about 100 and 1000-fold higher than our highest concentration and the experiment was conducted at different time of treatment. Induction of ERβ gene expression was observed in MCF-7-BUS cells treated with micromolar CPF [186], as well as in hypothalamus of male mice pre-/ postnatally administered with 6 mg/kg bw/day CPF [140]. We observed a down-regulation of ERβ, in both cell lines, upon CPF treatment, suggesting that a different mechanism may occur at lower concentrations, possibly related also to the sex-specific effects exerted by this pesticide [187].

CPF may alter mammary gland morphology, as observed in adult female rats administered with 0.01 mg and 1 mg/ kg bw/day in which increased number of ducts and alveolar structures occurred; the effect was associated to increased PgR expression and decreased E2 serum levels with no effect on ERα expression [188]. Increased branching of ducts and higher diameter and number of buds were observed in adult female rats administered with 0.1 and 2.5 mg/kg bw/day CPF [142]. In transactivation assays using cell lines not derived from mammary gland, CPF induced both agonistic or antagonistic activity toward ER [137,189,190] and AhR [137,189,191], no androgenic or anti-androgenic effects [137,138,192] and antagonistic activity versus PgR [137], and CPF agonism was effective only versus ERα and not ERβ [190], thus mostly confirming our observations in MCF-7 cells but not in MCF-12A cells where CPF affected only the ERβ expression.

Although the endocrine effects described do not overlap with our results, dose differences and species-specific effects may explain the discrepancy. In any case, our results support CPF as a potential risk factor for mammary gland development due to the estrogenic activity observed in both MCF-7 and MCF-12A cells.

Limited evidence is available for IMI endocrine disrupting activity. In particular, estrogenic activity was observed in transfected human breast cancer cells (MELN) in the micromolar range [151] whereas no androgenic activity was detected [193]. On the contrary, an anti-androgenic effect was observed in male mice following administration of 10 and 30 mg/kg bw IMI which reduced AR gene and protein expression in testis as well as E2 secretion at the highest dose [150]. A significant E2 reduction was observed also in female rats administered with 50-300 mg/kg bw/day IMI [194]. No effect on AhR expression was observed in liver of mice treated with 0.6 mg/kg bw/day [195]. IMI can also modify the activity of aromatase, the enzyme responsible of estrogen synthesis, in Hs578t breast cancer cells only at the lowest concentration (0.1 μM) [196]. Our results

overlapped with available literature concerning the modulation of AR and AhR by IMI, even though in a opposite way considering the two cell lines: indeed the receptors were repressed in MCF-12A but induced in MCF-7 cells. However, previous evidence supports the observed increase of E2 secretion only at the lowest concentration tested, as well as the induction of ERα gene expression. In our study, IMI repressed ERβ in MCF-7 cells and up-regulated in MCF-12A cells; moreover, IMI repressed PgR in both cell lines. Unfortunately, no data on IMI interaction with these receptors are available in literature. Overall, also IMI may affect mammary gland development deranging the expression of these endocrine endpoints, thus studies investigating IMI effects on this tissue are warranted since no data are available yet.

GLY activation of estrogen responsive elements was described in two different studies in hormone-dependent breast cancer cells (T47D) in the nano- and micromolar range [98,159]; both ERs gene expression was induced by 1 and 100 nM GLY at 6h but not at 24h [159] thus suggesting an early triggering effect. Conversely, no ERs transactivation was observed in other transfected cells [151,158]. However, GLY displayed anti-androgenic activity [158] and strongly inhibited aromatase activity [151] in the micromolar range. Available evidence of *in vivo* studies is contrasting as regards GLY effects on nuclear receptors. Postnatal administration of a GLY-based formulation (2 mg/kg bw/day GLY) to female rats affected mammary gland development inducing an increase of hyperplastic ducts displaying enhanced ERα and PgR protein expression [160]. On the contrary, PgR decreased expression was observed in the uterus of developmentally exposed female rats with a concomitant increase of ERα expression and E2 serum levels [197]. Differently from females, male rats exposed to GLY at 3.5 mg/kg bw/day displayed no effect on mammary gland morphology whereas ERα gene and protein expression were decreased; besides, AR protein expression was increased, whereas its gene expression was decreased [198]. GLY effects observed in MCF-7 cells and MCF-12A cells were

generally more similar to IMI as regards gene expression, however in MCF-7 cells it induced E2 dose-related decrease at high doses although not significant at all concentrations tested .

During puberty, the mammary gland development is promoted by estrogen and progesterone hormones which, by activating ERα and PgR, induce ductal elongation and alveolar formation, whereas ERβ promotes apoptosis and differentiation of the epithelial tissue [199]. Moreover, the action of AR and AhR is also important in mammary gland development as AR counteracts the extent of proliferation, ductal extension and bud morphology [94] and AhR, due to cross-talk with ERs and coactivator of Erα, regulates proliferation [96].
In MCF-12A cells, increase in ERα and ERβ and decrease in gene AR expression may severely affect this balance contributing to increased proliferation and alteration in mammary gland morphology with more ductal extension and altered buds morphology. However, the inhibition of AhR gene expression, acting as a coactivator of ERα, needs further studies to better elucidate how its modulation may affect mammary gland development.
The effects of the three pesticides on the MCF-7 tumorigenic cell line led to hypothesize that in the context of mammary gland development they are able to increase cell proliferation by up-regulating ERα, AR, and AhR but at the same time cell differentiation is not observed, probably because they are tumorigenic cells.
The effects observed in both cell lines may occur even if PgR is downregulated. Indeed, studies in mice lacking PgR demonstrated that females had normal glands at puberty but no alveolar formation at lactation [93], thus indicating a more relevant role of PgR during a later phase of mammary gland development [200,201].

In addition, E2 secretion which can be performed locally by aromatase, may further activate estrogenic pathways including vascular endothelial grow factor transcription [202], and thus mammary gland angiogenesis.

Overall, the results of our study showed that all three pesticides at concentrations really occurring in children influenced NRs gene expression involved in the mammary gland development, in both cell lines, even in a different way, suggesting a potential ability to affect mammary gland development.

The data obtained from the *in vitro* toxicological study showed that all three pesticides can act with endocrine disrupting activity in both cell lines with respect to considered toxicological endpoints, in particular the modulation of NRs gene expression. The next step will be to evaluate the impact on the functional aspect of the selected nuclear receptors by determining their protein expression. Overall, the present results prompt to further investigations to better clarify the different mechanisms underlying pesticides effects.

Furthermore, the ultimate aim of our *in vitro* study was to develop a suitable model reproducing the *in vivo* physiology of the mammary gland, therefore the selected cell lines were used to set up a 3D cell culture system for future studies. The 3D cell culture system recreates *in vitro* a situation more similar to the physiological *in vivo* due to the presence of ECM essential for cell-cell and cell-matrix interactions [124]. For this aim, two different 3D cell culture systems were used and then compared: the hydrogel system and the magnetic levitation system by using the MCF-7 and MCF-12A cells, respectively.

The procedure of setting up the hydrogel system was extremely laborious because the formation of the hydrogel and thus of the spheroids need several methodological steps with additional time consuming; moreover, the experimental phase can start only after the complete formation of the spheroids occurring in at least 7 days, with a possible increase of necrotic process in the core of the spheroids [203]. In agreement with previous studies [204], we

observed that the process of recovery of spheroids embedded in the hydrogel, as necessary step for the characterization of the cellular response (e.g., RNA and protein isolation), requires high manual precision to maintain cellular integrity needed to ensure the efficiency of the experiment. In addition, the characteristic of hydrogel to promote the formation of a number of spheroids, each one with a different size (fig. 30), makes difficult the reproducibility and the interpretation of data, as reported analogously for natural origin gels [127].

Considering these limitations, we decided to set up a different 3D cell culture by using a magnetic system characterized by the formation of a single spheroid through the addition of magnetic NanoShuttle™-PL during the cell growing in monolayer. In this system we used only the human breast cell line MCF-12A, for several reasons: it is non-tumorigenic, it consists of 3 different cell types reproducing a physiological situation of the mammary gland, and, finally, it expresses all NRs involved in the mammary gland development, as we demonstrated in the toxicological study. The procedure of spheroid formation by this approach is more simple and rapid than with hydrogel and allow to obtain a single spheroid within 24 hours, thus highly reducing experimental times. In addition, we applied the magnetic levitation, a specific technique for cell magnetization in which the spheroid levitates on the surface of the medium, and the resulting air-liquid contact promotes autonomous cell secretion of the ECM [130]. Under this condition, a more bioactive matrix is produced than that obtained in a synthetic condition as in hydrogel [205].

Therefore, the magnetic 3D model represents a better model for future studies concerning the evaluation of pesticide effects according to the study performed in 2D.

Conclusions

This is the first study providing evidence on toxicological effects of CPF, IMI, and GLY on both tumorigenic (MCF-7) and non-tumorigenic (MCF-12A) human mammary cell lines. The three pesticides exerted effects supporting

three different underlying modes of action, especially highlighted by the use of the cell lines, however no definitive conclusion could be drawn as regards mammary gland development. In any case, the results raise the concern on possible adverse effects induced by exposure to these compounds, especially in vulnerable population groups as children.

The MCF-12A cell line demonstrated to be a suitable model to evaluate the potential effects of pesticides as more similar to physiological conditions and more sensitive to exposure effects.

The 3D model obtained with magnetic system is a suitable tool to investigate the effects of chemicals in more reproducible conditions simulating the *in vivo* mammary gland physiology.

4 Epidemiological study

4.1 Background

4.1.1 Pesticide exposure: food intake and residential area

Humans are exposed to pesticide residues mainly through dietary intake of water and foods. Residue presence is yearly checked by the annual monitoring control plan [206], for being below the MRLs values established by European legislation [207]. Since pesticide exposure has been associated with several diseases [208], experimental and epidemiological studies are required to provide data on the association of pesticide exposure, even at very low levels, and health effects [209]. In this context, since children represent a vulnerable and susceptible group for whom neurological and developmental disorders have been associated with pesticide exposure, EFSA has paid particular attention to residue levels in foods for infants and young children, reviewing the approach used to set acceptable daily intake values and reviewing some MRLs.

In addition to dietary exposure, also the inhalation route is relevant in relation to the residential area by possibly contributing to pesticide exposure due to the presence of farmed fields potentially treated with pesticides. In particular, it has been observed that farm households had higher levels of pesticide residues associated to the consumption of contaminated fruit and vegetables [210], and children from agriculture areas showed urinary higher levels of atrazine, metolachlor, chlorpyrifos than those living in non-agricultural areas [116].

4.1.2 Case-Control Study

As reported in Coppola et al., [83] in PEACH project a case-control study was performed to measure pesticide exposure in girls living in agricultural areas of Central Italy and to correlate idiopathic premature thelarche with individual and environmental factors (dietary habits, agricultural practices, related use of pesticides, land use).

Some preparatory steps and methods are presented below to introduce the development of the FFQ set up during the doctoral fellowship.

Girls with idiopathic premature thelarche matched for age with healthy control girls (n = 60 + 60), were enrolled by paediatric endocrinologists at the Fermo and Civitanova Marche Hospitals and by family pediatricians of the Italian National Health System working in the same areas.
The pediatricians were requested to select eligible candidates during their routine activity, presenting them the study and asking them to sign the informed consent. Moreover, they explained to girls' mothers how to fill in the questionnaire/food diary, and how to collect first morning urine samples for the analytical determination of pesticide metabolites levels.

For the enrolment of girls with idiopathic premature thelarche, the paediatric endocrinologist verified the following conditions: the differential diagnosis of true early puberty and premature thelarche: age: 2–7 years, breast development (Tanner stage II), absence of other signs of puberty, normal stature, normal growth rate, age bone corresponding to chronological age or advanced of no more than 1 year, pelvic ultrasound consistent with pre-puberty (uterine longitudinal length and volume <3.5 cm and <1.8 mL), prepuberal Gonadotropin-releasing Hormone Stimulation Test (GnRH test, peak FSH value higher than LH, peak LH value <5 mIU/mL). The GnRH hormone test was performed by taking blood samples at 15th, 30th, 60th, and 120th minutes following intravenous administration of Relefact LH-RH (Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany.) at 100 μg/m2 diluted 1:10 with 0.9% physiological solution. In order to exclude other forms of precocious puberty, 17-β estradiol, alpha-fetoprotein, human chorionic gonadotropin, anti-Mullerian, thyroid and adrenal hormones were evaluated. All hormones were measured by the immune-chemiluminescence method according to manufacturer's protocols (Beckman Coulter, Brea, CA 92821, USA), with

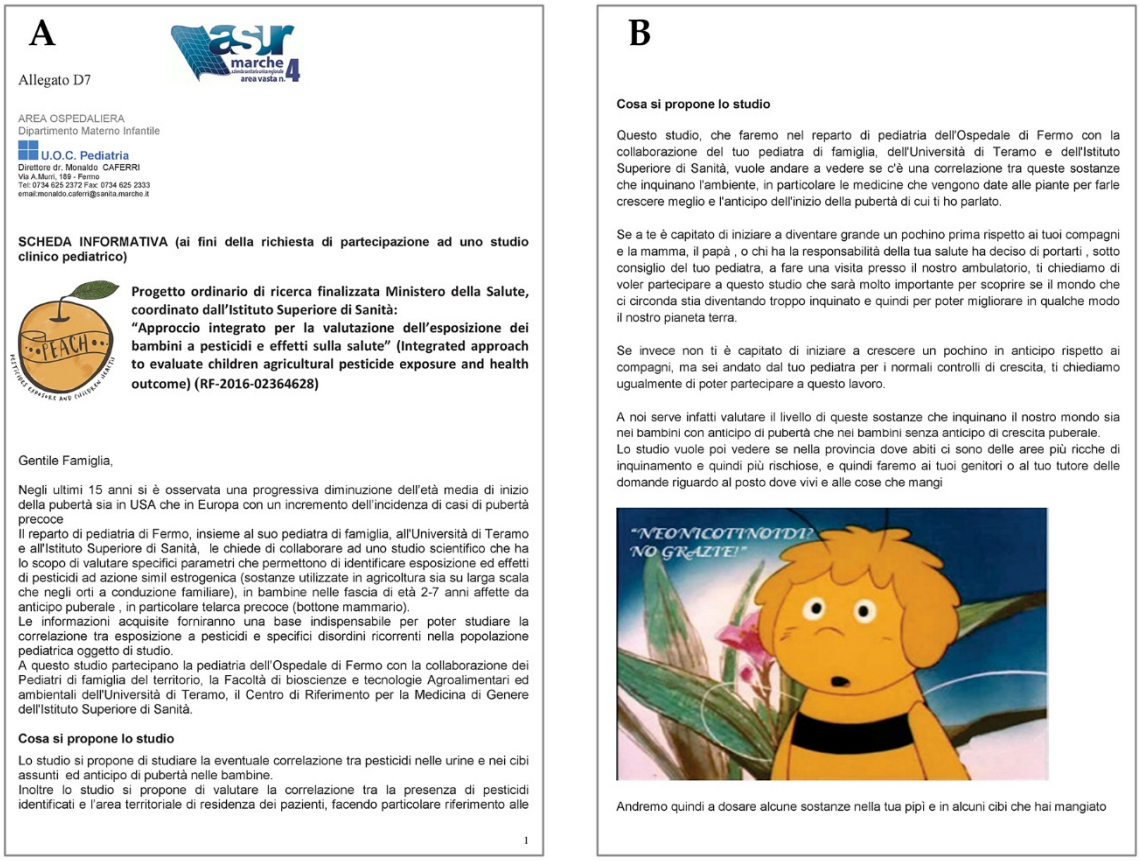
detection limit (LOD) = 0.2 UI/mL and linearity in the range of 0.2–200 UI/mL. Moreover, 10 < BMI (Body Mass Index) > 75 and no concomitant therapy were applied as criteria for the enrolment of both cases and controls.

Some documents were provided to pediatricians to be submitted to the family of the enrolled girls (both cases and controls), including:

1. An information sheet for the family of healthy controls (with the aim of encouraging participation), describing the purpose of the study, the aim of urine sampling and Food Frequency Questionnaire (FFQ), and the confidentiality and management of personal data;
2. Informed consent for parents (Fig. 32 A);
3. Information sheet for the child, like that presented to parents but with simple drawings and sentences and including informed consent (Fig. 32 B);
4. Indications for the enlisted person on how to fill in the FFQ and the food diary as well as the instructions for collecting the urine samples;
5. The FFQ and the food diary.

Each enrolled subject was asked to sign the consent, to collect a first morning urine sample and fill in the FFQ and the food diary during the day before the urine sampling.

For each enrolled subject, an alphanumeric code was created to accomplish the ethics committee requirements concerning data protection and anonymity and assigned to urine sample and FFQ.



A

Allegato D7

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SCHEDA INFORMATIVA (ai fini della richiesta di partecipazione ad uno studio clinico pediatrico)

Progetto ordinario di ricerca finalizzata Ministero della Salute, coordinato dall'Istituto Superiore di Sanità:
"Approccio integrato per la valutazione dell'esposizione dei bambini a pesticidi e effetti sulla salute" (Integrated approach to evaluate children agricultural pesticide exposure and health outcome) (RF-2016-02364628)

Gentile Famiglia,

Negli ultimi 15 anni si è osservata una progressiva diminuzione dell'età media di inizio della pubertà sia in USA che in Europa con un incremento dell'incidenza di casi di pubertà precoce
Il reparto di pediatria di Fermo, insieme al suo pediatra di famiglia, all'Università di Teramo e all'Istituto Superiore di Sanità, le chiede di collaborare ad uno studio scientifico che ha lo scopo di valutare specifici parametri che permettono di identificare esposizione ed effetti di pesticidi ad azione simil estrogenica (sostanze utilizzate in agricoltura sia su larga scala che negli orti a conduzione familiare), in bambine nelle fascia di età 2-7 anni affette da anticipo puberale , in particolare telarca precoce (bottone mammario).
Le informazioni acquisite forniranno una base indispensabile per poter studiare la correlazione tra esposizione a pesticidi e specifici disordini ricorrenti nella popolazione pediatrica oggetto di studio.
A questo studio partecipano la pediatria dell'Ospedale di Fermo con la collaborazione dei Pediatri di famiglia del territorio, la Facoltà di bioscienze e tecnologie Agroalimentari ed ambientali dell'Università di Teramo, il Centro di Riferimento per la Medicina di Genere dell'Istituto Superiore di Sanità.

Cosa si propone lo studio

Lo studio si propone di studiare la eventuale correlazione tra pesticidi nelle urine e nei cibi assunti ed anticipo di pubertà nelle bambine.
Inoltre lo studio si propone di valutare la correlazione tra la presenza di pesticidi identificati e l'area territoriale di residenza dei pazienti, facendo particolare riferimento alle

1

B

Cosa si propone lo studio

Questo studio, che faremo nel reparto di pediatria dell'Ospedale di Fermo con la collaborazione del tuo pediatra di famiglia, dell'Università di Teramo e dell'Istituto Superiore di Sanità, vuole andare a vedere se c'è una correlazione tra queste sostanze che inquinano l'ambiente, in particolare le medicine che vengono date alle piante per farle crescere meglio e l'anticipo dell'inizio della pubertà di cui ti ho parlato.

Se a te è capitato di iniziare a diventare grande un pochino prima rispetto ai tuoi compagni e la mamma, il papà , o chi ha la responsabilità della tua salute ha deciso di portarti , sotto consiglio del tuo pediatra, a fare una visita presso il nostro ambulatorio, ti chiediamo di voler partecipare a questo studio che sarà molto importante per scoprire se il mondo che ci circonda stia diventando troppo inquinato e quindi per poter migliorare in qualche modo il nostro pianeta terra.

Se invece non ti è capitato di iniziare a crescere un pochino in anticipo rispetto ai compagni, ma sei andato dal tuo pediatra per i normali controlli di crescita, ti chiediamo ugualmente di poter partecipare a questo lavoro.

A noi serve infatti valutare il livello di queste sostanze che inquinano il nostro mondo sia nei bambini con anticipo di pubertà che nei bambini senza anticipo di crescita puberale.
Lo studio vuole poi vedere se nella provincia dove abiti ci sono delle aree più ricche di inquinamento e quindi più rischiose, e quindi faremo ai tuoi genitori o al tuo tutore delle domande riguardo al posto dove vivi e alle cose che mangi

Andremo quindi a dosare alcune sostanze nella tua pipì e in alcuni cibi che hai mangiato

Figure 32. A Information sheet for the family and B Information sheet for the child.

The project was evaluated by ethics committees as the main activity of the project concerns the collection of information and samples from girls. The ethics committees of the Istituto Superiore di Sanità (Italian National Health Institute), as project coordinator, and Marche Region, as the beneficiary in charge of the case-control study, approved the project and the informed consent (RF-2016-02364628, Prot N_PRE-4203, 17 April 2018; ASUR CERM 2018-190, respectively).

4.1.3 Geographical Area Characterization and Agricultural Activities

Specific areas in the Centre of Italy (Fermo and Macerata Districts, Marche Region) are considered in the study, both characterized by agriculture practice in farms and in private vegetable gardens.

The province of Fermo is a 863 km² area in the Marche Region, Central-Eastern Italy, with about 175,000 inhabitants, that extends from the Adriatic coast to the Sibillini Mountains (over 2,000 meters above the sea level (a.s.l.). The main city is Fermo (about 38,000 citizens, 319 meters a.s.l.).

The province of Macerata is a 2,780 km² area in the Marche Region, Central-Eastern Italy, with about 314,178 inhabitants, that is wet to east from the Adriatic Sea and to west confines with the Umbria. The main city is Macerata (circa 40575 citizens, 315 meters a.s.l).

Both provinces are agricultural and manufacturing areas, particularly renown for shoemaking (Fig. 33).

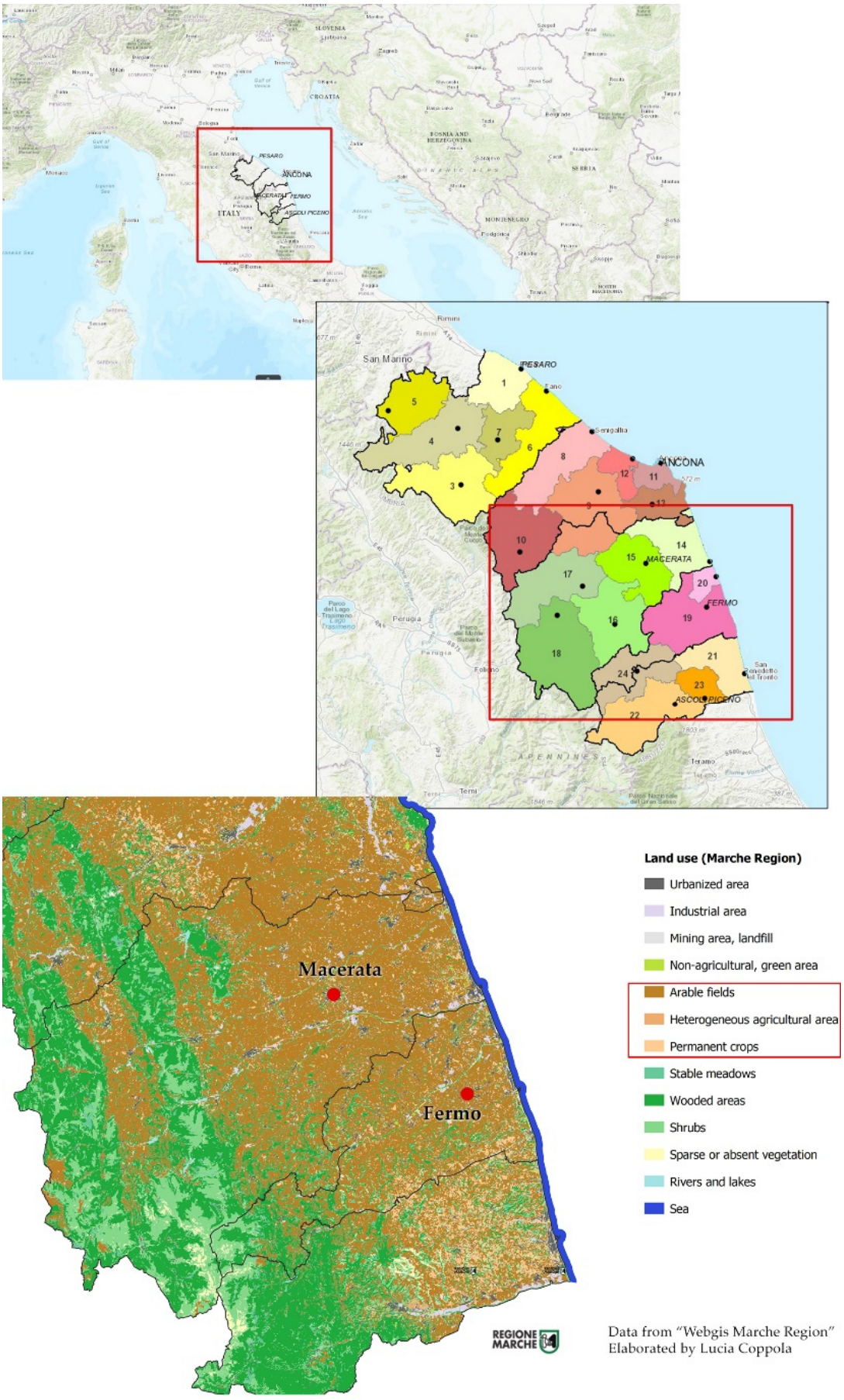


Figure 33. Land use details of Fermo and Macerata provinces.

For this study, data on the type of cultivations in the Fermo and Macerata areas and the agrochemicals used were obtained by the local agronomists. Specifically, the main crops in the areas include cereals (e.g., wheat, barley, oats, corn, sorghum), sunflowers, leaf and cruciferous vegetables (e.g., cabbage, cauliflower, broccoli, radish, spinach, chicory, chard, lettuce), horticultural tomato, stone fruit (e.g., cherry; peach, plum, apricot), pome fruit (e.g., apples, pears, loquat).

Among the several pesticides used in these types of cultivations, we selected 44 compounds and their metabolites as mainly used in the territory, and to be analysed in foods collected for this study (Table 2, 3).

Fungicides	**Insecticides**	**Acaricides**	**Herbicides**
Boscalid	Acetamipirid	Abamectina	Glyphosate
Cyproconazole	Aldicarb Sulfone	Clorpyriphos-metile	
Epoxiconazole	Aldicarb Sulfoxide	Clorpyriphos-ethil	
Fenbuconazole	Carbaryl	Dimethoate	
Fludioxonil	Clothianidin	Emamectin	
Mancozeb	Coumaphos	Omethoate	
Metiram	Formetanate	Phosmet	
Myclobutanil	Imidacloprid	Terbufos	
Penconazole	Isoprocarb	Terbufos sulfone	
Penncozeb	Malathion	Trichlorfon	
Prochloraz	Methiocarb		
Propiconazole	Pirimicarb		
Propineb	Thiacloprid		
Tebuconazole	Thiamethoxam		
Thiabendazole			
Thiram			
Tiofanate metile			
Tricyclazole			
Zineb			

Table 2. Pesticides analysed in food groups.

Metabolite	Parent	Metabolite	Parent
Ethylene thiourea (ETU)	Mancozeb	6-chloronicotinic acid (6CNA)	Acetamipirid
Ethylene thiourea (ETU)	Metiram	3-Chloro-4-methylumbelliferone (CMHC)	Coumaphos
		Diethylphosphate (DEP)	
		Diethylthiophosphate (DETP)	
Ethylene thiourea (ETU)	Penncozeb	6-chloronicotinic acid (6CNA)	Imidacloprid
2,4,6-Trichlorophenol	Prochloraz	Malaoxon	Malathion
		Malathion dicarboxylic acid (MDA)	
Propylenethiourea (PTU)	Propineb	Methiocarb sulfone e sulfoxide	Methiocarb
5-Hydroxythiabendazole	Thiabendazole	Pirimicarb-desmethyl	Pirimicarb
Ethylene thiourea (ETU)	Thiram	6-chloronicotinic acid (6CNA)	Thiacloprid
Ethylene thiourea (ETU)	Zineb	Dimethylphosphate (DMP)	Clorpyriphos-metile
		Dimethylthiophosphate (DMTP)	
Somma di B1a, B1b E B1A 8,9Z	Abamectina	3,5,6-Trichloro-2-pyridinol (TCP)	Clorpyriphos-ethil
		Diethylphosphate (DEP)	
		Diethylthiophosphate (DETP)	
Dimethylphosphate (DMP)	Phosmet	Diethylphosphate (DEP)	Terbufos
Dimethylthiophosphate (DMTP)		Diethylthiophosphate (DETP)	
Phosmet oxon			
2,4,6-Trichlorophenol	Glyphosate		
Aminomethylphosphonic (AMPA)			

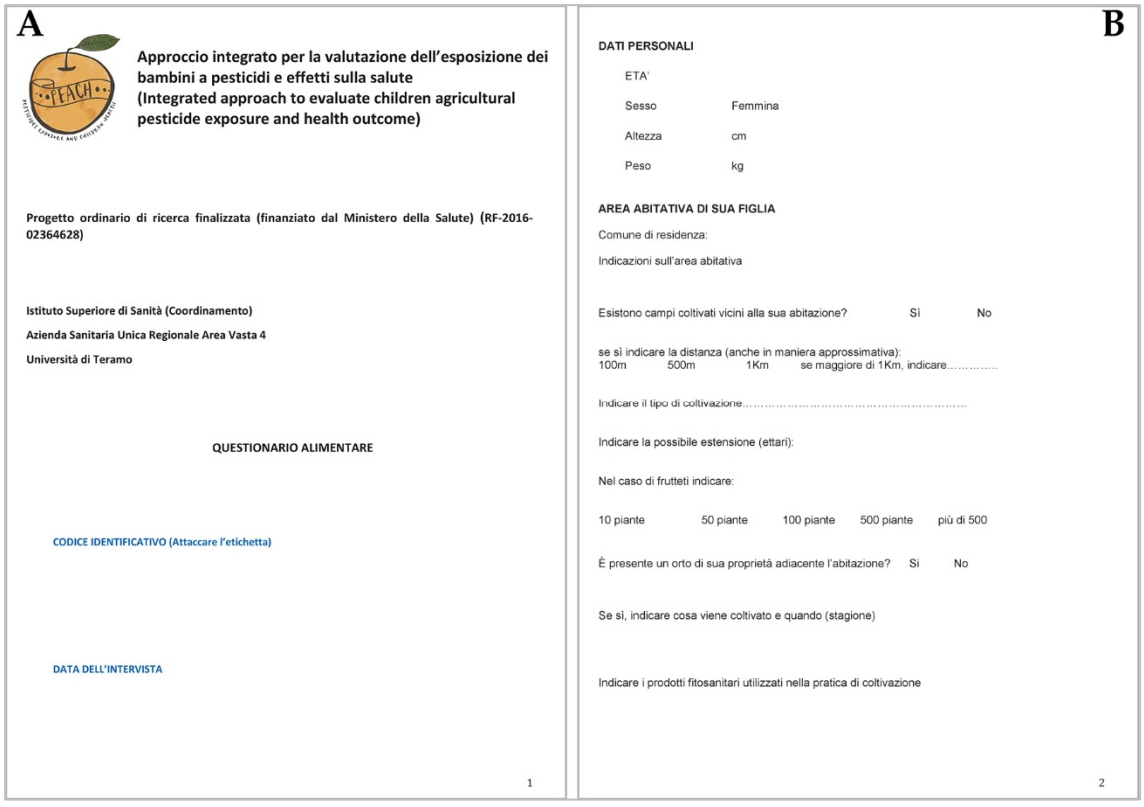
Table 3. Metabolite and parent pesticides analysed in urine samples.

4.2 Material and Methods

4.2.1 Development of Food Frequency Questionnaire and food diary

The questionnaire was constructed starting from the FFQ produced by the European Prospective Investigation into Cancer (EPIC) study [211] and the National Health and Nutrition Examination Survey (NHANES) and then adapted for our study. The FFQ was implemented including food categories reported in the EFSA standardized system of food classification and description (FoodEx 2 EFSA database) [212]. A questionnaire was prepared in a paper form to collect both personal and residence area data as well as food consumption information as reported in Coppola et al., [83] and described as following.

The questionnaire was structured in three parts. In the first part, personal information (e.g., age, body weight) and information on residential environment (e.g., proximity to cultivated fields, presence of private vegetable garden) are required (Fig. 34 A, B).


A

Approccio integrato per la valutazione dell'esposizione dei bambini a pesticidi e effetti sulla salute
(Integrated approach to evaluate children agricultural pesticide exposure and health outcome)

Progetto ordinario di ricerca finalizzata (finanziato dal Ministero della Salute) (RF-2016-02364628)

Istituto Superiore di Sanità (Coordinamento)
Azienda Sanitaria Unica Regionale Area Vasta 4
Università di Teramo

QUESTIONARIO ALIMENTARE

CODICE IDENTIFICATIVO (Attaccare l'etichetta)

DATA DELL'INTERVISTA

1

B

DATI PERSONALI

ETA'
Sesso Femmina
Altezza cm
Peso kg

AREA ABITATIVA DI SUA FIGLIA

Comune di residenza:
Indicazioni sull'area abitativa

Esistono campi coltivati vicini alla sua abitazione? Si No

se sì indicare la distanza (anche in maniera approssimativa):
100m 500m 1Km se maggiore di 1Km, indicare……

Indicare il tipo di coltivazione……

Indicare la possibile estensione (ettari):

Nel caso di frutteti indicare:

10 piante 50 piante 100 piante 500 piante più di 500

È presente un orto di sua proprietà adiacente l'abitazione? Si No

Se sì, indicare cosa viene coltivato e quando (stagione)

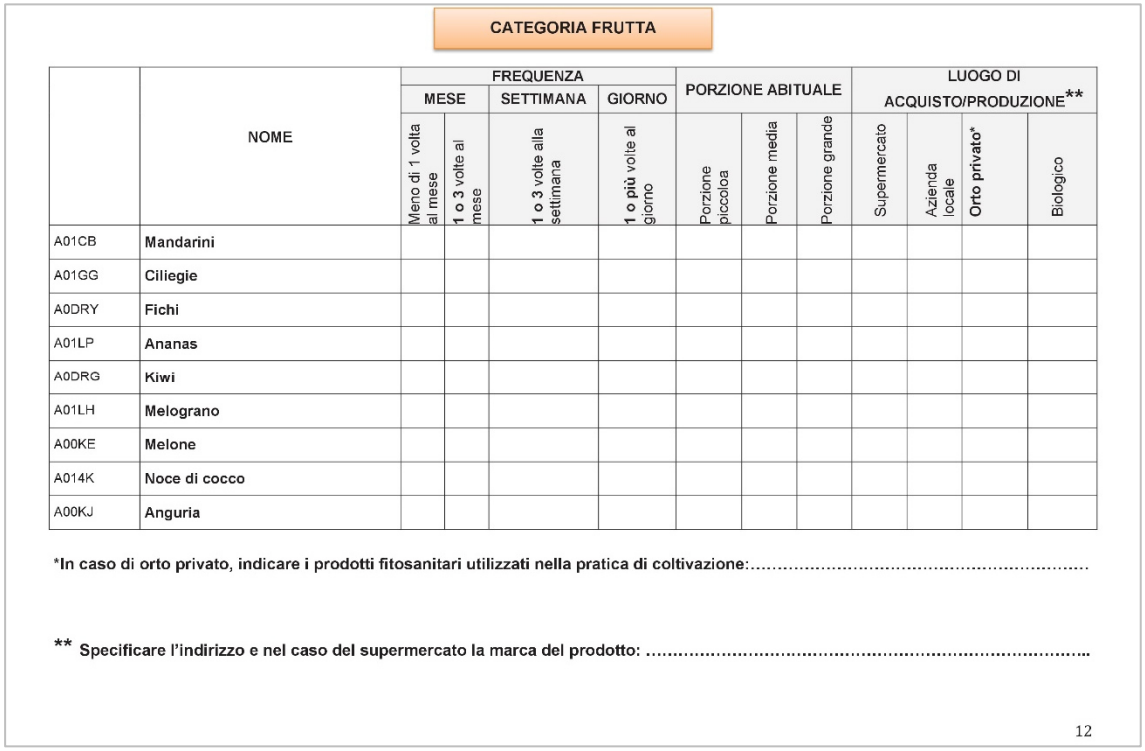
Indicare i prodotti fitosanitari utilizzati nella pratica di coltivazione

2

Figure 34. FFQ images related to personal and residential information (A and B).

The second part is represented by the FFQ and includes one table for each food category considered in the official national and European control activities (i.e., vegetables, fruit, cereals, fish, meat, dairy products, eggs, honey and oil) [32,213]. Food commodities listed within each category were selected considering: i) the information obtained by both the local agronomists about crops grown in the study area; ii) the information obtained by paediatricians involved in the study, about the food items consumed by the children aged 2–7 years. Each food commodity has been associated to the alphanumeric code assigned by the EFSA FoodEx 2 to facilitate data collection and subsequent food exposure analysis [212].

In the FFQ, each enrolled girl specified the commodities consumed by indicating the frequency of consumption (daily, weekly, monthly), the usual portion consumed (small, medium, large) but also the place of purchase/production (supermarket, farm or private garden) (Fig. 35). The small, medium or large portions take into account the indications provided by the Italian Society of Human Nutrition concerning the Reference Intake Levels of Nutrients and Energy for the Italian population (LARN) [214] which indicate, for example, for fruit a quantity of 150g as a standard or medium portion. The information about the place of purchase/production is fundamental to define the sampling plan.



CATEGORIA FRUTTA

	NOME	FREQUENZA				PORZIONE ABITUALE			LUOGO DI ACQUISTO/PRODUZIONE**			
		MESE		SETTIMANA	GIORNO							
		Meno di 1 volta al mese	**1 o 3** volte al mese	**1 o 3** volte alla settimana	**1 o più** volte al giorno	Porzione piccola	Porzione media	Porzione grande	Supermercato	Azienda locale	**Orto privato***	Biologico
A01CB	**Mandarini**											
A01GG	**Ciliegie**											
A0DRY	**Fichi**											
A01LP	**Ananas**											
A0DRG	**Kiwi**											
A01LH	**Melograno**											
A00KE	**Melone**											
A014K	**Noce di cocco**											
A00KJ	**Anguria**											

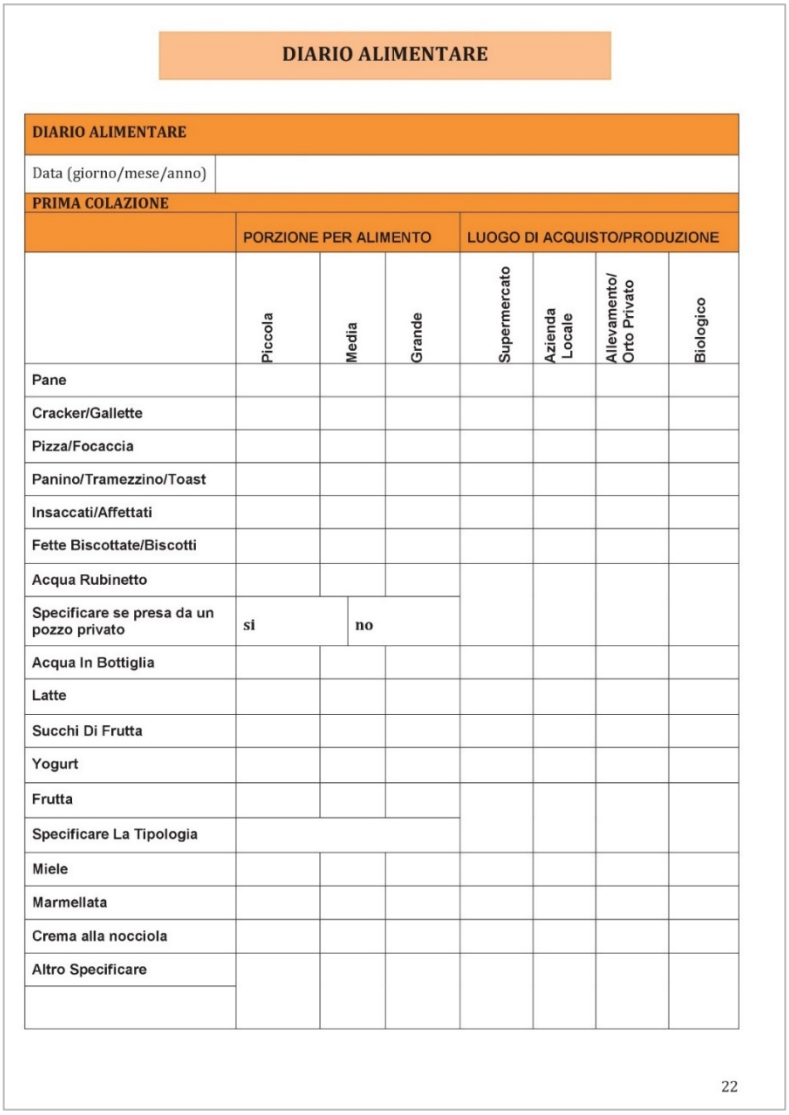
***In caso di orto privato, indicare i prodotti fitosanitari utilizzati nella pratica di coltivazione:**……

** **Specificare l'indirizzo e nel caso del supermercato la marca del prodotto:** ……

12

Figure 35. FFQ example. The image shows the food category "fruit".

The last part of the questionnaire is represented by a food diary collecting information on food consumed by the enrolled girls 24h before the urine sampling (Fig. 36).



DIARIO ALIMENTARE

DIARIO ALIMENTARE							
Data (giorno/mese/anno)							
PRIMA COLAZIONE							
	PORZIONE PER ALIMENTO			LUOGO DI ACQUISTO/PRODUZIONE			
	Piccola	Media	Grande	Supermercato	Azienda Locale	Allevamento/ Orto Privato	Biologico
Pane							
Cracker/Gallette							
Pizza/Focaccia							
Panino/Tramezzino/Toast							
Insaccati/Affettati							
Fette Biscottate/Biscotti							
Acqua Rubinetto							
Specificare se presa da un pozzo privato	si	no					
Acqua In Bottiglia							
Latte							
Succhi Di Frutta							
Yogurt							
Frutta							
Specificare La Tipologia							
Miele							
Marmellata							
Crema alla nocciola							
Altro Specificare							

22

Figure 36. Food diary to be completed on food consumed by enrolled girls 24h before urine collection.

The FFQ was validated by administering it to a small number of pediatricians and families. From the validation, some criticalities emerged, which helped to adjust some questions inherent information requested about the area of residence, the place of purchase and the brand of the product purchased. At the end of the revision process, the final version was adopted in the study.

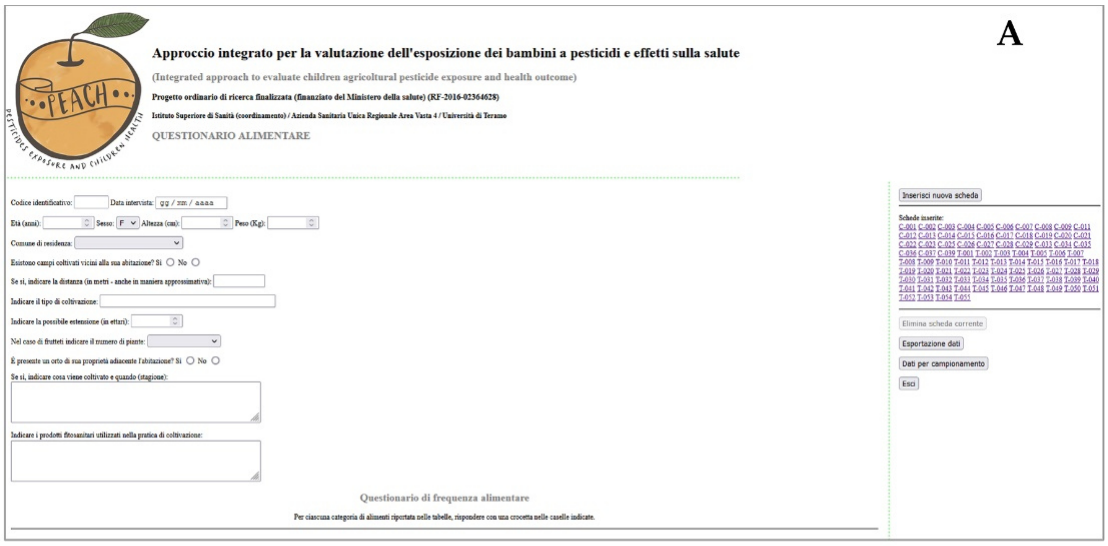
The FFQs filled in by the enrolled girls were checked for data consistency; data entry was then performed in an online database, developed in collaboration with the ISS Core Facilities Service (FAST) on the basis of the paper version. The online database was necessarily created to properly assist the data entry and

consistency check process. Indeed, due to the large number of information fields to fill in in a single questionnaire (much more than 1000), a general-purpose application software (i.e., spreadsheets or similar) was deemed inadequate for this task. Therefore, an ad hoc web platform was developed, providing a suitable and user-friendly interface, especially designed to minimize input errors and having full editing capabilities. The application was developed by a computational scientist in a HTML-Javascript-PHP-MySQL-Apache framework, exclusively using open source software. Using the application, data are first stored into a relational database on a server and subsequently exported (via dedicated functions) into files in a Tab-Separated Values (TSV) format, suitable for further processing through scientific statistical analysis software.

The online database has been validated through the insertion of some questionnaires and then implemented applying some changes:

- Yes/no answers to questions, such as "Are there cultivated fields... etc." and "Is there a vegetable garden... etc." have been converted into Yes/No "radio buttons" with mutually exclusive selection and with the possibility to leave them in an indeterminate state (=absent information);
- in addition to the above radio buttons, all the others presenting a mutually exclusive selection (i.e., all those relating to food frequency and usual portion) have been made unselectable with a second click;
- the headings with the description "rotated" vertically have been reported horizontally and abbreviated;
- foods items have been reported in the same order as they appear in the paper questionnaire and not in alphabetical order as in a previous version;
- the possibility of deleting an entire questionnaire from the database has been added through a command button, requiring a confirmation.

As last step, the data download function was implemented; the online database was then complete and ready for data entry (Fig. 37 A, B).



A

Approccio integrato per la valutazione dell'esposizione dei bambini a pesticidi e effetti sulla salute

(Integrated approach to evaluate children agricoltural pesticide exposure and health outcome)

Progetto ordinario di ricerca finalizzata (finanziato dal Ministero della salute) (RF-2016-02364628)

Istituto Superiore di Sanità (coordinamento) / Azienda Sanitaria Unica Regionale Area Vasta 4 / Università di Teramo

QUESTIONARIO ALIMENTARE

Codice identificativo: Data intervista: gg / mm / aaaa

Età (anni): Sesso: F Altezza (cm): Peso (Kg):

Comune di residenza:

Esistono campi coltivati vicini alla sua abitazione? Si No

Se si, indicare la distanza (in metri - anche in maniera approssimativa):

Indicare il tipo di coltivazione:

Indicare la possibile estensione (in ettari):

Nel caso di frutteti indicare il numero di piante:

È presente un orto di sua proprietà adiacente l'abitazione? Si No

Se si, indicare cosa viene coltivato e quando (stagione):

Indicare i prodotti fitosanitari utilizzati nella pratica di coltivazione:

Questionario di frequenza alimentare

Per ciascuna categoria di alimenti riportata nelle tabelle, rispondere con una crocetta nelle caselle indicate.

Inserisci nuova scheda

Schede inserite:

C-001 C-002 C-003 C-004 C-005 C-006 C-007 C-008 C-009 C-011 C-012 C-013 C-014 C-015 C-016 C-017 C-018 C-019 C-020 C-021 C-022 C-023 C-025 C-026 C-027 C-028 C-029 C-031 C-034 C-035 C-036 C-037 C-039 T-001 T-002 T-003 T-004 T-005 T-006 T-007 T-008 T-009 T-010 T-011 T-012 T-013 T-014 T-015 T-016 T-017 T-018 T-019 T-020 T-021 T-022 T-023 T-024 T-025 T-026 T-027 T-028 T-029 T-030 T-031 T-032 T-033 T-034 T-035 T-036 T-037 T-038 T-039 T-040 T-041 T-042 T-043 T-044 T-045 T-046 T-047 T-048 T-049 T-050 T-051 T-052 T-053 T-054 T-055

Elimina scheda corrente

Esportazione dati

Dati per campionamento

Esci

B

Categoria Verdura

ID	Alimento	Frequenza				Porzione abituale			Luogo di acquisto/produzione (**)			
		Mese		Settimana	Giorno							
		<1	1-3	1-3	≥1	Piccola	Media	Grande	Supermercato	Azienda locale	Orto privato (*)	Biologico
A00LD	Cicoria (inclusi radicchio, scarola, puntarelle, etc.)	○	○	○	○	○	○	○	☐	☐	☐	☐
A00FM	Broccoli (inclusi broccoletti, cime di rapa, cavolfiore, cavolo, verza, etc.)	○	○	○	○	○	○	○	☐	☐	☐	☐
A0DJS	Bieta	○	○	○	○	○	○	○	☐	☐	☐	☐
A0DPB	Carote	○	○	○	○	○	○	○	☐	☐	☐	☐
A00HQ	Pomodori	○	○	○	○	○	○	○	☐	☐	☐	☐
A0DLB	Lattuga (inclusi iceberg, lollo rossa, romana, etc.)	○	○	○	○	○	○	○	☐	☐	☐	☐
A00JR	Zucchine	○	○	○	○	○	○	○	☐	☐	☐	☐
A00MH	Spinaci	○	○	○	○	○	○	○	☐	☐	☐	☐
A011Z	Fagioli (freschi o secchi)	○	○	○	○	○	○	○	☐	☐	☐	☐
A01BB	Fagioli in scatola	○	○	○	○	○	○	○	☐	☐	☐	☐
A00JA	Peperoni	○	○	○	○	○	○	○	☐	☐	☐	☐
A00JC	Melanzane	○	○	○	○	○	○	○	☐	☐	☐	☐
A05LG	Finocchi	○	○	○	○	○	○	○	☐	☐	☐	☐
A00PB	Fagiolini	○	○	○	○	○	○	○	☐	☐	☐	☐
A0DFB	Lenticchie (fresche o secche)	○	○	○	○	○	○	○	☐	☐	☐	☐
A01BD	Lenticchie in scatola	○	○	○	○	○	○	○	☐	☐	☐	☐
A05DR	Zucca	○	○	○	○	○	○	○	☐	☐	☐	☐
A0BAV	Ceci (freschi o secchi)	○	○	○	○	○	○	○	☐	☐	☐	☐
A01BE	Ceci in scatola	○	○	○	○	○	○	○	☐	☐	☐	☐
A01HH	Olive	○	○	○	○	○	○	○	☐	☐	☐	☐
A00PX	Piselli (freschi o secchi)	○	○	○	○	○	○	○	☐	☐	☐	☐
A01BC	Piselli in scatola	○	○	○	○	○	○	○	☐	☐	☐	☐
A0ETG	Funghi	○	○	○	○	○	○	○	☐	☐	☐	☐
A00JM	Cetrioli	○	○	○	○	○	○	○	☐	☐	☐	☐
A00ZT	Patate	○	○	○	○	○	○	○	☐	☐	☐	☐
A04MA	Erbe aromatiche	○	○	○	○	○	○	○	☐	☐	☐	☐
A00GY	Aglio	○	○	○	○	○	○	○	☐	☐	☐	☐
A00HB	Cipolla	○	○	○	○	○	○	○	☐	☐	☐	☐
	ALTRO - SPECIFICARE	○	○	○	○	○	○	○	☐	☐	☐	☐

ALTRO - SPECIFICARE:

(*) In caso di orto privato, indicare i prodotti fitosanitari utilizzati nella pratica di coltivazione:

(**) Specificare l'indirizzo e, nel caso del supermercato, la marca del prodotto:

Figure 37. Example of pages (A, B) from the FFQ online database.

4.3 Results and discussion

4.3.1 Food Sampling Plan Construction

Data from a total of 120 FFQs including 60 controls and 60 cases was entered in the online database during the enrolment campaign. Data was then downloaded and analysed to define a food sampling plan focussed on foods locally produced and consumed by the girls. Data was then sorted by place of purchase/production, considering only local farms or private gardens, and by food category with the list of commodities. (Fig. 38). Successively, commodities were bought at the local farm, after geolocation, or asked to the garden owners to provide them.

	A	B	C	D	E	F
1	ID_Sche	Categoria	ID_Alimen	Alimento	Specifica_Al	Luogo
683	C-39	Verdura	A05LG	Finocchi		locale
684	C-39	Verdura	A05DR	Zucca		locale
685	C-39	Verdura	A01HH	Olive		locale
686	C-39	Verdura	A00PX	Piselli (freschi o secchi)		locale
715	C-5	Verdura	A05LG	Finocchi		locale
716	C-5	Verdura	A00PB	Fagiolini		locale
717	C-5	Verdura	A05DR	Zucca		privato
718	C-5	Verdura	A00ZT	Patate		locale
754	C-8	Verdura	A00HQ	Pomodori		privato
755	C-8	Verdura	A05LG	Finocchi		privato
756	C-8	Verdura	A01HH	Olive		privato
757	C-8	Verdura	A00JM	Cetrioli		privato
758	C-8	Verdura	A00ZT	Patate		privato
835	T-002	Verdura	A00HQ	Pomodori		privato
836	T-002	Verdura	A0DLB	Lattuga (inclusi iceberg, lollo rossa, ron		privato
848	T-003	Verdura	A00LD	Cicoria (inclusi radicchio, scarola, punta		locale
849	T-003	Verdura	A00FM	Broccoli (inclusi broccoletti, cime di rap		locale
850	T-003	Verdura	A0DJS	Bieta		locale,privato
851	T-003	Verdura	A0DPB	Carote		locale,privato
852	T-003	Verdura	A00JR	Zucchine		locale,privato
853	T-003	Verdura	A00MH	Spinaci		locale,privato
854	T-003	Verdura	A00JC	Melanzane		locale
856	T-003	Verdura	A00PX	Piselli (freschi o secchi)		locale,privato
860	T-003	Verdura	A00GY	Aglio		locale
896	T-006	Verdura	A00HQ	Pomodori		privato
897	T-006	Verdura	A00ZT	Patate		locale
913	T-011	Verdura	A00LD	Cicoria (inclusi radicchio, scarola, punta		locale
914	T-011	Verdura	A00FM	Broccoli (inclusi broccoletti, cime di rap		locale
915	T-011	Verdura	A011Z	Fagioli (freschi o secchi)		locale
916	T-011	Verdura	A05LG	Finocchi		locale
917	T-011	Verdura	A00ZT	Patate		locale
918	T-011	Verdura	A04MA	Erbe aromatiche		locale
919	T-011	Verdura	A00GY	Aglio		locale
920	T-011	Verdura	A00HB	Cipolla		locale
942	T-013	Verdura	A00HQ	Pomodori		locale,privato
943	T-013	Verdura	A0DLB	Lattuga (inclusi iceberg, lollo rossa, ron		privato

Figure 38. Example spreadsheet with data exported from online database. Data are sorted by place of purchase/production, considering only local farms or private gardens, and by food category.

In particular, the sampling of fruit and vegetables was performed twice a year according to the seasonality of different commodities production, as shown in the table 4. Sampling was performed on the whole in 17 local farms and 25 private gardens. So far, meat, eggs, oil, and honey categories, Chicken fresh meat (A01SP), Rabbit fresh meat (A01RQ), Hen eggs (A031G), and Olive oils (A036P) were sampled in 10 farms e 6 private gardens.

The sampled foods were frozen in separated containers according to their assignment as foods consumed by cases or controls; frozen samples were then sent to the laboratory for the chemical analysis of pesticide residues.

Summer		Winter	
FoodEx 2 code	**Food**	**FoodEx 2 code**	**Food**
A00HQ	Tomatoes and similar	A00LD	Escaroles and similar-
A00JR	Courgettes	A0DJS	Beetroot leaves
A00PB	Legumes with pod	A00FM	Broccoli and similar
A00ZT	Potatoes	A00MH	Spinaches and similar-
A00JA	Sweet peppers	A0DPB	Carrot
A05LG	Fennel (live plants)	A05DR	Pumpkins (live plants)
A00JM	Cucumbers	A01DN	Pears and similar-
A0DLB	Lettuces and similar	A0DRG	Kiwi
A00JC	Aubergines and similar-	A01GQ	Plums
A01DV	Grapes and similar fruits		
A01DH	Apples and similar		
A00KJ	Watermelons		
A01EA	Strawberries		
A00KE	Melons and similar		
A01GL	Peaches and similar		
A0DVX	Apricots and similar-		
A01GG	Cherries and similar		

Table 4. Fruit and vegetables sampled by seasonality on local farms and private gardens.

4.3.2 Dietary exposure assessment

Dietary exposure assessment to pesticides of the enrolled girls was performed using the EFSA Dietary Exposure (DietEx) tool [215] and considering: i) type and quantity of food items consumed, as reported in the FFQ; ii) the pesticide analytical levels determined in locally produced foods.

The equation that underlies the calculation of chemical exposure through the diet is shown below [216]:

$$Dietary\ exposure = \frac{\Sigma \text{ Food chemical concentration} \times \text{Food consumption}}{\text{Body weight}}$$

This methodology will be applied in the project when the analytical determination of pesticides will be completed. As a preliminary data and to illustrate the approach, we report the concentration of IMI detected in melon samples from local farms, being 0.045 mg/kg.

On the basis of FFQ data, we retrieved body weight information on all the subjects who reported to have consumed melon, also registering the portion of melon consumed and the analytically determined IMI concentration. Based on the above equation, the estimated mean dietary exposure value was $2.7*10^{-7}$ mg/kg body weight (bw) per day.

This value is very low compared to the ADI value for IMI equal to 0.06 mg/kg bw per day, [217], thus indicating that dietary exposure to IMI through the only consumption of a medium portion of melon does not represent a hazard to health.

Obviously, the final dietary intake estimation for this pesticide will be achieved when the analytical data on pesticide residues on all food categories will be available.

Conclusions

This study demonstrated that a properly modified FFQ is a key point in the epidemiological study and able to meet different requirements such as directing towards specific food sampling and supporting the estimation of dietary exposure for each food category (meat, fish, eggs, milk, honey, fruits, vegetables, cereals and oil) in girls, with the final aim to evaluate a possible association with health issues, such as the premature thelarche.

5 Conclusions

Overall, the results from our study, including both the *in vitro* toxicological study and the epidemiological study, provided relevant evidence on pesticide toxicology and suitable tools for their risk assessment.

The *in vitro* toxicological study demonstrated that all three pesticides i.e., CPF, IMI and GLY exerted non overlapping effects regarding to the endpoints evaluated but all acted with endocrine disrupting activity in both MCF-7 tumorigenic and MCF-12A non-tumorigenic cell lines. Both cell lines proved to be a suitable and sensitive model to highlight the effects of pesticides, in particular, MCF-12A cell line, as representative of an "effective cellular condition", was found to be a valid study model to evaluate the potential effects of endocrine disrupting chemicals.

The selected panel tests, including cell viability and proliferation, oxidative stress, energy metabolism, estrogen production and nuclear receptor gene expression (i.e., ERα, ERβ, AR, PgR and AhR), proved to be a sensitive tool to highlight early effects following exposure to the selected pesticides.

The effects were observed even at exposure concentrations in the nano-molar range and corresponding to a real exposure scenario in children, thus raising concern on the harmfulness of such widely used pesticides on human health, especially in developing organisms as children. Thus, although our results did not provide conclusive evidence on mammary gland development alteration, overall they confirmed and described novel effects, especially regarding the gene expression modulation of NRs, deserving attention.

The magnetic levitation 3D model obtained with MCF-12A cells could represents a suitable tool to study the effects of chemicals in conditions more closely mimicking mammary gland physiology *in vivo*.

As regards the epidemiological study, the implemented of FFQ has proven to be a suitable tool to direct proper sampling of locally produced foods and perform pesticides' dietary exposure assessment.

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Abbreviation list

2D	two-dimensional
3D	three-dimensional
ADI	Acceptable Daily Intake
AhR	Aryl hydrocarbon receptor
AMPA	Ainomethylphosphonic acid
AR	Androgen Receptor
ATP	Adenosine triphosphate
BMI	Body Mass Index
CPF	Chlorpyrifos
DHT	5-dihydrotestosterone
DietEx	Dietary Exposure
E2	17β-estradiol
ECHA	European Chemicals Agency
ECM	Extracellular matrix
ECVAM	European Centre for the Validation of Alternative Methods
ED	Endocrine Disruptors
EDCs	Endocrine-disrupting chemicals
EFSA	European Food Safety Authority
ER	Estrogen Receptor
FFQ	Food Frequency Questionnaire
FSH	Follicle-stimulating hormone
GLY	Glyphosate
GnRH	Gonadotropin-releasing Hormone
HI	Hazard identification
HPG	Hypothalamus-pituitary-gonadal
IARC	International Agency for Research on Cancer
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IMI	Imidacloprid
IPT	Idiopathic Premature Thelarche
LARN	Nutrients and Energy for the Italian population
LH	Luteinizing hormone
LO(A)EL	Lowest Observed Adverse Effect Level

MRL	Maximum Residue Level
nAChR	Nicotinic acetylcholine receptor
NOAEL	No Observed Effect Level
NRC	American National Research Council
NRs	Nuclear receptors
OECD	Organization for Economic Co-operation and Development
PEACH	Integrated approach to evaluate children agricultural pesticide exposure and health outcome
PgR	Progesterone Receptor
PRIMo	Pesticide Residues Intake Model
PS	Phosphatidylserine
RA	Risk assessment
ROS	Reactive Oxygen Species
T	Testosterone
TDI	Tolerable Daily Intake
THR	thyroid hormone receptor
UF	Uncertainty factor