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"Toxicogenomics in The Analysis of Endocrine Disrupting Chemicals Effects on Pancreatic Islet Function: The Bisphenol A Case"

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Abstract

Bisphenol A (BPA) is widely used in plastic products. It is classified as an Endocrine Disrupting Chemical and has been associated to several endocrine-related disorders. The widespread exposure and the health effects are of concern. Epidemiological studies and in vivo and in vitro experiments correlate the BPA exposure to alterations of pancreatic islets, liver and adipose tissue functions. Given these data, its prevalence in the environment and the presence in serum from humans worldwide, BPA may play a role in the rapid increase of incidence of metabolic disorders.

In this work we studied the effects of low doses of BPA on ex vivo primary murine pancreatic islets through a toxicogenomical approach. We found, through trascriptome analysis,that BPA is able to alter the expression of specific genes involved in mitochondrial functions, ROS detoxification and insulin exocytosis signal generation. Moreover, we demonstrated that BPA exposure impairs islets viability and promotes apoptosis in dose-dependent manner. Finally, we assessed that this apoptosis triggering is due to a BPA-dependent increase of ROS that activate the NF*k*B pathway. Taken together, our results suggest that exposure to low doses of BPA interferes with mitochondrial functions, which, in turn, leads to intracellular ROS increase, NF*k*B pathway activation and, finally, to apoptosis. Therefore, this work confirms the detrimental effects of BPA on pancreatic islet functions, shedding a new light on its mechanism of action.

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1 Introduction

1.1 Endocrine Disrupting Chemicals

Recently, it is emerging the idea that micro-traces of some environmental pollutants could modify organism responses, increase susceptibility to certain diseases, and have chronic effects visible only in long run. This has opened a new field in environmental toxicology with the identification of new class of compounds: **E**ndocrine **D**isrupting **C**hemicals (EDCs). The US Environmental Protection Agency (EPA) defines the EDCs as "Exogenous agent that interferes with the production, release, transport, metabolism, binding action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes". This group of molecules is highly heterogeneous and includes both synthetic and natural compounds, e.g. dioxin and dioxin-like compounds, polychlorinated biphenyls (PCBs) and Polybrominated biphenyls (PBBs) used in chemical and electronic industries, dichlorodiphenyltrichloroethane (DDT), chlorpyrifos and other pesticides, fungicides and weed killers, plasticizer and plastic constituent such as Bisphenol A (BPA) and phthalates, pharmaceuticals such as Diethylstilbestrol (DES), Plant Growth Regulators (PGRs) and phytoestrogens (e.g. genistein, daidzein, coumestrol, resveratrol), heavy metals and metalloids. The sources of exposure to EDCs are diverse and vary widely around , the most part come from industrial and metropolitan pollution but they may be also found in many everyday products– including plastic bottles, metal food cans, detergents, flame-retardants, food, toys, cosmetics (Fig. 1)[1], [2], [3]. Exposure occurs through drinking contaminated water, breathing contaminated air, ingesting food, or through transdermal absorption. Furthermore, in utero and neonatal exposure and transgenerational effects, through the germline cells, are gaining more and more interest.

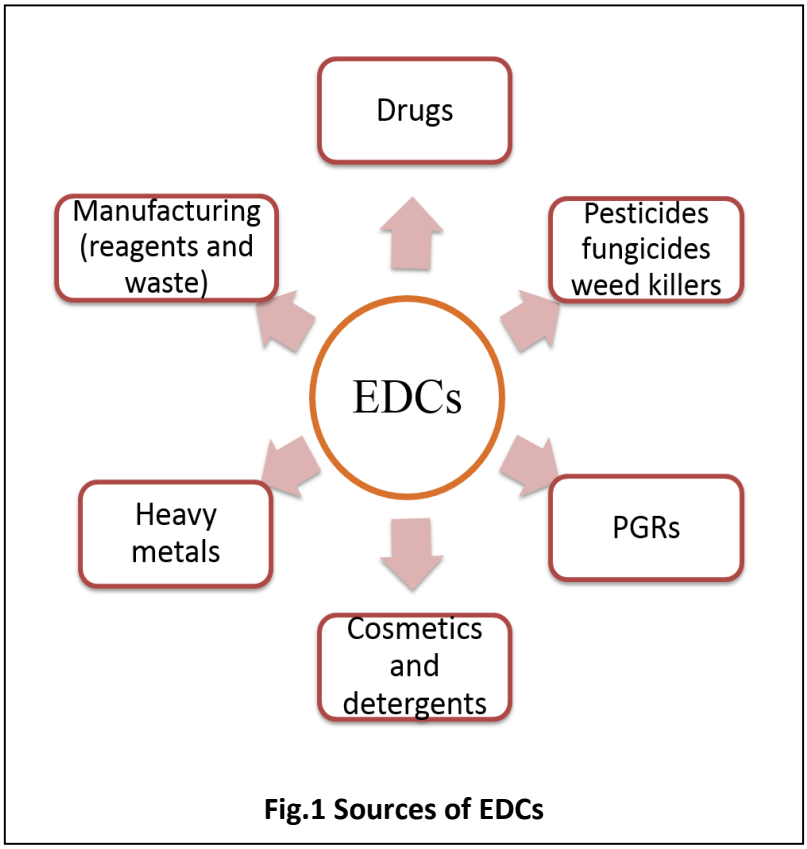


Fig.1 Sources of EDCs

Industrialized areas are typically characterized by contamination from a wide range of industrial chemicals that may leach into soil and groundwater and contaminate air. These complex mixtures enter the food chain and, owing to bioaccumulation and biomagnification, accumulate in animals higher up the food chain such as humans. Furthermore, several EDCs were designed to have long half-lives and, although this was beneficial for their industrial use, it has turned out to be quite detrimental to wildlife and humans. Because these substances do not decay easily, even substances that were banned decades ago remain in high levels in the environment. Some endocrine disruptors are detectable in so-called "pristine" environments at remote distances from the site they were produced, used, or released due to water and air currents, to become incorporated into the food chain in an otherwise uncontaminated region. Others, such as BPA, may not be as persistent, although recent evidence suggests longer half-lives but are so widespread in their use that there is a prevalent human exposure. In the human body, due to their lipophilicity, they accumulate in adipose tissue, where thay can be retrieved unmetabolized or metabolized into more toxic compounds than the parent molecules. From this tisuue they are slowly released

and can be detected as part of the body burden of virtually every tested individual animal or human [4], [5].

1.1.1 Low Dose

One of the main feature of EDCs is their ability to exert their effects at very low concentrations. Indeed, the term "low dose" has become common to indicate the physiologically and pathologically relevant dose of an EDC [6].It is well established in the endocrine literature that natural hormones act at extremely low serum concentrations, typically in the picomolar to nanomolar range. Many studies published in the peer-reviewed literature document that EDCs can act in the nanomolar to micromolar range, and some show activity at picomolar levels. The Low Dose is considerably lower than the doses usually used in standard toxicological tests and than of the previously set LOAEL and NOAEL. The Low Dose is also frequently comparable to environmental concentration and to concentration measured in human fluids of EDCs [7].

1.1.2 Non monotonic dose response curve

Another EDC distinctive feature is the non-monotonic dose response curve. Classically, it was assumed that curves describing dose and response are monotonic, i.e. dose escalation leads to a greater effect and the slope never reverses from positive to negative or vice versa. For EDCs non-monotonic curves, including "U-shaped" or "inverted-U-shaped" dose response relationships, have been described [8]. The reasons why dose responses to toxicants may be non-monotonic can be manifold: the activation of metabolizing enzymes or conjugation substrates may result in a U-shaped dose response for some endpoints, with effects at low and at high levels of exposure, and diminished or nonexistent effects at intermediate levels, due to metabolic clearance. Furthermore, adaptive responses through complex cell signaling pathways and feedback mechanisms could cause non-monotonic effects that are inconsistent with the traditionally expected dose-response curves based on extrapolation of high dose data

predicting a decreasing effect for lower doses. Finally, the EDCs could have multiple targets with different affinity either in an organism or in an organ or even in single cells. Therefore, when in low concentration, they may be able to bind only some targets, while at higher concentrations can bind other targets producing changes in the effects and causing non-monotonic dose-response curve.

1.1.3 EDCs - Mechanisms of Action

Our understanding of the mechanisms by which EDCs exert their effect has grown. They were originally thought to exert actions primarily through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, among others. Today, basic scientific research shows that the mechanisms are much broader than originally recognized. Thus, endocrine disruptors act via nuclear receptors, non-nuclear steroid hormone receptors (*e.g.*, membrane ERs), non-steroid receptors (*e.g.*, neurotransmitter receptors), orphan receptors [*e.g.*, aryl hydrocarbon receptor (AhR)], enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine system [9]. Binding these targets the EDCs can exert their effects in various ways such as having agonist or antagonist actions, competing for transport molecules, modifying natural hormones homeostasis (production and elimination), modifying hormone receptors expression, altering gene expression, disrupting epigenetic cells programming, disturbing intracellular transduction pathways [9].

A number of issues have proven to be key concepts to a full understanding of mechanisms of action and consequences of exposure to EDCs:

- Latency from exposure
- Age at exposure
- Transgenerational, epigenetic effects
- Cocktail effects
- Nontraditional dose-response dynamics

1.1.3.1 Age of exposure and critical windows

Exposures to EDCs have different effects depending on the life stage of the exposed, fetus, infant or adult. Effects resulting from adult exposure are generally reversible and are termed "activational". Effects resulting from exposure during organ development (beginning during prenatal development and continuing in postnatal life through puberty) may result in persistent alterations of the affected systems, even in the absence of subsequent exposure; these effects are termed "organizational" [10]. Some organizational effects are measurable immediately upon exposure and persist throughout the life [11]. Other organizational effects are undetectable at the time of exposure, but they become apparent in subsequent adulthood [10], [12] . Windows of vulnerability, also known as critical periods, during which the developing system is most sensitive to exposure, are common features of organizational effects [13]. Exposures occurring outside the critical periods will not elicit organizational effects. There is evidence that organizational effects of estrogenic endocrine disruptors such as BPA are mediated by epigenetic alterations in DNA [14]. Organizational and activational effects on the same tissue often differ qualitatively as well as in duration and in the dose required to elicit effects.

1.1.3.2 Transgenerational and epigenetic effects

EDCs may affect not only the exposed individual but also subsequent generations. Recent evidence suggests that the mechanism of transmission may involve in some cases the germline and may be non-genomic. That is, transmitted effects may not be due to mutation of the DNA sequence, but rather due to modifications of epigenetic state of the cells. Generally, an epigenetic effect is defined as heritable changes in gene expression or cellular phenotype without any alterations in the DNA sequence, and its mechanisms include DNA methylation, histone modifications and expression of non-coding RNAs (including microRNAs) [15]. Recent investigations have examined the relationships between exposure to environmental chemical xenobiotics and epigenetic effect induction and have reported that several toxicants have a potential role in modifying epigenetic marks [16]. In addition to epigenetic mechanism, reports shows that estrogens and xenoestrogens have the ability to cause genomic instability [17], [18], with improper chromosome segregation that can lead to chromosomic aberrations or aneuploidy in the progeny.

1.1.3.3 Cocktail effects

The large number of EDCs present in the environment raises questions about the effects of simultaneous exposure to multiple compounds. However, relatively few studies have discussed combination effects. For some authors effects of combined exposure to several (xeno)estrogens at equipotent concentrations were in high agreement with the predictions made with the model of concentration addition (based on the assumption that chemicals act via a similar mechanism) [19], [20]. Other endpoints, however, show far more complex outcomes after combined exposure.

1.1.3.4 EDCs – cell targets

Although EDCs can act through several mechanisms, most information is available about interference of EDCs with sex hormones, especially xenoestrogens.

1.1.3.4.1 Activation of the classical nuclear receptors.

EDCs can bind nuclear receptors (NR) and act as agonist or antagonist. They can control gene networks and modulate target cell activities through the activation of NRs, which bind responsive elements in the promoters of target genes and regulate target gene expression [19]. After activation by binding to a ligand resulting in a conformation change, receptors can act as transcription factors, interacting with coactivators and corepressors and with DNA sequences [21], [22]. However, the change in conformation differs in function of the ligand [23]–[27]. That is what has been observed: receptors bound to xenobiotic ligands do not have exactly the same influence on gene expression as receptors bound by endogenous ligands [28], [29]. This has been studied for several EDCs, such as octylphenol, nonylphenol, endosulfan, kepone [30] and BPA and for the phytoestrogen coumestrol which has opposite effects on the regulation of estrogen receptors compared to 17–β-estradiol (E2) [31].

1.1.3.4.2 Activation of membrane-bound receptors

Hormones can also induce non-genomic responses based on plasma membrane receptors (e.g. mERs, GPR30) and acting through second messenger-triggered signal cascades [32]. The rapid non-genomic mechanisms have been shown to interact with cytoplasm signal transduction molecules such as cAMP and adenylate cyclase, calcium, PI3K, PKB, and G-proteins or directly with secondary transcription factors such as AP-1, STATs, NFκB, and Sp1 [33][34]. These actions can result in several different effects, e.g. change in ions concentrations (Ca^{2+}, K^{+}) that can lead to cell motility or hormone exocytosis such as prolactin or insulin [35]. EDCs, such as dieldrin, endosulfan, nonylphenol, BPA, coumestrol, and DES are known to affect Ca^{2+} influx and PRL or insulin release.

1.1.3.4.3 Cytoplasmic interactions

Besides with nuclear-and plasma membrane-associated receptors, EDCs can also interact with targets within the cytosol. One of the best-studied examples is the

activation of Src/Ras/ERK (MAPK) pathway, in a mechanism linked to the proliferative effects of estrogens[36]. Another example is the modulation of nitric oxide (NO), with activation of endothelial NO synthase (eNOS, the enzyme responsible for NO production) [36].

1.1.3.4.4 Activation of Estrogen-Related Receptors.

A particular case is the bind of xenoestrogens to the Estrogen Related Receptors (ERRs). ERRs are a subfamily of orphan nuclear receptors closely related to ERα and ERβ. Three of these ERR are known: ERRα, ERRβ, and ERRγ. ER and ERR show a considerable amount of similarity in aminoacid sequence, but E2 does not bind ERRs. However, ERR can bind to estrogen response elements which suggests a possible overlap between ER and ERR action [35]. ERRs show spontaneous transcriptional activity, which is repressed by a few chemicals. For example, DES represses the molecular activities of ERRs. Another inverse agonist of ERRγ is 4-hydroxytamoxifen (4- OHT). On the other hand, bisphenol-A has been observed to have a distinct antagonist action to the inverse agonist activity of 4-OHT, thus preserving ERR-activity in the presence of 4-OHT [37].

1.1.3.4.5 Interference with hormones metabolism and hormonal feedback regulation.

Along with the direct influence of EDCs on endocrine targets, they can affect endogenous hormones production through negative and positive feedback [4] or influencing enzymes involved in the process of hormones synthesis. They can also compete with endogenous hormones for transport molecules.

For example, some EDCs, amongst others PCBs and BPA, can disrupt the link between neuroendocrine systems and peripheral endocrine systems for the control of homeostatic processes including reproduction, growth, metabolism, energy balance, and stress response. PCBs reduce the thyroxin and TSH (thyroid stimulating hormone) response to TRH (thyrotropin-releasing hormone), which result in metabolism and energy balance deregulation. Thyroid disruption also has consequences for neural

development [38]. Furthermore, the obesogenicity of DES has been supposed to involve action on the developing hypothalamic circuits, which are important for the energy balance [38].

An esplicative example of how EDCs influence the hormone synthesis is he case of xenoestrogen. Xenoestrogens have been shown to affect steroidogenic enzymes, including hydroxysteroid dehydrogenases, aromatase, sulphatases, and sulphotransferases. Mostly, steroidogenesis is inhibited by xenoestrogens [39]. Atrazine was observed to stimulate aromatase activity in some cell types, which leads to a higher synthesis of estradiol [40]. On the other hand, DDT and several metabolites were reported to inhibit aromatase activity in some cell types [39].

1.1.3.4.6 Cross-talk with other pathways after binding other receptors.

The expression of many genes involved in xenobiotic/drug metabolism and transport is regulated by at least three nuclear receptors or xenosensors: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR). These receptors establish crosstalk with other nuclear receptors or transcription factors controlling signalling pathways that are important to the homeostasis [41]. For example, many drugs and environmental pollutants activate PXR. A significant proportion (54%) of compounds with estrogenic activity or those able to bind ER were found to be PXR activators (estradiol, ethynylestradiol, BPA, tamoxifen, phthalates, alkylphenols) [42]. The PXR, which regulates several cytochrome P450 enzymes crucial in the oxidative metabolism of a wide range of chemicals can, thus, play a role in the inactivation of toxic or carcinogenic chemicals, in the activation of pro-carcinogens and could also act as a protector of the endocrine system from chemical perturbation [42]. The AhR is a ligand activated transcription factor and a key regulator of the cellular response to xenobiotic exposure. It belongs to the family of the bHLH-PAS proteins, and is ubiquitously expressed and well conserved throughout evolution. It is strongly activated by organic compounds such as dioxins, PCDF, PCB and BPA [43] showed that the agonist-activated AhR/Arnt heterodimer directly associates with

estrogen receptors ER−α and ER−β. This association resulted in the recruitment of unliganded ER and the coactivator p300 to estrogen responsive gene promoters, leading to activation of transcription and estrogenic effects.

1.1.4 Epidemiology of EDCs

The endocrine related diseases and disorders are strongly increasing in the last 50 years. The speed with which the increases in disease incidence has occurred in recent decades rules out genetic factors as the sole plausible explanation**.** Environmental component and other non-genetic factors are supposed to play a role. Despite considerable public interest and research activity, the scope and magnitude of the impact of putative EDCs on human health are largely unknown. Exposures in some cases, have been unusually high, such as those from chemical spills or sewage out falls; therefore they are not typical of the ambient exposures normally encountered by wildlife or human populations. So one of the main challenges was, and still is, to demonstrate whether the low environmental concentrations of EDCs are detrimental for health or not. In wildlife, a number of adverse effects have been linked to specific EDCs. Effects linked to endocrine disruption have been largely noted in invertebrates, reptiles, fish, birds and mammals [44]. Over the last decade, scientific understanding of the relationship between exposure to endocrine disruptors and health has advanced rapidly. There is a growing concern that maternal, fetal and childhood exposure to EDCs could play a larger role in the causation of many endocrine diseases and disorders than previously believed. This is supported by studies of wildlife populations and of laboratory animals showing associations between exposure to EDCs and adverse health effects and by the fact that the increased incidence and prevalence of several endocrine disorders cannot be explained by genetic factors alone.

There are several health problems worldwide with postulated association to the exposure to EDCs: decreased sperm counts/quality, increased incidence of hypospadias and cryptorchidism, altered birth sex ratios, miscarriage, increased

incidence of cancer, altered development, brain and behavior defects, impaired immune function, attention deficit hyperactivity disorder, hyper allergic diseases, asthma, obesity, heart disease and type two diabetes. Just some of the several recognized epidemiological associations between EDCs and pathologies and related published papers are listed in table 1.

TABLE 1 Epidemiological associations between EDCs and disease

DISORDER	EPIDEMIOLOGICAL ASSOCIATED EDCs
Abnormal sex ratio	DES [45], PCBs [46], Dioxins [47]
Negative pregnancy outcomes	Lead [48], BPA [43][47], Pesticides [50]–[52], Chlorpyrifos [53], DDT/DDE [54]
PCOS	BPA [55]
Female sub-fecundity	Lead [56], [57], Pesticides [58]–[62], DDT/DDE [63]–[65], PCBS [63], [66]–[68]
Female altered puberty beginning	DDT/DDE [69], [70], Dioxins [71], Lead [72], [73], PBBs [74], PCBs [72]
Male urogenital malformations	DES [75]–[77], DDT/DDE/DDE [78], PCBs [79], Phthalate [80]
Poor sperm quality	PCBs [81], [82], Dioxins [47], Phthalates [83], [84]
Altered menstrual cycle	Lead [48], Pesticides [85], DDT/DDE [86][69], PCBs [86], [87], Dioxins [88], Phytoestrogens [89]
Breast cancer	DES [90]–[92], Dioxins [93]
Prostate cancer	DES[90]–[92], Dioxins [93], Pesticides [94], [95], PCBs [96]
Thyroid cancer	Pesticides [97], [98], Dioxins [97], PCBs [98]
Metabolic disorders	BPA Phthalates [101], DDT/DDE [102]

However, whether or not endocrine disrupting compounds (EDCs) could be a contributing factor remains the subject of intense scrutiny and other determinants such as diet, stress, and body weight, likely, also play a role [4], [103]–[106].

The most commonly studied EDCs are DDT, PCBs, dioxins and BPA[104], [105]

1.2 Bisphenol A

BPA (2, 2-bis (4-hydroxyphenyl) propane) is a small (228 Da) molecule, composed by two phenolic functional groups [108]–[113]. It is prepared by the combination of two equivalents of phenol with one equivalent of acetone; it was first synthesized by A. P.

Dian in in 1891 and was later investigated in the 1930s during the search for synthetic estrogens [113].

The BPA is used as monomer in polymerization reaction to produce polycarbonate plastics and epoxy resins and in the synthesis of polyesters, polysulfone, polyvinylchloride, and polyethylene terephthalate. Moreover, due to its ability to give plasticity and toughness, it is used as additive in production of nearly all plastics or plastic based materials. The BPA is among the highest-production-volume chemicals in the world, with an annual production of over 2 million tonnes [108], [113].

Therefore it is widely present in a multitude of everyday products, such as drinking water tanks and pipes, medical and dental devices (such as catheters and dental sealants), eyeglass lenses, CDs and DVDs, household electronics, paints, adhesives, food and beverage cans (as lining film), childhood products (such as toys and baby-bottles), and in almost all plastic tools or products [113]–[116].

The BPA is within these products as a polymer. In consequence of mechanical, chemical o physical stress (e.g. detergents, heat or aging), the BPA polymers break down, because of hydrolysis of ester bonds (Fig. 2), and the monomers can seep into water, foods and dust particles, contaminating the environment and providing ample

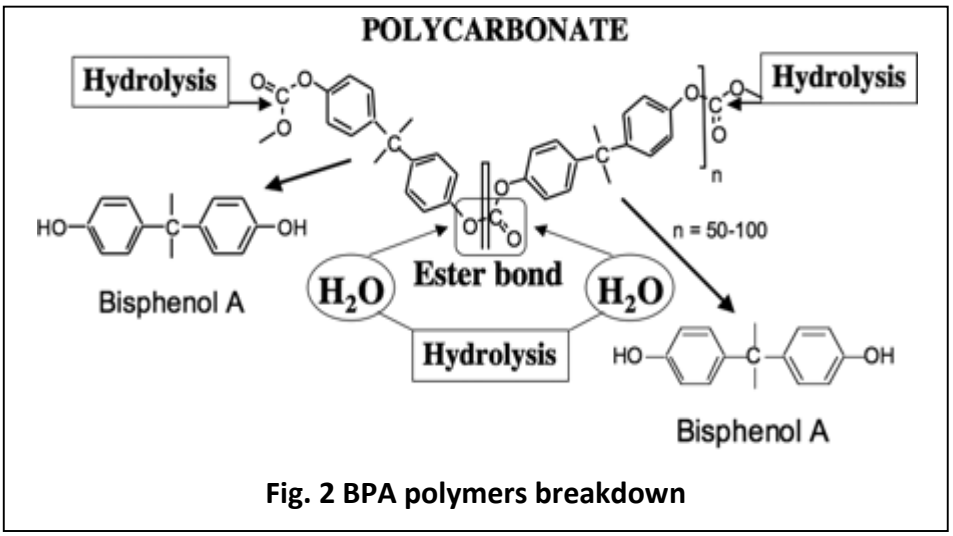


Fig. 2 BPA polymers breakdown

entry ways for BPA into humans and animals. This process is commonly known as “leaching” [109], [111], [115], [116].

The primary source of exposure to BPA for most people is through the diet. While inhalation and transdermal absorption are other possible routes of exposure, BPA in food and beverages accounts for the majority of daily human exposure.

Therefore, exposure to BPA is nearly ubiquitous. Measurements by American Centers for Disease Control (CDC) established that 92.6% of American citizens 6 years or older have detectable BPA levels in their urine and its concentration in human serum ranges from 0.2 to 1.6 ng/ml (0.9-7 nM) [119]–[121].

A comprehensive, cross-sectional study of dust, indoor and outdoor air, and solid and liquid food in preschool-aged children suggests that dietary sources constitute 99% of BPA exposure [119]. For example, in a randomized control study, the researchers found that consumption of one serving of canned soup daily over 5 days was associated with a more than 1000% increase in urinary BPA [122]. Moreover, since BPA is a lipophilic compound it can also accumulate in fat, with detectable levels found in 50% of breast adipose tissue samples from women, suggesting that the compound accumulates in fat and other physiologic compartments [78].

Concerning the widespread exposure and the potential risk of this compound, in the 1980s LOAEL for BPA was determined at 50mg/kg bw/day and the NOAEL at 5mg/ kg bw/day. These doses were established in a 2-year carcinogenesis study conducted in adult rodents exposed daily to high doses of BPA. Nevertheless, in 1988, the EPA calculated a ‘reference dose’’ or safe dose of 50 μg/kg bw/day in a series of studies in which the changes of body weight in animals fed diets containing BPA were analyzed [123]. This reference dose remains the current safety standard for BPA today despite new knowledge about BPA, including the numerous reports of non-monotonic dose response effects of BPA, and despite the numerous scientific evidence supports that

BPA can interfere with the endocrine signaling pathways at doses below the calculated safe dose [117], [123]

1.2.1 BPA- Mechanisms of Action

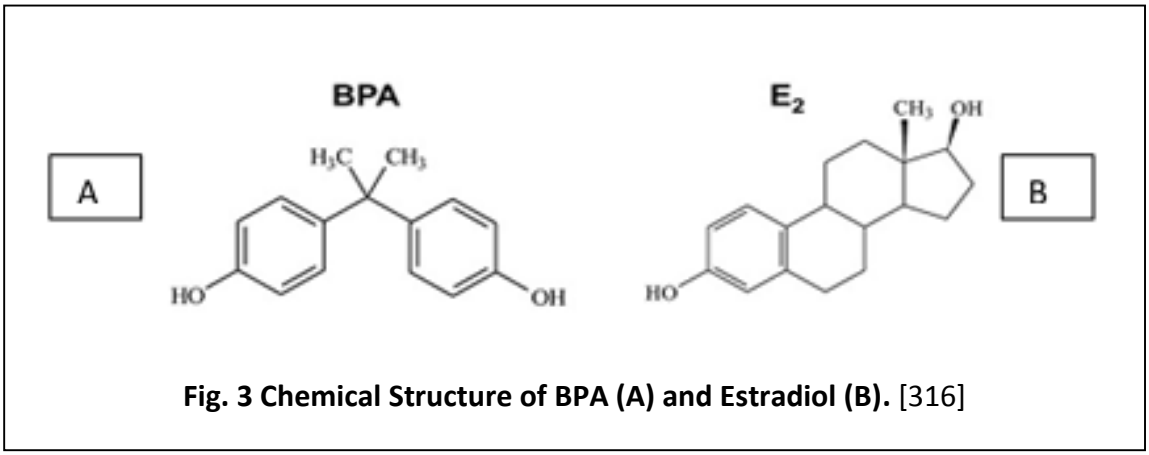


Fig. 3 Chemical Structure of BPA (A) and Estradiol (B). [316]

The BPA is basically known for being a xenoestrogen. Indeed, before its extensive use in chemical industry, it was originally investigated as synthetic estrogen but abandoned for pharmaceutical use because of the discovery in 1938 of DES with greater estrogenic effect [124]. The core structure of BPA resembles that of natural 17β-estradiol (E2) (Fig. 4) and fits in the ER binding pocket. This was confirmed by biochemical assays that examined the kinetics of BPA binding to ER and determined that BPA binds both ERα and ERβ [123][124].

Therefore, the most literature converges on its effects on estrogens-responsive tissues or organs (specifically on reproductive system), mediated by classic nuclear ERs or novel transmembrane (GPR30) or membrane-bound ERs.

Until recently, BPA was considered a weak environmental estrogen because of its affinity for the nuclear ERs is approximately 10000-fold weaker compared to estradiol [126]. However, recent studies confirmed that BPA is a SERM and can have agonist or antagonistic action on ERs. These actions, through a variety a pathways, can induce (or inhibit) cellular responses at very low concentrations, below the levels where BPA

is expected to bind to the classical nuclear ERs (reviewed in [127]). Extensive biochemical studies of ERα have identified two distinct gene transactivating regions, termed AF1 (found in the amino terminus) and AF2 (located in the carboxyl terminus) [125].ER agonists are subdivided based on their ability to activate these regions. Binding of BPA to the ERs alters their ability to recruit co-activators and this may contribute to the complex tissue-specific responses to exposure, as well as to the dual action (agonistic and antagonistic) of the BPA [128]. For instance, BPA is able to alter the gene expression in cells containing ERβ where TIF2 is the main coactivator, but may be equally effective in cells expressing either ERα or ERβ if the steroid receptor coactivator-1a is present [129].

Several membrane steroid receptors have been described, including a membrane-bound form of ERs (mER) that is similar but not identical to the nuclear ERα [128][129] and a transmembrane ER called G protein-coupled receptor 30 (GPR30) [132]. BPA has been shown to bind to both mER and GPR30. Studies have determined that these membrane-bound receptors are capable of non-genomic steroid actions [130]–[133] (also reviewed in [128]). For example, GH3/B6 pituitary cells, which naturally express mER, respond to low-level BPA exposure by producing a calcium flux, which leads to prolactin release [131].

The antiandrogenic properties of BPA are still somewhat in dispute. There have been mixed results with mammalian cell reporter assays, *i.e.*, some groups measured antiandrogenic activity of BPA with a half-maximal response at 2.14–3.2 μM [132][133], whereas others were unable to demonstrate any antagonist activity [136].

Additional studies indicate that BPA also binds to an orphan nuclear receptor called estrogen-related receptor-γ (ERR-γ) [137]. Although the endogenous ligand for ERR-γ remains unknown, the human receptor behaves as a constitutive activator of transcription and may play a role in differentiation and maturation of the fetal brain [37].

In addition to its estrogenic activity, there is some evidence that BPA binds to thyroid hormone receptor, acting as a thyroid hormone antagonist by preventing the binding of T3. The affinity of BPA for this receptor several-fold lower than its affinity for the ERs, nevertheless, at very low concentrations, it is able to inhibit the TR-induced gene expression recruiting the co-repressor N-COR [138]. Moreover, in a recent study we showed that very low concentration of BPA (10^{-9}M or lower) is able to affect the pituitary-thyroid axis directly altering, in vivo and in vitro, the expression of genes involved in thyroid hormones synthesis (i.e. thyroblobulin, sodium iodide symporter) or their expression regulators (i.e PAX8 protein). This effect is not mediated neither by ERs nor by TRs, but mainly by retinoid acid receptors (RARs) and NF-*k*B [139].

BPA also binds the aryl hydrocarbon receptor (AhR) [135] but the effects remain unknown at this time. Because AhR can cross-talk with other receptors including ERs and the androgen receptor, endocrine-related endpoints may be affected by its activation (reviewed in [140]). It is also reported that BPA interacts with and activates human PXR (pregnane X receptor), a nuclear receptor that functions as a regulator of xenobiotics, acting as a potent agonist [141].

It has been reported that BPA can affect cell-signaling mechanisms by increasing intracellular calcium and phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), and nuclear translocation of nuclear factor NF-*k*B [142].

Growing evidence suggests that BPA is associated with oxidative stress [141][142]. Indeed, it has been revealed that BPA can disturb oxidative homeostasis through direct or indirect pathways, including mitochondrial function [145], modulation of antioxidant enzymes and increase thiobarbituric acid-reactive substances in the brain, kidney and testis of mice exposed throughout embryonic/fetal route [146]. Oxidative stress has been implicated in aging and many pathological disorders, such as ischemic diseases, neurodegenerative diseases, diabetes, and cancer, although the underlying mechanisms are not always completely understood [147].

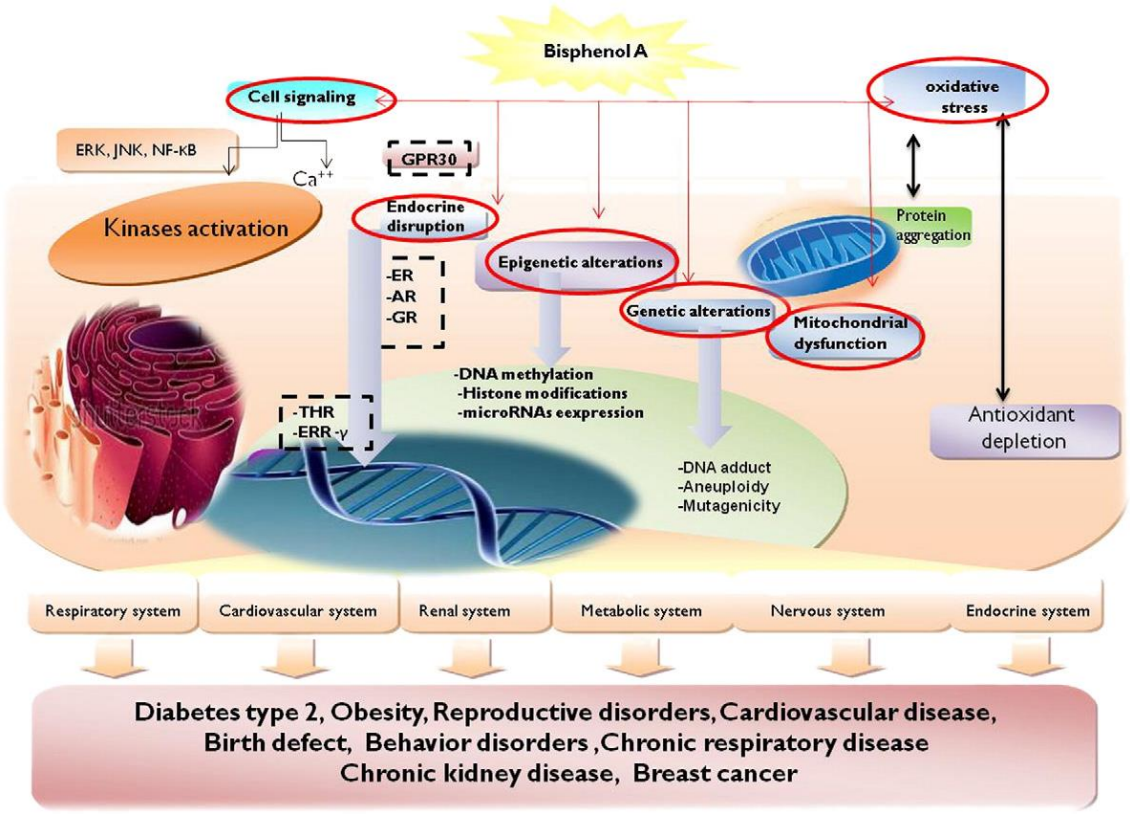


Fig. 4 Cellular and molecular mechanisms of action of BPA in human disease induction. *AR: androgen receptor, ER: estrogen receptor, ERK: extracellular signal-regulated kinase, ERR-γ: estrogen-related receptor gamma, GPR 30: G protein-coupled receptor 30, GR: glucocorticoid receptor, JNK: c-Jun N-terminal kinase, NF-κb: nuclear factor-kappa B THR: thyroid hormone receptor.* [9]

Finally, BPA can alter cell function and response modifying epigenetic state of the cells. This is of particular concern when happen in germinal cells and in perinatal developmental tissue. In fact, epigenome alteration is probably the process that allow the BPA to change offspring phenotype, predisposing individuals to develop certain diseases in adulthood. Specifically it has been demonstrated that low dose of BPA ($2x10^{-8}$ – $2x10^{-4}$ M) alters the gene expression of epigenetic regulatory factors such as DNA methyltransferases (DNMTs) and methyl-CpG binding protein 2 (MECP2) in a dose-dependent mechanism [148]. Moreover, BPA increases the expression of the histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2) level [149], which is involved in maintaining the transcriptional repressive state of genes over successive cell generations [150]. In addition, BPA exposure has been shown to alter microRNA expression, in the fetal ewe ovary [151] and in human placental cell lines (miR-146a)

[152] suggesting a potential impact in reproductive system and developmental pathways.

1.2.2 BPA - Health Issues

Several publications associate low doses BPA exposure to endocrine-related disorders, anomalies and diseases. For example, since 2007, more than 50 epidemiology studies have examined various health outcomes in relation to BPA exposure and the number of experimental studies in vivo or in vitro is on the rise. The most of these studies use a dose or a concentration of BPA equal or below the EPA cut-off $1x10^{-7}$M or 50ug/Kg/day. Nevertheless many studies examining doses at or below $1x10^{-9}$M report significant biological effects [153]–[166] challenging the hypothesis that BPA only has "weak" endocrine disrupting activity and suggesting that it may be reasonable shift down the reference safe dose set by EPA.

In sum, the literature indicates there are reproducible effects of BPA exposures on a number of endpoints including reproductive system issue, hormonal related cancers, behaviors, liver function, thyroid hormone signaling and metabolic disorders.

All experimental studies examined in the sections below, unless otherwise specified, use concentrations or doses equal to or lower than the EPA reference dose.

1.2.2.1 Reproductive system

Epidemiological studies indicate that BPA is related to decreased sperm quality (low concentration, count, vitality and motility) [167][168], male genital anomalies (shorter anogenital distance and cryptochidism) [169], [170], altered blood sex hormones levels (estrogens, androgens and gonadotropins) [169], [171]–[173][174], polycystic ovary syndrome [121], [175], [176] and endometrial disorders (e.g. endometriosis) [177], [178]. Investigations conducted in fertility centers have shown that high urinary level

of BPA are associated with poor ovarian response to hormone stimulation [179], [180]decreased probability of in vitro fertilization (55% lower for every doubling of BPA level) [181] and high probability of implantation failure [182]. Moreover, a study of Sugiura-Ogasawara et al. [49] demonstrated that women who experienced recurrent miscarriages had significantly higher total serum BPA than the healthy controls from the same town. Of the miscarriage patients who subsequently had successful pregnancies, there was a trend of less serum BPA. Probably, this increase in miscarriages is due to chromosomal anomalies (aberrations and aneuploidies), induced by BPA in oocytes [49], [183], [184].Several in vitro and in vivo studies confirm these negative outcomes in reproductive performances and in the disorders of the reproductive apparatus. Studies on cell lines or on ex vivo primary cells isolated from the reproductive tract identified several effects of BPA ranging from altered hormone secretion [185] to altered gene expression [186]–[188] and altered proliferation [157], [159], [189], [190]. Animal studies showed that BPA alters estrous cycles, number of oocytes, receptors expression and cell proliferation in multiple compartments of the uterus, testis weight at puberty and in adulthood and prostate size [113]. Furthermore, BPA was shown to induce hypermethylation of estrogen receptor promoter regions in rat testis, indicating in the epigenetic changes one of the possible mechanisms of BPA induced adverse effects on spermatogenesis and fertility [191].

1.2.2.2 Hormonal cancers

The studies on effects of low doses of BPA on cancer induction are controversial, except for breast cancers. However, the idea that BPA can play a role in tumorigenesis and neoplastic progression of hormonal-dependent cancers is well accepted. Indeed, studies in rodents have shown that BPA induces mammary pre-neoplastic or neoplastic lesions [192]–[195]. Two studies in vivo and in vitro showed that low BPA doses can accelerate mammary tumorigenesis and metastasis in MMTV-erbB2 mice [196] and can induce a profile of tumor aggressiveness in high-risk cells from breast cancer

patients [197]. Another recent study revealed the presence of carcinomas in the mammary glands of rats exposed to low doses of BPA only during early development in the absence of any additional carcinogen treatment [198].

Furthermore, there is evidence from rodent models and human prostate cell lines that BPA can influence prostate carcinogenesis, modulating cancer cell proliferation and stimulate disease progression. The recent reports have provided evidence that early life exposure to BPA may increase susceptibility to hormonal carcinogenesis in the prostate gland, possibly by developmentally reprogramming carcinogenic risk [199]. Studies using a rat model showed that brief neonatal exposure to a low dose of BPA significantly increased the incidence and grade of prostatic intraepithelial neoplasia following adult estrogen exposure. This model of sensitivity to hormonal carcinogenesis is relevant to humans in that relative estradiol levels increase in the aging male and may contribute to prostate disease risk [200]. The above studies further identified alterations in DNA methylation patterns in multiple cell signaling genes in BPA-exposed prostates which suggest that environmentally relevant doses of BPA 'imprint' the developing prostate through epigenetic alterations [199].

In vivo analyses of BPA impact on human prostate tumor growth and recurrence was performed utilizing a xenograft model [201]. Tumor size increased in response to BPA administration and mice in the BPA cohort demonstrated an earlier rise in PSA.

Finally, Hiroi et al. have suggested the presence of associations between BPA exposure and complex endometrial hyperplasia and endometrial cancer [178].

1.2.2.3 Brain morphology, neuroendocrine signaling and behavior

The most meaningful studies on the effects on brain development, neuroendocrine signaling, and behavior concern the in utero and perinatal exposure to BPA. The exposure of pups or of pregnant rats or mice to BPA results in an increased expression

of ERs [202] and ER-α labeled neurons [203] in diverse brain areas and in a reduced number of dopamine containing neurons [204]. CD-1 mice developmental exposure to BPA result in a significant change in the locus coeruleus [205], [206] while maternal exposure disrupt normal neocortical development in fetuses by accelerating neuronal differentiation/migration [207]. Moreover, five studies have demonstrated low dose effects of adult BPA exposures on brain endpoints such as synapse formation and remodeling [208], [209]. Some of these effects were observed only in response to additional hormone treatments (e.g., BPA attenuates the effects of estrogens on synapse formation/ remodeling). Epidemiological studies examining neurobehavioral outcomes correlates pre-natal exposure to BPA exposure and neurobehavioral outcomes, such as aggressivity and hyperactivity [210] or anxious or depressed behavior with poor emotional control [211]. These observations are in agreement with results of behavioral tests conducted on laboratory rodents exposed to low doses of BPA such as anxious behavior , increase of aggressiveness, impaired learning, hyper-reactivity to painful or fear-provoking stimuli, altered adult play and other socio-sexual behaviors [212]–[217].

1.2.2.4 Liver function

Studies in vitro and in vivo showed the effects of low doses of BPA (10^{-9}M) are able to enhance the expression of liver enzymes and marker of oxidative stress and to alter the hepatic gene expression and potential markers of liver function [218]–[221]. Furthermore, three epidemiological studies correlate high level of urinary BPA with elevations in liver enzymes (e.g. alkaline phosphatase, lactate dehydrogenase and γ-glutamyltransferase) [222]–[224].

1.2.2.5 Thyroid function

Various studies have investigated associations between BPA and thyroid dysfunction. This is of particular concern because thyroid hormones play an important role in controlling metabolism and brain development. In rats prenatal BPA exposure was associated with decreased TSH [225]and increased level of T3/T4 [226] in pups. Several cohorts studies suggest associations between elevated urinary BPA and low circulating TSH and or high T3/T4 [173], [227]; the altered levels of TSH was found also in newborn, which mothers were exposed to BPA [225]. Further, another epidemiological study found that individuals with increased thyroid function (hyperthyroid and sub-clinical hyperthyroid) has significantly higher BPA urinary levels than individuals with normal thyroid function (euthyroid), indicating higher BPA is associated with increased thyroid function [228]. All these studies are in agreement with our findings on enhanced expression of genes involved in thyroid hormones synthesis and in cell proliferation induced by low concentrations of BPA in vitro and in vivo [140].

1.2.2.6 Metabolism

The endocrine system is involved in the control of metabolism, and important organs such as the pancreas and adipose tissue are endocrine organs. The thyroid gland is important in setting the basal metabolic rate, and the adrenal gland plays a role in glucose and lipid metabolism. Consequently, there is good reason to consider that endocrine disruption, and BPA, may be associated with disorders of metabolic function.

1.3 BPA and Metabolic Disorders

The three main metabolic disorders are the metabolic syndrome, the obesity and the diabetes mellitus.

The metabolic syndrome is a condition characterized by the presence of three out of five determinants: abdominal adiposity, hypertension, low high-density lipoprotein, elevated triglycerides and abnormal fasting glucose and often includes insulin resistance, hyperinsulinemia and dyslipidemia. There are different definitions of the syndrome, so there are different measures of its prevalence in population, ranging from 1% to 30%. In Italy the metabolic syndrome affects 25% of and 27% of women, equivalent to about 14 million people [229].

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, leading to reduced life expectancy and/or increased health problems [230]. People are considered obese when their body mass index (BMI) a measurement obtained by dividing a person's weight by the square of the person's height, exceeds 30 kg/m2. Authorities view it as one of the most serious public health problems of the 21st century [231]. Its prevalence is rising dramatically in both wealthy and poor countries, indeed worldwide obesity has nearly doubled since 1980. In the 2008 the WHO estimates that 1.4 billion adults are overweight (35%of world population). Of these 500 million adults (greater than 10%) are obese, with higher rates among women than men [232].

Type II diabetes is a pathology characterized by chronic hyperglycemia in the context of insulin resistance, hyperinsulinemia and relative loss of β-cellular mass. This is in contrast to diabetes mellitus type 1, in which there is an absolute lack of insulin due to breakdown of islet cells in the pancreas [233]. Type 2 diabetes makes up about 90% of cases of diabetes, with the other 10% due primarily to diabetes mellitus type 1 and gestational diabetes. Its prevalence, and related mortality, are rising, so that WHO recognize the diabetes as a global epidemic. Actually, there are approximately 285

million people with diabetes (6% world population) and it estimates that there will be 600 million in 2030. In Europe, the diabetes causes the 10% of deaths in people ranging 40 to 60 years [234].

These metabolic disorders are three distinct pathological or sub-pathological conditions deeply interrelated. The conditions comprising the metabolic syndrome constitute major risk factors for type 2 diabetes; conversely, obesity is a risk factor for occurrence of the metabolic syndrome and diabetes. The metabolic syndrome is often defined as a pre-diabetic state. In effect, normo-glycemic subjects with metabolic syndrome present a 5-fold increased risk in diabetes developing [235], whereas in subjects with impaired fasting glucose and/or impaired glucose tolerance there is an additional 5-7 fold risk increase [236].

Causal relationships between the metabolic syndrome, obesity and diabetes are controversial; however, these three disorders share risk factors, most of their etiology and many related health issues. Furthermore, they are related to glucose metabolism and to its control by the endocrine system and endocrine organs.

The theory that environmental chemicals could explain a global metabolic disorders epidemic was proposed in 2002 [237]. The commonly held causes, overeating and lack of exercise, were considered insufficient to explain the observed increases in these debases rates. The chemical causation hypothesis was supported by the rapid onset of the increases, which appeared unlikely to be explained by genetic causes. Moreover, recent experimental studies have suggested that BPA affects glucose metabolism through diverse mechanisms including insulin resistance, pancreatic β-cell dysfunction, adipogenesis, inflammation and oxidative stress, which presumed a plausible connections between BPA and diabetes [123], [238]. Indeed, It has been reported that higher BPA exposure, reflected in higher urinary concentrations of BPA recorded in the general adult population of the United States (1455 adults aged 18 through 74 years), may be associated with diabetes [222]. More recently Shankar and Teppala (2011) and Silver et al. (2011) found the same conclusion in a representative

sample of U.S. adult cohort [239], [240]. A positive association between urinary BPA levels and pre-diabetes in 3516 subjects free of diabetes risk factors (body mass index, alcohol intake, blood pressure and serum cholesterol) has been reported. In subgroup analysis, this association was stronger among women and obese subjects [241]. Experimental evidences of BPA involvement in endocrine disruption of energy metabolism control, specifically in the disruption of endocrine pancreas function, are further discussed in sections below.

1.3.1 The Pancreas

The most important organ that control the glucose metabolism is the endocrine pancreas.

The pancreas is both an exocrine and endocrine gland, it lies behind the peritoneum between the greater curvature of the stomach and the duodenum. It is a 15 cm long elongated structure and weighs approximately 85 to 100g. The exocrine portion consists of acini, which produce pancreatic juice, and a duct system that carries the pancreatic juice to the small intestine and the endocrine portion, consisting of pancreatic islets that are able to produce hormones that enter the circulatory system. The pancreatic islets are dispersed among the ducts and acini of the pancreas, with quantities ranging from 500,000 to 1 million, are able to secrete at least five hormones

and paracrine products, the most important of which are insulin, glucagon, and somatostatin [242].

1.3.1.1 Islets of Langerhans

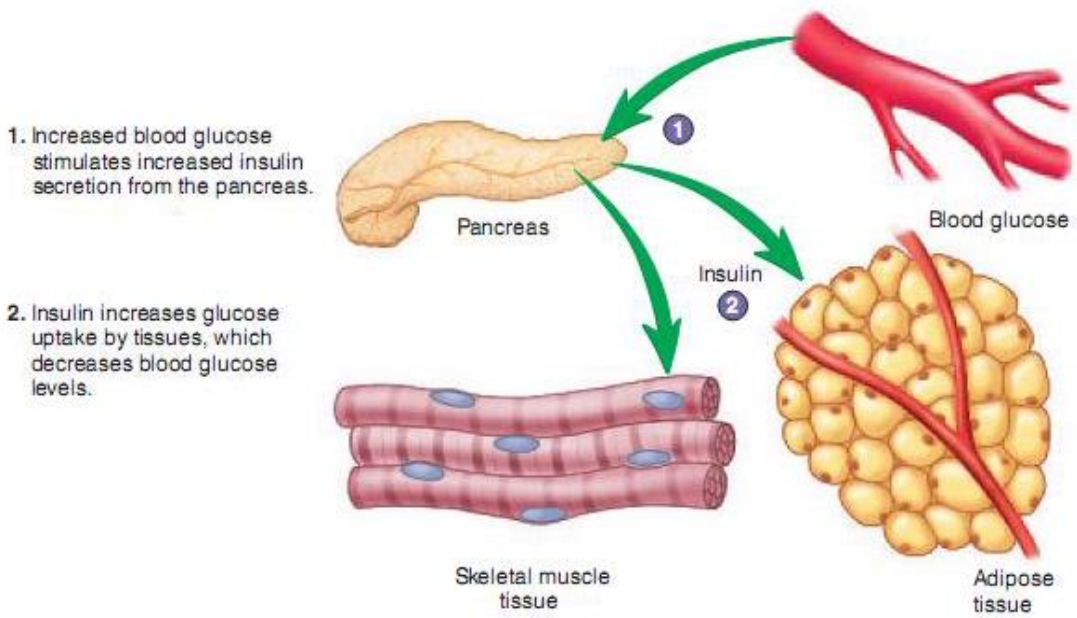


Fig. 5 Simplified mechanism of the role and targets of insulin. [242]

The islets of Langerhans or pancreatic islets are scattered throughout the pancreas and constitute about 1%-2% of the pancreas. They contain several different cell types – including endocrine cells, endothelial cells, nerves and fibroblasts – a range in size from just a few islet cells to complex several thousand islet cells with a diameter up to 300-400 μm. Each islet containing between 1000 and 3000 cells and contain insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP cells and ghrelin-producing epsilon cells [243].

The main function of pancreatic β-cells is the biosynthesis and release of insulin, which is controlled by many signaling molecules including neurotransmitters, hormones and nutrients, among which glucose is the most important. Indeed β-cells function as glucose sensors with the crucial task of precisely adjusting insulin release to blood glucose levels, a process in which mitochondria play a central role. Altered nutrient

storage and usage, hyperglycemia, and ultimately diabetes, appear when loss or dysfunction of the β-cells fall below a critical threshold [244][245].

1.3.1.2 Animal model – the mouse

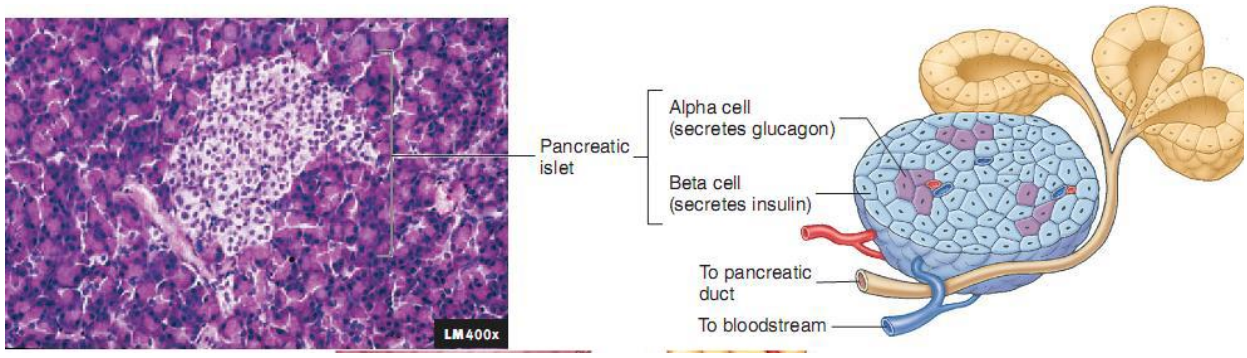


Fig. 6 Structure and morphology of the pancreatic islet. [242]

In the mouse the pancreas is described as a diffuse pink gland located between stomach, duodenum and colon. It extends posteriorly in the duodenal loop, lying close to the mesenteric attachment. It is divided into irregular lobes and lobules. Several excretory ducts are present, some joining the bile duct where this crosses the pancreas before entering the duodenum. It is both an exocrine and endocrine gland, the first is a complex tubulealveolar gland lacking a connective tissue capsule but surrounded by loose vascular connective tissue of the mesentery and separated into lobes and lobules by septa of loose fibroelastic tissue. The endocrine portions is constituted by the islets of Langerhans, are always closely associated with the septal ducts and blood vessels. The percentage of the different cell subpopulations and the islet cytoarchitecture vary between species. In rodent islets, β-cells are the most abundant, 60–80% of the total number of cells and α-cells constitute 15–20%; in human islets the proportion varies, with the percentage of α-cells being higher (35–45%) and the β-cell percentage lower (55–65%). In humans α and β-cells are distributed evenly throughout the islet, suggesting that paracrine interactions between both types of cells may be vital, on the

other hand in rodents β-cells constitute the core of the islets while non β-cells are distributed in the periphery [244][246].

1.3.2 Regulation of Blood Glucose by insulin

The regulation of blood glucose levels requires insulin. Blood glucose levels can increase dramatically when too little insulin is secreted or when insulin receptors do not respond to it; consequently the pancreas is a vital organ for regulating glucose metabolism in the body. The pancreatic β-cells express the glucose receptors GLUT-1 and GLUT-2, the first is more expressed in human while the second in rodents. Glucose is transported into β-cells, induced by the increase of plasma glucose levels and leads to insulin exocytosis, which manipulates the glucose levels [247]. The combination of glucose receptors, transports and phosphorylation proteins forms the " glucosensor unit" that controls with feedback mechanism the maximum secretory response and the expression of GLUTs on the plasma membrane.

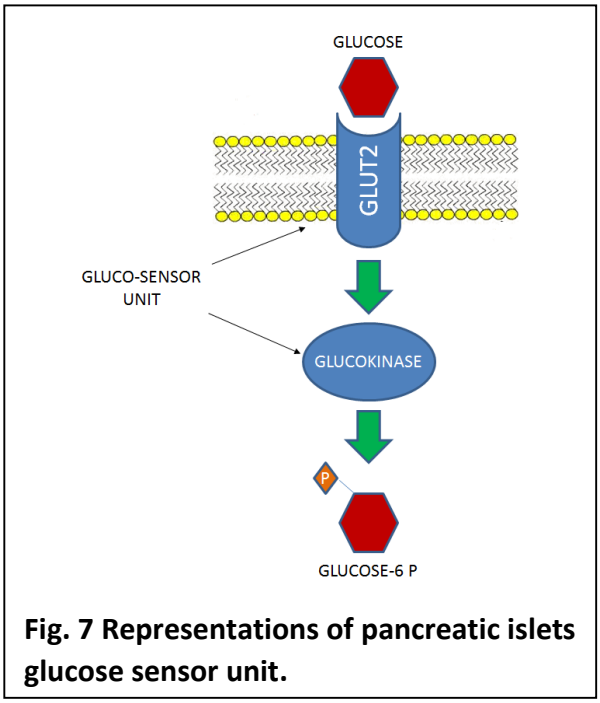


Fig. 7 Representations of pancreatic islets glucose sensor unit.

The secretory response of β-cells depends on their electrical activity. The β-cell membrane contains specific transporters for glucose, which promote its entering into the cell by facilitated diffusion. Once inside the cell, it is phosphorylated to glucose-6-phosphate by glucokinase, thereby initiating glycolysis and, subsequently, oxidized. Thereafter, mitochondrial metabolism generates ATP, one of the products of this oxidation step, which appears to be the key factor that regulates insulin secretion. K+ channels in the β-cell membrane are regulated by changes in ATP levels, when there is its increase (inside the β cell) the K^+ channels close depolarizing the β-cell membrane. When depolarization occurs, membrane bound Ca^{2+} channels open and, subsequently, Ca^{2+} flows into the β cell. Then, the increases in intracellular Ca^{2+} concentration cause exocytosis of the insulin-containing secretory granules with the insulin being secreted into pancreatic venous blood and, then, delivered to the systemic circulation. This process is responsible of the short-term insulinic response (10 minutes) that lead to exocytosis of ready releasable granules bound to the membrane. When there is a persistent glucose stimulus, additional signals are necessary to produce a sustained secretion of insulin. They participate in the amplifying pathway formerly referred to as the K^+-ATP-channel-independent stimulation of insulin secretion.

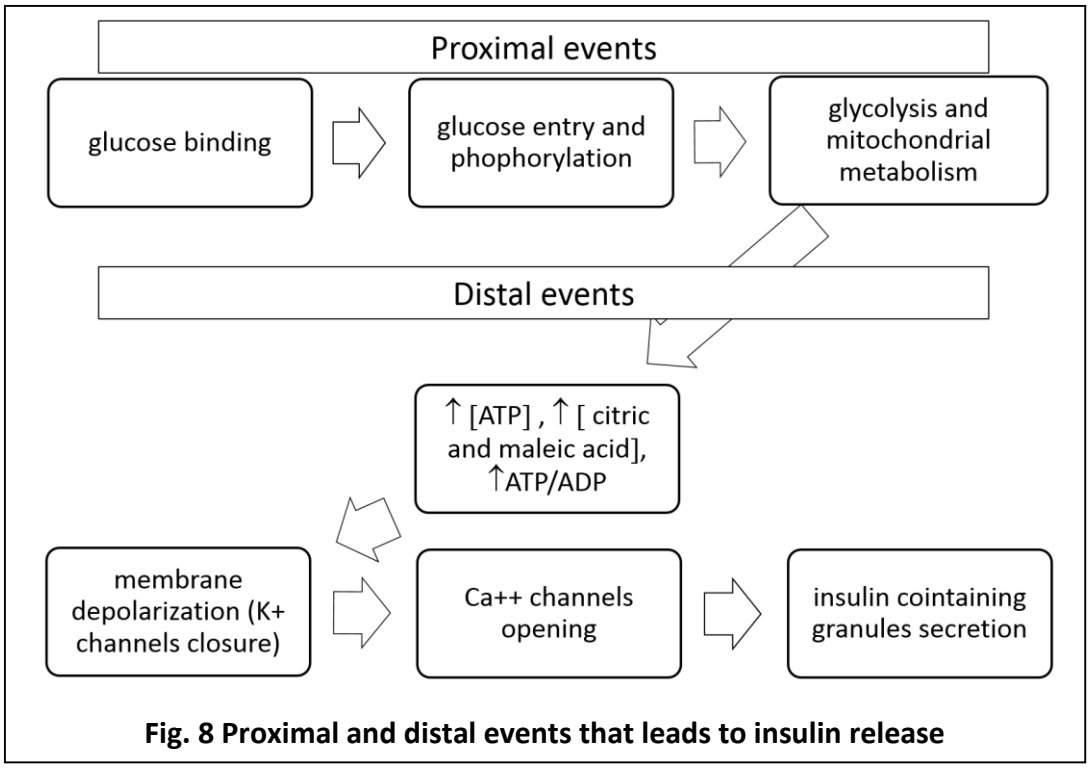


Fig. 8 Proximal and distal events that leads to insulin release

In contrast to the transient secretion induced by Ca^{2+} raising agents, the sustained insulin release depends on the generation of metabolic factors. This kind of insulinic response is more slow and progressive and can be induced only by high levels glycaemia persistent for long time [244], [248].

1.3.3 - Mitochondria

Mitochondria are key organelles that generate the largest part of cellular ATP and represent the central crossroad of metabolic pathways [248]. The metabolic profiling of β-cell identified mitochondria as key components for their function, as sensors and as generators of metabolic signals controlling insulin secretion [248]. In most tissues, cytosolic conversion of pyruvate to lactate by lactate-dehydrogenase ensures the NADH oxidation, however in β-cells this task is performed mainly through mitochondrial NADH shuttles, transferring glycolysis-derived electrons to mitochondria. This favors the transfer of pyruvate into the mitochondria, which is followed by a catabolism of glucose-derived pyruvate that induces mitochondrial activation and subsequently generation of ATP [245].

A unifying theme is that production of reactive oxygen species (ROS) induced by metabolic stress represents a common pathway of injury in the cascade of events that ultimately results in β-cell failure [249]. Indeed, mitochondrial electron transport chain is the major site of ROS production within the cell. ROS formation is coupled with this electron transportation as a by-product of normal mitochondrial respiration through the one-electron reduction of molecular oxygen; furthermore mitochondria are also one of the primary targets of ROS. The mitochondrial genome is vulnerable to oxidative stress and consecutive susceptible to damages too more extent than nuclear DNA, due to the lack of protective histones and low repair mechanisms [248], [249]. Short transient exposure to oxidative stress is sufficient to impair glucose-stimulated insulin secretion in pancreatic islets. ROS may affect insulin-secreting cells, resulting in mitochondrial

inactivation, thereby interrupting transduction of signals and, as such, affecting insulin secretion. It was observed that one single acute oxidative stress may induce β-cell dysfunction, in a lasting over days manner, a fact that can be explained by persistent damages in mitochondrial components, accompanied by subsequent generation of endogenous ROS of mitochondrial origin [245], [248].

1.3.4 Role of Estrogens in Glucose Metabolism

The (xeno)estrogens can modulate the energy balance influencing the activity of estrogens responsive endocrine organs that play a role in metabolism.

They are part of the refined mechanism that enables the organism to adjust the metabolism to different energy needs. For example, the estrogens increase the glycaemia and the glucose usage during pregnancy. Nevertheless, inappropriate levels of estrogens, even in addition with xenoestrogens can disrupt this fragile endocrine control leading to pathological consequence (Fig. 10).

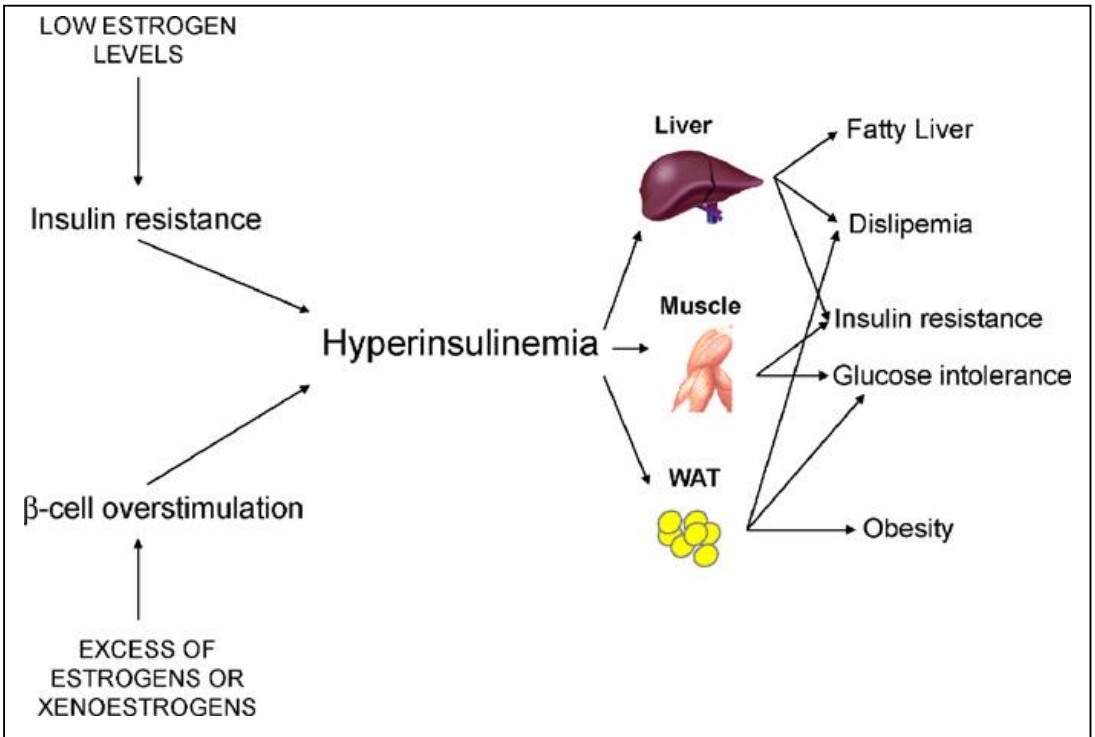


Fig. 9 Action of excessive insulin signaling in different tissues. [244]

The involvement of estrogens in energy balance has been confirmed in studies on their receptors. ER-α knockout as well as aromatase knockout (ArKO) mice are obese and insulin resistant [250][251]. This insulin-resistant state may be caused by the action of ER-α, since this receptor is related to glucose metabolism in different tissues including skeletal muscle, adipose tissue, liver, brain and endocrine pancreas [252].

ER-α modulates the GLUT4 expression in skeletal muscle and its transcript is extremely reduced in ER-α −/− mice [253]. The ER-α is also involved in modulating hepatic insulin sensitivity. Actually, ER-α −/− mice have severe hepatic insulin resistance as well as a concomitant alteration of glucose uptake by skeletal muscle [254]. Additionally, estrogens control the distribution of body fat and adipose tissue metabolism, involving both ER-α and ER-β [255][256][257]. Indeed, it is known that estrogens can regulate the amount of white adipose tissue (WAT) in females and males. It has been demonstrated that the absence of ER-α produces adipocyte hyperplasia and hypertrophy in WAT and is accompanied by insulin resistance and glucose intolerance [255][258].

In the brain, the disruption of the ER-α in the ventromedial nucleus of the hypothalamus leads to weight gain, increased visceral adiposity, hyperphagia, hyperglycaemia and impaired energy expenditure in female mice .

The role of ERs in the physiology of the endocrine pancreas is still greatly undetermined. Plasma insulin levels are increased in pregnant rats in response to increased levels of sex steroids [252]. E2 at concentrations comparable to those in pregnancy enhances insulin secretion in perfused rat pancreas [259]. In humans, E2 reverses the effect of menopause on glucose and insulin metabolism, resulting in increased pancreatic insulin secretion as well as improved insulin resistance [260], [261]. In mice, E2 acting partly through ER-α protects pancreatic β cells from apoptosis induced by oxidative stress [262]. In in vitro studies on murine pancreatic

islet treatment with low doses of E2 increases pancreatic insulin expression, content and secretion through ER-α [263].

The novel membrane bound estrogen receptor is responsible for the rapid insulinotropic effect of E2 when applied at physiological concentrations, both *in vitro* and *in vivo* [264]. Once bound to its membrane receptor, estrogen triggers the synthesis of cGMP, which in turn activates protein kinase G (PKG). The ATP-dependent potassium channels (KATP), then, close in a PKG-dependent manner causing the plasma membrane depolarization and enhancing [Ca^{2+}] signals [265]. As a result, insulin secretion is increased [266]. This receptor, activated by E2, abolishes glucagon release inhibiting [Ca^{2+}] oscillations induced at low glucose concentrations [267]. Moreover, the increase of [Ca^{2+}] by E2 in β cells is involved in the rapid activation of the transcription factor CREB, transforming an extranuclear initiated signal into a nuclear response [268].

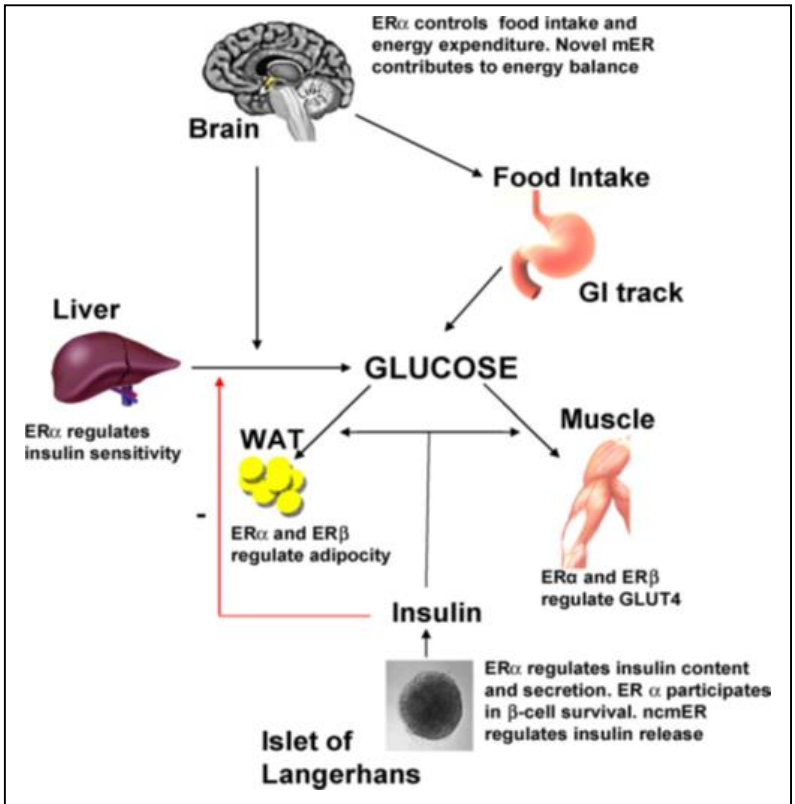


Fig. 10 Estrogen Receptors role in glucose homeostasis. [252]

Although estrogens receptors represent the main link between estrogens and metabolism, other feasible players currently emerging are liver X receptors (LXRs). LXRs are well recognized as important regulators of cholesterol homeostasis as well as modulators of lipid and carbohydrate metabolism. It is already known that estrogens can regulate LXRs in metabolic tissues [296]. In the liver, the activation of LXRs leads to the suppression of the gluconeogenic program. In white adipose tissue, the expression of GLUT4, which promotes glucose uptake, is upregulated after LXR activation . Besides its action on peripheral tissues, a novel role for LXR in the pancreas has recently been described. The LXR agonist T09011317 increase glucose dependent insulin secretion. On the other hand, the absence of LXR in mice leads to glucose intolerance and to an accumulation of lipid droplets in pancreatic islets [252].

1.3.5 BPA - Metabolism Effects

The BPA exerts its main effects on energy metabolism influencing the activity of white adipose tissue (WAT) and endocrine pancreas. BPA has many effects on WAT ranging from alteration of inflammatory cytokines and adiponectin release to lipid accumulation and changes of proteins [108], [269]–[271]. The adiponectin is a hormone produced by adipocytes that has different effect depending on the receptors bound, AdipoR1 or AdipoR2 [272]. It increases the fat acids oxidation and glucose metabolism in the skeletal muscle and reduces the hepatic gluconeogenesis enhancing the insulin sensibility [273]. Specifically, BPA inhibited adiponectin secretion from human adipose explants with a U-shaped NMDR curve: at concentrations of $1x10^{-10}$ M and $1x10^{-9}$ M inhibited the release, whereas doses of $1x10^{-8}$ M and $1x10^{-7}$ M were indistinguishable from unexposed controls [113]. Furthermore, higher concentrations of BPA ($1x10^{-6}$ M) are able to stimulate IL-6 and TNFα release, two inflammatory cytokines. considering that low circulating adiponectin levels and elevated inflammatory cytokines are strongly associated with increased risks of obesity-related diseases, these data suggest a that BPA can be considered an obsogen [108]. It is also described that BPA is able to enter fibroblasts in the differentiation process and to

enhance the adipocyte conversion in combination with insulin suggesting that in vivo prolonged exposure to BPA might increase body fat mass and as such be involved the development of obesity [274].

Several experimental studies, in vitro and in vivo, shown that BPA is able to influence the pancreatic islet function. Nadal et al. demonstrated in vitro on isolated islet that low doses of BPA (ranging from $1x10^{-10}$ to $1x10^{-6}$M) are equal to E2 in provoking increase of islet insulin content and release, as well as in increase of the insulin gene expression in an inverted U-shape dose response manner. This effect is mediated by ERs, as it is blocked by the co-incubation with the well-known ERs antagonist ICI 182,780[263], [264].

Moreover, BPA has been shown to bind to both mER and GPR30 in β-islet. Several studies have determined that these membrane-bound receptors are capable of non-genomic steroid actions [113], [252], [268]. With the same mechanism previously described, low concentrations of BPA ($1x10^{-10}$, $1x10^{-9}$M) induce the closure of K^+-ATP dependent channels, the increase of intracellular [Ca^{2+}] and insulin release. The increase of intracellular Ca^{2+} leads also to activation of CREB, an ubiquitous transcription factor, that has many target gene among the others the insulin gene [268]. These results were confirmed by in vivo studies on mice. The administration of low doses of E2 in or BPA (10 or 100 ug/kg) induce a rapid (30 minutes) increase of insulin secretion ERs independent, accompanied by a glycaemia reduction. More prolonged treatment (4 days) increase the insulin content and release, raise the insulin response to high levels of blood glucose and alter the glycemic and insulinic curve after IGTT (intraperitoneal glucose tolerance test) and ITT (insulin tolerance test). The results of these three works (reviewed in [238]) suggest a potential role of BPA in establishment of hyperinsulinemia, insulin resistance and impaired glucose tolerance. To confirm this, more prolonged exposures, lasting 8 days to 10 weeks, were tested and verified to alter blood glucose and insulin levels, glucose and insulin tolerance, food intake, triglyceride levels, and responses to sugar challenges [220], [275], [276].

More recently a new link between BPA and metabolic disorders that involve the demonstrated ability of BPA to generate oxidative stress and inflammation [277] has been proposed. This hypothesis suggests that BPA, through these mechanisms, promotes insulin resistance and alters adipocyte activity and proliferation. Furthermore, it would also be able to impair pancreatic islet function and vitality damaging their mitochondrial activity.

A study on effect of BPA on mitochondrial activity, oxidization and inflammation was conducted on HepG2 cells, an hepatic cell line containing the entire battery of detoxification enzymes, with concentration of BPA ranging from $1x10^{-12}$ to $2x10^{-4}$ M [218]. The results shown that BPA induce a mitochondria-dependent ROS (reactive oxygen species) generation and lipoperoxidation, which led to a late cytosolic oxidative stress, sustained in time (72h) and showing a non-monotonic dose-response pattern (an inverted U-shape dose response). Mitochondria-related oxidative stress mainly results from a dysfunction of the respiratory chain [278]. In fact, the cells exposed to BPA shown also a membrane hyperpolarization. This state is usually associated with ROS generation, due to a poor electron flux leading to a direct reaction with oxygen [279]. It is worth noting that the increase of ROS associated with the increase of membrane potential is relevant for liver cell injury [278].

A recent study conducted on rat insulinoma cell line (INS-1) [280] shown that low dose of BPA (ranging from $2x10^{-9}$ to $2x10^{-6}$M) induce changes in mitochondrial mass, morphology, membrane depolarization and it reduces the ATP production. Moreover, exposure results in a dose dependent increase in the proportion of early apoptotic and apoptotic cells. A higher expression of BAX gene was observed as well as a reduced expression of BCL-2. The mitochondrial and cytosolic fractions were prepared from INS-1 cells to investigate whether the release of cytochrome c was involved in the apoptotic process induced by BPA. Indeed, cytochrome c amount was reduced with increasing BPA concentrations in isolated mitochondria but significantly elevated in the cytosolic fractions in INS-1. Thus, BPA promoted the translocation of cytochrome

c from the mitochondria into the cytosol in INS-1 cells. Another study, on ex vivo rat pancreatic islets, partially confirm these results [281] reporting, also, mitochondrial abnormalities at higher BPA concentrations ($2.5x10^{-5}$M). Recently, it has been reported that oxidative stress induced by BPA can accelerate toxic aggregation of human islet amyloid polypeptide (hIAPP, a hormone synthesized and secreted by the pancreatic β-cells) leading to β-cell death [111].

Collectively, all these results indicate that low doses of BPA can adversely affect metabolic pathways that are revealed in vitro cultured pancreatic islets and in vivo in rodents, suggesting that the associations between BPA exposures and metabolic disorders, in some human populations, can be obvious adverse endpoint.

1.4 Thesis Aim

The widespread exposure to BPA and the already proven effects on the human organism are of concern. BPA is considered as an EDC able to play a role in the onset of metabolic disorders as acting on multiple organs and tissues involved in glucose homeostasis.

The overall objective of this study is to identify the effects of BPA on ex vivo murine pancreatic islets and dissect its endocrine disruption mechanism in the regulation of islets function using a toxicogenomic approach. We chose to study the effects of three different concentrations of BPA, $1x10^{-4}$M, known to be cytotoxic, $1x10^{-6}$M and $1x10^{-9}$M to mimic worker and worldwide population exposure, respectively. We focused on the effects of 1x10-9 M BPA, as it can be considered the environmental concentration. We specifically aim to evaluate:

- the influence on the viability of the islet cells
- the alterations inducted in the trascriptome
- the specific cellular functions impaired
- the pathways involved in endocrine disruption

2 Materials and Methods

2.1 Mice

C57/B6 male mice (Biogem s.c.a.r.l., Animal Facility [Biogem AMF], Ariano Irpino [AV], Italy) were used given the animal experimentation background, more specifically the existent data related to the pancreatic influence from BPA. Mice were housed in polysulfone cages with ad libitum access to water and normal diet under specific pathogen-free facility at 22°C ± 2° temperature and 55% ± 15% relative humidity with 12h light:12h darkness cycle and 18 ± 2 changes of air per hour. All experimental procedures on animal were executed in Biogem AMF rooms. The procedures, equipment and environments used in this study comply with the Legislative Decree of 27 January 1992 No. 116, which transposes the European Commission's Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. All aspects of the study regarding animal welfare were approved by the Ethics Committee of the Biogem scar.l. and the Ministry of Health.

2.2 Isolation, purification and culture of mouse pancreatic islets

Primary cultures of mouse β-Islets were prepared following a Gotoh et al. modified protocol [282]. Briefly, after mouse sacrifice (CO2 inhalation), the pancreas was exposed and injected with 3mls of collagenase solution (0.8mg/ml of Collagenase P [Roche Dignostic GmbH] in HBSS [Sigma-Aldrich]) (Fig. 12). Then, the pancreas was excised and digested by 37.7 °C incubation for 12 minutes. After 2 washing step, the islets were purified by differential centrifugation on Histopaque 1077 (Sigma-Aldrich) and HBSS density gradient (Fig. 12, E). The islet yield and quality was checked on an inverted microscope and the islets were handpicked and cultured at 37°C with 5% CO2.

In RPMI 1640 (Sigma-Aldrich) + 10% Fetal Bovine Serum (FBS, Sigma-Aldrich) + 11mM glucose (Sigma-Aldrich) + 1% Pen/Strep (Sigma-Aldrich). 250-300 islets per 6cm dish with 3mls of media were used, in order to avoid stressing them. When indicated, isolated islets were treated with 1x10-4, 1x10-6, 1x10-9 M BPA, E2, 50mM, 25 mM glucose, 3mM N-Acetyl-l-cysteine, 10µM BMS 345541, 10µM LY294002, 10µM ICI182780, or vehicle (DMSO, Sigma-Aldrich) and incubated for 24h, 48h or 7 days.

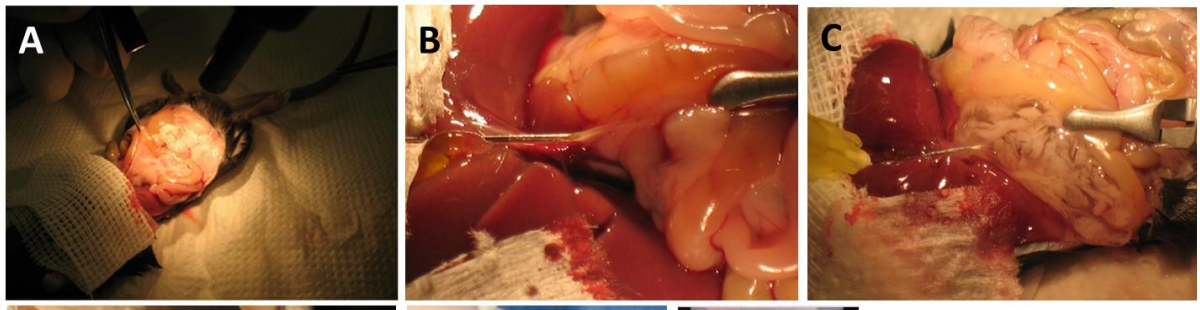


Fig.11 Murine pancreas ex vivo perfusion and islets isolation: *(A, B, C) pancreas exposition and injection; (D, E) pancreas removal; (F): Islets gradient purification.*

2.3 Islets disaggregation

Dispersion of cells with Ca2+-free medium was done immediately after the isolation of islets. Islets were incubated for 5 minutes at 37°C in 0.25% trypsin-EDTA (Sigma-Aldrich), then washed and resuspended in culture medium and plated.

2.4 Isolation, purification and culture of mouse hepatocytes

Primary hepatocytes were obtained by collagenase perfusion of anesthetized mice. After anesthesia and laparotomy a PE10 catheter was introduced in portal vein. The

liver was perfused with a pre-digestion solution (HBSS w/o Mg and Ca + 0.5mM EGTA + 25mM Hepes + 1% Pen/Strep[Sigma-Aldrich], pH 7.4) pre-warmed to 37 ºC for 10 min at the rate of 5 mL/min, afterwards perfused with pre-warmed digestion solution (William's E Medium + 15mM Hepes + 1% Pen/Strep + 0.32 mg/ml Collagenase type IV [Sigma-Aldrich], pH 7.4) at the same rate for 10 minutes.

The digested liver was excised and hepatocytes were released with gentle shaking of the digested liver into 15 ml of hepatocyte isolation medium (William's E + 4% FBS + 1% Pen/Strep [Sigma-Aldrich]). The digested was filtered (70mM) and twice washed. In order to access the quality of the perfusion the cell viability was assessed via trypan blue staining and then the cells were plated in collagen type I (Sigma-Aldrich) coated dishes with hepatocyte isolation medium. After allowing the cells to attach for approximately 6 hours at 37°C, they were washed once with PBS and the hepatocyte isolation medium was replaced with hepatocyte growth medium (William's E + 4% heat inactivated FBS + 1% Pen/Strep + 50ng/ml Epidermal Growth Factor + 1mg/ml insulin + 10mg/ml Transferrin + 1.3 mg/ml Hydrocortisone [Sigma-Aldrich]). When indicated, hepatocyte were treated with 1x10-9 M BPA, or vehicle (DMSO, Sigma-Aldrich) and incubated for 24h, 48h. No longer treatments were performed due to in vitro mesenchymal transition of the hepatocytes.

2.5 MTT assay

In order to access the cell proliferation, cell viability, and cytotoxicity the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on hepatocytes or dispersed islets in a 96-well plates. The MTT reagent in PBS was added to the cells (final concentration of 0.5 mg/ml) and the cultures were incubated for 4 h at 37°C. The reaction was stopped removing the reagent and adding 100 µl of acidified isopropanol (0,04% HCl) to each well. To dissolve the tetrazolium crystals the plates were mixed on a plate shaker for 10 min at room temperature Absorbance at 595 nm was read in EnVision™ 2103 Multilabel Reader (Perkin Elmer).

2.6 RNA Extraction

RNA was extracted from isolated islets or hepatocytes using a commercial kit (Trizol reagent, Invitrogen) according to the manufacturer's instructions. A further step of RNA purification was made with a phenol-chloroform procedure. The RNA concentration and purity was determined with the NanoDrop spectrophotometer ND-1000. For qRT-PCR, 1 mg of total RNA was reverse-transcribed by using the QuantiTect Reverse Transcription Kit (Qiagen) in a total volume of 20ml, according to the manufacturer's instructions.

2.7 Microarray

cRNA was generated by using the Affymetrix One-Cycle Target Labeling and Control Reagent kit (Affymetrix Inc), according to the manufacturer's instructions, starting from 5 μg of total RNA. Biotinylated cRNA was hybridized to the Mouse MOE 430 2.0 Genome Arrays (Affymetrix). Chips were washed and scanned on the Affymetrix Complete GeneChip System, generating digitized image data (DAT) files.The datasets obtained were analyzed with GeneSpring GX 12 Software (Agilent Technologies). Robust multichip average (RMA) algorithm [283] was used for summarization and normalization. Hybridization quality was assessed by spiked-in controls. Principal Component Analysis (PCA) was performed to check data quality that resulted adequate for all samples. Transcripts were filtered by their signal intensity values, selecting transcripts with intensity values between 20 and 100 percentile in at least 1 out of each set samples for differential analysis.

Differentially expressed transcripts between exposed islets vs controls were filtered for absolute fold-change ≥ 1.5 and corrected p-value ≤ 0.05. Statistical analysis was performed using Oneway ANOVA adjusted for multiple comparison by the Benjamini-Hochberg method.

Functional annotation for differentially expressed transcripts was performed using Ingenuity Pathway Analysis (IPA; http://www.ingenuity.com), a web-based tool for the identification of biological functions as well as canonical pathways that are most significant to the dataset. Fisher Exact test was used to calculate the p-value determining the likelihood that the association between the set of focus genes in the dataset and a given process or pathway is due to chance alone. Corrected p-value calculation (based on the Benjamini-Hochberg method) controlled the error rate in analysis results and focus in on the most significant biological functions associated with DEG.

2.8 Real Time PCRs

The qRT-PCR experiments were conducted using Applied Biosystem 7300 Real-Time PCR System and Power SYBR Green Master Mix (Applied Biosystems). Result analysis was performed following real-time relative quantization guidelines through the relative standard curve method as suggested by Applied Biosystems. Briefly, the amplification efficiency of each primer pair was calculated from triplicate relative standard curves for each primer pair using the equation $E = 10^{-1/slope}$ and then used to convert Ct values obtained from each reaction into relative-expression units. Data obtained in this way were then normalized on the relative expression of three reference genes (Gapdh, Tubulin and b-2-microglobulin [B2M]) [284]. Primers were designed using NCBI Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) Primer sequences used in the study are listed in Table 2.

TABLE 2 Primers sequences

Gene	Forward primer	Reverse primer
Bax	ACAGATCATGAAGACAGGGG	CAAAGTAGAAGAGGGCAACC
Sod2	CGTGAACAATCTCAACGCCACCGA	CCTCCAGCAACTCTCCTTTGGGTT
Uqrcb	GCGGGCCGATCTGCTGTTTC	GCCTCATAGTCAGGTCCAGGGCT
Gpx3	AAACAGGAGCCAGGCGAGAACT	CCCGTTCACATCTCCTTTCTCAAA
Ttc35	AGCAGGTCATGATTGCAGCCCT	ACGCTTTCTGGCAGCAGTGTT
Vapa	GAGATGTGTGTTTGAAATGCCGA	GGTCCGTCTTGTTTGGATGC
Ndufs4	TGGCTACAGCTGCCGTTTCCG	GGTCAGCGGTTGATGCCCAA
Zfand2a	ACCCGTGAGTGCCAGGTGAT	AACAGTGCTTCCCCAAGTCAGGA
Iars	GACTTGGAGGAGGTAGTGTGC	GATGGGATGGTCAGGTGGTC
Atp1b1	CTTCCGTCCTAATGACCCCA	TGATTGATGTCGCCCCGTTC
Atp6v1f	ATCGAAGACACTTTCAGGCAA	ATGCTCCTTGGACGGGATCT
Gapdh	ACCACAGTCCATGCCATCAC	CACCACCCTGTTGCTGTAGCC
Tub	CAACACCTTCTTCAGTGAGACAGG	TACATGATCTCCTTGCCAATGGT
B2M	CCGAACATACTGAACTGCTA	TGCTATTTCTTTCTGCGTGC

2.9 ATPlite assay

In order to assess the cells ATP production a luminescence assay based on firefly luciferase (ATPlite^{TM} assay, Perkin Helmer) was performed on hepatocytes or dispersed islets in 96-well black plates according to manufacturer's instructions. The ATPLite assay involved adding a 50 µl lysis solution to each well of a 96-well plate followed by a 50 µl substrate solution. The plates were dark-adapted, and luminescence was measured in Orion II microplate luminometer (Berthold Detection Systems GmbH) giving comparative adenosine triphosphate levels.

2.10 TUNEL assay

Apoptotic cells were visualized by using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (In Situ Cell Death Kit, Fluorescein, Roche Applied Science) according to manufacturer's instructions. In brief, after islets dispersion, the cells were plated in an 8-well slide chamber and treated for 48h with diverse concentration of BPA or with only vehicle for three controls, untreated (C), Negative Control (CN), and Positive Control (CP). At the end of the treatment the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% sodium citrate and 0.1% Triton X-100. Then, the samples were incubated in the TUNEL reaction mixture for 1 h at 37 °C in dark. For CP, the cells were previously treated with DNase I for 10 min at 15 to 25ºC, and for CN, the cells were incubated with label solution without terminal transferase, instead of the TUNEL reaction mixture. After the reaction was completed, the slides were rinsed with PBS, counterstained with a 1μg/mL DAPI solution and mounted. The number of apoptotic nuclei and total number of nuclei were determined under a fluorescence microscope

2.11 Statistical and bioinformatics analyses

The statistical analyses have been performed with Student's t test using Office Excel for Window, unless otherwise indicated. In all cases, probability p-values below 0.05 were considered significant. Data from at least three independent experiments were considered for the statistical analysis. To retrieve the hypothetical transcriptional factors binding sites in the genes promoter, their sequence ranging from −1000/+50 bp has been loaded in Genomatix suite software (Genomatix Software GmbH, http://www.genomatix.de/) and analyzed at the relative profile score of the 80% [285].

3 Results

3.1 Low doses of BPA does not alter the murine pancreatic islets morphology

To assess the influence of BPA on murine pancreatic islets vitality and morphology, after the isolation and plating they were treated, for 48 hours, with different doses of BPA, $1x10^{-4}$, $1x10^{-6}$, $1x10^{-9}$ M and to $5x10^{-2}$ M of glucose, as positive control. The exposure to BPA did not induce macroscopic changes in the morphology of the islets at the lower dose ($1x10^{-9}$ M, Fig 12 D). The central necrotic core, typical of ex- vivo cultured islets, was clearly visible and border was clearly defined (Fig. 12 A and D). Nevertheless, at higher concentrations and in positive control this core was not visible, probably already expelled as the presence of large amount of debris near the islets suggests (Fig 12 B, C and E). Moreover, the morphology of these islets seems more granulated and less compact than the controls.

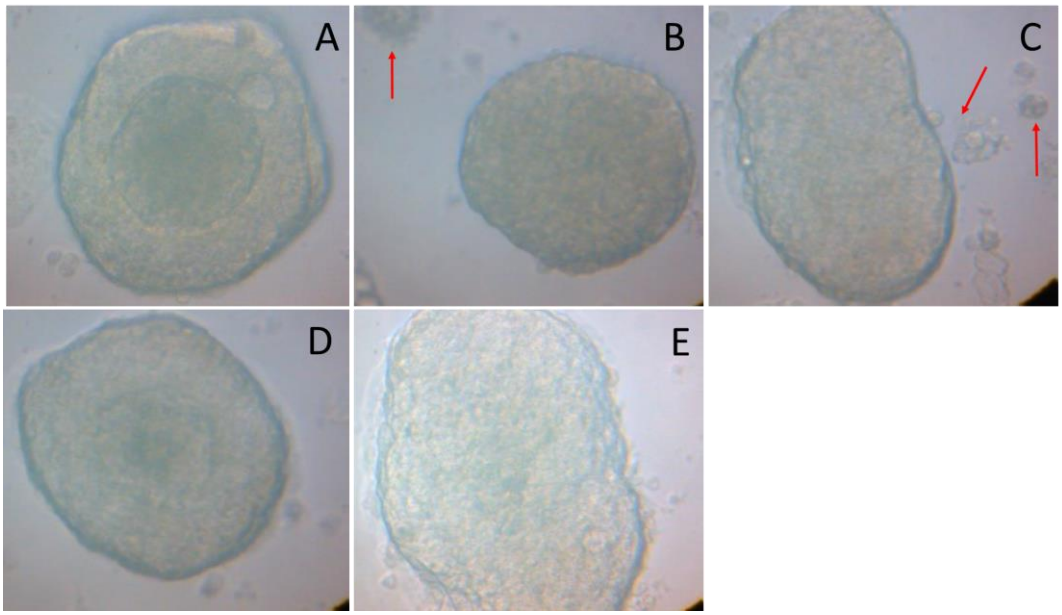


Fig. 12 Morphology of pancreatic islets after 48h treatments with BPA at diverse concentrations. Representative images of pancreatic islets at 40x magnification. Islets were exposed to: vehicle only (DMSO) **(A)**, $1x10^{-4}$M **(B)**, $1x10^{-6}$ M **(C)**, $1x10^{-9}$M **(D)** BPA or $5x10^{-2}$M glucose **(E)**. Red arrows show the ejected necrotic central

3.2 BPA impairs murine pancreatic islet cells viability

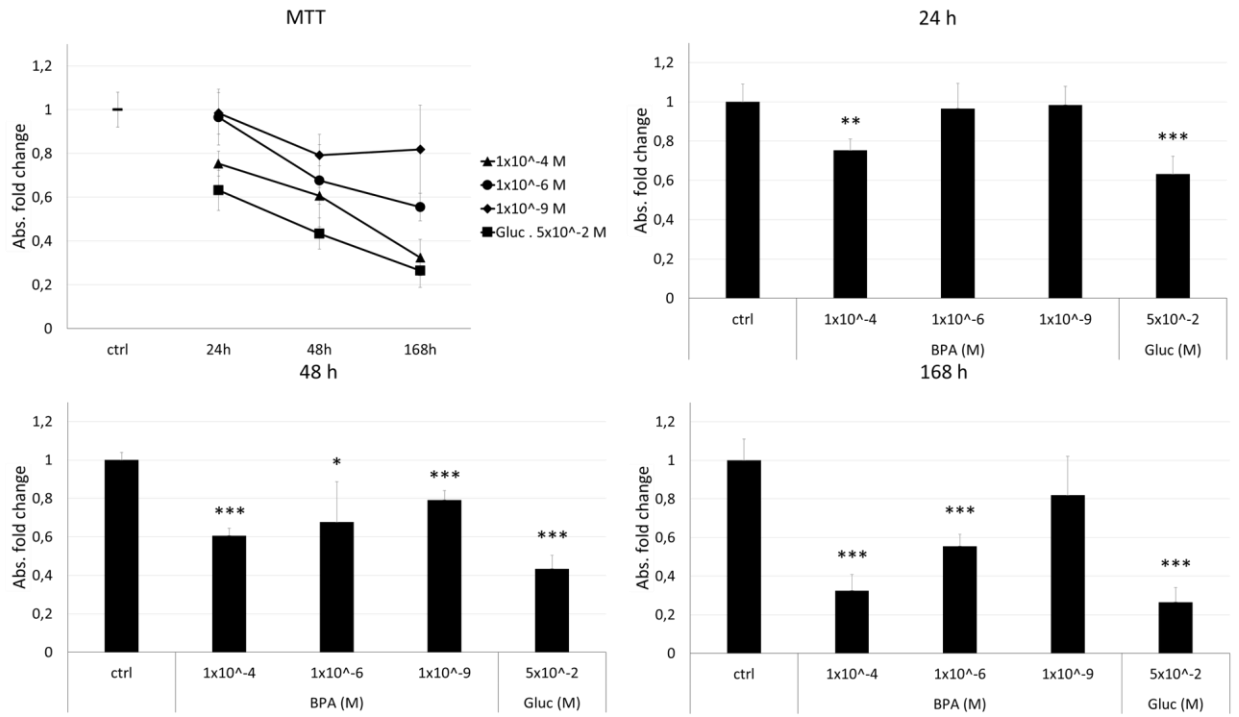


Fig. 13 Murine islets cell viability as determined by MTT assay. *Islet cells were cultured in the presence or absence of 1x10^{-4} M, 1x10^{-6} M, 1x10^{-9} BPA or 5x10^{-2} M glucose for 24h* ***(B)****, 48h* ***(C)****,168h* ***(D)****.* ***(A)*** *is a comparison of time response curve at diverse concentrations of BPA or glucose.* Data are reported as fold change value calculated as ratio between average absorbance in control and treated cells *as the mean ± standard deviation of at least three independent experiments.*
** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with untreated control cells*

The MTT assay was used to investigate the effects of BPA at diverse concentrations on islet cells viability; treatment with to 5x10^{-2} M of glucose was used as positive control. The treatment was performed on disperses pancreatic islets and lasted 24h, 48h or 7 days. As it is visible in the Figure 13 A, BPA affected the viability of dispersed islets in a dose dependent manner. The concentration of 1x10^{-4} M had the strongest effect, completely comparable to that exerted by glucose 5x10^{-2} M. Treatment with BPA at 1x10^{-6} M had an intermediate effect while lowest concentration had a mild effect. Specifically, cells exposed to 1x10^{-6} and 1x10^{-9}M BPA for 24 hours did not exhibit any significant change in viability, whereas at 1x10^{-4} M BPA and 5x10^{-2} M glucose cell viability was significantly decreased to 75 or 63%, of that observed in

control, respectively (Fig. 13 B). When the incubation time was extended to 48 hours, $1x10^{-4}$ M, $1x10^{-6}$ and $1x10^{-9}$ M BPA significantly reduced the viability of pancreatic cells to 61, 68, 79 % of that observed in unstimulated control cells (Fig. 13 C). After 7 days treatment (Fig. 13 D), glucose and $1x10^{-4}$ M BPA strongly affected the viability (23 and 32%) and a further decrease was also observed for cells treated with $1x10^{-6}$ M BPA (55%). No further decrease (82%) was observed upon treatment with 1x10-9 M BPA .

3.3 Microarray analysis: Low concentration of BPA alters murine pancreatic islets gene expressions

As stated in literature, pancreatic islets exposure to BPA is associated with multiple effects. Therefore, we performed a gene expression profile analyses (microarray) to have a global look of the transcriptional responses of the islets to low dose BPA. The explanted islets were treated for 48 hours with $1x10^{-9}$ M BPA or with only vehicle. The RNA was purified and subjected to microarray analysis. To minimize the effects of inter-individual variations we organized a system of pools of pancreas and then of RNA, following the scheme in figure 14. The use of mice from an inbred strain and of the pool system allowed minimizing the number of required animals.

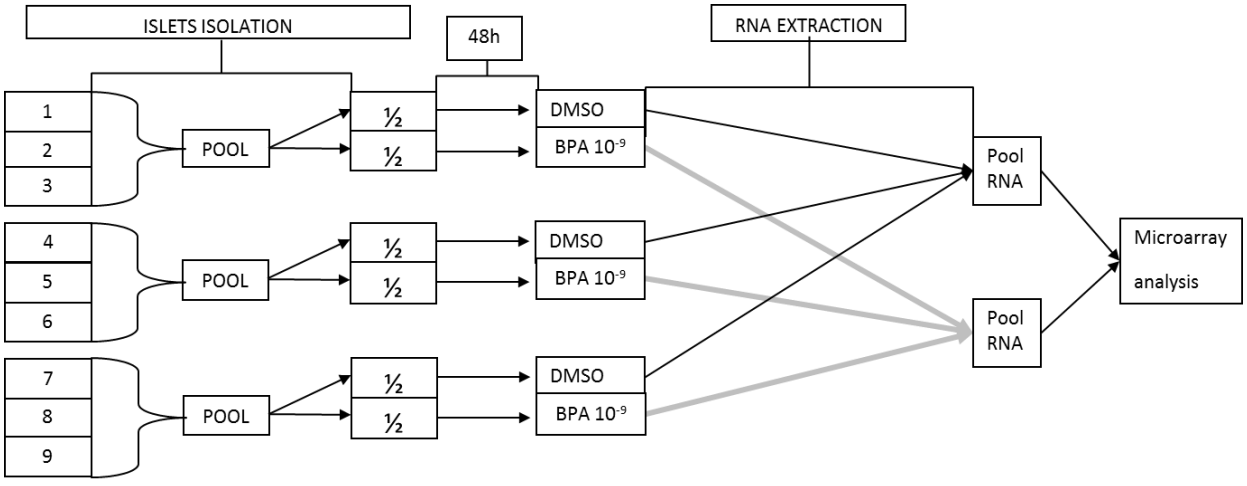


Fig. 14 Pool system for microarray analysis. *To minimize the effects of mice inter-individual variations, for each group, the islets from three pancreas were pooled, then re-divided in two parts for treatments. After collecting the two RNA for each group, treated and controls were pooled again and addressed to microarray analysis*

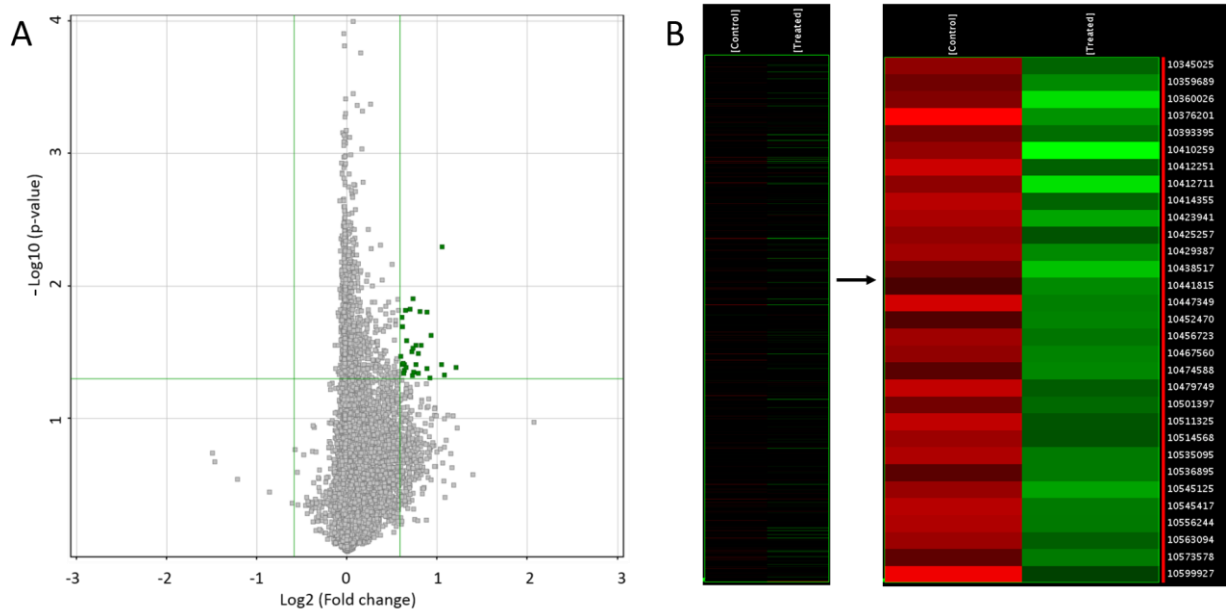


Fig. 15 Transcriptome alteration of murine pancreatic islets after BPA exposure.
***(A)**: Volcano plots of microarray data in 48h 1x10^{-9}M BPA treated islets compared to untreated control islets. The y-axis value is the negative logarithm base 10 of the corrected p value. A green horizontal line on the plot represents the user-defined significant threshold for p value. The x-axis is shown as the logarithm base 2 of the fold change in expression level between treated and control. The vertical green lines on the plot represent the user defined thresholds for fold change. Green dots down-regulated genes. **(B)**: HeatMap from the same microarray data. In the right, there is a representative magnification of the genes that were found to be deregulated.*

The microarray data were analyzed using the GeneSpring Software. A Volcano Plot of the data obtained is reported in Figure 15 A; it is a representation of the differential gene expressions between the control and the treated conditions. The Volcano plot allows the visualization of the relationship between fold-change and statistical significance [286]. A change of at least 1,5 fold (up or down) threshold was considered meaningful for the experiment. Surprisingly, the analysis gave only a small group of 29 **D**ifferential **E**xpressed **G**enes (DEGs, green color) and all of them were down-regulated. However, as visible in figure 15 A, the down-regulation was the general trend. Figure 15 B shows the heat map made from the analysis of the microarray data. The expression levels are represented by the pattern of colors as it is visible in the magnification done, where the genes having lower expression levels than the control samples, represented in green, are highlighted.

The DEGs and their respective fold changes and p-values are listed in the Table 3. Given the referred threshold of 1.5 fold-change, 29 genes (or pseudogenes) were found differentially expressed. More precisely a fold change ranged from -1.50 and -2.30 was found. Some of these genes codify for mitochondrial localized proteins involved in respiratory chain, in ROS detoxification process or in generation of insulin exocytosis signal (ATP synthesis and membrane hyperpolarization). Moreover, there are also genes involved in more generic mechanisms of the cell, such as polymerases, ribosomal proteins and MAPK.

TABLE 3: Differential Expressed Genes

Gene symbol	Fold change	p-value	Genedescription	Refseq acc. numb.
Uqcrb	-2,3	0,04	ubiquinol-cytochrome c reductase binding protein	NM_026219
Gpx3	-2,07	0,01	glutathione peroxidase 3	NM_001083929
Ttc35	-1,91	0,02	tetratricopeptide repeat domain 35	NM_025736
Alg3	-1,88	0,05	asparagine-linked glycosylation 3 homolog	NM_145939
Rpl23	-1,84	0,04	ribosomal protein L23	NM_022891
Cript	-1,84	0,02	cysteine-rich PDZ-binding protein	NM_019936
Ptplb	-1,76	0,03	protein tyrosine phosphatase-like	NM_023587
Mat2a	-1,75	0,02	methionine adenosyltransferase II, alpha	NM_145569
Zfand2a	-1,73	0,05	zinc finger, AN1-type domain 2°	NM_133349
Snora23	-1,72	0,03	small nucleolar RNA, H/ACA box 23	NR_033336
Ndufs4	-1,69	0,03	NADH dehydrogenase (ubiquinone) Fe-S protein 4	NM_010887
Tm9sf3	-1,69	0,04	transmembrane 9 superfamily member 3	NM_133352
BC031181	-1,67	0,04		
Mapk1ip1l	-1,66	0,03	mitogen-activated protein kinase 1 interacting protein 1-like	NM_178684
Aff2	-1,66	0,01	AF4/FMR2 family, member 2	NM_008032
Rpp38	-1,65	0,05	ribonuclease P/MRP 38 subunit (human)	NM_001013376
Atp1b1	-1,64	0,03	ATPase, Na+/K+ transporting, beta 1 polypeptide	NM_009721
Rps20	-1,62	0,01	ribosomal protein S20	NM_026147
2900064A13Rik	-1,58	0,03	RIKEN cDNA 2900064A13 gene	
Rps11	-1,57	0,04	ribosomal protein S11	NM_013725
Iars	-1,56	0,02	isoleucine-tRNA synthetase	NM_172015
Sod2	-1,55	0,04	superoxide dismutase 2, mitochondrial	NM_013671
MRF-1	-1,55	0,04	splicing factor, arginine/serine-rich 2 (SC-35)	NM_011358
Vapa	-1,54	0,05	vesicle-associated membrane protein, associated protein A	NM_013933
BC056474	-1,54	0,04		
Atp6v1f	-1,53	0,02	ATPase, H+ transporting, lysosomal V1 subunit F	NM_025381
Tm2d1	-1,53	0,04	TM2 domain containing 1	NM_053157
Tmem167b	-1,52	0,02	transmembrane protein 167B	NM_026198
Polr2f	-1,51	0,03	polymerase (RNA) II (DNA directed) polypeptide F	NM_027231

3.4 Microarray analysis: Low concentration of BPA impairs pancreatic murine islets mitochondrial functions

Additionally, to identify if the deregulated genes were involved in any fundamentally and biologically specific disorders, we performed an IPA Canonical Pathway analysis using the list of DEGs. As expected, this analysis gave only two deregulated Canonical Pathways and both pertaining to mitochondrial function: oxidative phosphorylation and mitochondrial dysfunction (Fig. 16).

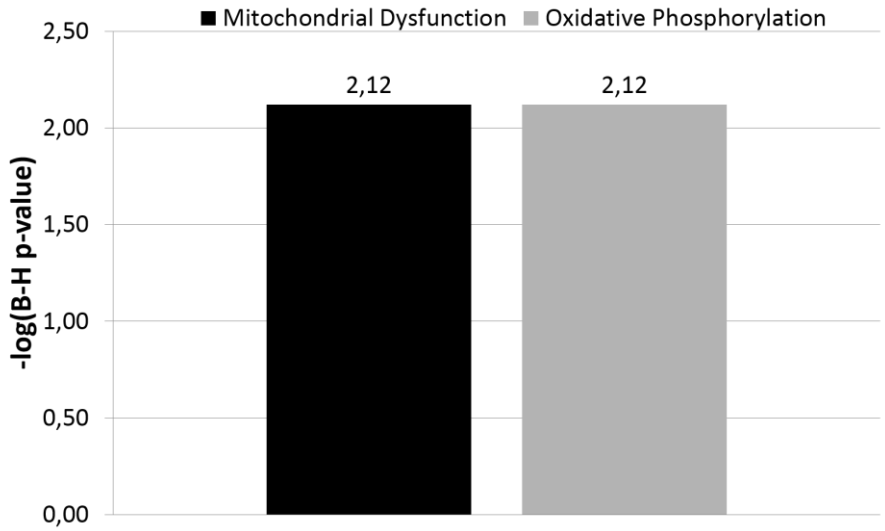
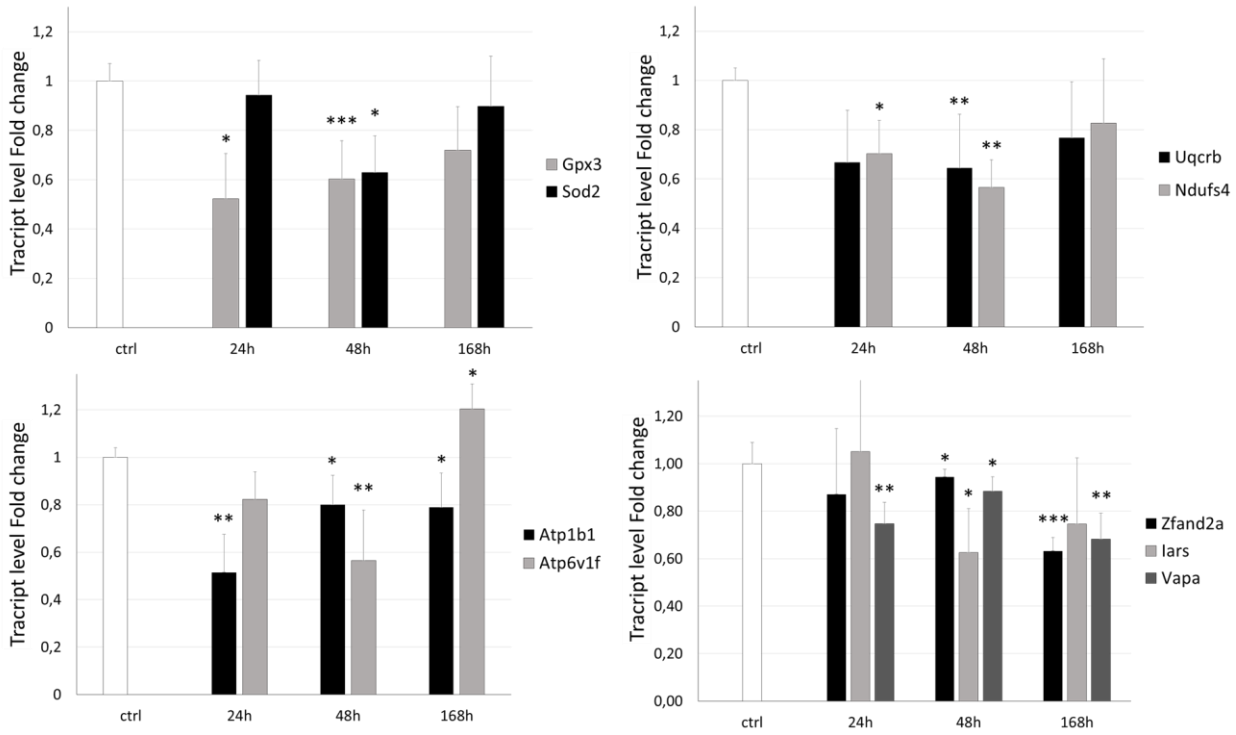


Fig. 16 IPA Analysis of DEGs. *Most significant Canonical Pathways deregulated in pancreatic murine islets exposed to 1x10^{-9}M BPA for 48h*

3.5 Microarray validation: Low concentration of BPA alters genes involved in specific cell functions

Microarray results can be influenced by each step of the complex assay, from array manufacturing to sample preparation (extraction, labeling and hybridization) and image analysis. So a validation of expression differences accomplished by an alternative method is required. Quantitative Real time PCR (qRT-PCR) was the strategy chosen to perform this validation since it is rapid and applicable to samples

with limited amount of RNA [287]. We analyzed the gene expression of 9 out of 29 genes in a new experimental set. The genes were chosen according to properties or functions of the proteins for which they encode. Uqrcb (NM_026219) and Ndufs4 (NM_010887) encode for protein involved in the electrons transport chain; they are sub-units of complex III and complex I, respectively [103][104]. Gpx3 (NM_001083929) and Sod2 (NM_013671) encode well-known proteins involved in protection of the cell against free radicals. Gpx3 catalyzes the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduced glutathione and functions in the protection of cells against oxidative damage [290]. Sod2 is a mitochondrial matrix enzyme that scavenges oxygen radicals produced by the extensive oxidation-reduction and electron transport reactions occurring in mitochondria [291]. Atp1b1 (NM_009721) encodes a beta subunit of the Na+/K+ ATPase pump that is involved in membrane depolarization and in the generation of exocytosis signal [292]. Atp6v1f (NM_013933) encodes a component of ATPase, which is able to generate ATP using the H+ gradient across the mitochondrial membrane [293]. Zfand2a (NM_133349) encodes the protein Airap that enhances the proteasome function during cell stress and is involved in disposal of protein aggregates [294]. Vapa (NM_013933) is a type IV membrane protein present in plasma membrane and intracellular vesicles or granules. It is involved in vesicle trafficking, membrane fusion and tight junction formation [295]. Iars (NM_172015) encode isoleucine-tRNA synthetase that catalyze the aminoacylation of tRNA by their cognate amino acid. Changes in the aminoacylation properties of tRNA-Ile (a loss of isoleucylation efficiency or acquisition of mischarging properties) have been associated to a glycogen and lipid accumulation and to decreased reactivity for cytochrome c oxidase in the muscle [296].



F**ig.17 Gene expression alteration of selcted genes in murine pancreatic islets as determined by qRT-PCR.** Islets were cultured in presence or absence of $1x10^{-9}$ M BPA for 24 h, 48 h or 168 h. The selected DEGs are grouped by functional categories. ROS detoxification, Gpx3 and Sod2 **(A)**. Respiratory chain subunits, Uqcrb and Ndufs4 **(B)** ATP-dependent pump subunits **(C)**. Protein synthesis and degradation, Vapa, Iars, Zfand2a **(D)**. *Data are reported as fold change values calculated as ratio between average gene expression in control and treated cells. The qRT-PCR results* are expressed *as the mean ± standard deviation of at least three independent experiments.*

** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with untreated control cells*

To validate the microarray results, we prepared new sets of samples with the aim to analyze also the kinetic of the cellular level regulation of the above reported transcripts upon exposure to low dose of BPA. In the Figure 17 are represented the qRT-PCR results obtained using RNA prepared from islets treated with $1x10^{-9}$ M BPA at three different time-points of treatment, 24, 48 and 178 hours. The reported data confirmed their downregulation with significant p-values at 48 hours as resulted from microarray. Most of them had a gene expression inhibition equal or greater to that found with the analysis of microarray, ranging from fold change of -1.56 to -1.78 (Atp6v1f, Gpx3, Sod2, Iars, Ndufs4 and Uqcrb, Fig 17 A, B, C, and D), whereas 3 genes had a milder down-regulation (from -1.06 to -1.25, Atp1b1, Vapa, Zfand2a, Fig. 17 C and D). At 24

hours of treatment, 2 genes already are strongly downreguleted, over the cut-off of -1.5 (Atp1b1 -1.96, Gpx3 -1.92, Fig 17 A and C). Furthermore, 2 genes had a milder downregulation (Ndufs4 -1.43, Vapa -1.33 , Fig. 17 B and D), whereas 5 genes had no significant expression inhibition (Sod2, Uqcrb, Atp6v1f, Iars, Zfand2a, Fig 17 A, B, C

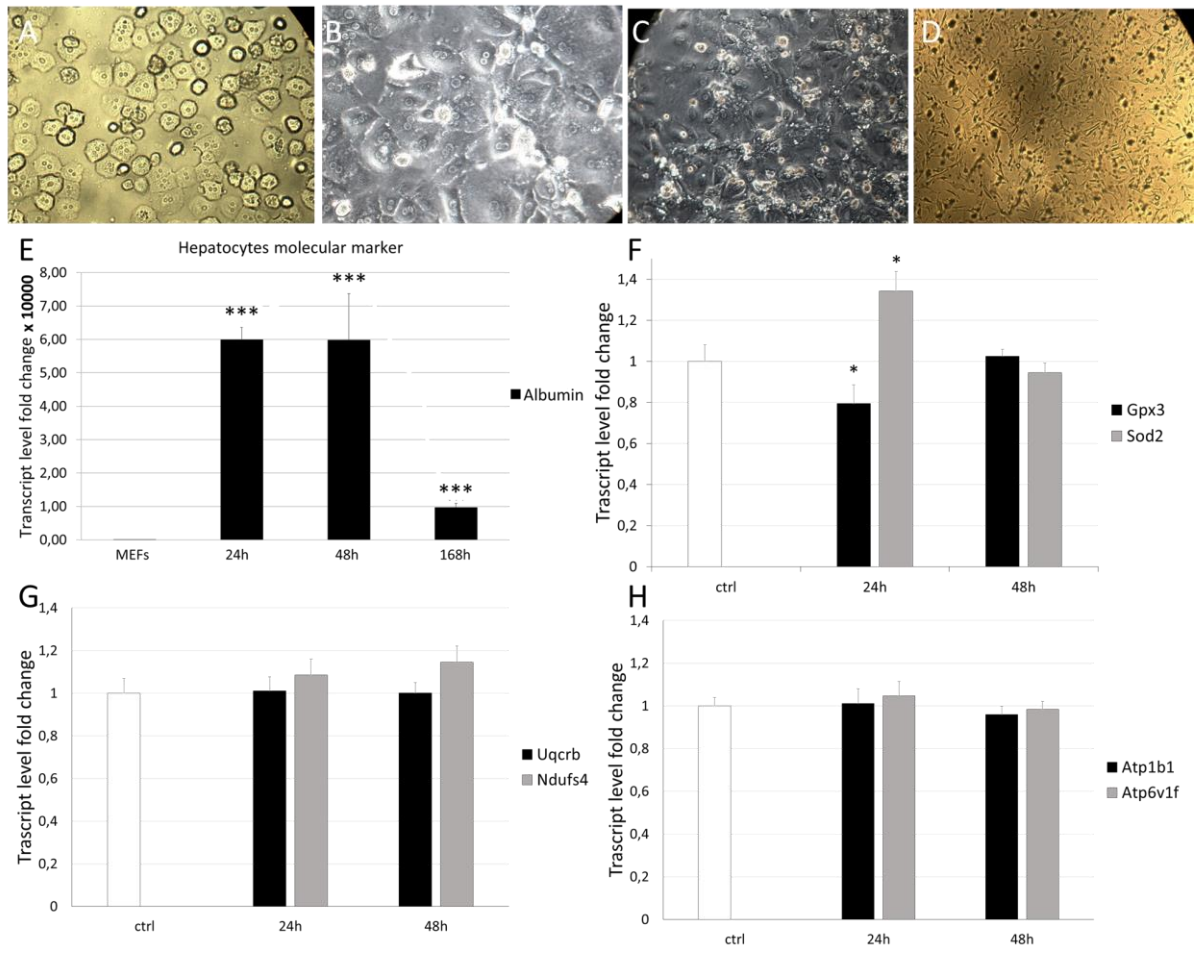


Fig. 18 Hepatocyte primary cultures and their gene expression alteration.

A, B, C, D: *Primary culture of murine liver cells. After 12 h of culture, parenchymal hepatocytes are attached to the dish's surface, and showed typical hexagonal cobblestone-like morphology with one or two round nuclei (**A**, 20X magnification). After 24 h, they spread on the dish's surface, establish intercellular contacts and maintain their morphology (**B**, 20X). After 48 h, they begin to assume a tapered shape but rounded nuclei are still visible (**C**, 10X). Parenchymal hepatocytes lost their epithelial cell morphology within 7 days in culture, and transformed into more flattened fibroblast-like cells (**D**, 10X).* ***E:*** *Albumin gene expression quantification as hepatocyte molecular marker by qRT-PCR. The data are reported as fold change values as ratio between average expression of albumin gene in MEFs and in diverse time cultured hepatocytes.* ***F, G, H:*** *expression alteration of selected genes in murine hepatocytes. Hepatocytes were cultured in presence or absence of 1x10-9M BPA for 24 h or 48 h. Data are reported as fold change values calculated as ratio between average gene expression in control and treated cells. The qRT-PCR results* are expressed *as the mean ± standard deviation of at least three independent experiments.*

** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with untreated control cells*

and D). After 7 days of treatment the number of genes that had a significantly gene expression inhibition was reduced (Gpx3, Vapa, Zfand2a). The most of them had no significant variation and one, Atp6v1f, had even a mild upregulation in gene expression.

3.6 BPA has specific effects on murine pancreatic islets

To ensure that the alteration of the transcriptome was a specific effect of BPA on pancreatic islets, we decided to check the gene expression of the same genes on cells from another organ involved in glucose homeostasis, primary ex vivo murine hepatocytes. An in vivo and in vitro study, using hepatocellular carcinoma cell line (Hep2G) and C57/b6 mice injected with low doses, showed that BPA could alter liver enzyme (AST and ALT) and cytokines release (TNF-α and IL-6) [297]. In vivo data suggest that BPA is able to induce oxidative damage in liver after long and repeated exposure [298]. Moreover, another study demonstrated that BPA could alter the mitochondrial function in Hep2G cells enhancing ROS production [218]. The hepatocytes ware treated with $1x10^{-9}$ M BPA for 24h and 48h. The 168h treatment was not performed because of in vitro mesenchymal transition of ex vivo hepatocytes [299] (Fig. 18 A, B, C and D). This fibroblast-like conversion was confirmed comparing the mRNA levels of Albumin gene in mouse embryonic fibroblasts (MEFs) with those in hepatocytes after 24h, 48h and 168h in culture by a qRT-PCR. After 7 days, hepatocytes had an Albumin gene expression 6 times lesser than 24h and 48h cultured hepatocytes (Fig. 18 E). As is visible in figure 18 F, G and H, the DEGs found in murine pancreatic islets, in this system were very stable and did not appear to be influenced by $1x10^{-9}$ M BPA treatment neither at 24h nor at 48h, with the exception of Sod2 and Gpx3 genes at 24h that appeared upregulated and downregulated, respectively. Thus, this data suggest that the effects detected in pancreatic islets transcriptome are specific and that the alterations on liver function, especially those on

mitochondria, observed by others authors are dependent to other mechanisms of disruption.

3.7 Low concentrations of BPA have no effect on ATP production

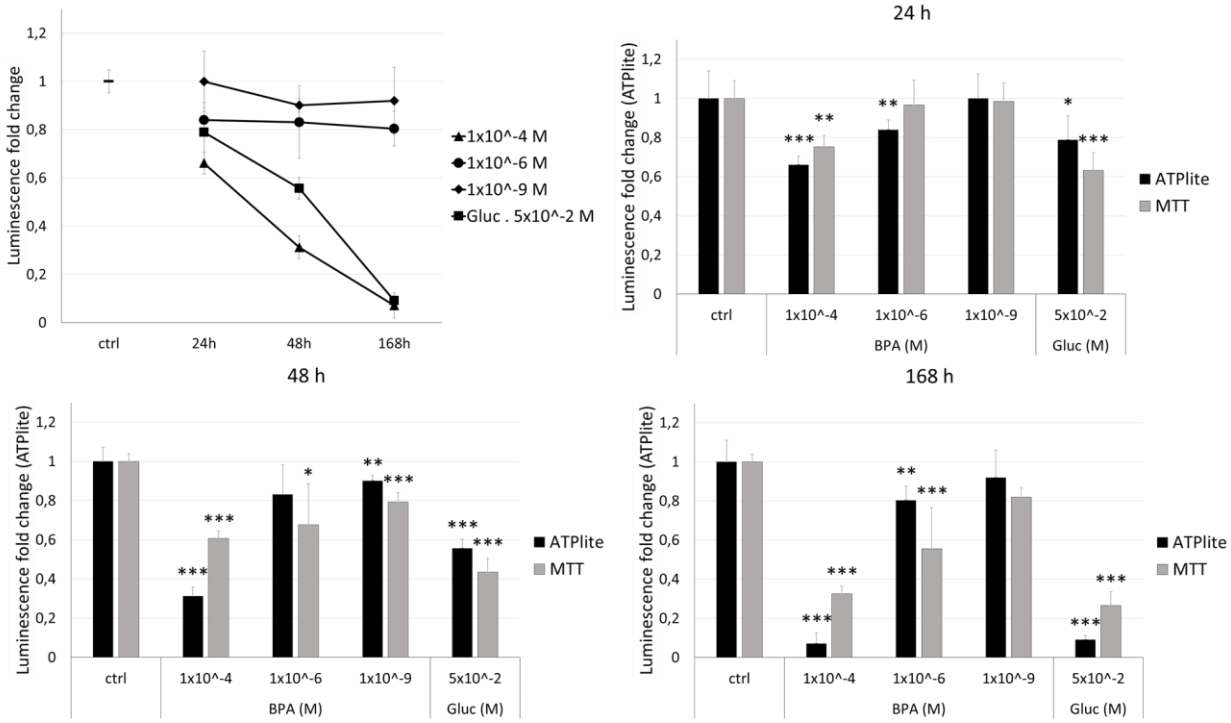


Fig. 19 Murine islets cell intracellular ATP concentration as determined by ATPlite assay. *Islet cells were cultured in the presence or absence of 1x10^{-4} M, 1x10^{-6} M, 1x10^{-9} BPA or 5x10^{-2} M glucose for 24h **(B)**, 48h **(C)**,168h **(D)**. **(A)** is a comparison of time response curve at diverse concentrations of BPA or glucose. The data are compared with previously reported MTT assay results. Data are reported as fold change value calculated as ratio between average luminescence in control and treated cells as the mean ± standard deviation of 3–6 replicates from at least three independent experiments.*
** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with untreated control cells*

Considering the role that some of the reported genes have to the ATP production [103][104][108], we performed experiments to have a direct measure of the ATP production. ATP intracellular level was determined by the ATPLite^{TM} (PerkinElmer) and treatment with to 5x10^{-2} M glucose was used as positive control. The dispersed pancreatic islets were treated with diverse concentrations of BPA for 24h, 48h or 168h. As expected, since this assay is directly linked to the number of viable cells, the results

followed the same trend of those obtained with MTT assay, with a linear dose response curve (Fig. 19 A). In this case, the treatment with $1x10^{-4}$ M BPA affects the cells viability more than the treatment with $5x10^{-2}$ M glucose, while the time-response curves of treatments with $1x10^{-6}$ M and $1x10^{-9}$ M BPA are closer. However, comparing the results of ATPLite™ assay with those of MTT is possible to see that there is not a significant reduction in ATP production, except at the highest concentration (Fig. 19 B, C and D).

3.8 BPA promotes apoptosis

The already reported results suggest that the exposure to BPA is able to affect the viability of the pancreatic cells. Furthermore, given the relevance as key organelles of the mitochondria for β-cell function, it is well known that their dysfunction or defects can trigger the apoptosis [245]. In this sense, we decided to assess the apoptosis at the single cell level with a TUNEL assay, by IF, on dispersed islets. For the realization of the TUNEL assay, we treated the samples $1x10^{-4}$, $1x10^{-6}$, $1x10^{-9}$ M BPA. The cells were stained with DAPI, to evidence the nuclei, and with the TUNEL reagent (Fig. 20). In the untreated samples there is no strong staining from TUNEL, only a weak background which indicates the absence of apoptosis (Fig. 20 A). In the panels representing the exposition to $1x10^{-4}$, $1x10^{-6}$ M BPA (Fig. 20 B and C) a considerable signal of apoptosis is visible, with 25% of the cells displaying a strong apoptotic signal. In the cells exposed to $1x10^{-9}$ M BPA (Fig 20 D), we could observe that apoptosis was also present to a less extent then the higher concentrations.

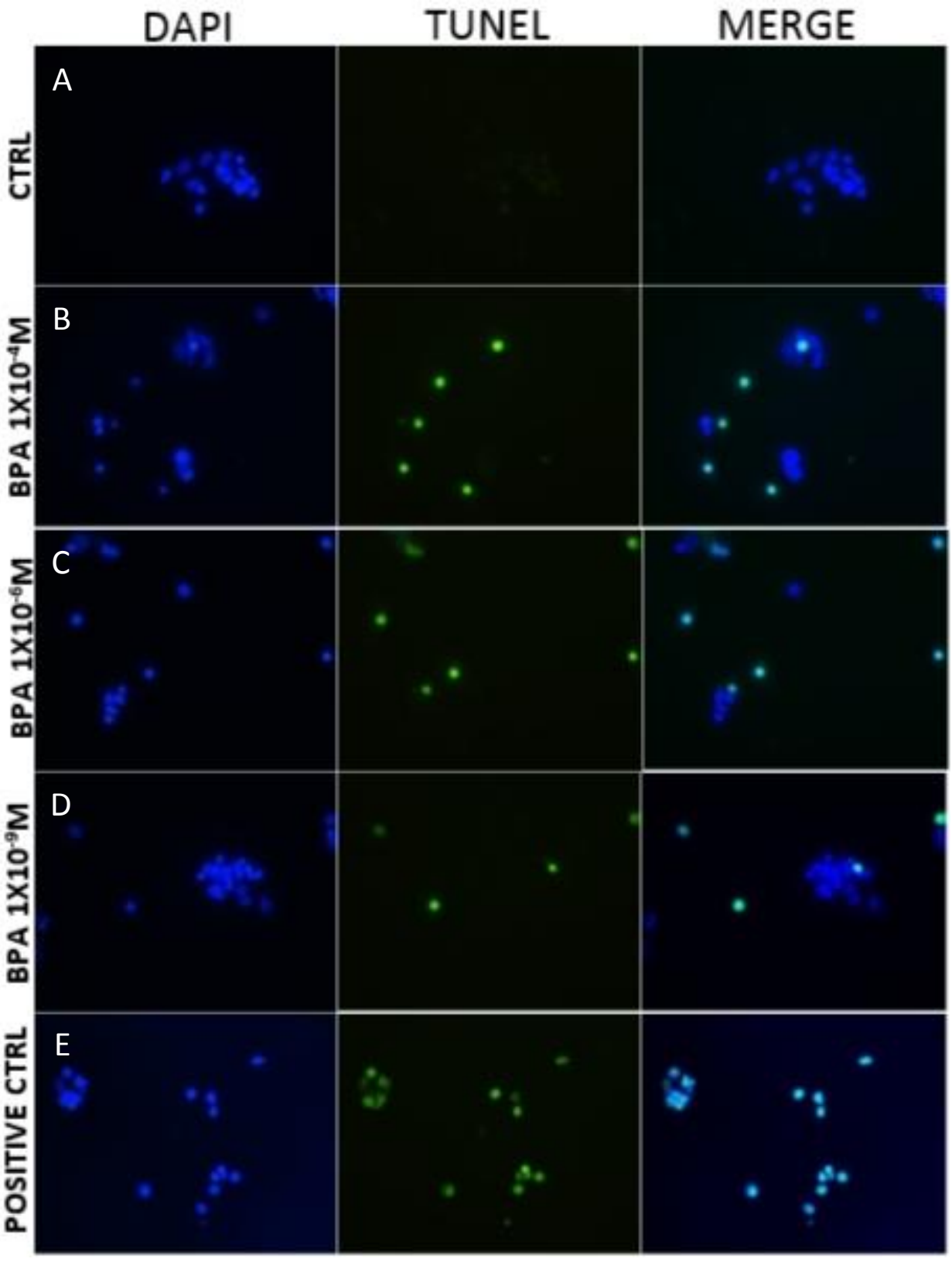


Fig. 20 Pancreatic islet cells apoptosis induced by treatment with diverse concentration of BPA for 48 h as determined by IF-TUNEL assay. Representative images of apoptotic cells stained with DAPI, to *evidence nuclei, TUNEL reagent, to notice apoptosis and merged stains at 40x magnification. Islet cells were exposed to: vehicle only (DMSO)* ***(A)****, 1x10^{-4}M* ***(B)****, 1x10^{-6} M* ***(C)****, 1x10^{-9}M* ***(D)*** *BPA or DNase 2000U (Positive Control)* ***(E)****.*

3.9 BPA, but not E2, enhances Bax gene transcription specifically in murine pancreatic islets

After the TUNEL assay and the evidence that $1x10^{-9}$ M BPA could induce apoptosis in murine pancreatic islet cells, the analysis of the expression of a pro-apoptotic gene during was the chosen approach. We opted to determine the expression of Bax gene since it has been reported that induces cell death by acting on the mitochondria [300] and appears to be an essential gateway to mitochondrial dysfunction required for cell death in response to diverse stimuli [301]. We assessed the Bax transcript level in RNA prepared from islets or hepatocytes treated with $1x10^{-9}$ M BPA for 24 hours, 48 hours and 7 days. At this concentration in islets, a significant increase in the cellular amount of Bax mRNA in all the time-point analyzed was observed (Fig. 21 A). As expected, according to MTT results, the highest effect was observed at 48 hours with an up-regulation of 2.5 time compared to control, while at 24h and 7 days the effect was lower but still significant (1.30 and 1.38, respectively). Moreover, the incubation of islets with the same concentration of E2 did not replicate this effect. Conversely, E2 protected the islets from apoptosis (-1.37, Fig. 21 B). In addition, to confirm he specificity of the effects $1x10^{-9}$ M BPA in our model, we performed the same assay on murine hepatocytes treated for 24h and 48h. As is visible in figure 21 C, the BPA induced a very mild increase of the gene expression, while at 48h this effect was no longer visible (Fig. 21 C).

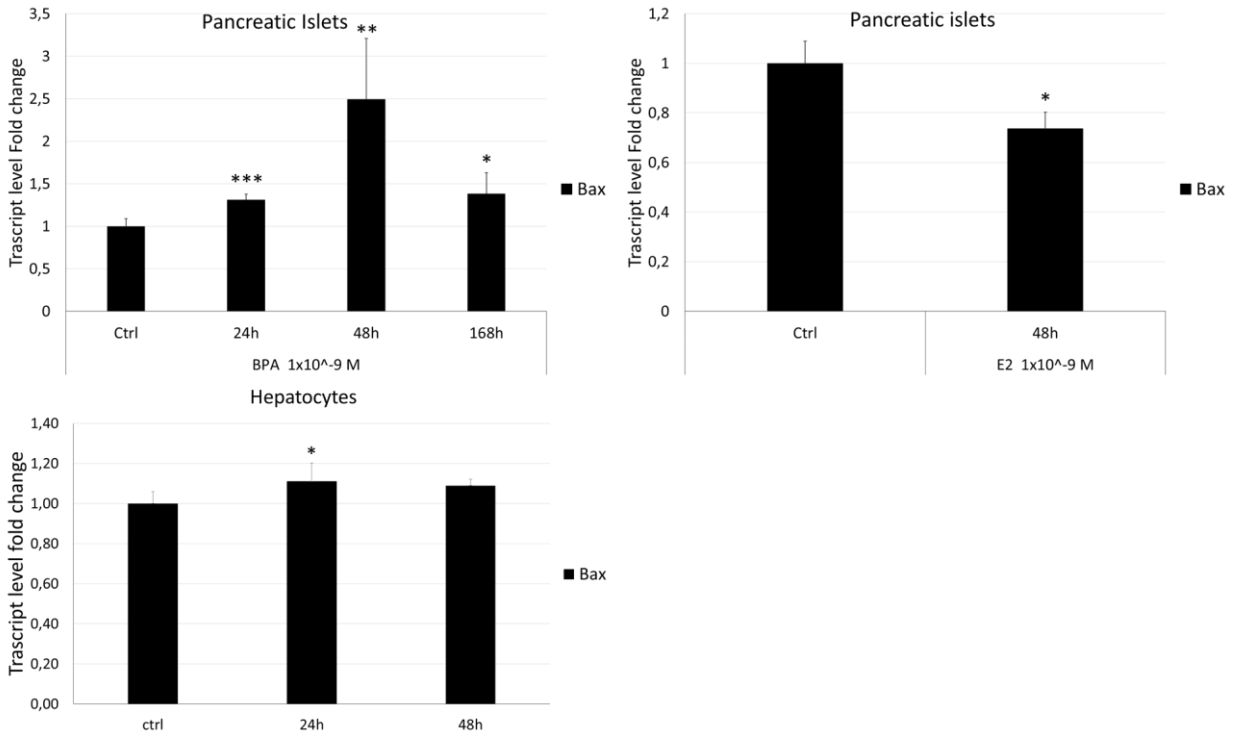


Fig. 21 Expression alterations of Bax gene in murine pancreatic islets and hepatocytes as determined by qRT-PCR. ***A:*** *islets were cultured in presence or absence of 1x10-9M BPA for 24 h or 48 h or 7 days.* ***B:*** *islets were cultured in presence or absence of 1x10-9M E2 for 48 h s.* ***C:*** *primary hepatocytes were cultured in presence or absence of 1x10-9M BPA for 24 h or 48 h. Data are reported as fold change values calculated as ratio between average gene expression in control and treated islets or cells. The qRT-PCR results are expressed as the mean ± standard deviation of at least three independent experiments.*
** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with untreated control cells*

3.10 BPA, in murine pancreatic islets, induces apoptosis enhancing ROS level and triggering NF-*k*B pathway

Recently we published that, in rat thyroid follicular cells, FRTL-5, low concentrations of BPA (ranging from $1x10^{-9}$ to $1x10^{-4}$ M) enhanced the expression of the thyroglobulin (Tg) gene acting on its promoter. Interestingly, two different antiestrogens, ICI 182780 (ICI) and tamoxifen (TAM) were not able to affect the BPA activity on the Tg-promoter, even if the estrogen receptor signaling was inducible by estrogen in this cellular setting. Upon the analysis of Tg promoter organization, we developed two FRTL-5 reporter cell lines stably expressing the luciferase gene under the control of a

NF-*k*B and RAR/RXR dependent promoters. We shown that BPA is able to induce the NF-*k*B and RAR/RXR signalling in our experimental condition [139]. Therefore, these results suggest that that BPA could exert its activity on this cell line not exclusively through ER signaling but involving also other cellular pathways. The activity of other nuclear receptors can be deregulated by BPA by different mechanisms (reviewed in [302]). Thus, we asked whether the BPA treatment could alter the pancreatic gene expression and induce apoptosis acting on the same or other transcription pathways. To this aim, we have identified by bioinformatic analysis (Genomatix database) the transcriptional factors whose binding sites can be predicted in the promoter region (-1000/+50)bp of our genes of interest (Atp1b1, Atp6v1f, Sod2, Gpx3, Uqcrb, Ndufs4 and Bax). Among the others, binding sites for NF-*k*B, retinoic acid receptors (RAR/RXR) and Fork head domain factors (FKHD) have been found (table 4). These binding sites were present in the promoter of all genes analyzed (Fig. 22). Specifically NF-*k*B had 8 match, RAR/RXR 29 and FKHD 41.

TABLE 4 Binding sites promoter analysis

Matrix Family	p-value	Total matches	Sod2	Ndufs4	Uqcrb	Gpx3	Atp1b1	Atp6v1f	Bax
NF*k*B	0,02	12	4	1	1	1	3	1	1
RAR/RXR	0,03	33	4	1	4	7	6	6	5
FKHD	0,03	47	6	6	12	8	2	7	6

Considering our previous experience, the bioinformatic analysis, the already shown ability of BPA to induce apoptosis and ROS increase [218][145] and their ability to activate NFκB related transcriptional pathway [303], we decide to focus our attention on three diverse pathways and on ROS production. We treated pancreatic murine islets for 48h with $1x10^{-9}$M BPA and/or with 3mM N-Acetyl-l-cysteine (NAC), 10μM BMS 345541 (BMS), 10 μM LY294002 (LY) and 1μM ICI. NAC is a ROS scavenger that showed the ability to prevent insulin secretion inducted by ROS in rat pancreatic islets [304].

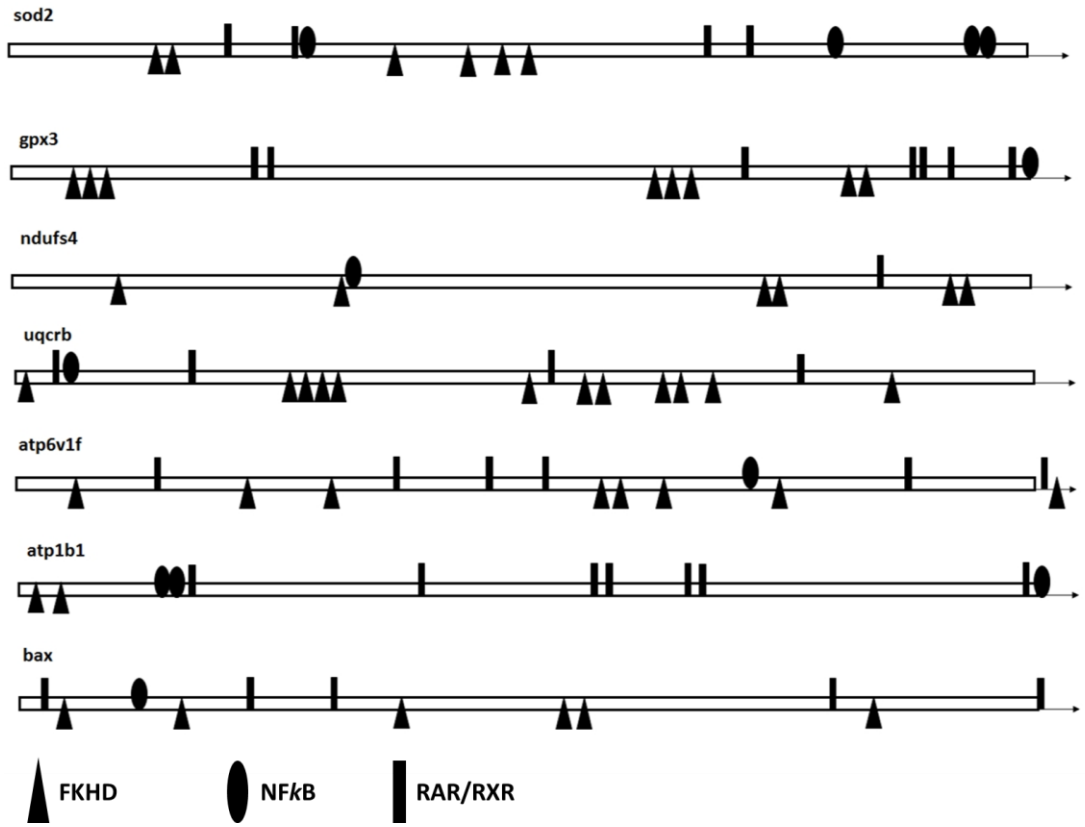


Fig. 22 Promoters bioninformatic analysis. *Schematic representation of the transcription binding sites predicted by Genomatix software in selected gene promoter fragments −1000/+50 bp.*

BMS is an IκB kinase (IKK) inhibitor able to block the activation of NFκB pathway; Chen et al., 2011, showed that it is able to improve islets xenograft survival inhibiting NFκB pathway [305]. LY is a selective inhibitor of PI3K/Akt pathway and downstream FKHD activation. Its ability to amplify insulin release in rat pancreatic islets has been reported [306]. The already mentioned ICI was used by Alonso-Magdalena et al., 2008, to block insulin release inducted by low concentrations of BPA [263].

At the end of treatment, the RNA was purified and Bax transcript quantified by qRT-PCR. As reported in Fig. 23, the co-incubation with BPA and ICI or LY did not affect the upregulation of Bax, meaning that the apoptotic effect is not induced by the activation of ERs or through Akt pathway. Conversely, the presence of NAC and BMS significantly decreased the induction of Bax transcript (Fig. 23). Specifically, islets treated with BMS alone or BMS and BPA presented Bax mRNA level -1.25 and -1.5

times lesser than untreated islets, respectively. While islets treated with NAC alone or NAC and BPA had -1.1 and 1.25 fold changes compared to control islets, respectively. The data suggest that BPA could affect the islets viability by increasing the intracellular ROS levels and activating Nf*k*B pathways

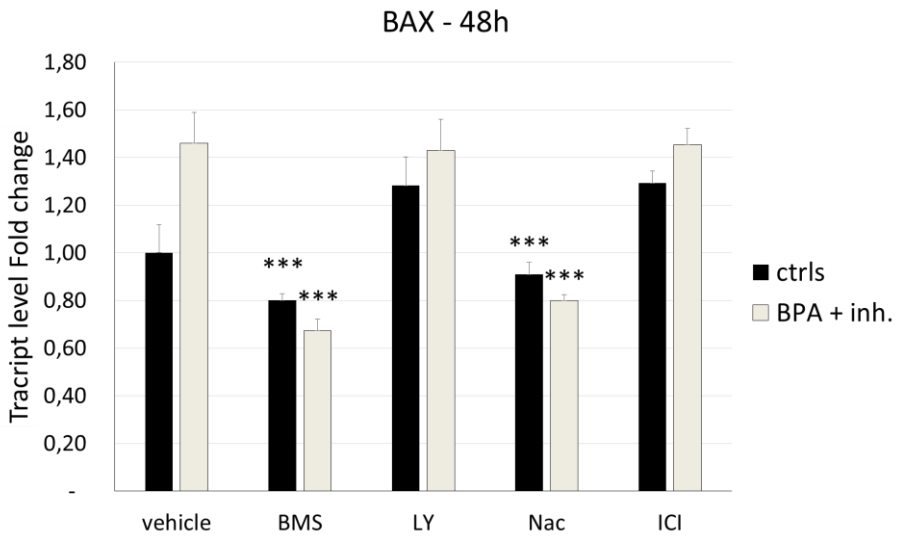


Fig. 23 Expression alterations of Bax gene in murine pancreatic islets subsequently exposure to BPA and Inhibitors as determined by qRT-PCR. *Islets were cultured in presence or absence of $1x10^{-9}$M BPA, $3x10^{-3}$M NAC, $1x10^{-5}$M BMS, $1x10^{-5}$M LY or $1x10^{-6}$M ICI for 48 h. Data are reported as fold change values calculated as ratio between average gene expression in control and treated islets. The results are expressed as the mean ± standard deviation of at least three independent experiments.*
** p-value<0.05; **p-value<0.01; ***p-value<0.001 compared with BPA only treated islets*

4 Discussion

BPA is a polycarbonate used in numerous consumer products, including food and water containers, lining of food and beverage metal cans and medical tubing, becoming an important contaminant due to its ubiquitous presence and the increased exposure [106][108].

BPA exposure is associated with multiple diseases, such as reproductive system, nervous system and sexual dysfunctions as well as increased risk of cancer and heart disease [179][180]. Epidemiological correlation between BPA and the occurrence of diabetes has been found [307]. In vitro and in vivo studies have shown that exposure to low doses of BPA elicits alteration in insulinemia, pancreatic islets function and glucose homeostasis [238]. Moreover, in WAT, BPA is able to suppress the adiponectin and increase inflammatory cytokines (Tnf-α, IL-6,) release [108]. Given these effects, its prevalence in the environment and the presence in serum from humans worldwide, BPA may play a role in the rapid increase of incidence of metabolic disorders.

In this work, we studied the effects of BPA on ex vivo primary murine pancreatic islets through a toxicogenomical approach. We reported that exposure could decrease islet cells viability, alter the transcriptome, interfere with mitochondrial activity and other specific cell functions and increase the apoptosis in a dose-dependent manner. More importantly we found that BPA apoptosis pathway triggering is dependent to intracellular ROS levels elevation and activation of NF*k*B pathway.

We chose to study the effects of three different concentrations of BPA, $1x10^{-4}$ M, known to be cytotoxic, $1x10^{-6}$M and $1x10^{-9}$M to mimic worker and worldwide population exposure, respectively [182][183]. We focused on the effects of $1x10^{-9}$ M BPA, as it can be considered the environmental concentration. Moreover, the lower concentration used in this study can be considered a ‘low dose’ because estimates of

circulating levels of BPA at the LOAEL define an equivalent low-dose concentration as $2.19x10^{-7}$M in vitro culture studies [125][126].

To begin we inspected the effects of BPA on morphology of the pancreatic islets, after 48 hours of treatment. We could not observe relevant diameter or other gross alterations. On the other hand, from the three chosen concentrations we were able to observe that both $1x10^{-4}$ M, and $1x10^{-6}$ M BPA treatments appear to induce some changes in the morphology with the whole islet gaining a granulated aspect and a precocious loss of the necrotic central core (Fig 12). The formation of a necrotic core is typical of ex vivo cultured islets and is due to hypoxia of the cells in the central area. The islet throw out this core after few days in culture. Probably, the treatment with BPA loosens the compactness of the cells and allows a faster ejection of the necrotic core.

To investigate the cytotoxic effect of BPA we performed an MTT assay. We found that BPA significantly reduced islet cells viability in a dose- and time-dependent manner. Noteworthy, that $1x10^{-9}$M BPA decrease the viability of 20% after 48 hours of treatment (Fig. 13), even if longer incubations did not induced further decrease, this suggest that low concentration of BPA could have an impact on islets viability and be detrimental for their health status.

The transcriptome analysis has been successfully applied in testing low doses EDCs in biological systems to identify non-standard toxicological endpoints [310]. This is particular powerful in the analysis of compounds, such as BPA, capable exerting effects at low doses and with a non-monotonic dose-response curve. The toxicogenomical approach through RNA microarray analysis allowed us to have a snapshot of the global status of the islets, indicating the main functions impaired and the further direction to follow to identify the processes and the pathways affected by BPA exposure.

The analysis of microarray data gave a short list of 29 downregulated DEGs (table 3). Among genes involved in generic cell functions, there was a large part of genes encoding for mitochondrial localized proteins, indicating the mitochondrion as the key organelle affected by BPA exposure. This was confirmed by IPA analysis that identified mitochondrial dysfunction and oxidative phosphorylation as the most deregulated canonical pathways (Fig 16). Mitochondria are critical for the regulation of β-cell mass and maintenance of β-cell function through the coupling of glucose stimulus to insulin release [170][185]. Indeed, a recent study indicated that Wistar rat offspring perinatally exposed to 50 μg/kg/day BPA present profound mitochondrial structural defects in β-cells that precede any observable alterations in glucose homeostasis [311]. Moreover, exposure of isolated rat islets to $1.1x10^{-7}$ M BPA has also been reported to cause mitochondrial swelling, to alter the expression of typical mitochondrial genes, to decrease the activity of the mitochondrial respiratory enzyme COX IV, and to reduce ATP content [281]. Nevertheless, mitochondrial dysfunction at so low concentration of BPA in analysis of endocrine pancreatic islets was not yet found. Further experiments will be performed to analyze the proper mitochondrial functionality. Specifically we are current testing the BPA effects on mitochondrial morphology and abundance, on mitochondrial membrane potential and on mitochondrial ROS production.

From the list of genes obtained, we decided to further analyze 9 genes according to the function of the protein for which encode. For better understand the onset and the duration of these mitochondrial effects, we investigated, by qRT- PCR analysis, two more time-points, 24 hours and 7 days, beside the 48 hours for the microarray validation. The results showed $1x10^{-9}$M BPA produced greater effects in a range from 24 to 48 hours, while longer exposition did not produce further changes in gene expression (Fig. 17). Actually, after 7 days of treatment, the transcript level of most of the genes goes back up. This contradictory data could be explained with the shelf life in vitro of explanted primary islets. After 7 days of culture, they may lost many of their

characteristics, their metabolic and communication functionality. Most of the genes had the highest inhibition at 48 hours, confirming the correct choice of this time point for the microarray experiments, while only 2 genes out of 9 had a greater inhibition at 24 hours.

Among the genes we decided to further analyze, Ndufs4 and Uqcrb, both encoding for sub-unit of the mitochondrial respiratory chain. The down-regulation of these two genes (Fig. 17 B), as well as of ATP6v1f (Fig. 17 C), pointed to an impaired oxidative phosphorylation that may cause defects in glucose stimulated ATP production. In β-cells, the levels of ATP directly regulate the ATP dependent K+ channels that in turn regulates the ability of the cell to secrete insulin. Adding that Atp1b1, also down-regulated in our experiments (Fig. 17 C), is a subunit of K+ channels, we can hypothesize that BPA-induced mitochondrial damage might play a critical role in the development of pancreatic islets dysfunction. To support this hypothesis other authors epidemiologically linked type II diabetes and deregulation of the expression of gene involved in oxidative phosphorylation [312]. Actually, we tried to verify if there was a reduction of intracellular levels of ATP but we obtained significant results only at highest concentration (Fig. 19). Probably a low concentration of BPA, such $1x10^{-9}$M, is not able to produce this functional defect but predispose the cells to develop this type of damage. We are currently testing this hypothesis by examining the response of the islet cells to a co-incubation with $1x10^{-9}$M BPA and $3x10^{-2}$M glucose. We hypothesize that BPA exposure (or pre-exposure) could affect the ability of islet cells to have an appropriate response to an overcharge of their function.

Furthermore, down-regulation of expression of gene encoding proteins involved in the respiratory chain could lead to leakage of electrons and to higher production of ROS and other free radicals. Interestingly, we also found deregulated the expression of two genes, Sod2 and Gpx3 (Fig.17 A), which encode proteins exactly assigned to ROS and free radicals scavenging. Therefore, the BPA could damage the islets with a dual effect, enhancing the oxidative stress and lowering the cell defensive system and, thus,

promoting the apoptosis. Indeed the experiments shown in fig 20 showed that NAC (a well-known antioxidant) is able to decrease the expression of the pro-apoptotic Bax gene.

Iars encode isoleucine-tRNA synthetase. Isoleucine is an amino acid of predominant importance in human mitochondria [313]. For these reasons a loss of isoleucylation efficiency are expected to lead to important impairment of protein synthesis [296]. The downregulation of this gene expression, inducted by BPA (Fig. 17 D), could enhance the misfolding of mitochondrial localized proteins and promote their aggregation.

Finally, BPA showed to decrease the expression of Vapa (Fig. 17 D), which protein is involved I tight junction formation. Its downregulation could be linked to the observed reduced compactness of islets (Fig. 12), a clear sign of non-healthy islets.

As we expected, a low concentration of BPA, such as $1x10^{-9}$M, does not produce large alterations but may affect several cellular system and make the islets not able to efficaciously response to more insults. This bad predisposition is important in multifactorial and complex pathologies such as metabolic disorders.

One potential consequence of mitochondrial damage is the increase in apoptosis. Mitochondrial dysfunction ultimately leads to apoptosis and contributes to the development of diabetes [245]. In this sense, here we demonstrated that BPA exhibit a dose dependent role in the apoptosis of the islet cells, as it is visible in the figure 20, probably via activation of the mitochondrial pathway, as the up-regulation of Bax gene expression suggest (Fig. 21).

Federici et al. demonstrated in in vitro human islets cultured that high glucose induced apoptosis is consecutive to an increasing of Bax mRNA levels [314]. In our experiments, the expression of Bax was increased in all the time points tested when compared to control. In agreement with the MTT and the TUNEL assay, at 48 hours Bax reached 2.5-fold expression evidencing the pathway through which the apoptosis occurred.

The data of this study clearly point to a specific mechanism of action of BPA in pancreatic islet cells (Figs 18 e 21). Previous studies clearly demonstrated that BPA is a xenoextrogen acting through nuclear ERs and membrane bound ERs [238], but the data in figure 23 suggest that, in pancreatic islets ERs are not involved in the triggering of apoptosis. Indeed we propose that BPA, besides its estrogenic actions, promotes cell death increasing intracellular ROS and activating NF*k*B pathway. These results are in agreement with the findings that BPA exposure increase ROS cellular amount regulating NF*k*B pathway [315]. Figure 23, we showed that BMS, inhibitor of NF*k*B pathway, and NAC (antioxidant compound) are able to block the Bax transcript cellular level upon exposure to BPA (in co-treatment conditions). They exert to less extent the same effects also in BPA untreated islets, suggesting that the NF*k*B pathway is activated in the stressing condition deriving from the purification and culture of the islets.

Further functional experiments will be necessary to completely elucidate the mechanisms through which BPA promotes apoptosis in murine pancreatic islets. The results of this work are an in vitro (even if ex vivo) conceptual prove of the detrimental effects of BPA on pancreatic islets. Nevertheless, data from in vivo studies and longer exposure to low doses of BPA are required. To this aim, we are programming to transplant BPA treated islets in obese and diabetic mouse models that will be further treated with BPA intraperitoneally injected or oral administered to mimic the effects of environmental concentrations of BPA in metabolic disorders susceptible population. Furthermore, all the results will be confirmed in vivo in a zebrafish transgenic cell line carrying the GFP coding sequence under the control of the insulin promoter. Thus, the islets can be recognized and picked up from animal treated with environmental doses of BPA starting from fertilization and exposed for long time.

5 Conclusions

This work is a contribution to clarify the role of the endocrine disruption by BPA in the function of pancreatic islets. Data from the current study overwhelmingly indicate that BPA affects the viability and function of normal murine pancreatic islets and accelerates their rate of apoptosis in a dose-dependent manner. This is adding new knowledge on the role of BPA on diabetes development and progression that, until now, has been focused on the insulin resistance. We suggest that exposure to BPA alters the expression of key genes, induces mitochondrial defects, raises the intracellular ROS levels and ultimately triggers the apoptosis of islet cells. Despite the published role of estrogen and BPA on the secretion of insulin, here we show that the regulation of transcript level by BPA is not carried out through the estrogen receptors but involve the NF*k*B pathway, probably among others. The demonstration that BPA at environmental dose is able to affects the islets functions and viability are in agreement with the link between metabolic dysfunctions and BPA exposure proposed in literature and confirm that BPA could be an important environmental factor in metabolic disorders onset.

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