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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professor Doutora Concetta Ambrosino (Universidade de Sannio) e do Professor Doutor Carlos Duarte (Universidade de Coimbra)

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“Believe you can and you’re halfway there”

Theodore Roosevelt

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Index

Abreviations

% - Percentage

µg – Microgram

µl – Microliter

µM - Micromolar

AhR - Aryl hydrocarbon receptor

AR – Androgen Receptor

ATP - adenosina trifosfato

BPA - Bisphenol A

CAR - Calcium-sensing receptor

CDC - Centers for Disease Control

CREB - cAMP-responsive element binding protein

DAPI - 4',6-diamidino-2-phenylindole

DDT - Dichlorodiphenyltrichloroethane

DES – Diethylstilbestrol

DNA – Desoxiribonucleic acid

E2 - 17β-estradiol

EDCs - Endocrine disruptor compounds

EPA – Environmental protection agency

ER – Estrogen Receptor

ERR – Estrogen Related Receptor

FDA – Food and Drug Administration

GPR30 - G protein-coupled receptor 30

GR – Glucocorticoid Receptor

IKK - IkappaB kinase

IL-6 – Interleukin 6

IPA - Ingenuity Pathway Analysis

LOAEL – Lowest-observable-adverse effect-level

M – Molar

mER - membrane-bound ER

mg – Milligram

ml – Millilitre

mM – Millimolar

mRNA - Messenger Ribonucleic acid

Nac - N-Acetyl-l-cysteine

ncmER - non-classical membrane estrogen receptors

NHANES - National Health and Nutrition Examination Survey

NL – Natural Ligand

nM - Nanomolar

NMDR – Non monotonic dose respond curvencm

ºC – Celsius degrees

PBBs - Polybrominated biphenyls

PBDEs - Polybrominated diphenyl ethers

PCBs - Polychlorinated biphenyl

PXR - pregnane X receptor

RNA - Ribonucleic acid

ROS – Reactive oxygen Species

TNFα -Tumor necrosis factor-alpha

XEs -Xenoestrogens

Abstract

Exposure to low levels of EDCs may be of concern. There is a ubiquitous presence of EDCs in today's environment and a frequent detection of their presence in blood and urine during population surveys. The near omnipresence of the exposures combined with the nontrivial potential health effects due to a sensitivity to low levels of EDCs will directly influence the health of current and future populations. Bisphenol A is a small molecule which is used as a monomer in polymerization reaction to produce polycarbonate plastics, epoxy resins and other polymer materials for manufacturing plastic utensils and is among the highest-production-volume chemicals in the world. In a process commonly referred to as “leaching”, BPA seeps into the contents of various food and water packages and into dust particles, providing ample entry ways for BPA into physiological systems of animals and humans. Measurements by the CDC revealed detectable levels of BPA in the urine samples of 92.6% of more than 2500 participants of the cross sectional NHANES study, with the adjusted mean BPA levels being reported at 4.5 ng/ml in children, 3.0 ng/ml in adolescents and 2.5 ng/ml in adults. BPA exposure is associated with multiple diseases within multiple biological systems, with recent studies, especially regarding model-animals suggesting that BPA exposure may have a significant role in the development of obesity, insulin resistance, and subsequently have relevance in the development of diabetes mellitus.

In this work we evaluated the role of endocrine disruption in the regulation of pancreatic beta cell activity. For that we analyzed the effect of BPA at the concentration of 1 nM on mouse pancreatic Islets of Langerhans, mouse embryonic fibroblast (MEF) and hepatocytes, given the specific role that each of this cellular types may have in the homeostasis of the organism, through an transcriptomical approach.

Through the analysis of the microarray data we were able to conclude that at 48 hours BPA at the concentration of 1 nM was able to deregulate the expression of a small group of genes, related to oxidative phosphorylation and mitochondrial dysfunction, specifically in β-Islets. Given this information it was decided to access the cellular viability and ATP production and the apoptosis at the singular cell level with the same treatment conditions, and our data showed that there is reduction of the cellular viability and ATP production and the apoptosis signal was visible through IF. Finally after an

analysis of the expression of Bax that was significantly up-regulated. A preliminary treatment with possible inhibitors of the apoptosis promoted by BPA was prepared and the Bax expression was evaluated. Surprisingly there was an inhibition of the expression of Bax when the IKK inhibitor BMS-345541 was used, suggesting a possible pathway for the promotion of apoptosis by BPA that goes through the NFkB.

Since BPA showed a role in the promotion of apoptosis in mouse β-Islets. we could conclude that our results were consistent with the association between the development of Diabetes and the exposition to environmentally relevant doses of BPA present in literature.

Resumo

A exposição a niveis reduzidos de disruptores endocrinos é uma questão relevante. Existe uma ubiquidade destes EDCs no ambiente e a detecção frequente da sua presença em exames ao sangue e urina de diversas populações. A omnipresença das exposições combinada com um risco elevado para a saúde devido a uma sensibilidade a níveis reduzidos destes EDCs irá afectar a saúde de várias gerações. O Bisfenol A é um pequena molécula utilizada em reacções de polimerização para a produção de plásticos policarbonatos, resinas e outros polímeros usados no fabrico de utensílios plásticos e hoje em dia encontra-se entre os químicos mais produzidos no planeta. Num processo denominado "leaching", o BPA tem a capacidade de se infiltrar no conteúdo dos seus recipientes (comida, água) e em partículas de pó, providenciando uma ampla possibilidade de entrada em sistemas fisiológicos. Num estudo realizado pelo CDC no qual participaram mais de 2500 indivíduos verificou-se que 92.6% dos indivíduos possuíam níveis detectáveis de BPA na urina, sendo que os níveis médios ajustados variavam com a idade, sendo reportado como 4.5ng/ml em crianças, 3.0ng/ml em adolescentes e 2.5ng/mlem adultos. A exposição ao BPA encontra-se associada a múltiplas doenças em diversos sistemas biológicos, sendo que, estudos recentes atribuem a esta exposição um papel relevante no desenvolvimento de obesidade, resistência à insulina e subsequentemente o aparecimento de Diabetes Mellitus.

Neste trabalho, avaliamos o papel da disrupção endócrina na regulação da actividade de células β-pancreáticas. Para esta análise foi realizada uma avaliação do efeito do BPA a uma concentração de 1 nM em Islets de Langerhans de murganho, MEF e hepatócitos, devido ao papel que cada um destes tipos celulares desempenha ao nível homeostasia do organismo, através duma abordagem de avaliação transcriptómica. A análise dos microarrays permitiu inferir que após uma exposição de 48 horas a uma concentração de 1nM o BPA causou a desregulação de um pequeno grupo de genes relacionados com a fosforilação oxidativa e disfunção mitocondrial, especificamente em β-Islets. Portanto foi decidido avaliar se existiria um efeito na viabilidade celular, produção de ATP e a apoptose ao nível celular singular (através de Imunofluorescência), tendo os resultados revelado reduções de viabilidade celular e produção de ATP e a presença de sinal apoptótico com as mesmas condições de tratamento. Por fim, após a

verificação do aumento da expressão do gene pró-apoptótico Bax foi realizado um pré-tratamento com possíveis inibidores de vias apoptóticas com intuito de descobrir através da qual o BPA actua, e uma nova verificação da expressão do Bax foi realizada, sendo que apenas o inibidor selectivo BMS-345541 causou uma diminuição da expressão do Bax, sugerindo uma acção que passa através no factor de transcrição NFkb.

Pudemos concluir que os resultados obtidos são consistentes com a associação entre a exposição ao BPA e o desenvolvimento de diabetes que se encontra descrito na literatura uma vez que a exposição a uma dose de 1 nM de BPA promoveu a apoptose em β-Islets de murganho.

Chapter I – Introduction

1.1 - Toxicogenomics

The ability to discern the mechanisms of toxicity that are related to health issues is an important challenge faced by scientists, public-health decision makers and regulatory authorities, whose aim, is to protect humans and the environment from exposures to hazardous drugs, chemicals and environmental stressors (Waters and Fostel, 2004).

The term 'toxic agent' can be defined as any substance that causes harmful effects on living organisms, but in general, such hazardous effects are substantially dependent on the chemical's exposure level (Kiyosawa et al, 2010).

Toxicology - the study of poisons – is focused on the substances and exposures that cause adverse effects in living organisms. A vital part of these studies is the empirical and contextual characterization of adverse effects at the level of the organism, the tissue, the cell and intracellular molecular systems. Therefore, traditional studies in toxicology measure the effect of an agent on an organism's food consumption and digestion, on its body and organ weight, on a microscopic histopathology, and on cell viability, immortalization, necrosis and apoptosis (Waters and Fostel, 2004).

Nowadays, traditional toxicity testing approaches are inadequate to meet the challenge of current toxicity assessment requirements. Tens of thousands of chemicals are used annually in industry that have no toxicological data associated with them, and this number is increasing (Vulpe and North, 2010). During the past two decades, and especially since the publication of the sequence of the human genome, rapid and unprecedented advances in molecular biology have been achieved. This resulted in a dramatic increase of our knowledge in the field of genomics, (Gatzidou et al, 2007) which is defined as the systematic study on a whole-genome scale for the identification of genetic contributions to human conditions, (Zhang et al, 2011) using other words genomics represents the study of the structure, function and nucleotide sequences of component genes of the genome, in order to determine how genes interact and influence biological pathways, networks and cellular physiology. The link between conventional toxicological research and functional genomics resulted in the emergence of toxicogenomics (Gatzidou et al, 2007).

Toxicogenomics is defined as "the application of global mRNA, protein and metabolites analysis related- technologies to study the effects of hazards on organisms", therefore it intends to studies the interactions between the genome and the adverse

biological effects caused by exogenous agents such as environmental stressors, toxins, drugs and chemicals (Vulpe and North 2010). These technologies can be applied into two broad and overlapping classes: mechanistic or investigative, and predictive toxicology. Mechanistic or investigative toxicology is the field of toxicology focused on the biochemical and biological responses in a particular type of toxicity giving important information for the risk assessment of different compounds; it is used to characterize toxicological findings at the molecular level, generate hypotheses about the mechanism of toxicity, identify potential safety biomarkers, contribute to the elucidation of species specificity and at the end also to support the risk assessment of new chemical entities. Predictive toxicology, in the present context, refers to the field of toxicology focused on the identification of a compound to be potentially toxic; in addiction this term is often used to refer to studies that attempt to extrapolate toxic reactions from preclinical species to humans (Gatzidou et al, 2007; Sahu, 2008; Semizarov and Blomme, 2008).

This field has three principal goals: to understand the relationship between environmental stress and human disease susceptibility; to identify useful biomarkers of disease and exposure to toxic substances; and to elucidate the molecular mechanisms of toxicity (Waters and Fostel, 2004).

Chemical-specific changes in the transcriptome profile leads to changes in the proteome profile, the metabolome profile and eventually the tissue-level phenotypes. Thus, it is natural that the transcriptome profile would contain a significant degree of information for biological conditions at the moment, which may lead to a profound understanding of chemical-induced molecular perturbations (Kiyosawa et al, 2010).

1.2- Endocrine system

The term endocrine is derived from the Greek words *endo*, meaning within, and *crino*, to separate. The term implies that cells of endocrine glands secret chemical signals that influence tissues that are separated from endocrine glands by some distance. (Seeley et al, 2004)

The endocrine system is a complex collection of hormone producing glands, which differ from most of the other organ systems of the body due to the fact that the various glands are not anatomically continuous. However, they do form a system in the functional sense and are able to co-ordinate the control and regulation of a multitude of biochemical processes that occur in the body, such as metabolism, growth,

cardiovascular function, and digestion, as well as more specialized functions such as behaviour, sexual differentiation during embryogenesis, sexual maturation during puberty, and reproduction in adulthood. The endocrine glands consist of pineal, pituitary, thyroid, parathyroid, thymus, adrenals, pancreas, ovaries (female), and testes (males), in addition to minor glands distributed in multiple tissues (Harvey and Cockburn, 1999; Vander et al, 2001; Naciff and Daston, 2004).

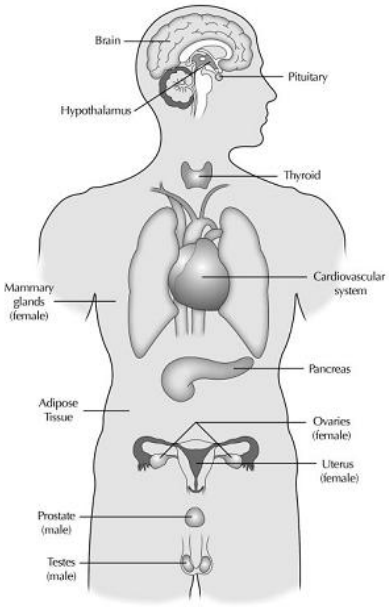

Figure 1 - Model representative of the endocrine systems (Diamanti-Kandarakis et al, 2009)

These glands produce hormones, term derived from the Greek *hormon*, meaning to set in motion. A hormone is traditionally defined as a chemical signal or ligand that is produced in minute amounts by a collection of cells, is secreted into interstitial spaces, and enters the circulatory system, where it is transported some distance in order to act an influence, in a specific manner, those tissues, denominated target tissues (Seeley et al, 2004). Hormones are secreted by endocrine glands when there is a need for them in their target organs. Therefore, the cells of endocrine glands must respond to changes in blood or perhaps to the presence of other hormones in the blood. These stimuli are the information they use to increase or decrease secretion of their own hormones, consequently, when a hormone brings about its effect, the stimulus is reversed, and secretion of the hormone decreases until the stimulus reoccurs, being this denominated a negative feedback mechanism (Scalon and Sanders, 2007).

Endogenous hormones act through several mechanisms. The classical mechanism of action for steroid hormones such as estrogens, androgens, thyroid and progesterone involves binding of the hormone to its receptor, the interaction of this hormone-receptor complex with other cofactor in a cell, and the activation or inactivation of transcription of a target gene. In addition, hormone signalling also involves the synthesis, degradation, or inactivation of hormones by specific enzymes, any or all of which may be targeted by endocrine disruptor compounds (EDCs). Labelling a chemical as an ‘‘endocrine disruptor’’ involves using the term to very broadly cover disruption of the synthesis and transport of chemical messengers (autocrine, paracrine, endocrine, neurotransmitters), as well as their intracellular signalling pathways and receptor systems that regulate cell function and intercellular communication. (Naciff and Daston, 2004; Gore, 2007; Vom Saal et al, 2012).

1.3 – The 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. (Saladin, 2003; Seeley, et al 2004).

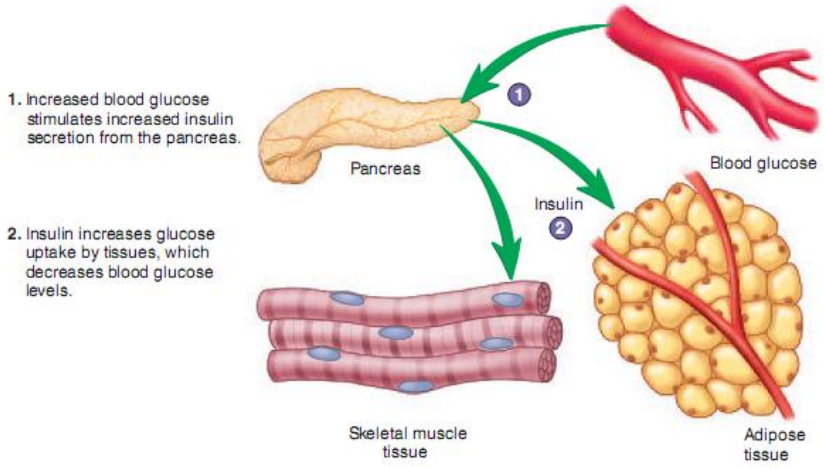

Figure 2 - Simplified mechanism of the role and targets of insulin (Seeley et al, 2003)

1.3.1 - Islets of Langerhans

The islets of Langerhans or pancreatic islets are scattered throughout the pancreas and constitute about 1%-2% of the pancreas and 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 beta cells, glucagon-producing alpha cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP cells and ghrelin-producing epsilon cells (Costanzo, 2006; Seino and Bell, 2007).

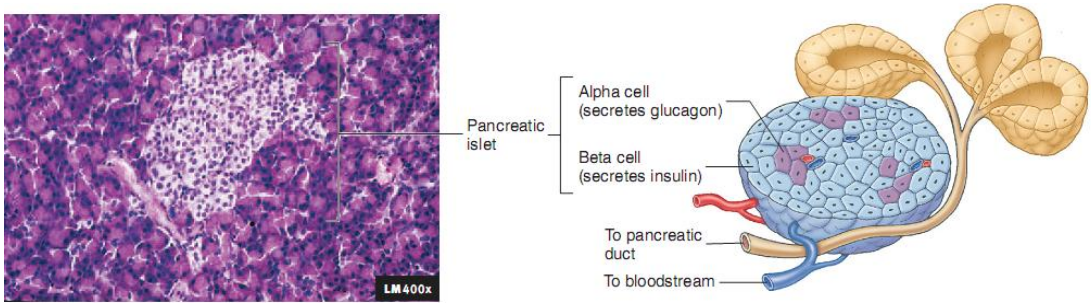

Figure 3 - Struture and morphology of the β-Islet (Seeley et al, 2003)

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 (Nadal et al, 2009; Supale et al, 2012)

1.4 – 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. Glucose is transported into β-cells, induced by the increase of

plasma glucose levels and leads to insulin exocytosis, which manipulates the glucose levels (Han and Park, 2011). The secretory response of β-cells depends on their electrical activity. The β-cell membrane contains specific transporters for glucose, which promote his entering into the cell by facilitated diffusion, once inside the cell, it's 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 an increase (inside the β cell), the K^+ channels close, which depolarizes the β-cell membrane. In the β-cell membrane Ca^{2+} channels are also present and they are regulated by changes in voltage, becoming open through depolarization and close through hyperpolarization. When depolarization occurs, Ca^{2+} channels open and, subsequently Ca^{2+} flows into the β cell. Finally, 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. Relevantly, additional signals are necessary to reproduce the sustained secretion elicited by glucose. They participate in the amplifying pathway formerly referred to as the K_{ATP}-channel-independent stimulation of insulin secretion. In contrast to the transient secretion induced by Ca^{2+} raising agents, the sustained insulin release depends on the generation of metabolic factors. C peptide is secreted in equimolar amounts with insulin and is excreted unchanged in the urine. Therefore, the excretion rate of C peptide can be used to assess and monitor endogenous β-cell function (Costanzo, 2007; Nadal et al, 2009; Maechler et al, 2010)

1.5 - Mitochondria

Mitochondria are key organelles that generate the largest part of cellular ATP and represent the central crossroad of metabolic pathways. (Maechler et al, 2010). 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 (Maechler et al, 2010). 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 (Supale et al, 2012).

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 (Ma et al, 2012) and 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 are more extensive than those in nuclear DNA due to the lack of protective histones and low repair mechanisms (Maechler et al, 2010; Ma et al, 2012). 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, in fact 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 (Maechler et al, 2010).

1.6 - Animal Model – The Mouse

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 and the endocrine portions is constituted by the islets of Langerhans, are always closely associated with the septal ducts and blood

vessels. (Green, 1968) 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%). Moreover, 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 (Nadal et al, 2009).

1.7 - Endocrine Disruption

1.7.1 - EDCs

An endocrine-disrupting compound was defined by the U.S. Environmental Protection Agency (EPA) a "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process.".

It is well recognized that EDCs pose a potential risk affecting both wildlife and human health on a global scale. The issue of endocrine disruption has been something of a *cause célèbre* since it was first identified as an issue about 25 years ago. Few scientists had previously suspected that certain synthetic chemicals might be able to interfere with the workings of the endocrine system at low concentrations. However, in the mid-1990s, Theo Colborn and others brought this subject to the attention of a wide audience when it became clear that many different wildlife species were experiencing effects that were attributable to damaged hormone signalling (Matthiessen, 2013).

The group of molecules identified as endocrine disruptors is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [Bisphenol A (BPA)], plasticizers (phtalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltricholoethane (DDT)], fungicides (vinclozolin), and pharmaceutical agents [diethylstillbestrol (DES)] (Diamanti-Kandarakis et al, 2009) and according to the Chemical Abstracts Service (CAS) the

numbers of synthetic chemicals, such as pharmaceuticals, cosmetics, pesticides, food additives and industrial chemicals have increased dramatically over the last few decades (Cheng et al, 2013). As of December 6, 2012, there were 70 million chemicals registered (www.cas.org). The most commonly studied EDCs are dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), phthalates, and bisphenol A (BPA) (Bonefeld-Jorgensen et al, 2007; Bernal and Jirtle, 2010).

At the environmental level, wildlife is vulnerable to the endocrine disrupting effects of EDCs. Effects linked to endocrine disruption have been largely noted in invertebrates, reptiles, fish, birds and mammals and the human species. The species, at the top of the food chain are particularly exposed to health risks from environmental polluting chemicals owing to bioaccumulation and biomagnification of such substances in the food chain. Drinking or ingesting contaminated water or food, breathing contaminated air or contacting contaminated soil represent the main pathways of exposure to environmental polluting chemicals, a number of EDCs appear in drinking water as a result of effluent from manufacturing plants and agricultural centers entering into streams. Some EDCs are present in personal hygiene products as well as in food and beverage containers (Mnif et al, 2011; Bechi et al, 2013; Rogers et al, 2013). 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. In general, EDCs are also considered to accumulate in the body because of their chemical stability. Since these substances do not decay easily, may not be metabolized, or may be metabolized into more toxic compounds than the parent molecule, even substances that were banned decades ago, remain in high levels in the environment, and they can be detected as part of the body burden of virtually every tested individual, animal or human (Diamanti-Kandarakis et al, 2009; Sasaya et al, 2012).

1.7.2 – Epidemiological evidences

There are several health problems worldwide with postulated association to the exposure to EDCs: decreased sperm counts, increased incidence of hypospadias and cryptorchidism, altered birth sex ratios, miscarriage, increased incidence of cancer, altered development, brain and behaviour defects, impaired immune function, attention

deficit hyperactivity disorder, hyper allergic diseases, asthma, obesity, heart disease and type two diabetes. Epidemiological evidence that human reproductive health is declining, particularly in Western nations, continues to mount, sperm counts in Western countries appear to have declined by half in the past 50 years and female fecundity is declining, even among young women. Within the United States, median age at menarche, breast development, and sexual precocity has steadily advanced and similar trends have been noted in Europe and among children adopted from developing countries by Western parents. The cause is likely complex and multi-faceted, but rapidity of the increase in reproductive and behavioral disorders suggests an environmental component. 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 (Safe, 2005; Diamanti-Kandarakis et al, 2009; Myers et al, 2009; Patisaul and Adewale, 2009; Bernal and Jirtle, 2010).

1.8 - Molecular Mechanisms of Action

The molecular mechanisms of this endocrine disruption, however, remain poorly understood (Bechi et al, 2013). Effects of these compounds are known to occur in multiple endocrine axes such as estrogen, androgen, thyroid hormone, prolactin and insulin systems. The putative effects are wide ranging and the mechanisms of action are concomitantly diverse (Diamanti-Kandarakis et al, 2009; Ding et al, 2010). A large number of these EDCs act via the estrogen receptor (ER), imperfectly mimicking and interfering with the physiologic actions of endogenous estrogens. Xenoestrogens (XEs) can bind to ERs , the complex recognizes DNA response elements and alters gene expression; in the non-genomic pathway XEs can bind to membrane-bound ERs and rapidly initiate signaling cascades that culminate in kinase and phosphatase activations, ultimately influencing cellular function by post-translational modifications of a variety of proteins (Viñas et al, 2012).

Estrogens and xenoestrogens exert a great variety of actions in almost every cell type and through diverse cellular and molecular pathways (Ropero et al, 2008). EDCs were originally thought to exert actions primarily through nuclear hormone receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs) and

retinoid receptors, nonetheless today scientific research shows that the mechanisms are much broader than originally recognized (Schug et al, 2011). As example divergent pathways including, but not limited to peroxisome proliferator-activated receptor γ and actions through other nuclear receptors, steroidogenic enzymes, neurotransmitter receptors and systems, and the mimic the natural hormone's action (agonist action), furthermore these compounds may bind to these receptors without activating them, blocking the receptors and inhibiting their action, and on the other hand, EDCs may interfere with the synthesis, transport, metabolism and elimination of hormones, thereby decreasing the concentration of natural hormones. In addition to these endocrine active properties, some EDCs have been shown to disrupt epigenomic programming. Thus, EDCs act via nuclear receptors, non-nuclear steroid hormone receptors, non-steroid receptors, and orphan receptors, transcriptional coactivators, enzymatic pathways involved in steroid biosynthesis or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al, 2009; Bernal and Jirtle, 2010; Mnif et al, 2011). Through these interactions and acting as agonists or antagonists, EDCs are able to alter the activity of response elements of genes, block natural hormones from binding to their receptors, or in some cases increase the perceived amount of endogenous hormone in the body by acting as a hormone mimic to its receptor. The modifications of the pattern of gene expression on particular cell types, from target tissues or organs as response to a toxic exposure can be highlighted. Some chemicals elicit toxic responses by first damaging cellular components or DNA, which provoke alterations in the expression of appropriate repair genes, while that others modulate endocrine systems or cellular replication disturb directly by triggering signal transduction systems, either at the membrane or in the nucleus, leading to alteration of gene expression (Naciff and Daston, 2004; Rogers et al, 2012).

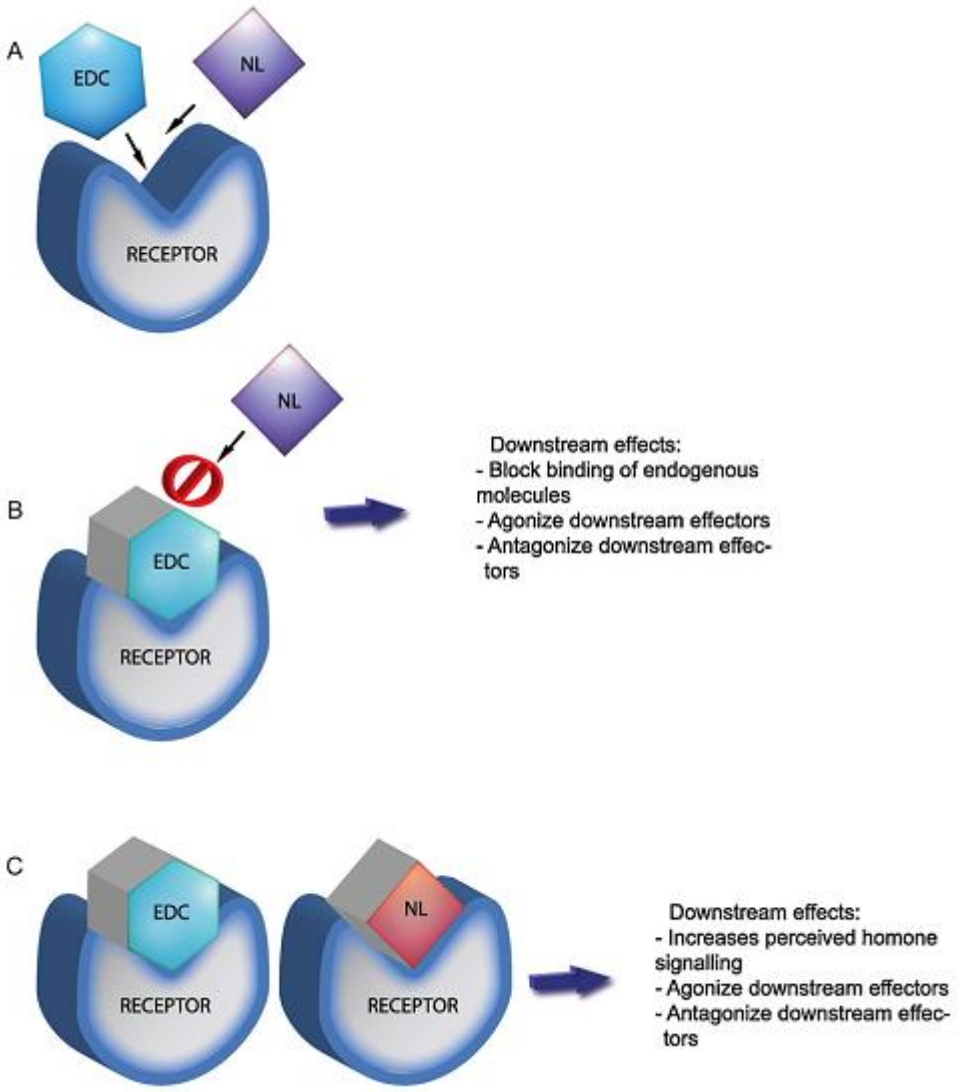

Figure 4 - Representation of the interactions between endocrine disruptors and receptor function. (A) EDCs can compete with Natural hormones for receptor binding. (B) Binding of EDCs can result in altered downstream signaling. (C) EDCs may bind unbound receptors while natural hormones are also bound which results in agonistic or antagonistic downstream signaling (Rogers et al, 2013)

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

- Age at exposure - Exposure of an adult to an EDC may have very different consequences from exposure to a developing fetus or infant. In fact, the field of endocrine disruption has embraced the terminology "the fetal basis of adult disease" to describe interactions of the environment of a developing organism and the external environment with the individual's genes to determine the propensity of that individual to develop a disease or dysfunction later in life.

- Latency from exposure - The consequences of developmental exposure may not be immediately apparent early in life but may be manifested in adulthood or during aging.

- Cocktail effects - Contamination of environments is rarely due to a single compound. Furthermore, effects of different classes of EDCs may be additive or even synergistic.

- Transgenerational, epigenetic effects - EDCs may affect not only the exposed individual but also subsequent generations. Recent evidence suggests that the mechanism of transmission may in some cases involve 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 factors that regulate gene expression such as DNA methylation and histone acetylation.

- Nontraditional dose-response dynamics - Even infinitesimally low levels of exposure, indeed any level of exposure at all, may cause endocrine or reproductive abnormalities, particularly if exposure occurs during a critical developmental window. Surprisingly, low doses may even exert more potent effects than higher doses. EDCs may exert nontraditional dose-response curves, such as inverted-U or U-shaped curves.

(Diamanti-Kandarakis et al, 2009)

1.9 -Bisphenol A

1.9.1 - BPA

BPA (2, 2-bis (4-hydroxyphenyl) propane) is a small (228 Da) molecule which is used as a monomer in polymerization reaction to produce polycarbonate plastics, epoxy resins and other polymer materials for manufacturing plastic utensils and is among the highest-production-volume chemicals in the world, with an annual production of over 2 million tonnes (Ben-Jonathan et al, 2009; Ryan et al, 2010; Lam et al, 2011; Gong et al, 2013; Molina et al, 2013). BPA is formed by two phenol functional groups, and 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 (Vandenberg et al, 2009)

BPA is widely used in the production of packaging for food and drinks, paints, adhesives, drinking water pipe linings, dental sealants, flame-retardant, medical tubing, toys and eyeglass lenses (Qin et al, 2013; Ribeiro-Varandas, 2013; Teng et al, 2013). In a process commonly referred to as "leaching", BPA seeps into the contents of various food and water packages and into dust particles, providing ample entry ways for BPA into physiological systems of animals and humans. Moreover BPA is contained in items that we come in contact at home and in the workplace including the coating of CDs, DVDs, electrical and electronic equipment, automobiles, sports safety equipment, recycled paper and carbonless paper, nevertheless humans are exposed to BPA mainly through oral and inhalation routes.

1.9.2 - Leaching

Numerous studies found that BPA leaches from polycarbonate baby bottles and reusable water bottles. Other polycarbonate containers intended to be used as reusable food containers, food-contact items such as polyvinyl chloride stretch films, and some paper and card- board used as food containers have been examined for their BPA content. Metallic food cans are protected from rusting and corrosion by the application of epoxy resins as inner coatings. Many of these resins are synthesized by the condensation of BPA with epichlorhydrin to create BPA diglycidyl ether. The relevant problem arises from factors as the incomplete polymerization, the aging, heating and contact with acids and bases, including those commonly found in cleaning supplies and detergents, that may cause the BPA polymers to break apart, and as such thing happens,

residual BPA leaches and has the potential to contaminate stored foods (Vanderberg et al, 2009; Rubin, 2011; Gong et al, 2013; Jayashree et al, 2013).

Several studies show that BPA is released from consumer products leading to detectable levels of BPA in food, drinking water, waste water, air and dust. A comprehensive, cross-sectional study of dust, indoor and outdoor air, and solid and liquid food in preschool-aged children suggested that dietary sources constitute 99% of BPA exposure. Other studies identified BPA in human serum, urine, amniotic fluid, umbilical cord blood, placental and adipose tissue and milk of lactating mothers, even though at varying levels. BPA is rapidly excreted in urine, with a half-life in the range of 4 to 43 hours, however, 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. (Bondesson et al, 2009; Diamanti-Kandarakis et al, 2009; Huc et al, 2012, Trasande et al, 2012).

Given the prevalence of BPA in our environment, it is not surprising that measurable levels have been detected in the majority of individuals examined to date. Measurements by the Centers for Disease Control (CDC) revealed detectable levels of BPA in the urine samples of 92.6% of more than 2500 participants of the cross sectional NHANES (National Health and Nutrition Examination Survey) study. The adjusted mean BPA levels reported were 4.5 ng/ml in children (6–11 years of age), 3.0 ng/ml in adolescents (12–19 years of age) and 2.5 ng/ml in adults with over 20 years old (Ruby and Soto, 2011). BPA metabolites were also found in more than 90% of the urine samples collected from the general populations of the United States and Italy, and estimated to be present in 95% of the United States citizens with concentrations in human serum ranging from 0.2 to 1.6 ng/mL (0.88–7.0 nM) (Huc et al, 2012; Teng et al, 2013), another study has identified the strong presence of BPA and in the blood samples of most Hong Kong citizens, with a mean concentration of 0.95 ng/mL (4.1 nM) (Wan et al 2013).

Estrogens and xenoestrogens exert a great variety of actions in almost every cell type and through diverse cellular and molecular pathways and BPA has well characterized estrogenic and other endocrine disrupting activities that are mediated via multiple molecular mechanisms. The core structure of BPA resembles that of natural 17β-estradiol (E2) (Le et al, 2008; Ropero et al, 2008; Sheng et al, 2013)

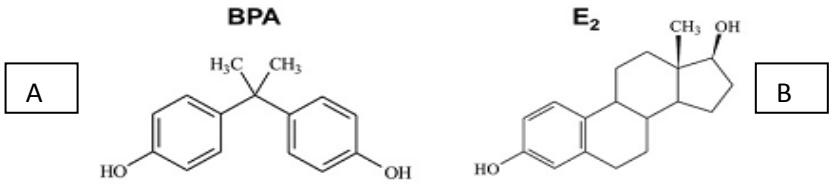

Figure 5 - Chemical Structure of BPA (A) and Estradiol (B) Dong et al, 2011

1.10 - LOAEL

Concerning the potential risk of this compound, in the 1980s the lowest-observable-adverse effect-level (LOAEL) for BPA was determined at 50mg/kg bw/day, and the Environmental Protection Agency (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 (Alonso-Magdalena at, 2012). The LOAEL for BPA was established in a 2 year carcinogenesis study conducted in adult rodents exposed daily to high doses of BPA. The established LOAEL was then divided by an uncertainty factor of 1000, in order to provide a safety margin below the permitted daily exposure limits. Therefore, the first safety standard set by the EPA in 1988 and adapted by the FDA as a reference dose for BPA was calculated to be 50 µg/kg BW/day. 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 (Rubin, 2011; Alonso-Magdalena at, 2012).

1.11 – Associated Dysfunctions

BPA exposure is associated with multiple diseases within multiple biological systems. Various disorders can be highlighted, BPA is able to accelerate growth and puberty, alter the ovarian cycle in females, interfere with embryonic development, and to induce aneuploidy, it has harmful effects on the multipotent neural progenitor and increases depression-like behaviour in rats. Moreover, a relationship between BPA blood levels, obesity, polycystic ovary syndrome, repeated miscarriage, and endometrial hyperplasia has been found. Exposure to BPA has also been correlated with the incidence of diverse types of tumors (Dong et al, 2011; Pupo et al, 2012). A study from

Alonso-Magdalena suggest that the endocrine disruptor BPA should be evaluated as a possible risk factor for gestational diabetes, type 2 diabetes, and cardiovascular disease associated with metabolic syndrome (Alonso-Magdalena et al, 2010). In vivo studies using much lower doses of BPA than the LOAEL have shown that it affects sexual maturation, induces a decrease in daily sperm count and fertility, disrupts chromosome alignment, affects synaptogenesis and it rapidly increases plasma insulin, altering blood glucose concentration (Alonso-Magdalena et al, 2006). Studies regarding *Xenopus tadpoles* and *Japanese medaka* showed skewed sex ratios, with tendency of feminization upon exposition to BPA, suggesting that it may act as an endocrine disruptor (Pupo et al, 2012; Rogers et al, 2013).

The effects of BPA display a discrepancy with dose and time of exposure variations. Some of the effects due to developmental exposure to levels of BPA at or below the LOAEL or the human safe dose include: altered time of puberty; altered estrogenic cycles; prostate changes; altered mammary gland development and evidence of intraductal hyperplasia and preneoplastic mammary gland lesions in adulthood; changes in the uterus and ovary; alterations in brain sexual dimorphisms; additional changes in the brain and in brain steroid receptor levels and receptor transcripts; changes in behavior including reports of hyperactivity; increased aggressiveness; altered socio-sexual behavior; altered cognitive and anxiolytic behaviors; increased susceptibility to drugs of addiction; altered body weight and body composition and altered glucose homeostasis (Rubin, 2011).

Meaningfully, it has been argued that the shape of the dose-response curve, particularly for chemicals with hormonal activity, is very different at high concentrations than the one obtained at low concentrations, BPA often exhibits a lack of linear dose-dependent relationship, showing instead U-shaped or inverted U-shaped curves. Consequently, extrapolation from an action or lack of action, of BPA at high doses to its presumed bioactivity at low doses is unwarranted (Naciff and Daston, 2004; Hugo et al, 2008). For many years, when assessing the effects of possible endocrine disruptors, toxicologists have relied on the principle that "the dose makes the poison," implying that higher doses were expected to cause greater harm. Thus, effects that are not seen at high doses are not expected at low doses. In contrast, multiple studies have found that neither the threshold nor the linear non-threshold models are applicable to the responses of hormones in which biphasic dose responses have been observed for many

different endpoints at many levels of organization. These U-shaped and inverted U-shaped dose response curves are considered "non monotonic" and are used as evidence that very low doses of natural and synthetic hormones can affect endpoints such as cell proliferation and organ development (Vandenberg et al 2009; Schug et al, 2011). Non monotonic dose–response curves result from multiple mechanisms. Hormones and hormone-mimicking chemicals act through receptors in target cells. Very low doses can stimulate the production of more receptors (receptor up-regulation), resulting in an increase in responses, whereas higher doses (within the typical toxicological range of chemical testing) can inhibit receptors (receptor down-regulation), resulting in a decrease in responses (Myers et al, 2009).

Despite the controversy surrounding the "low dose" concept, there are several reasons why dose response curves to toxicants may be non-monotonic, the induction of metabolizing enzymes, conjugation of substrates, down regulation of receptors at higher hormone levels and also the integration of two or more monotonic dose response curves that occur through different pathways affecting a common endpoint with opposing effects. Additionally, adaptive responses through complex cell signalling pathways and feedback mechanisms could cause non-monotonic effects that are inconsistent with traditional dose-response curves (Schug et al, 2011).

NMDR curves have been observed after exposure of cultured cells to BPA. For instance, the response of GH3/B6 pituitary cells to BPA followed a U-shaped NMDR curve, where doses of 10^{-12}M, 10^{-11}M, and 10^{-8} M elicited significant responses and doses of 10^{-10}M and 10^{-9} M did not. LNCaP prostate cancer cells responded to BPA in a similar manner with maximal proliferation induced by 10^{-9} M. Additionally, BPA inhibited adiponectin secretion from human adipose explants with a U-shaped NMDR curve; concentrations of 10^{-10} M and 10^{-9} M inhibited release, whereas doses of 10^{-8} M and 10^{-7} M were indistinguishable from unexposed controls (Vandenberg et al 2009). Another example of a clear 'U'-shaped dose-response curve was found treating breast, subcutaneous and visceral adipose tissue explants as well as isolated mature adipocytes with BPA, in the same study it was reported that at 1 and 10mM concentrations BPA was able to inhibit adiponectin release and was also found to a 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 (Ben-Jonathan et al, 2009). It is also described that BPA is able to enter fibroblasts in the differentiation process and 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 (Masuno et al, 2002), it also influences 3T3-F442A adipocytes by increasing basal and insulin stimulated glucose transport (Sakurai et al, 2004) Pancreatic islet cells exposed to BPA have their insulin release affected, displaying an inverted U-shaped NMDR curve where only doses of 10^{-9} M and 10^{-10} M significantly increase insulin release (Vandenberg et al 2009).

1.12 - BPA and diabetes

Obesity increased in prevalence to a considerable extent during the last half of the 20th century in both adults and children (Elobeid and Allison, 2008). It has deleterious effects on human health by increasing the risk of associated metabolic abnormalities such as insulin resistance, hyperinsulinemia, hypertension, and dislipidemia, all components of the metabolic syndrome which constitute, in turn, one of the major risk factors for the development of among other pathologies, diabetes mellitus type 2 (Diamanti-Kandarakis et al, 2009).

Recent studies, especially regarding model-animals suggest that BPA exposure may have a significant role in weight gain, contribution to the development of obesity, insulin resistance, and subsequently have relevance in the development of diabetes mellitus (Shankar and Teppala, 2011). In experimental studies, it has been shown to interfere and disrupt metabolic mechanisms, suggesting that it may have a relevant role in the body mass increase at environmentally relevant doses (Trasand et al, 2012)

A multitude of factors will influence whether an individual develops obesity, as such, genetic, nutritional and environmental factors are known to impact hunger and satiety, basal metabolic rate, carbohydrate and lipid flux, and the regulation of adipocyte proliferation and differentiation and developmental programming of metabolic set points (Grün and Blumberg, 2009) and although most attention has focused on high caloric diet and sedentary lifestyle as the root causes, the role of environmental factors is gaining credence (Hugo et al, 2008).

Recent epidemiological evidence suggests a correlation between BPA and the occurrence of diabetes. A study performed in China, regarding 3400 subjects, showed a strong association between urinary levels of BPA and diabetes, where an increase of 37% of the incidence of diabetes was found in subjects where the concentration of BPA

was above 1,43 ng/ml , against a reference concentration equal or lower than 0,47ng/ml (Gong et al, 2013). Increasing levels of urinary BPA were also positively associated with metabolic syndrome in a representative sample of US adults, independently from factors as age, gender, race/ethnicity, smoking, alcohol intake, moderate physical activity levels and urinary creatine level (Teleppala et al, 2012).

Relevantly, severe of the diabetes hallmarks that have correlations with impaired pancreatic β-cell function have been found in normal mice exposed to BPA (Gong et al, 2013) and studies in rodents have also demonstrated that exposure to BPA elicits alterations in glucose homeostasis (Batista et al, 2012). Pancreatic islets are thought to play a key role in the pathophysiology of Type 1 and Type 2 diabetes through the failure of islet beta cells to secrete sufficient quantities of insulin to regulate blood glucose (Carter et al, 2009).

1.13 - Associated Receptors

BPA is a xenoestrogen, and its estrogenic effect alters pancreatic β-cell function and induces glucose intolerance and insulin resistance in male mice (Alonso-Magdalena, 2010). There are many mechanisms proposed for the BPA action, it is well established that BPA can exert effects by binding at the nuclear steroid receptors, ERα and ERβ, induce estrogenic signals that modify estrogen-responsive gene expression.

The estrogen receptors ERα and ERβ have been both involved in energy balance, although up to now, evidence points to ERα as the main mediator. BPA has an affinity approximately 1:2000 of that of 17β-E2 for ER and therefore it will activate ERs at concentrations within the micromolar level, which are higher than those commonly found in the environment. The role of ERs in the physiology of the endocrine pancreas is still greatly undetermined, although recent studies have contributed with important functional evidence. The effects of estradiol (E2) in some physiological aspects of the islet of Langerhans have been known for a long time. There is an influence in the plasma insulin levels which become increased in pregnant rats in response to increased levels of sex steroids. The presence of E2 at concentrations similar to those in pregnancy enhances insulin secretion in perfused rat pancreas, acting partly through ERα, protects pancreatic β-cells from apoptosis induced by oxidative stress. In human,

it contributes to the reversion of the effect of menopause on glucose and insulin metabolism, resulting in increased pancreatic insulin secretion as well as improved insulin resistance. Several membrane steroid receptors have been described, it was demonstrated that both1 nM concentration of 17β-E2 and BPA produced calcium-dependent activation of CREB in mice pancreatic islets of Langerhans, evidencing that low-doses of BPA, can modulate gene transcription via an alternative pathway, functional "non-classical" membrane estrogen receptors (ncmER), independently from classical mechanism involving nuclear ERs (Quesada et al, 2002). Other receptors described include a membrane-bound form of ER similar to the nuclear ER (mER) and a transmembrane ER, called G protein-coupled receptor 30 (GPR30). BPA has been shown to bind to both mER and GPR30, and studies have determined that these membrane-bound receptors are capable of non- genomic steroid actions (Quesada et al, 2002; Ropero et al, 2008; Vanderberg et al, 2009).

The "non-classical membrane ER" (ncmER) has been described as capable of mediating actions at concentrations as low as 0.1nM of BPA in the endocrine pancreas. This action is also related to the activation of the transcription factor calcium-dependent cAMP-responsive element binding protein (CREB). BPA and estradiol were able to activate CREB with the same potency (Wetherill et al, 2007). 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 (Sui et al, 2012).

Xenoestrogens may also alter the ERs ability to recruit co-activators, a factor that may be important for differences in tissue- dependent responses (Vanderberg et al, 2009).

Objectives

Exposure to low levels of EDCs may be of concern. This is seemingly ubiquitous in today's environment, and EDCs are detectable in nearly all human blood samples. BPA is linked to a wide variety of endocrine dysfunction. BPA exposure increases the risk of mammary cancer, obesity, diabetes, and reproductive and neuroendocrine disorders. (Elobeid and Allison, 2008; Diamanti-Kandarakis et al, 2009) The endocrine disruption by BPA represents a real risk factor, and the studies regarding endocrine disruption conducted so far resulted in conflicting data.

Therefore the overall objective of the project is to dissect the mechanism of endocrine disruption of BPA, in the regulation of beta cells activity using a toxicogenomic approach.

We specifically aimed to: evaluate the effect of BPA on Pancreatic Islets of Langerhans, mouse embryonic fibroblast (MEF) and hepatocytes, given the specific role that each of this cellular types may have in the homeostasis of the organism; Control the specificity of the effect for each cellular type; Evaluate the influence of BPA in the cellular viability and production of ATP; and determine the pathway through which BPA is capable of inducing the dead on β-Islets.

Chapter II - Material and Methods

2.1 -Animal Model

During the study C57/B6 mice (Biogem s.c.a.r.l., Ariano Irpino (AV), Italia.) were used given the animal experimentation background, more specifically the existent data related to the pancreatic influence from BPA and also the acceptance from the committee on internal animal care.

2.2 - Isolation and purification of mouse β-Islets

The primary goal of isolating pancreatic islets, whether for in vivo transplantation or in vitro studies, is to obtain viable purified islets that respond in a manner consistent with their function in vivo. The key elements of a successful islet isolation procedure are: enzymatically digesting the tissues connecting the islets to the exocrine tissue, separating islets from non-islet tissue, and culturing isolated islets in an environment that maintains cell viability (Carter el al, 2009). Primary cultures of mouse β-Islets were prepared as previously, described by Gotoh et al.

Male C57/b6 mice were sacrificed by cervical dislocation, placed with the abdominal side facing and the skin was sterilized with ethanol 70%, afterwards an incision was made in order to expose the liver and intestines.

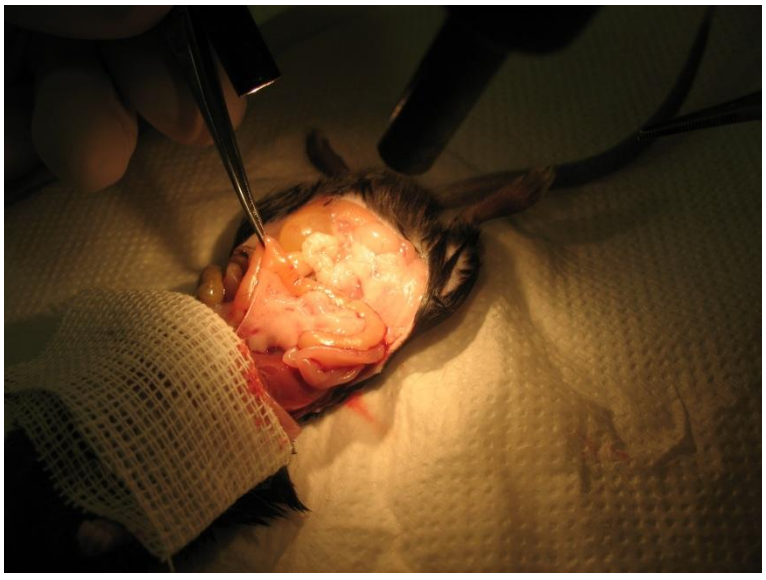

Figure 6 - Ex-Vivo Pancreatic perfusion : Exposing the common bile duct

Subsequently the ampulla was found and clamped in the duodenum wall to block the bile pathway to the duodenum.

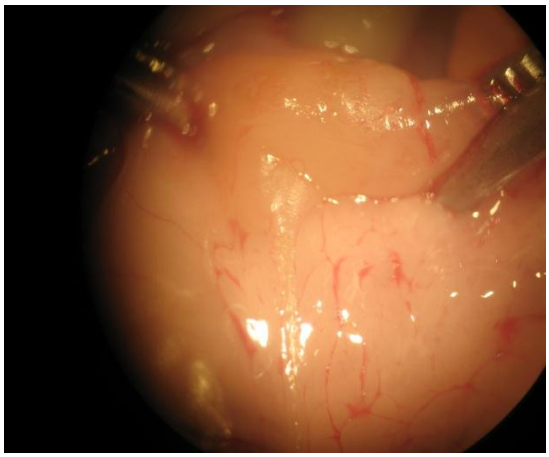

Figure 8 - Ex-Vivo Pancreatic perfusion : Searching for the Clamp adequated place

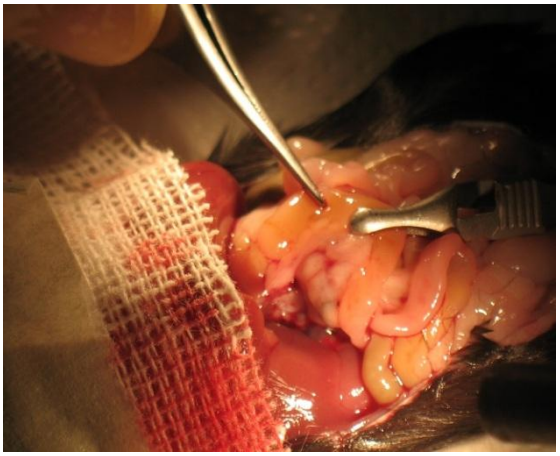

Figure 7 - Ex-Vivo Pancreatic perfusion : Clamp placed

The next and most delicate step, performed under the microscope, consisted in the insertion of a 5 mL syringe mounted with a 30G1/2-G needle into the common bile duct through the joint of the hepatic and cystic duct. As soon as approximately the middle of the common bile duct was reached, the pancreas was injected very slowly and carefully with 3mL of solution II.

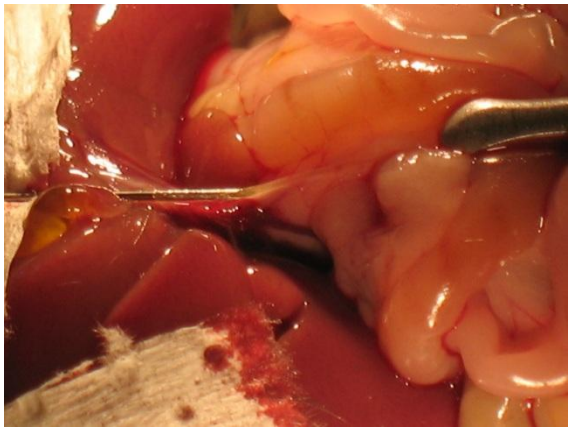

Figure 9 - Ex-Vivo Pancreatic perfusion :

The insertion of the needle

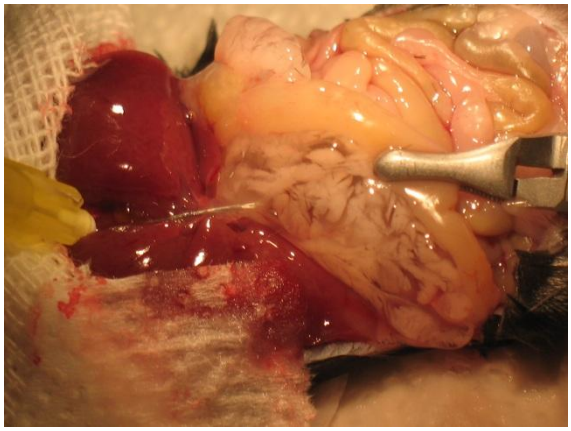

Figure 10 - Ex-Vivo Pancreatic perfusion :

The Perfusion

The pancreas was then removed and placed in a 50 mL tube. The excised pancreas was then digested at 37°C, without being cut into pieces or mechanically digested, for 15 min, being briefly shake by hand every 5 min.

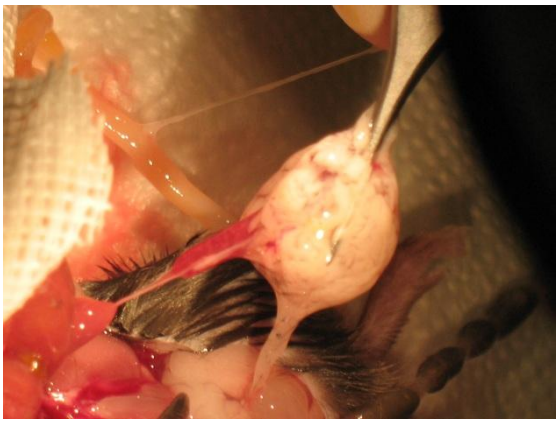

Figure 12 Ex-Vivo Pancreatic perfusion : Isolating Pancreas

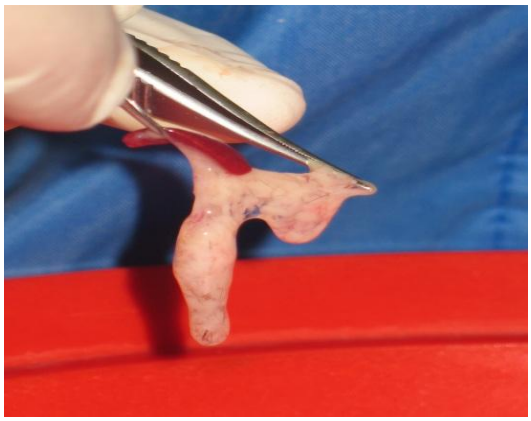

Figure 11- - Ex-Vivo Pancreatic perfusion : Pancreas After Isolation

Once the incubation period was over the tube was shaken by hand for 15 seconds to favour the disaggregation. Then, the tube was placed in ice with the purpose of terminating the digestion and 50 mL of RPMI complete medium were added. The solution was centrifuged for 5 min at 100g and 4ºC, and afterwards the pellet was resuspended in 25 mL of RPMI complete medium, filtrated to a new tube and the volume filled to 50 mL. The tube was then re-centrifuged for 5 min at 100g and 4º. The supernatant was discarded and the pellet was then resuspended in 10 mL of Histopaque 1077, at that moment 10 mL of Hank's were stratified very slowly in the falcon, using a syringe, this was followed by a centrifugation without acceleration and without brake at the speed of 900g and 20ºC. By the end of the centrifugation the ring of Islets was aspirated from interface between the Hank's and the Histopaque, without the use of glass pipettes to avoid losing Islets due to attachment.

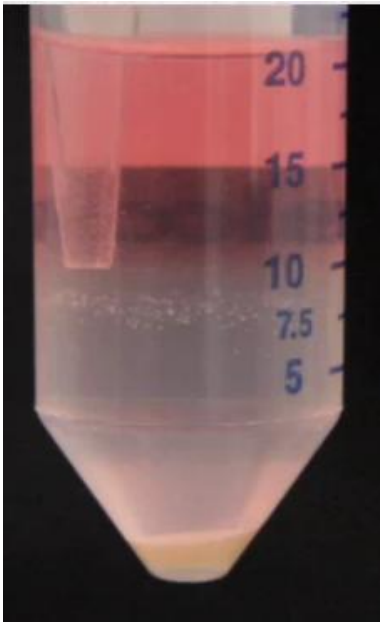

Figure 13 - Ex-Vivo Pancreatic perfusion : Ring of Islets after Gradient centrifugation

After collecting the Islets, the volume was filled to 25 mL, centrifuged at 100g and 4 ºC for 5 minutes, and then the wash was repeated. Subsequently the supernatant was eliminated and the pellet was re-suspended in an adequate amount for plating. The islet yield and quality was checked on an inverted microscope. Culturing too many islets in the same dish causes the islets to become necrotic and degrade so, for the treatments 250-300 islets per 6cm dish with 2-3mls of media were used, in order to avoid stressing the islets.

The advantages of perfusing the pancreas through the CBD are based on the allowance of the collagenase to access the islets using anatomical structures, and the reduction of mechanical damage to the islet through a stationary digestion.

2.3 - Islet Disaggregation

Dispersion of cells with Ca2+ -free medium was done immediately after the isolation of islets. All the islets were transferred into a tube containing 5 ml 0.25% trypsin-EDTA (Invitrogen, Paisley, UK). This was followed by a 5 minutes wait, and afterwards a centrifugation at 300g for 5 minutes. Four ml of supernatant were then removed and the islets dissociated by re-suspending the pellet very aggressively in the 1 ml that remained. In order to remove the supernatant (because Ca2+ -free medium and trypsin are both toxic after a while for cells), the tubes were toped up to 10 ml with RPMI 1640 and centrifuged at 300g for 5 minutes to spin down the cells, then the supernatant was removed and the cells were re-suspended in RPMI and plated.

2.4 - Culture

After performing islet isolation, proper culture conditions are imperative to ensuring that the islets are able to recover from the insult of collagenase digestion. An interesting notion is that there is no direct correlation between the β-cell apoptosis and glucose concentration. An increase in glucose concentration from 3 to 11 mM even promotes survival of β-cells. However, further elevation of glucose concentration increases β-cell apoptosis. Hence, RPMI 1640 medium with 11 mm glucose is optimal for culturing of pancreatic β-cells (Efanova, 1998; Carter el al, 2009). Overnight incubation of 16–20 hours provides islets time to recover from the harsh process of collagenase digestion. Recovery in a sterile incubator at 37°C with 5% CO_2 infusion and humidified air is necessary for islet function prior to performing viability or functional assessment assays. To maintain islets for long-term culture, the optimal islet density is four islets per square centimeter in order to prevent competition for nutrients (Carter el al, 2009). Visual inspection of the islets can provide some rudimentary information regarding health. When viewed with a scanning objective lens under a light microscope, islets appear spherical and golden-brown, approximately 50–250 μm in diameter. These features, particularly the darker colour of islets in comparison to the relatively transparent exocrine tissue, allow a rapid identification of islets. Supplementing visual inspection with additional techniques can provide quantification of islet viability and functionality (Carter el al, 2009).

2.5 - Hepatocyte In vivo Perfusion

Primary hepatocytes provide scientists with a valuable tool for evaluating metabolic, biochemical, and molecular functions in a physiologically relevant, readily controlled in vitro experimental system. However, as is the case for all primary cells, there are unique considerations that must be taken into account to minimize batch-to-batch variability and ensure the quality, reliability, and reproducibility of data (Zhang et al, 2010). Primary hepatocytes were obtained by collagenase perfusion of live mice. They were prepared for the perfusion through the exposition of the Portal-Vein, the initial procedure is similar to the described previously for the pancreatic perfusion, however there is a relevant difference, for the hepatic perfusion the mice were maintained alive and anesthetized. The liver was perfused through the Portal-Vein with

calcium and magnesium-free Hanks' buffered salt solution pre-warmed to 37 ºC for 4 min at the rate of 4 mL/min, afterwards perfused with Sigma Type IV Collagenase (0,32mg/ml) at the same rate for 5 min. The digested liver was then gently, but as fast possible, excised and hepatocytes were released with gentle shaking of the digested liver into 15 ml of chilled (4 °C) William's Complete Medium. The isolated material was then filtered through a 70-μm nylon filter, washed twice with the same medium by centrifugation at 50*g* for 3 and 5 min at 4 °C. After washing, the cells were resuspended in 10 hepatocyte isolation medium. In order to access the quality of the perfusion the cell viability was assessed via trypan blue staining and then the cells were plated. After allowing the cells to attach for approximately 2 hours at 37°C, they were washed once with PBS and the hepatocyte isolation medium was replaced with William's Supplemented Medium.

2.6 - Cell treatment

The isolated and cultured β-islets, Hepatocytes and the MEF cells from different animals were treated with an environmental significant dose of Bisphenol A. In order to perform this treatment, Bisphenol A was used in 10^{-9} M dose for different times, in order to comprehend time/effects relationships.

2.7 - Prime Design/ Test

The primers were chosen according to literature or designed and their putative quality tested with the use of Primer3Plus.

Primers	Forward	Reverse
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

The PCR products were analyzed by agarose gel electrophoresis at 1% with incorporated ethidium bromide. The electrophoretic run occurred at 100 V, in an electrophoretic tank containing TAE (1X) and the gel was visualized with an UV transiluminator, using the software QuantityOne 4.6.1.

2.8 - Cell Collecting

To extract RNA from the cells in culture, the culture medium was removed and the plate was washed with 1X PBS. Afterwards the cells were incubated 5 min at 37ºC with trypsin 0.25% to assist the detachment from the plate. The cells were recovered to a microtube and the plate was washed 1X PBS to remove any remaining cells. The cells were then pelleted in a refrigerated microcentrifuge at 4ºC for 3 minutes at 12000 rpm.

2.9 - RNA Extraction

To perform the total RNA isolation, Trizol Reagent (Invitrogen) protocol was followed as company's recommendations. 1 ml of Trizol Reagent was added to each tube, and the samples were allowed to completely dissociate by passing the mixture through a needle and by remaining for 5 min at 15 to 30ºC. Then, 0,2 mL of chloroform were added and the tubes were shaken by hand for 15 seconds, and incubated at 15 to 30ºC for 3 minutes. The following step was a centrifugation at 12,000g for 15 min at 4ºC. The aqueous phase was recovered to a new tube and 500 µl isopropanol were added, mixed and kept at room temperature for 10 minutes, at that moment the samples were centrifuged at 12,000g at 4°C for 10 minutes. The pellet was washed with 1 ml 70% ethanol, allowed to dry re-suspended in an adequate volume of H_2O-DEPC. The

RNA concentration and purity was determined using the NanoDrop spectrophotometer, and then stored at -80°C for future use.

2.10 - Phenol-Chloroform

The RNA samples volume was filled to 400 µl with H_2O-DEPC and a equivalent volume of phenol-chloroform acid was added. Then a 10 min a 4°C a 12000g centrifuge was made and the aqueous phase was recovered to a clean microtube. An 800 µl volume of isopropanol and 10 µg of glycogen were added. Subsequently the samples were incubated at -20ºC for at least 20 min and centrifuged at 16000 rpm for 30 minutes. Then the supernatant was removed and 500 µl of Ethanol 75% were added. A new centrifugation was made, at 7500 rpm for 5 minutes and the ethanol was removed, finally remained at RT for sensibly 7 min allowing it to dry and then re-suspended in and adequate volume of warm H_2O-DEPC. The RNA concentration and purity was re-determined using the NanoDrop spectrophotometer, as described previously.

2.11 - Nanodrop

The nucleic acids quantification was made with resorting to NanoDrop spectrophotometer ND-1000. The equipment was initialized and calibrated using 1 µl of H_2O milliQ. The blank was made with DEPC H_2O and then, the purity values (ratio between the absorbance at 260 nm and 280 nm and between the absorbance at 260 nm and 230 nm), and the concentration of nucleic acids was measured in ng/µl, depositing 1 µl of each sample in the instrument.

2.12 - Microarray

The RNA was then prepared, purified, controlled and used for gene expression profiling analyses using the Affimetrix platform. The microarray was prepared in the GECO Laboratory from the Biogem Institute, and performed using a Mouse Afymetrix Gene Chip 2.0.

The analysis of the data was performed using the GeneSpring Software. Ingenuity® Pathway Analysis (IPA) software was also accessed to obtain the information about gene prediction.

2.13 - Real Time PCR

The Real Time experiment was conducted in Applied Biosystem 7300 Real-Time PCR System, Power SYBR Green Master Mix was used (Applied Biosystems) and the analyses were done with SDS Enterprise Database software.

2.14 - Cellular Viability

In order to access the cell proliferation, cell viability, and cytotoxicity the MTT and the ATPLite assay were performed. Both assays were performed on cells treated with glucose at the concentration of 50 mM and BPA in the concentrations of 10-4, 10-6 and 10-9.

2.14.1 - MTT

First an adequate number of cells were plated on a 96-well plate with the respective medium. Measurements were made at 24h, 48h and 7 days. On the measuring day, 10 µl of MTT reagent were added to each well. Incubation at 37ºC, inside the cell incubator, with the reagent was made for 2 hours. Afterwards the medium was removed and 100 µl of acidified isopropanol (0,04%) were placed on each well. The plate was then incubated at room temperature with agitation for 15 minutes. To conclude de assay absorbance at 570 nm was read in EnVision™ 2103 Multilabel Reader (Perkin Elmer).

2.14.2 - ATPlite

First an adequate number of cells were plated on Black 96-well plate with the respective medium. Measurements were made at 48h. On the measuring day, 50 µL of mammalian cell lysis solution were added to the each well, which already contained 100 µL of medium. The microplate was then shaken for 5 minutes in an orbital shaker at 700 rpm. Afterwards, 50 µL of substrate solution were added to the wells and the microplate was shaken for 5 minutes in an orbital shaker at 700 rpm. To finish the plate was dark adapted for 10 minutes and the luminescence was measured in Orion II microplate luminometer (Berthhold) using the Simplicity 4.2 software.

2.15 - TUNEL

To access the apoptosis at the single cell level, the TUNEL assay was used. This technology is based on the detection of cleavages of genomic DNA that may result in double stranded low molecular weight DNA fragments, as well as single strand breaks in high molecular weight DNA. Those DNA breaks are identified through the labeling by terminal deoxynucleotidyl transferase, which catalysis polymerization of labeled nucleotides to the free 3'-OH DNA ends in a template-independent manner. The cells were plated in an 8-well slide chamber and treated for 48h with BPA at the concentration of 10-4, 10-6, 10-9, Glucose at 50 mM, and three controls (Negative Control; Positive Control; Experimental Control). After 48h the samples were fixed with freshly prepared Fixation Solution for 1h at 15 to 25ºC and once the incubation time was over the slides were rinsed with PBS. The cells were then fixated incubated in permeabilisation solution for 2 min on ice. Then the cells were incubated with the TUNEL reaction mixture. For the positive control the cells were DNase I for 10 min at 15 to 25ºC, and for the negative control the cells were incubated with label solution without terminal transferase, instead of the TUNEL reaction mixture. Afterwards the cells were incubated for 60 min at 37ºC in a humidified atmosphere in the dark. When the incubation time ended the slides were washed with PBS and counterstained with DAPI, mounted with and observed under a fluorescence microscope.

Chapter III - Results

3.1- Assessing the morphology of β-Islets 48 hours after the ex-vivo perfusion exposed to diferent doses of BPA

To access the influence of BPA on β-Islets vitality and morphology, after the isolation and plating the β-Islets were treated with different doses of BPA, 10^{-4}, 10^{-6} and 10^{-9} mM and also a sample exposed to 50 mM of glucose, as positive control. When exposed to BPA there were no remarkable differences between in the number of viable Islets after the 48 hours, and the morphology of the Islet doesn't appear to be influenced, Figure 6, In the same experimental settings, the reduction of the number of attached viable β-Islets was visible upon exposure to 50 mM glucose at (Figure 6 B).

At higher magnification (Figure 7) we could see that a high glucose concentration inducted alterations in the structure of the Islet (the control a clearly-defined regular border). Furthermore, the Islets exposed to BPA at 10-4 and 10-6 mM concentrations also had some changes in the morphology that became more irregular and with a granulated aspect . The exposition to BPA at 10-9 mM doesn't appear to induce visible morphological changes.

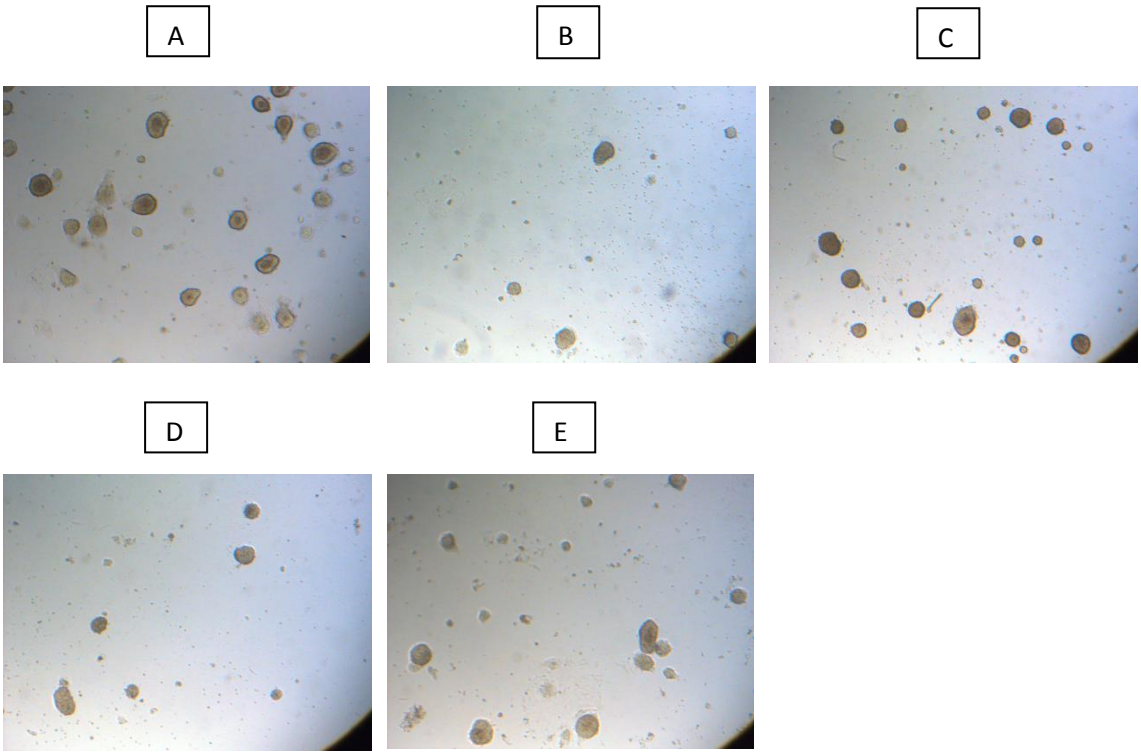

Figure 14- Morphology of β-Islets 48 hours after the ex-vivo perfusion. (A) Control; (B) Exposed to 50 mM Glucose ; (C) Exposed to 10^{-4} mM BPA; (D) Exposed to 10^{-6} mM BPA; (E) Exposed to 10^{-9} mM BPA

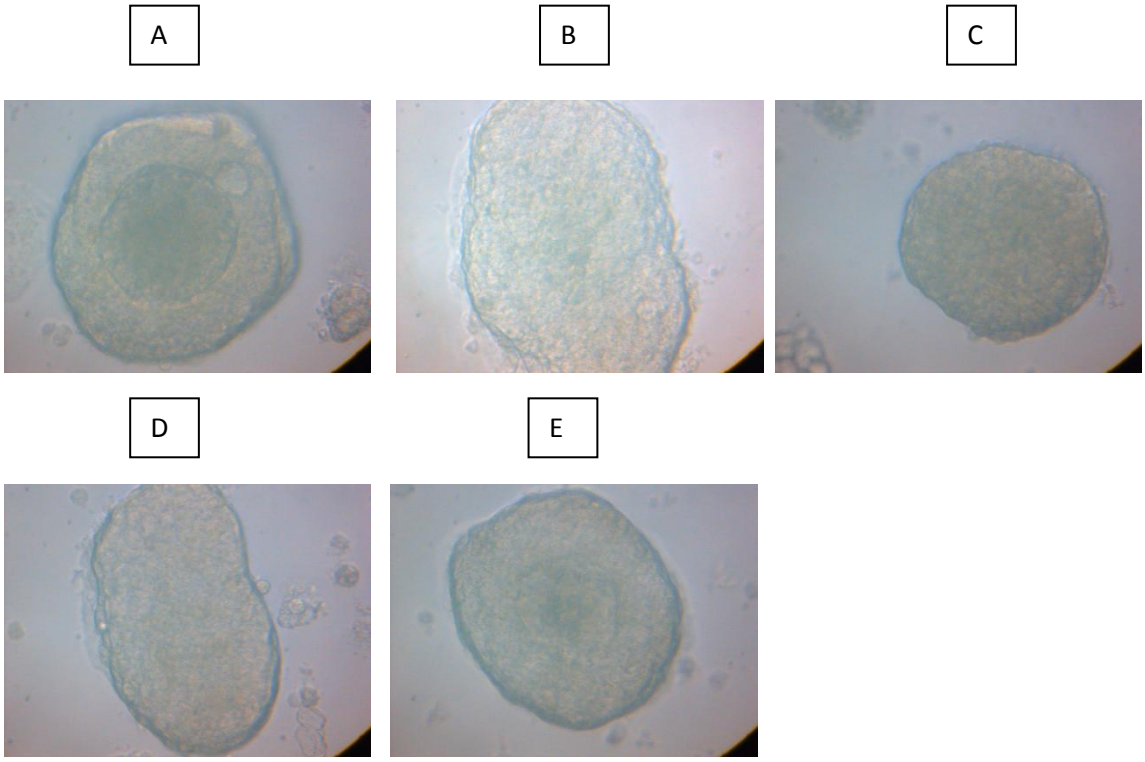

Figure 15- Morphology of β-Islets 48 hours after the ex-vivo perfusion. (A) Control; (B) Exposed to 50 mM Glucose ; (C) Exposed to 10-4 mM BPA; (D) Exposed to 10-6 mM BPA; (E) Exposed to 10-9 mM BPA

3.2 - Affimetrix Mouse Microarray

As it was reviewed, in literature BPA exposure is associated with multiple diseases within multiple biological systems, so the chosen step was the realization of a microarray for three different tissues types, mouse embryonic fibroblast (MEF), Hepatocytes and β-Islets. DNA microarrays can simultaneously measure the expression level of thousands of genes within a particular mRNA sample. For the realization of this microarray, for each specific primary tissue the cells were obtained from 9 different animals and pooled 3 to 3. The microarray data were analyzed using the GeneSpring Software. The Figure 8A is constituted by a Volcano Plot of the data obtained. The Volcano plot allows the visualization of the relationship between fold-change and statistical significance (Tarca et al, 2006). There is a representation of the differential expression between the control condition and the 48 hours 10^{-9} BPA treated for hepatocytes (A), MEF (B) and β-Islets (C). A change of at least 1,5 fold (up or down) threshold was considered meaningful for the experiment, and as its observable the first two show no statistically significant differences in the expression between control and

treated samples. However, it's visible in the Figure 8 C that for the treatment performed on β-Islets there is a small group of genes that have statistically significant expression differences between control and treated samples, that group of genes is assigned with the green color.

In the Figure 9 it's possible to see the heat maps made from the analysis of the microarray data from mouse embryonic fibroblast (MEF), Hepatocytes and β-Islets, the expression levels are represented by the pattern of colors, as is visible in the magnification done (Figure 9 D) in the we were able to see that for the treated β-Islets, there is a group genes represented by the green color have low expression levels when compared to the control samples.

Surprisingly, the shown data indicated that BPA doesn't have effects on cellular systems playing a role in diabetes other than the β-islet that, therefore, where used to further investigate the possible role of BPA on diabetes onset and progression.

3.2 A – Vulcano Plot

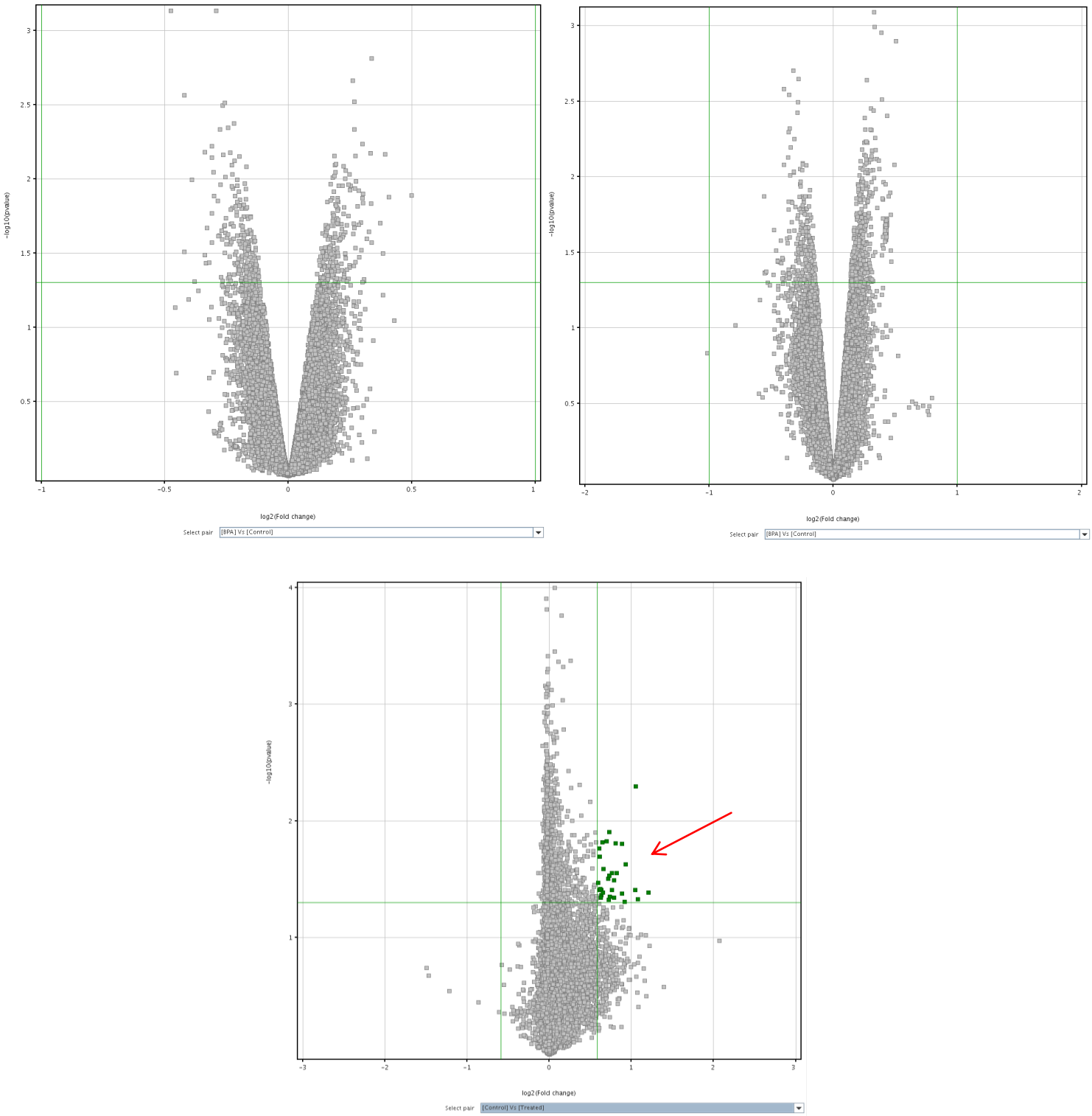

Figure 16 - Vulcano Plot from the Microarray performed on 10-9 M BPA treated for 48 hours Hepatocytes (A), Mef (B) and β-Islets (C)

3.2 B - Heatmap

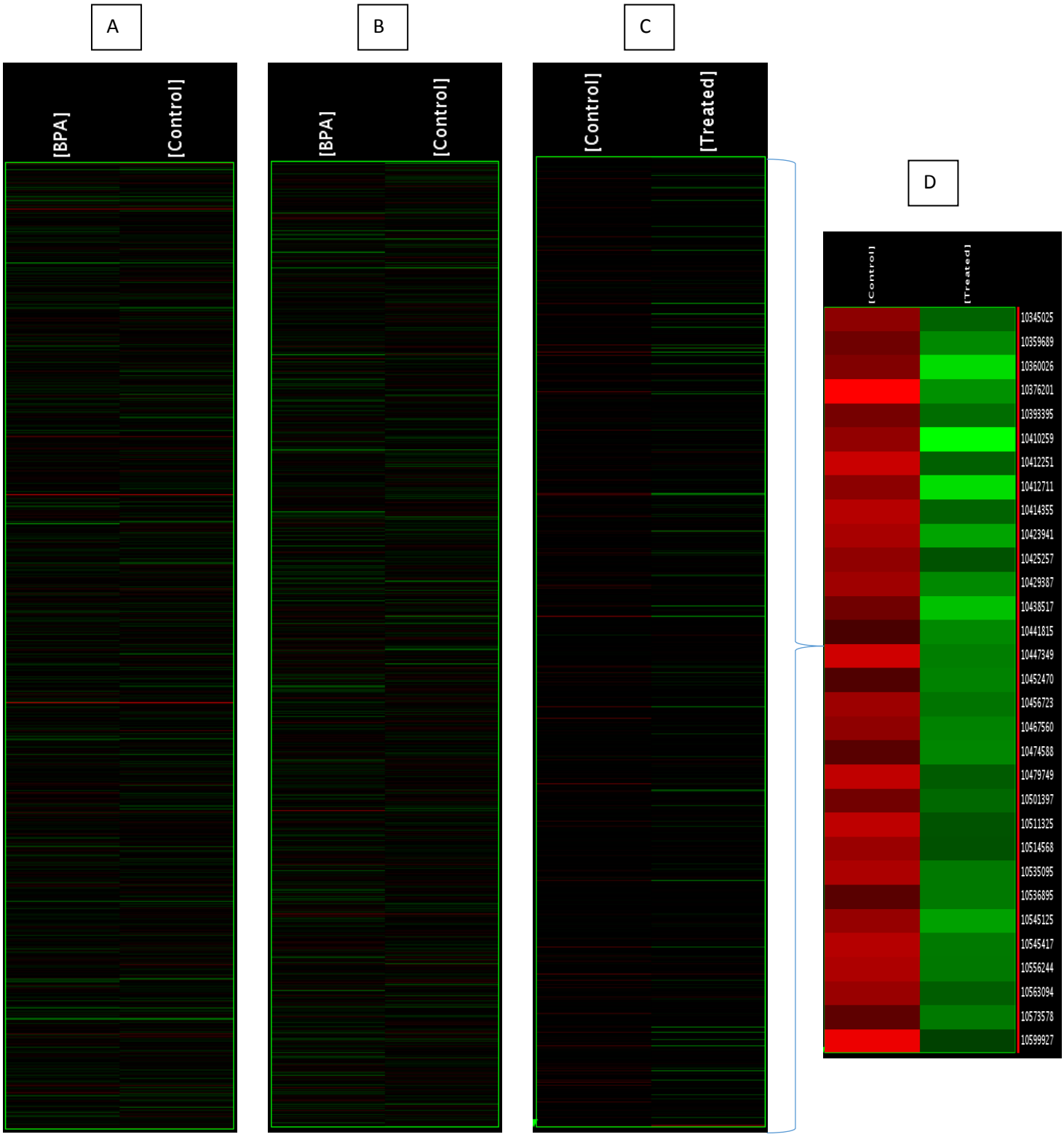

Figure 17 - HeatMap from the microarray performed on 48 hours treated Hepatocytes (A), Mef (B) and β-Islets (C) with 10^{-9} mM BPA. In the right (D) there is a representative magnification of the genes that were found to be deregulated on β-Islets.

3.3 - Deregulated Genes

The deregulated genes and their respective fold change is represented in the Table 1, as it was previously referred, a change of at least 1.5 fold (up or down), between treated and control, threshold was considered meaningful for the experiment. Given this threshold 22 genes were found to have at least 1.5 fold change. More precisely the fold change ranged from -1.5068305 and -2.301309 was found. Interestingly all of the 22 genes that had a statistically significant fold change were down-regulated comparing to the control condition.

Table 1 - Deregulated genes obtained from the 10^{-9} BPA treated, 48 hours, β-Islets affymetrix microarray

Symbol	Entrez Gene Name	Fold Change
AFF2	AF4/FMR2 family, member 2	-1.6556648
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-1.6410968
ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F	-1.5293903
CRIPT	cysteine-rich PDZ-binding protein	-1.8414806
GPX3	glutathione peroxidase 3 (plasma)	-2.0668383
MAPK1IP1L	mitogen-activated protein kinase 1 interacting protein 1-like	-1.6596464
MAT2A	methionine adenosyltransferase II, alpha	-1.7497746
POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	-1.5068305
RPL23	ribosomal protein L23	-1.843149
RPP38	ribonuclease P/MRP 38kDa subunit	-1.6528158
Rps20	ribosomal protein S20	-1.618454
TTC35	tetratricopeptide repeat domain 35	-1.9058185
ZFAND2A	zinc finger, AN1-type domain 2A	-1.7261531
ALG3	asparagine-linked glycosylation 3, alpha-1,3-mannosyltransferase homolog (S. cerevisiae)	-1.8824571
IARS	isoleucyl-tRNA synthetase	-1.5639364
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	-1.6941891
PTPLB	protein tyrosine phosphatase-like (proline instead of	-1.7596369

	catalytic arginine), member b	
RPS11	ribosomal protein S11	-1.5685618
SOD2	superoxide dismutase 2, mitochondrial	-1.5526665
TM2D1	TM2 domain containing 1	-1.5257024
UQCRB	ubiquinol-cytochrome c reductase binding protein	-2.301309
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa	-1.5426713

3.4 - Transcription Factors

In order to proceed with the analysis of importance of the genes that were found deregulated, the data obtained was processed and analyzed with the IPA Software. Firstly, the determination of the transcription regulators (TF) involved in the altered gene expression of the reported gene was done and they are listed in Table 2. The p-value and the respective targeted genes that were part of our initial deregulated genes list are also reported.

Table 2 - Transcription Factors associated with the deregulated genes (referred in the right column)

Transcription Regulator	p-value of overlap	Target molecules in dataset
HTT	4.87E-03	GPX3,NDUFS4,SOD2,UQCRB,VAPA
NR3C2 (includes EG:110784)	7.21E-03	ATP1B1,GPX3
KLF11	9.09E-03	SOD2
FOXO3	1.82E-02	IARS,SOD2
MXI1	2.53E-02	IARS
TFAP2B	2.53E-02	SOD2
SP2	2.71E-02	MAT2A
MYC	3.15E-02	MAT2A,RPL23,Rps20,SOD2
MED30	3.24E-02	SOD2
HOXA13	3.42E-02	VAPA
SP4	3.42E-02	MAT2A
Ikb	3.94E-02	MAT2A
Stat3-Stat3	3.94E-02	SOD2
NPM1	4.12E-02	SOD2
DDIT3	4.82E-02	SOD2

3.5 - Canonical Pathways

Additionally, to identify if the deregulated genes were involved in any fundamentally and biologically specific disorders, we performed IPA Canonical

Pathway analysis using the list of differentially expressed genes which resulted in the determination of two statically significant Canonical pathways, mitochondrial dysfunction and oxidative phosphorylation as it's visible in the Figure 12.

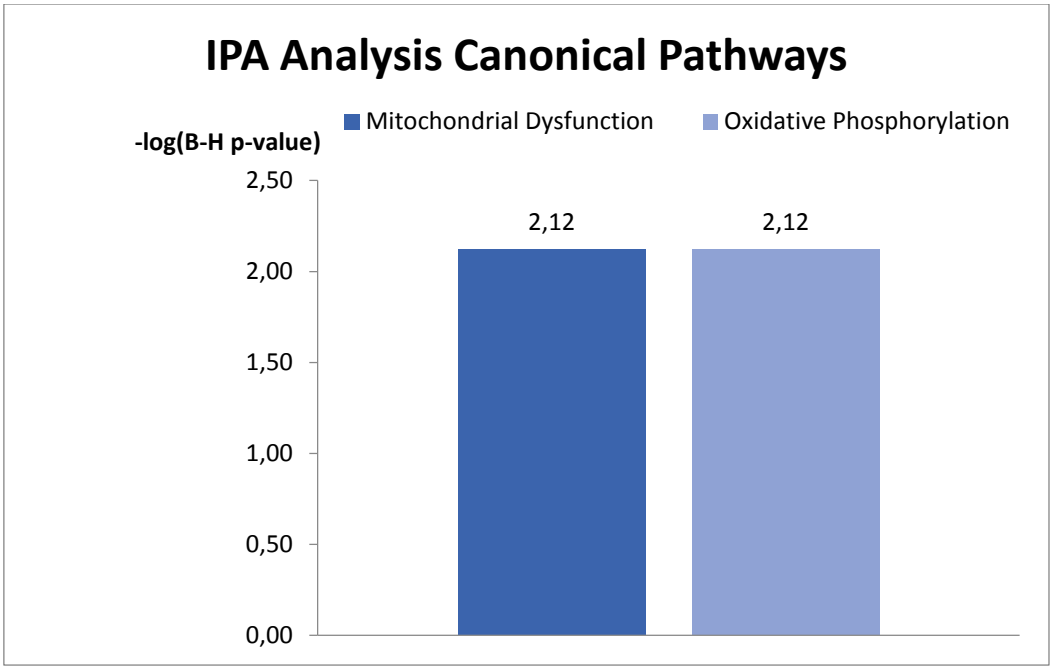

Figure 18 - IPA Analysis Canonical Pathways Prediction

3.6– Networks

In order to gain further insight into the mechanisms responsible for this gene expression alteration, ingenuity pathway analysis (IPA) was performed, which determined whether the predicted targets formed a network of interactions. The analyses resulted in a list of 4 network eligible genes. To construct the network, we included additional nodes which were not predicted as targets by the microarray. In Table 3 the list of genes included in the 4 different networks is available, where the array predicted genes are assigned with a *, the IPA also predicts a score for the predicted networks, as such in the Figure 19 and 20 there are represented the most significant networks identified by Ingenuity pathway analysis (IPA), with the green molecules representing downregulated genes. Ellipse, square, triangle, trapezoid, lozenge and circle represent transcription regulator, cytokine, kinase, transporter, enzyme and other molecules,

respectively. Arrows connecting molecules indicate one molecule acts on another and lines indicate one molecule binds to another.

Table 3 - Putative Molecule Networks List derived from IPA Analysis and the respective calculated score and Top Functions

Network	Molecules in Network	Score	Focus	Top Functions
1	ACTR3, AFF2*, ATP1B1*, ATP6V1F*, BAZ1A,BCCIP, CRIP2, CRIPT*, EIF4E, EPCAM, FAM158A, FXYD1, GPX3*, GRB2, HNF4A, KRAS,MAPK1IP1L*, MAT2A, MYC, PAF1, POLR2F*, POLR2K,POLR3D, POP7, RPL23*, RPP30, RPP38*, Rps20*, SEPHS1, SP2, tretinoin, TTC35*, UBE21, ZFAND2A*	33	13	Cell Cycle, reproductive System Development and Functions, Cancer
2	ALG3*, APP, CYTB, DARS, DUSP10, IARS*, IL4, INPP1, KL,MAP3K7, mir-23, ND2, ND3, ND4,ND5, ND6,NDUFS4*, NDUFS6, nitrate, NOXO1, PSME1, PTPLB*, RPS11*, RPS18, SLC19A2, SOD2*, TM2D1*, TMEM131, TNF, UQCRB*, UQCRC1, UQCRHL, UQCRQ, VAPA*, VEGFA	20	9	Free Radical Scavenging, cellular Development, Hematological System Development and Function
3	TM9SF3*, UNC93B1	3	1	Infectious Disease, Genetic Disorder, Inflammatory Disease
4	STAT4, TMEM167B*	3	1	Cell Cycle, Cell Death, Hematological System Development and Function

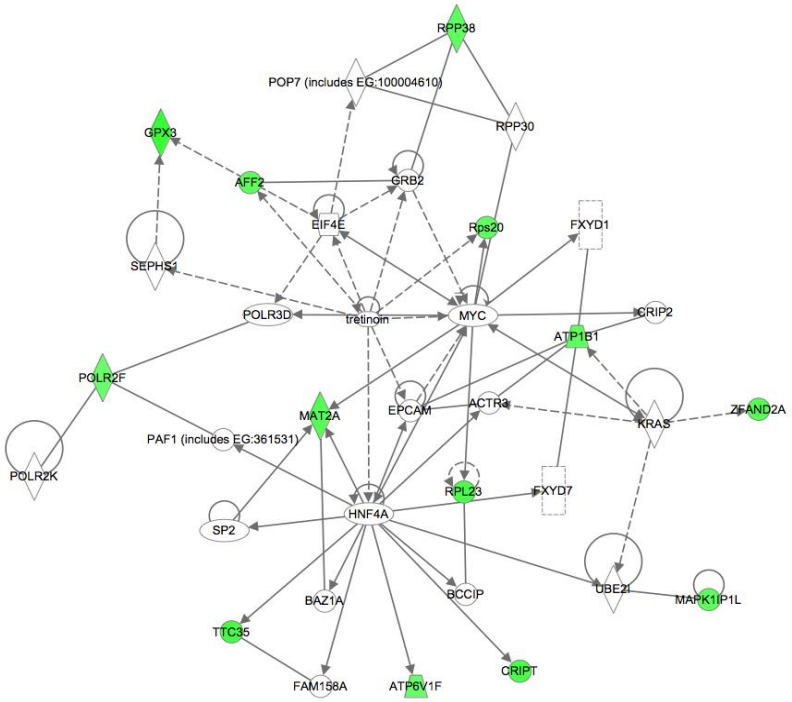

Figure 19 -Putative Molecule Network 1 derived from IPA Analysis - symbols in the figure represent the following: Solid line: Direct interaction. Dashed line. Indirect interaction.

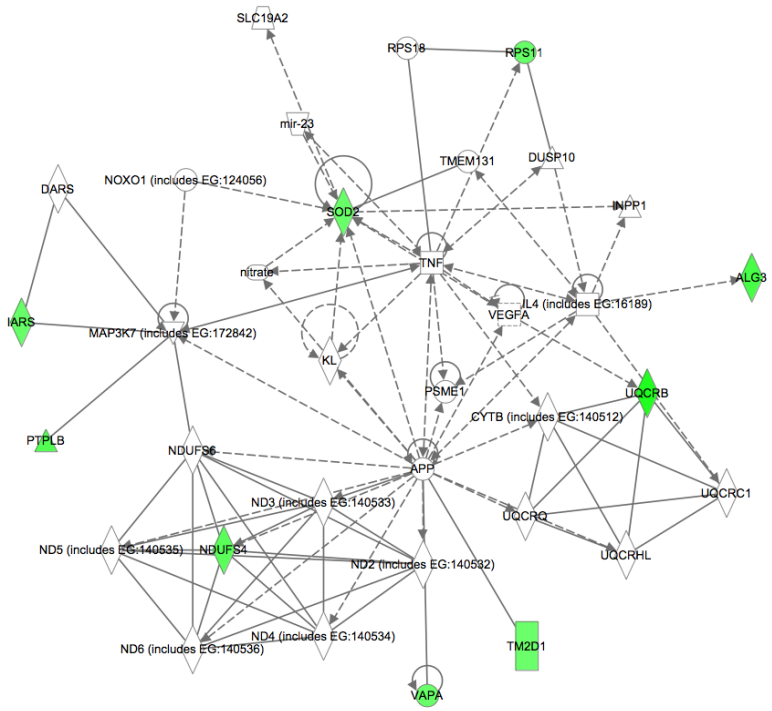

Figure 20 - Putative Molecule Network 2 derived from IPA Analysis symbols in the figure represent the following: Solid line: Direct interaction. Dashed line. Indirect interaction.

3.7 Real Time Gene Validation

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 alternate method is required. Real time PCR was the strategy chosen to perform this validation since it is rapid and quantitative method and applicable to samples with limited amount of RNA (Rajeevan, 2001). The Real-Time PCR validation was done normalizing the results with B2M and Tubulin using the Delta Delta Ct Method. In the Figure 13 are presented the real time results obtained using β-Islets, we analyzed the gene expression from 11 of the 22 genes that we found down regulated on the microarray. Four different time-points of treatment were analyzed, 12 hours, 24 hours, 48 hours and 7 days. As it's observable with exception made to the gene Ttc35 all of the genes appeared inhibited when treated with BPA at the concentration of 10^{-9} mM at 48 hours, the time point used

in the microarray. Furthermore there was a general tendency of down regulation for all of the genes in every time point.

Additionally to ensure that the deregulated gene expression had a specific character for β-Islets, we decided to validate the gene expression of those genes on Mef (Figure 14) and on Hepatocytes (Figure 15), for same four time points used in the validation performed for the β-Islets. As it's observable, in contrast with the treated β-Islets, with the with exception made to Gpx3 on the Mef cell line, that exhibited a strong variation, all of the genes chosen for the validation were very stable and their expression doesn't appear influenced by BPA at the concentration of 10^{-9} mM.

The Table 4 constitutes a comparative resume of the fold change found in all 3 cell types after 48 hours of treatment with BPA at 10-9 mM.

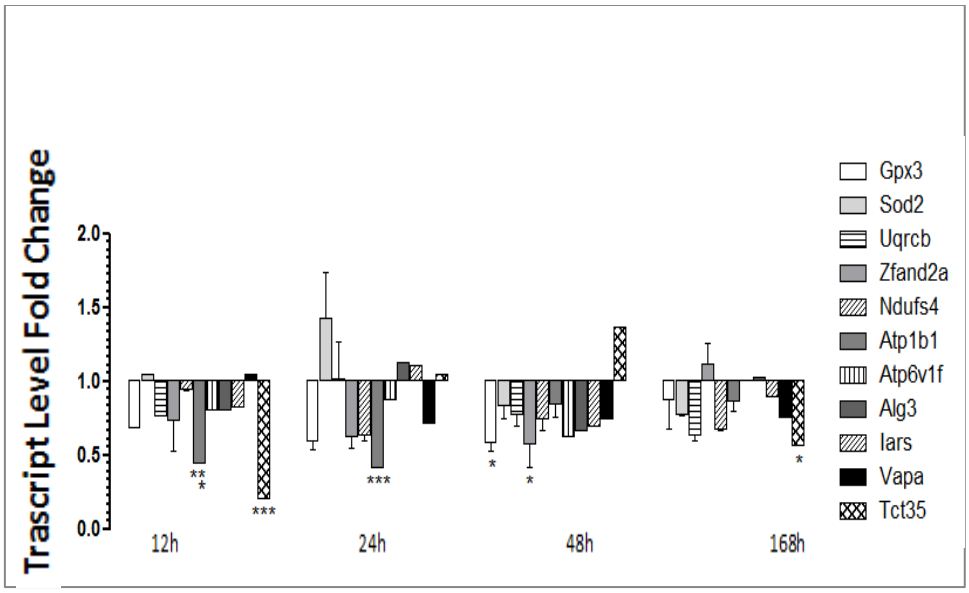

*Figure 21 - Real-Time Validation for β-Islet treated with BPA 10^{-9} for 12 hours, 24 hours, 48 hours and 7 days. Data represents mean+SEM of at least 3 independent experiments. * p<0.05; **p<0.01; ***p<0.001. (versus control) Condition (Two way ANOVA followed by Bouferroni Post hoc test)*

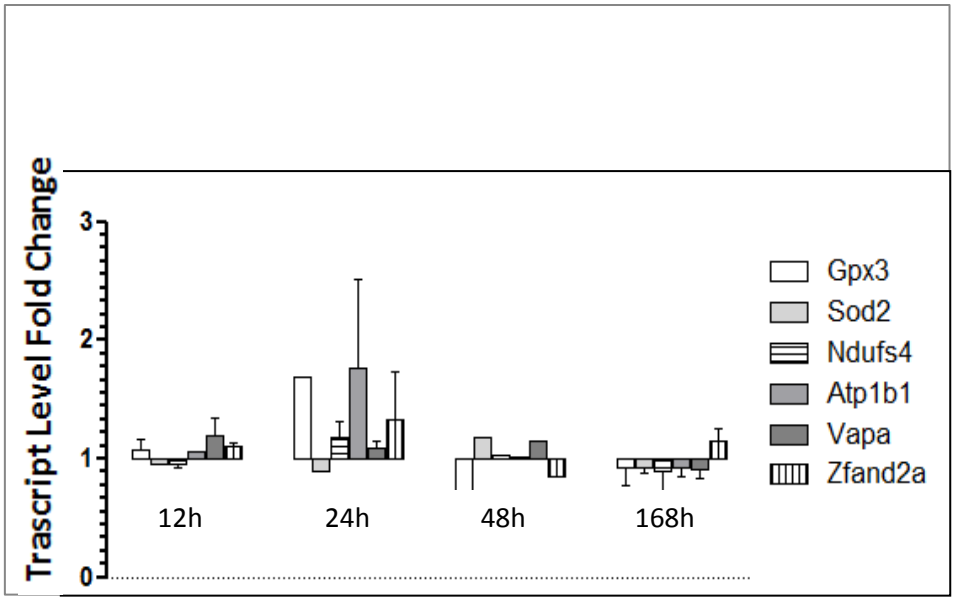

Figure 22 - Real-Time Validation for Mef treated with BPA 10^{-9} for 12 hours, 24 hours, 48 hours and 7 days. Data represents mean+SEM of at least 2 independent experiments. (versus control) Condition (Two way ANOVA followed by Bouferroni Post hoc test)

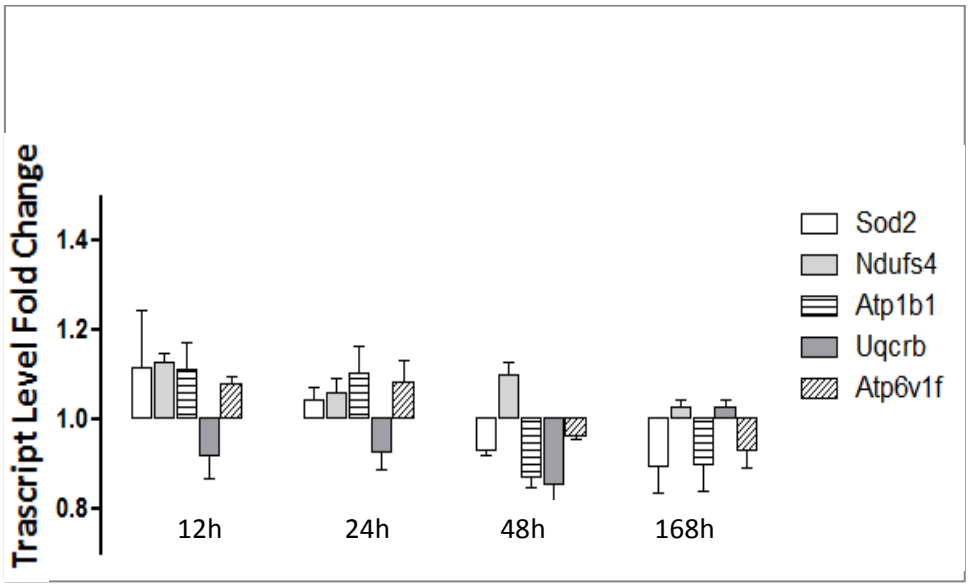

Figure 23 - Real-Time Validation for Hepatocytes treated with BPA 10^{-9} for 12 hours, 24 hours, 48 hours and 7 day. Data represents mean+SEM of at least 3 independent experiments. (versus control) Condition (Two way ANOVA followed by Bouferroni Post hoc test)

Table 4 - Gene Fold Change obtained from Real-Time PCR analysis performed on β-Islets, Hepatocytes and Mef treated with 10-9 mM BPA for 48 hours.

Cell Type	Islets	Mef	Hepatocytes
Gpx3	0,55	0,48	
Sod2	0,75	1,18	0,93
Uqrcb	0,8	-	0,86
Zfand2a	0,79	0,86	-
Ndufs4	0,67	1,03	0,87
Atp1b1	0,77	1,01	0,96
Atp6v1f	0,58	-	0,89
Alg3	0,61	-	-
Iars	0,59	-	-
Vapa	0,67	1,14	
Ttc35	1,7	-	-

3.8 Citotoxic Activity

The canonical pathways found through the Ingenuity® Pathway Analysis analysis associated the exposition to BPA with mitochondrial dysfunction and oxidative phosphorylation. Consequently we have verified if the BPA exposition resulted in an alteration of cell mortality as well as in ATP

3.8.1 – MTT

The MTT assay was used to investigate the cytotoxic effect of BPA at diverse concentrations. The treatment of performed on disperses β-Islets and lasted 48 hours. As it's visible in the Figure 16 BPA exerts cytotoxic activity on dispersed β-Islets in a dose dependent manner, with the concentration of 10-4 mM having the stronger effect (0,63), 10-6 mM an intermediate effect (0,67) and the 10-9 mM resulting in the lower effect (0,77). As a positive control we used a concentration of 50 mM of glucose in which we found that the viability decreased 50% (0,5).

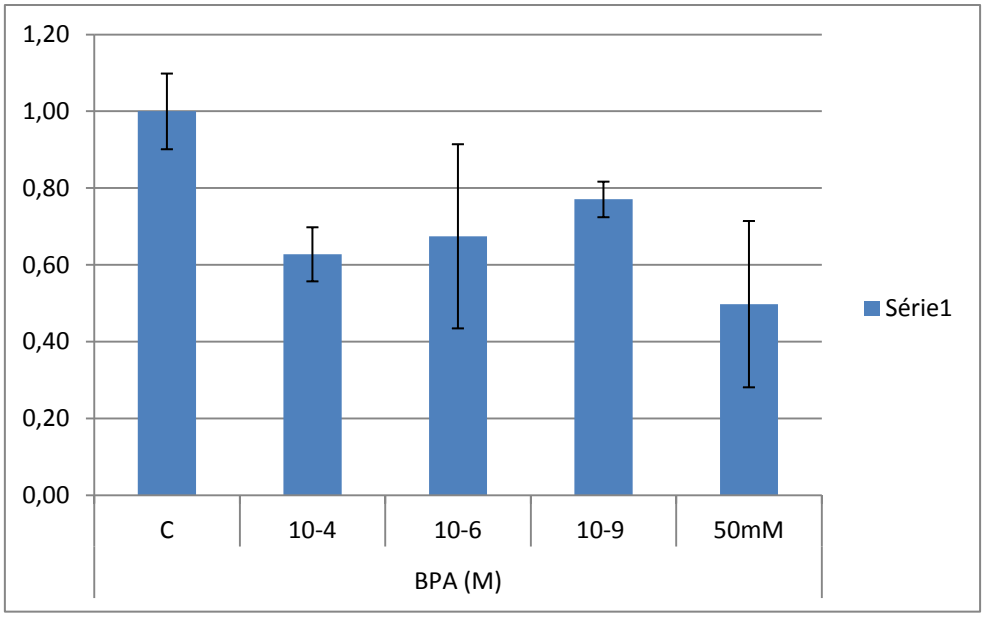

Figure 24 - MTT Assay - The data comes from a n=3 , with the 3 doses of BPA and the 50 mM of Glucose being confronted with the control

3.8.2 – Atplite

To have a direct measure of the ATP production, ATP intracellular level was determined by the ATPLite™ (PerkinElmer). The results showed a dose dependent reduction of ATP production that was 0,77 at 10-9M when compared with control. Results obtained with this technique were in agreement with the results obtained previously with the MTT, with BPA exerting cytotoxic activity in a dose dependent manner, as it's visible in the Figure 17.

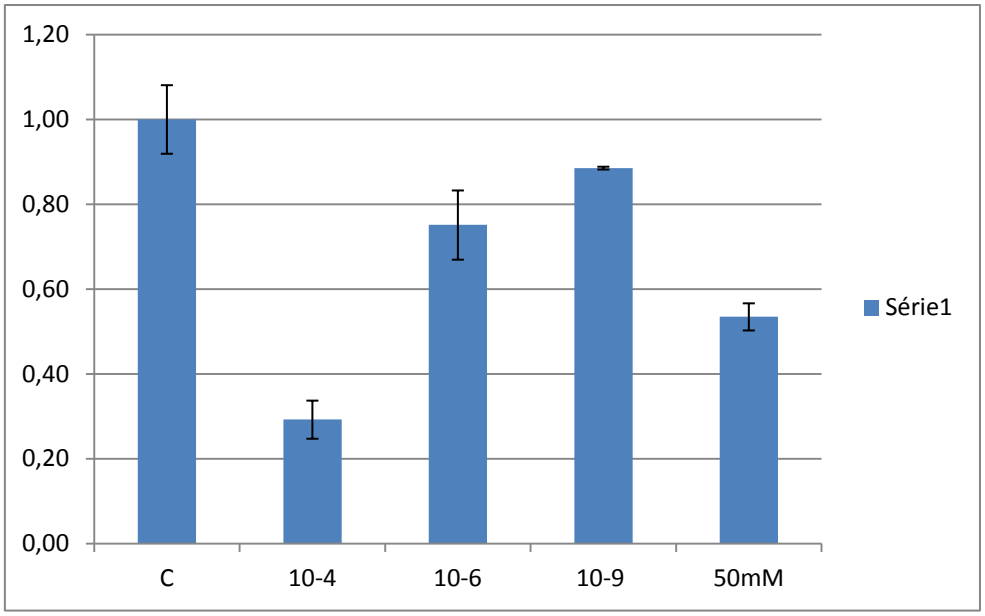

Figure 25 - ATPlite Assay - The data comes from a n=2 , with the 3 doses of BPA and the 50 mM of Glucose being confronted with the control

3.9 – TUNEL Assay

As previously referred results obtained from the Cellular metabolic activity assays indicated that the exposition to BPA is able to affect the viability of the Cells. In this sense we decided to assess the apoptosis at the single cell level and for that we performed TUNEL assay, by IF, on dispersed Islets. For the realization of the TUNEL assay apart from the 10-9 mM of BPA exposition, that was the concentration of main interest for us, BPA at 10-4, 10-6 and Glucose at 25mM were also tested. This choice was necessary due to the restricted surviving time of beta islets in culture, together with the absence of information about the necessary time to make it morphologically detectable. The cells were stained with DAPI, to evidence the nuclei (Figures 16 A to 21 A), with the TUNEL reagent (16 B to 21 B) and in Figures 16 C to 21 C the merge from the previous, two for each treatment, were shown. In the Figure 16 it's observable that in the untreated samples there is no strong staining from TUNEL, only a weak background which indicates the absence of apoptosis. For the Figures representing the exposition to BPA 10-4, 10-6 mM (Figures 17 and 18 respectively) a considerable signal of apoptosis is visible, with ¼ of the cells displaying a strong apoptotic signal. In the Figure 19 we could observe that apoptosis was also present, however to a less extent then the higher concentrations. The 25 mM Glucose treatment yielded a result that was

very similar to the exposition to BPA 10-4/10-6 as it's visible in the Figure 20. The figure 21 constituted the positive control in which the cells were treated with DNase 2000U before the staining, and where we can see 100% positive apoptotic cells.

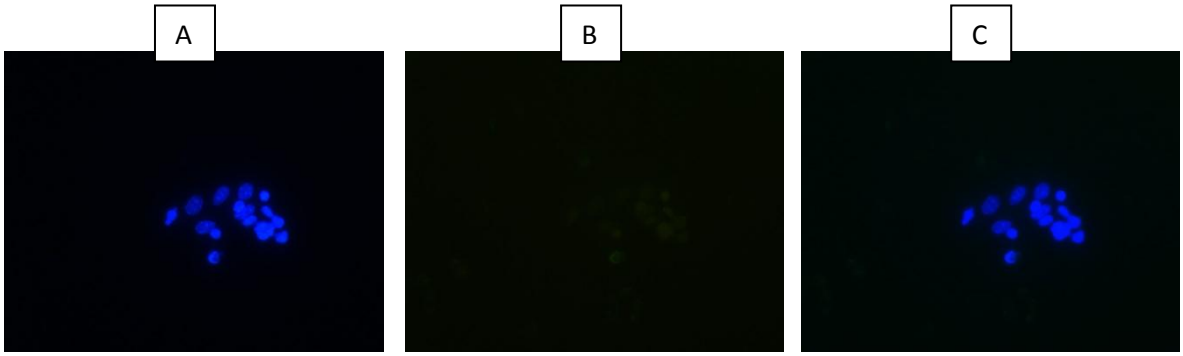

Figure 26 - Dispersed β-Islets non-treated (control) for 48 hours stained with DAPI (A); TUNEL Reagent (B) and Merged stains (C)

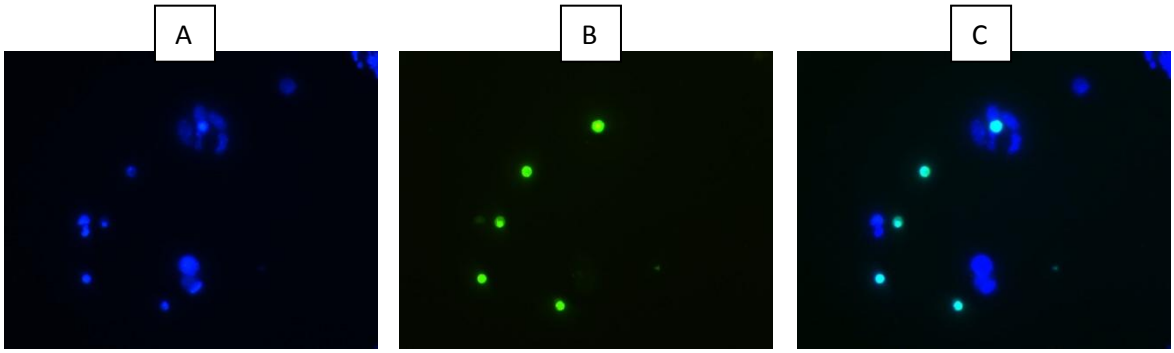

Figure 28 - Dispersed β-Islets treated with 10-4 BPA for 48 hours stained with DAPI (A); TUNEL Reagent (B) and Merged stains (C)

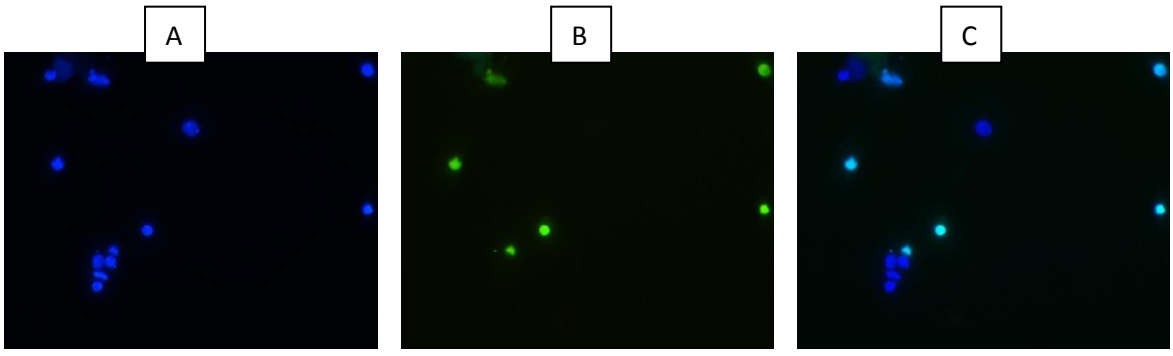

Figure 27 - Dispersed β-Islets treated with 10-6BPA for 48 hours stained with DAPI (A); TUNEL Reagent (B) and Merged stains (C)

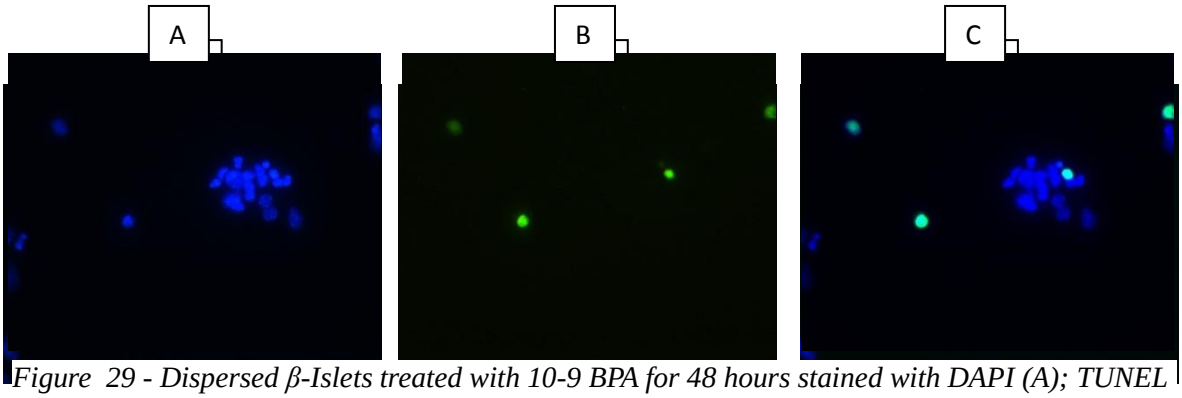

Figure 29 - Dispersed β-Islets treated with 10-9 BPA for 48 hours stained with DAPI (A); TUNEL
Figure 30 - Dispersed β-Islets treated with 25m of Glucose for 48 hours stained with DAPI (A); TUNEL Reagent (B) and Merged stains (C)

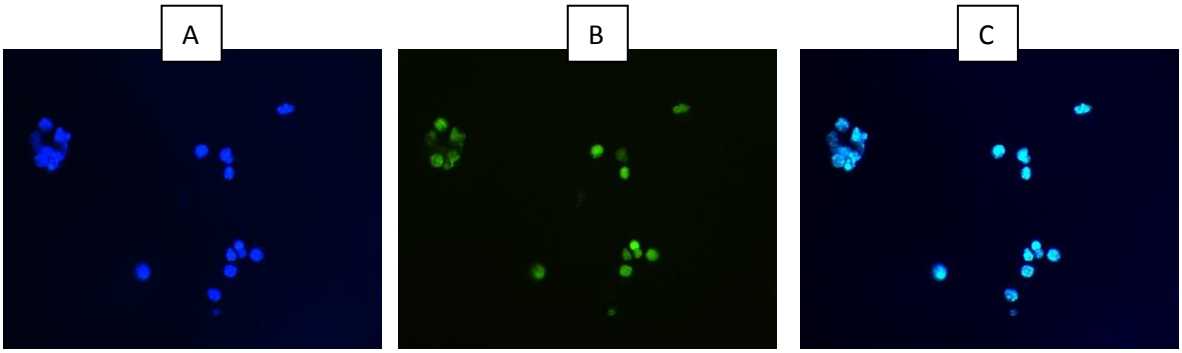

Figure 31 - Dispersed β-Islets treated with DNase 2000U (Positive Control) for 48 hours stained with DAPI (A); TUNEL Reagent (B) and Merged stains (C)

3.10 – Real Time Analysis – Bax

After the TUNEL assay performed on dispersed β-Islets, and the evidence that BPA at the concentration of 10-9 mM can induce apoptosis in this cells, the analysis of the expression of pro-apoptotic genes during the treatment with BPA was the chosen approach to understand to which extent the BPA influences the Islets of Langerhans. Apoptosis is a genetically controlled cell suicide pathway which plays an essential role in deleting excess, unwanted or damaged cells during development and tissue homeostasis and Bax is a pro-apoptotic protein that induces cell death through homodimerization and heterodimerization with bcl-2 and other members of the bcl-2 protein family. Consequently, we opted to determine the expression of Bax within different time points of treatment with BPA. We evaluated the expression of the gene Bax in β-Islets treated with BPA at 10-9 nM at 12 hours, 24 hours, 48 hours and 7 days is represented. The data is normalized for B2M and Tubulin given the Delta Delta Ct Method. In the Figure 22 it's observable that Bax is upregulated when compared to control in all of the time points that were controlled. Moreover at 48 hours was a 2,27 fold expression was reached, in agreement with the results obtained from the TUNEL assay.

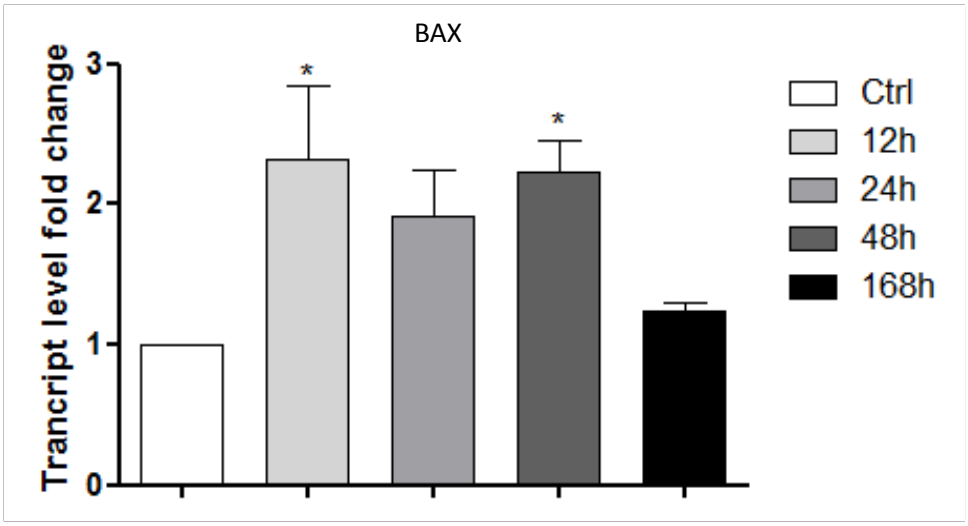

*Figure 32 -Real-Time analysis of Bax expression for β-Islets treated with BPA 10-9 for 12 hours, 24 hours, 48 hours and 7 days. Data represents mean+SEM of at least 3 independent experiments * p<0,05 Condition (one way ANOVA)*

3.11 – Pathway Inhibitors

To get some details about the apoptosis pathway that we found, and taking in account the deregulated genes and respective transcription factors we decided to perform a treatment with four possible compounds capable of inhibiting diverse pathways through which the BPA may act and exert the cytotoxic effects that were found. The chosen compounds were the antioxidant - N-Acetyl-l-cysteine (Nac), the IkappaB kinase (IKK) inhibitor – BMS 345541, the inhibitor of the MAPK - LY and the conventional Estrogen Receptor inhibitor - ICI. After 48 hours of treatment an analysis of the expression of BAX was made through Real-Time PCR and, as it's visible in the Figure 23, only BMS 345541 appeared to inhibit the expression of the gene BAX.

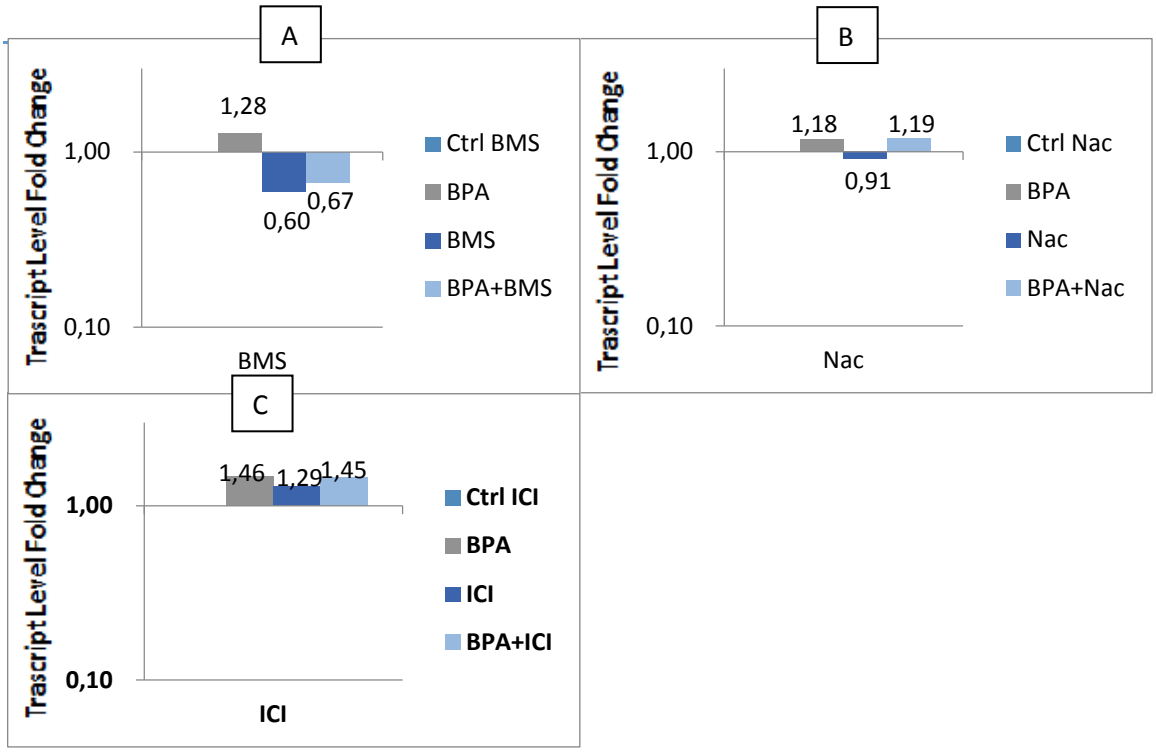

Figure 33 - Real-Time analysis of the expression of Bax in β-Islets treated with BPA 10^{-9} for 48 hours and simultaneously BMS (A); Nac (B); ICI (C)

Chapter IV - Discussion

Exposure to low levels of EDCs may be of concern. This is seemingly ubiquitous in today's environment, and EDCs are detectable in nearly all human blood samples, and even some of the shorter-lived potential endocrine disruptors are frequently detected in general population surveys as residues in blood or urine. The near omnipresence of the exposures combined with the nontrivial potential health effects justifies further research; the sensitivity of the human epigenome to low levels of EDCs will directly influence the health of current and future populations (Elobeid and Allison, 2008; Bernal and Jirtle, 2010). 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 (Ben-Jonathan et al, 2009; Lam et al, 2011).

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 (Gong et al, 2013). Studies in rodents have demonstrated that exposure to BPA elicits alterations in glucose homeostasis and correlation between BPA and the occurrence of diabetes has been found (Batista et al, 2012; Gong et al, 2013). Given its prevalence in the environment, presence in serum from humans worldwide, suppression of adiponectin and increased IL-6 and TNFα release, BPA may be, bona fide, the endocrine disruptor that adversely affects metabolic homeostasis (Ben-Jonathan, 2009).

Therefore, the endocrine disruption by BPA represents a real risk factor and, since the studies conducted so far resulted in conflicting data, future studies are required in order to determine the role of this compound in the development of multiple disorders namely, obesity, diabetes, and other related to metabolic disorders.

Thus, in this work we purposed to dissect the mechanisms of the endocrine disruptor Bisphenol A, in the regulation of the transcripts in 3 cellular settings related to diabetes onset and glucose production: Pancreatic Islets of Langerhans, mouse embryonic fibroblast (MEF) and hepatocytes. With Diabetes Mellitus (DM) being classified into four main groups, Type 1 DM results from lack of insulin production due to selective autoimmune destruction of pancreatic β-cells; Type 2 DM which is caused by insulin resistance in the main target tissues (liver, muscle, and fat), is also associated with alterations in hepatic metabolism, that leads to overproduction of glucose and lipids, which in turn is associated with the development of glucose intolerance and dyslipidaemias and indubitably inadequate compensatory insulin secretion response by

β-cells; other specific type of diabetes that include genetic defects of the β-cell; and gestational diabetes mellitus (GDM), characterized by glucose intolerance with onset or first recognition during pregnancy (Mellado-Gil et al, 2012; Sajan and Farese, 2012).

Methodologically, we chose a Systems Biology approach that presented some operative problem as the amount of protein required for a classical proteomic approach (2D-GEL and MS/MS analyses) is very high and requiring the sacrifice of a large number of mice. Therefore, we decide to explore firstly and definitely the role of BPA by transcriptomic approach, validate the data also functionally in order to properly use the animals. Furthermore, we obtained the Danio Rerio transgenic line (INS- GFP, control strain and reference by P. Argenton). The INS-GFP strain allows the handpicking of the β-Islets, so the Danio Rerio are going to be subjected to an in-vivo treatment with an environmental relevant dose of BPA, and subsequently the β-Islets are going to be picked and the analysis done for the mouse β-Islets (treated in-vitro) are going to be executed. The Danio Rerio transgenic line arrived one month ago and it will be ready for the experiments in the next months.

To begin with, as it's visible in Figure 14 and 15 the influence of BPA in the morphology of the β-Islets, after 48 hours of treatment, was inspected. We couldn't observe relevant diameter alterations, but on the other hand from the three chosen concentrations we were able to observe that both 10-4 and 10-6 mM BPA treatments appear to induce some changes in the morphology of the Islet, with the border becoming less defined and with the whole Islet gaining a granulated aspect. The treatment with 50 mM glucose induced stronger but similar morphological changes and the exposition to BPA at 10-9 mM doesn't appear to induce visible morphological changes.

To investigate possible mechanisms leading to defects in the function of the β-Islets we decided to use a toxicogenomical approach, that is based on the application of global mRNA, protein and metabolites analysis related-technologies to study the effects of hazards on organisms (Vulpe and North 2010), for that reason after the treatment with BPA at 10-9 mM for 48 hours of 9 β-Islets samples, 9 primary hepatocyte samples and nine MEF samples obtained from 9 different animals for each cellular type (with the respective control samples aswell) three microarray experiments were performed.

In contrast to DNA itself, the transcription of DNA to RNA is very labile and specific to tissues and to cells within tissues because gene expression is responsive to

the cellular and extra-cellular environment. RNA microarrays make it possible to take snapshots of profiles of gene expression throughout the genome for different groups and in different tissues (Plomin and Schalkwyk, 2007). In this work an evaluation of the effect of BPA on the gene expression profile, control *versus* treated at 10-9 mM was performed in the three different mentioned primary cell cultures. The analysis of the data obtained from the array experiments was performed with the GeneSpring software and with a change of at least 1.5 fold (up or down) threshold being considered meaningful for the experiment the result pointed out to a tissue specific effect. After the analysis we could observe that it wasn't detected any statistically significant variation of gene expression in the MEF and hepatocyte treatment. However, the microarray performed on the treated β-Islets retrieved a short list of genes that yielded statistically significant variation of gene expression for a group of 22 genes, represented on table 1.

Qin et al, 2013 reported that short-term exposure of BPA at environmentally relevant concentrations disturbs the upper hierarchies of hypothalamus-pituitary-gonadal axis at the transcription level, Dairkee et al, showed that BPA exposure induces aberrant expression of multiple checkpoints that regulate cell survival, proliferation and apoptosis and Saili et al, 2013 concluded that following a 0.1 μM BPA exposure a suppression of the expression of several genes involved in nervous system development and function occurred. However we couldn't find previous works evaluating gene expression influence by BPA at environmental relevant concentrations on β-Islets.

The IPA Software was used in the determination of the canonical pathways, allowing the identification of the involvement of the deregulated genes in any specific disorder. The analysis resulted in the determination of two statically significant Canonical pathways, mitochondrial dysfunction and oxidative phosphorylation as it's visible in the Figure 18. Moon et al, 2012 observed that a 10 nM and 100 nM dose of BPA induced mitochondrial dysfunction in the liver, on the other hand we found that 1nM BPA dose doesn't cause significant gene deregulation in primary hepatocyte cultures, yet as referred the predicted canonical pathway on β-Islets also pointed out to mitochondrial dysfunction. To obtain the maxim information possible about the data obtained from the IPA Software was also used to predict putative networks of interaction for the deregulated genes, the data presented in table 3 is referent to the 4 predicted networks, and the two networks that obtained the highest score are shown in the Figure 19 and Figure 20.

As previously said, within the list of genes that were influenced by BPA we found ATP6v1F to be down regulated. This gene encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. NDUFS4 or NADH ubiquinone oxidoreductase expression was also deregulated, it constitutes the first multisubunit enzyme complex of the mitochondrial respiratory chain and plays a vital role in cellular ATP production. Olsson et al, 2011 examined the association between if there was a differential expression from genes involved in the Oxidative Phosphorylation in human β-Islets of patients with Type II Diabetes, and concluded that there was a decreased expression of genes like ATP6V1H, ATP6V1E2, ATP6V1C1 NDUFA10, NDUFA5, NDUFS1, NDUFS5, among others. Despite not finding genes with decreased expression in common with our work, there are genes closely related and this fact goes in agreement with the association between Diabetes and the exposition to BPA that is documented in literature. We also found the superoxide dismutase 2 transcript. This gene codifies the enzyme SOD2 and its main function is to convert superoxide anion into hydrogen peroxide, which is subsequently converted to water by catalases leading to the reduction of reactive oxygen species levels. Reduced expression levels of SOD2 have been shown to result in increased mitochondria DNA damage l (Hurt et al, 2007). Although we couldn't find information about the influence of BPA on the SOD2 activity, Kabuto et al 2003, within a study in vivo observed that the SOD activity increased in liver and on brain, lung, kidney, and fat body didn't change significantly when male ICR mice were exposed to BPA with in a 5 days treatment through intraperitoneally administered doses of both 25 and 50 mg/kg/day. Still, neither the pancreatic tissue nor the evaluation of the mitochondrial form of SOD expression in the other tissues was made.

Mitochondrial dysfunction is associated with β-cell function, ultimately leads to apoptosis and contributes to the development of diabetes (Supale et al, 2012). It has been well documented that BPA can trigger apoptosis in various cells and tissues through mitochondrial signaling pathways (Lyn et al, 2013). Song et al, 2012 also observed that BPA significantly attenuated rat isolated Islets viability with a of 10 nM dose for 24 hours. Lyn et al, 2013, using a rat insulinoma cell (INS-1) line observed that after 24 and 48 hours of exposition BPA at 2 nM decreased cell viability and increased apoptosis in a dose-dependent manner. The utilization of a mouse insulinoma β-TC6 cell line was also a part of our approach at start, however the analysis of the influence of

BPA at 1 nM retrieved results that weren't similar to the ones obtained for the primary β-Islets, since there was neither a significant deregulation of the expression of the set of genes nor a decrease in the cell viability (data not shown). This fact pointed out the need of continuing with the use of primary cells.

Due to this direct relationship we decided to verify if BPA exposition elicited any alterations on cell viability. Two distinct techniques were used to evaluate the influence of BPA in the cellular viability, the MTT and the ATPlite. Despite not being numerically the same, with the 1 nM presenting a 0.77 viability for the MTT assay and 0,89 for the ATPlite assay, the results obtained from the both assays were concordant and reflected that BPA had a dose dependent cytotoxicity. Moreover, ATPlite assay is a direct measure of the ATP production that, as expected, was reduced. The difference among the two assays suggested it would be interesting to further determine the ATP production at shorter time exposure, which is now under evaluation.

In this sense we decided to assess the apoptosis at the single cell level, through a realization of the TUNEL assay using treated (BPA at 10-4, 10-6, 10-9 and Glucose at 25mM) and observation by Immunofluorescence. The various doses of treatment were used because there is not much information regarding the time of exposition to BPA needed to obtain a morphological detection of apoptosis. We used a 48 hours exposition to BPA and the results obtained confirmed the previously described cell viability assays, with BPA exhibiting a dose dependent role in the apoptosis of the dispersed β-Islets, as it's visible in the figures 27 to 29. The higher dose of BPA 100 μM had an effect that was similar to the 25 mM glucose exposition and showed the highest cytotoxicity, the 1 μM treatment dose reflected an intermediate phenotype and the 1 nM dose displayed a minor but present, apoptosis signal.

In healthy cells, BAX (inactive) is located in the cytosol or is loosely attached to membranes, but in response to stress signals, BAX enters into the mitochondria and induces cell death through homodimerization and heterodimerization with bcl-2 and other members of the bcl-2 protein family, resulting in downstream mitochondrial dysfunction. It is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome C (Ruiz-Vela et al, 2005). Therefore, we determined the expression of Bax within different time points of treatment with BPA at 10-9 nM at 12 hours, 24 hours, 48 hours and 7 days. As expected from the previous

results, the expression of Bax was increased in all the time points tested when compared to control. In agreement with the MTT/ATPlite and the TUNEL assay, at 48 hours Bax reached 2.37 fold expression and so, evidencing the pathway through which the apoptosis occurred became mandatory. To do so, a treatment with compounds capable of inhibiting apoptotic was done. The treatment with the inhibitors started 1 hour before the BPA treatment, and the chosen compounds were the Nac, BMS 345541 and ICI.

The antiestrogen ICI 182, 780 was used to observe if these effects were mediated through classic estrogenic pathway. N-acetyl-L-cysteine (NAC) was used due to his role as identifier and tester for ROS inducers, and the capacity of inhibiting ROS (Halasi et al, 2013). BMS 345541 down-regulates the IKK activity, which contributes to the degradation of substrate IκB proteins. The IκB is responsible for retain the transcription factor NF-κB in the cytoplasm, avoiding its entrance in the nucleus and subsequent transcription. Papaccio et al, 2005 observed that in β-Cells the activation of NF-κB, regulates MnSOD gene expression to protect β-islet from cytokine damage. However, the progression of β-cell deterioration leads to an induction of apoptosis through NF-κB. The analysis of the expression of BAX was made through Real-Time PCR and, as it's visible in the Figure 35 and only BMS 345541 inhibited the expression of the gene BAX, evidencing a possible pathway for the action of BPA on β-Islets. This result is preliminary and based on a single experiment, due to limited time; therefore its reproducibility must be evaluated. Nonetheless, it is an encouraging result.

Conclusion

This work is foremost a contribution to clarify the role of the endocrine disruption by BPA in the activity of β-Cells. BPA is a compound with ubiquitous presence of in the environment and above all with possibility of leaching that it presents it becomes a risk factor for various disorders in multiple axes.

The demonstration that a 1nM dose that is environmentally relevant (since higher concentrations have been reported in human urine and blood) is able to disrupt the β-cell function allows us to conclude that the preliminarily discerned pathway goes in concordance with the association between the development of Diabetes and the exposition BPA present in literature, since BPA showed a role in the promotion of apoptosis in mouse β-Islets.

Moreover from the three primary cultures analyzed we found this action as specific to β-Islets, therefore understanding how's possible to reduce this effect on β-Islets may be the following step.

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