Effects of bisphenol A on metabolism and evidences of a mode of action mediated through endocrine disruption

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TITLE[1]

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KEYWORDS

Bisphenol A, BPA, endocrine disruption, obesity, type 2 diabetes, insulin

FUNDING/Declaration of interest

This review was carried out in the framework of an assessment performed by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES). The authors declare no conflict of interest.

HIGHLIGHT

- BPA decreases insulin synthesis and secretion, after prenatal or adult exposure
- BPA decreases insulin action, after prenatal, perinatal and adult exposure
- There is good evidence of BPA toxicity on pancreatic β -cells
- Estrogen receptor mechanism (β/α or GPR30 types) may be involved
- Epigenetic mechanisms are also suggested

[1] BAT: brown adipose tissue; BMI: body mass index; bw: body weight; DES: diethylstilbestrol; DM: Diabete mellitus; ED: endocrine disruptor; eGFR: estimated glomerular filtration ; ERR: estrogen related receptor; ERRγ or ERRgamma: Estrogen-related receptor gamma; Erα or; ERalpha: estrogen receptor α ; ERβ or ERbeta: estrogen receptor beta ; FABP4: fatty acid binding protein 4; GD: gestation day; GLUT4: Glucose transporter type 4; GPER or GPR30: G protein-coupled estrogen receptor or G protein-coupled receptor 30, the membranous form of estrogen receptor; GR: glucocorticoid receptor (NR3C1); GSH:Glutathione ; GSIS: Glucose-Stimulated Insulin Secretion ; GTT: glucose tolerance test; HFD: High Fat Diet; IAPP: Islet Amyloid PolyPeptide ; ipGTT: intraperitoneal glucose tolerance tests; ipITT: intraperitoneal insulin tolerance tests; IR: insulin receptor; IRS-1: insulin receptor substrate 1; IS: insulin sensitivity; MDI: induction medium containing methylisobutylxanthine, dexamethasone, insulin; used in protocols to induce differentiation of 3T3-L1 cells into adipocyte-like cells; MoA: mode of action; MSCs: Mesenchymal Stromal Cells; NEFA: Non-Esterified Fatty Acids ; NHANES: US National Health and Nutrition Examination Survey; NHS: nurses' health study; NHSII: nurses' health study II; PI3K: phosphoinositide 3-kinase; PND: postnatal day; PPARγ: Peroxisome Proliferator Activated Receptor gamma ; PPRE: PPARγ; Response Element ; ROSI: Rosiglitazone; SOD: superoxide dismutase; TBT: Tributyltin ; TD2M: Type-2 diabetes mellitus ; TDI: tolerable daily intake; TG: triglyceride; TO: T0070907, used as a PPARγ antagonist; WAT: White Adipose Tissue; WB: Western blott

ABSTRACT (146 words)

Based on rodent studies after prenatal and/or perinatal or adult exposure, there is now evidence that BPA may increase metabolic disturbances eventually leading to type-2 diabetes development *via* an ED MoA. In particular, BPA has been shown to alter insulin synthesis and/or release by pancreatic β-cells, and insulin signaling within insulin-sensitive organs (i.e., liver, muscle, adipose tissues). This resulted in variations in the expression of specific hepatic or adipose tissue markers, which are indicative of a state of insulin resistance. These effects are considered by experts to be hallmarks of adverse hormonal effects, each leading to insulin resistance within the different insulin-sensitive tissues.

Although epidemiological studies are inconclusive, these effects are considered relevant for humans, because similarities exist in homeostatic regulation of insulin production and sensitivity between rodents and humans and because evidence was also shown through *in vitro* experimental data using human cells or tissues.

1. Introduction

Effects of BPA exposure on metabolism and obesity have recently been assessed in EU reports (ANSES, 2014; EFSA, 2015; ECHA, 2015). Metabolic disturbances reported with BPA include diabetogenic as well as obesogenic effects (ANSES, 2014). In particular, BPA has been shown to alter insulin synthesis and/or release by β-pancreatic cells, and insulin signalisation within insulin-sensitive organs (i.e., liver, muscle, adipose tissues). This resulted in variations in the expression of specific hepatic or adipose tissue markers, which are indicative of a state of insulin resistance and considered to be hallmarks of hormonal adverse effects. The analysis of ED-mediated effect of BPA on metabolism has therefore focused on alteration of insulin production and action.

The aim of this article was to review the data available at present that are in favour of a causal link between exposure to BPA and effects on insulin synthesis or sensitivity mediated by an endocrine disruptor (ED) MoA. The level of evidence will be weight to investigate how BPA can fulfill the requirements for a regulatory identification as an endocrine disrupting chemical (EDC). A brief overview on insulin resistance, its evolution towards type 2 diabetes and its hormonal control is first introduced. The present analysis was performed in close collaboration with the ANSES' Thematic Working group on Endocrine Disruptors. The studies were considered on the basis of their relevance, reliability and adequacy for the analysis and were qualitatively weighted based on collective expert judgement. Scientific studies considered irrelevant due to major deficiencies in their design and/or reporting are not included in the analysis. Human data were analysed together. Experimental data were confronted to each others with specific consideration of the periods of exposure in particular. The conclusion of the WoE was based on the combination of human and experimental in vivo and in vitro data.

Previous assessments relied mainly on publications reporting effects of BPA on mice by subcutaneous route (Alonso-Magdalena *et al.*, 2010) or oral route (Ryan *et al.*, 2010b; Miyawaki *et al.*, 2007) or on rats by oral route (Somm *et al.*, 2009). These studies suggest that rodents exposed in adulthood or during gestation undergo metabolic changes in various organs such as the liver, adipose tissue and pancreas (ANSES, 2014). In 2014, effects of BPA on lipogenesis (based on *in vivo* and *in vitro* data), after pre- or perinatal exposure or exposure in adulthood, have been considered by ANSES to be "recognized effects" whereas effects on glucose metabolism after pre- or perinatal exposure were considred as "controversial affects" (ANSES, 2014). Thus, it was concluded that there is some evidence that BPA disrupted glucose homeostasis that may be explained by an endocrine-mediated mode of action (MoA). The whole database was however not consistent with two other studies from Van Esterik *et al.* (2014) and Delclos *et al.* (2014) which did not provide evidence for strong effects indicative of BPA as an endocrine disruptor (ED). Two of the studied doses in Delclos *et al.* (2014) are the same as those in the study by Miyawaki *et al.* (2007) (260 and 2,600 µg/kg bw/day) used as a key study in the ANSES restriction dossier (2014). However, these two studies differ on many methodological points, which may explain why BPA had no effects on metabolism at these two doses in the Delclos study (2014) whereas Miyawaki *et al.* (2007) reported effects on body weight, adipose tissue weight, serum leptin levels, triglyceridemia, non-esterified fatty acids and glucose. These differences involve the animal model (rats in the Delclos study and mice in the study by Miyawaki *et al.*, 2007), the exposure route, the administration mode (gavage *versus* drinking water)and vehicle used*versus*, the exposure period (*post-coitum* day 6 to postnatal day 90 *versus post-coitum* day 6 to postnatal day 30), age of examination (adult *versus* juvenile stage), and diet (standard diet *versus* high-fat diet (30% kcal) in Delclos and Miyawaki study respectively).

In this article we did not detail the studies that were considered in these previous reports and only a summary of these studies is given in Table 1.

Table1: Summary of the studies examining the effects of bisphenol A on metabolism as quoted in the BPA restriction Dossier (ANSES, 2014)

Reference	Species/ strain	Routes of exposure	Dose Exposure period	Effects NOAEL/LOAEL
Alonso-Magdalena *et al.*, 2010	Mice	Sub-cutaneous	0 - 10 and 100 µg/kg bw/day GD9 to GD16	In F1 offspring, 6-month males had ↘ glucose tolerance, ↗ insulin resistance, and ↗ plasma levels of insulin, leptin, triglycerides and glycerol, altered calcium signalingin islets of Langherans ↘ BrdU incorporation into insulin-producing β cells, whereas their surface was unchanged. In mothers, ↗ insulin resistance induced by gestation and ↘ glucose tolerance. Dose-dependent ↗ in plasma levels of insulin, leptin, triglycerides and glycerol. ↘ insulin-stimulated Akt phosphorylation in gastrocnemius skeletal muscle and liver. 4 months post-partum: higher BW, higher concentrations of insulin, leptin, triglycerides and glycerol
Ryan *et al.*, 2010	CD-1 mice	Oral	0.25 µg/kg bw/day GD0 to PND21	In F1 offspring, ↗ BW in males and females at 3 weeks ↗ body length in males at 4 weeks, these biometric differences disappearing in adulthood. No significant effects on glucose tolerance were observed.
Somm *et al.*, 2009	Sprague Dawley rats	Oral	70 µg/kg bw/day GD6 - PND21	**At birth**: BPA treatment during gestation did not affect sex-ratio or litter size. Newborns (♀ and ♂): ↗ weight **PND21** ↗ BW in females Increased parametrial fat associated with adipocyte hypertrophy and overexpression of lipogenic genes and lipogenic enzymes In the liver, increased RNA levels of C/EBP-α, SREBP-1C, ACC and FAS k. Circulating lipids and glucose were normal. **4 to 14 weeks**: no difference in BW observed between BPA-treated males and control animals on standard chow diet. ↗ BW in BPA-exposed males fed a high-fat diet. ↗ BW in females for the 2 tested diets. In males fed a high-fat diet, normal glucose tolerance test results. Conclusion: Perinatal exposure to BPA. ↗ Adipogenesis at weaning in ♀. In adult ♂, ↗ BW observed if high-fat diet.
Miyawaki *et al.*, 2007	ICR mice fed a high-fat diet	Oral	0.26 and 2.72 mg/kg bw/d *via* drinking water GD10 until weaning	**PND31:** ↗ BW in BPA-exposed (low and high dose groups) females fed a high-fat diet. ↗ adipose tissue weight in BPA-exposed (low dose group) females fed a high-fat diet. ↗ BW and adipose tissue weight in BPA-exposed (high dose group) males fed a high-fat diet. ↗ leptin in BPA-exposed (low dose group) females

				fed a high-fat diet ↗ total cholesterol in BPA-exposed (low and high dose groups) females fed a high-fat diet. No change in glycemia in females ↗ non esterified fatty acids in BPA-exposed (low dose group) males fed a high-fat diet ↗ triglycerides in BPA-exposed (low and high dose groups) males fed a high-fat diet. ↘ glycemia in BPA-exposed (low dose group) males fed a high-fat diet

New experimental *in vivo* studies collected until May 2016 have explored effects of BPA on metabolism and body weight. Importantly, they tend to reinforce the observations previously reported by ANSES. In addition, several of them pointed that one way of BPA-induced metabolic disorders was through interfering in the balanced interplay between insulin secretion by the β pancreatic cells and insulin action in liver and the adipose tissue and that it occurred through endocrine disruption.

Energy homeostasis is highly hormonally regulated both centrally to regulate food behavior and peripherally to maintain glycaemia at physiological levels between the fed and fasting states to meet energy demands. Normoglycaemia is maintained by the balanced interplay between insulin secretion and insulin action. Insulin is produced and released by the ß-cells of the pancreatic islets also containing the α-cells producing glucagon which counteracts insulin action (i.e., it elevates blood glucose). The endocrine pancreas that represents 1% of the pancreas also includes δ cells secreting somatostatin (involved in the regulation of α- and β-cell activities), γ cells, secreting pancreatic polypeptide (involved in the regulation of both endocrine and exocrine pancreas secretions), and ε-cells producing ghrelin, a protein that stimulates hunger.

Insulin signaling transduction is a highly complex network system characterized by extensive points of regulation throughout the signal transducing pathway downstream the binding to insulin receptor with a cascade of protein phosphorylations and cross-talks within the different signaling cascades (Guo S, 2014,). Insulin resistance defines a metabolic state in which an insulin-sensitive organ does not respond properly to insulin. Manifestations of the insulin resistance syndrome include increased body weight, dyslipidemia, hyperglycaemia and glucose intolerance, hyperinsulinemia, low-grade inflammation but also hypertension, ultimately leading to a spectrum of metabolic diseases including type 2 diabetes, nonalcoholic fatty liver disease, cancer and cardiovascular diseases. Importantly, several mechanisms can contribute to the etiology of insulin resistance depending on primary organ(s) targeted. For example, insulin resistance in muscle, liver, fat and pancreas are central in the metabolic alterations leading to type 2 diabetes (Figure 1).

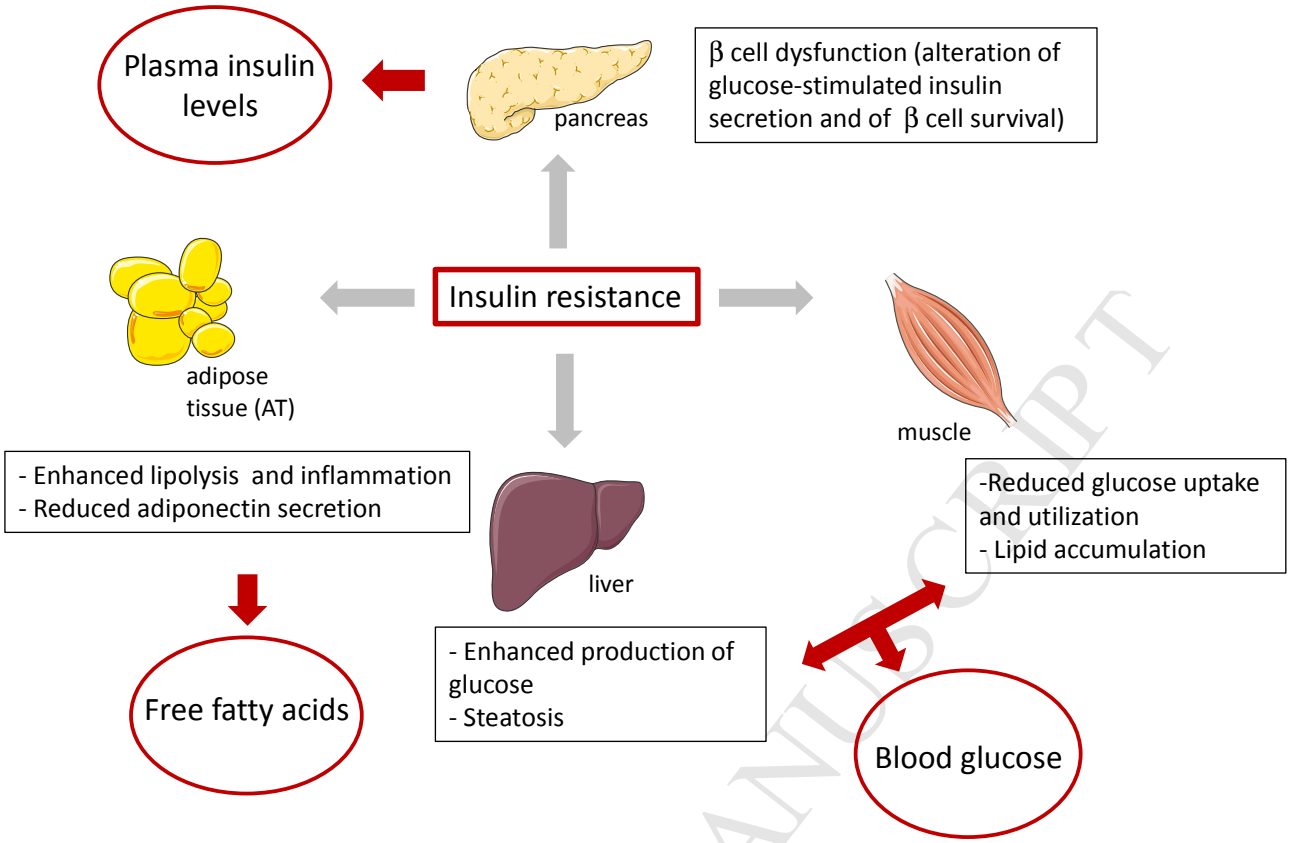

Figure 1: Metabolic disturbances characterizing the insulin resistance state with its specific outcomes

Reduction of insulin secretion and/or insulin action (insulin resistance) leads to an increase of lipolysis (and consequently an increase in plasma concentration of free fatty acids), an increase in hepatic production of glucose and a decrease in muscle glucose uptake (and consequently an increase in blood glucose concentration). Dyslipidemia resulting from enhanced free fatty acids leads to lipotoxicity with enhanced accumulation of lipids in the liver (steatosis) and the muscle. Within the adipose tissue and in addition to sustained lipolysis, insulin resistance is marked by a low-grade and chronic inflammation with high levels of secretion of pro-inflammatory cytokines such as TNF α and interleukin 6 reducing plasma levels of adiponectin. Adaptive mechanisms such as enhanced insulin secretion (hyperinsulinemia) prevent hyperglycaemia. However chronic hyperactivity of ß-cells exhausts adaptive capacities of the pancreas leading to a decrease of insulin production in response to glucose and enhancement of insulin resistance with further augmentation in free fatty acids in plasma through autoamplification processes, eventually leading to type 2 diabetes. Interestingly, through binding to receptors expressed in the various metabolic tissues, adiponectin whose secretion is inversely correlated with enhanced fat mass (obesity) improves pancreatic β cell function, enhances peripheral insulin sensitivity, suppresses hepatic glucose production and reduces inflammation, all events which are hallmarks of insulin resistance, making of this hormone a marker of insulin sensitivity (Ruan and Dong 2016). The coordination of the insulin response largely depends on cross-talks between the liver, the adipose tissues and the muscle through at least the secretion of hormones, the hepatokines in the liver, adipokines in the adipose tissues and myokines in the muscle, which contribute to modulate insulin sensitivity, while incretins, which are enteric hormones, stimulate insulin secretion (Drucker DJ., 2006). Glucocorticoids, thyroid hormones and sexual steroids also contribute at regulating energy homeostasis at physiological levels of hormones while metabolic disorders occur at non physiological levels (e.g., hypothyroidism, Cushing syndrome with excess glucocorticoids, androgen excess in women (polycystic ovary syndrome), androgen deprivation in men (hypogonadism), estrogen excess (pill, gestation) or deprivation

(menopause)) (Nadal *et al*., 2017).. Hence, sex steroids operate in a sex- and tissue-specific manner where both deprivation and excess for both estrogens and androgens are deleterious for males while only androgen excess has been shown to be deleterious in females (Mauvais-Jarvis *et al.*, 2013; Navarro *et al.*, 2015; Mauvais-Jarvis, 2015). Specifically, involvement of estrogens has been largely described in the regulation of metabolism through the modulation of food intake, body weight, glucose/insulin balance, body fat distribution, lipogenesis and lipolysis, and energy consumption as evidenced with the phenotype of obesity and insulin resistance in both male and female mice deficient for the estrogen receptor alpha (ERα). Further, it is well documented that estrogens at physiological levels confer metabolic protection to women (Mauvais-Jarvis 2015). In men, androgens also contribute at reducing hepatic steatosis (Lin *et al.*, 2008; Zhang *et al.* 2013), preventing visceral fat accumulation, and at improving β cell function (Xu *et al.*, 2017).

2. Experimental data

This review is based on an evaluation of recent *in vivo* and *in vitro* experimental studies investigating specific aspects related to alterations in insulin secretion by -pancreatic β cells, or alterations of insulin action (signaling mechanisms) upon the insulin-sensitive organs.

2.1. Effects of BPA on glycaemia and insulin synthesis

2.1.1. Adverse effect of BPA on the endocrine function of the pancreas

This section focuses on the effect of BPA on the pancreatic insulin synthesis and secretion, focusing on new publications from 2013 to November 2016. These papers are presented successively and summarized in Table . Some papers present the effect of BPA both on the pancreas and on the insulin-sensitive tissues. In order to avoid redundancy, they will also be presented in the present section.

It has been well described that β-cell mass is critical for proper functioning of the endocrine pancreas as well as insulin biosynthesis and secretion. Importantly, all three estrogen receptors (Erα, ERβ and the G protein coupled ER (GPER)) have been identified in rodent and human β cells, where they play essential roles in islet survival and function (Nadal *et al*., 2011; Tiano & Mauvais-Jarvis, 2012). Therefore, because of the estrogeno-mimetic action of BPA (Dodds EC and Lawson W, 1936) its exposure is suspected likely to interfere with endocrine pancreas development and function. Further, it is also considered that early life may be a critical window of susceptibility to BPA exposure, knowing that fetal pancreatic development occurs both in fetal and neonatal stages in rodents. Of note, in humans the major development is completed prenatally (Jennings *et al.*, 2015).

In vivo data and early life exposure

Several papers have suggested that endocrine pancreas dysfunction in adults animals may result from early life exposure in accordance with the Barker hypothesis on the developmental origins of adult diseases in humans (Barker, 2007). In their study in C57BL/6 mice fed with a BPA diet (25 mg/kg bw/day in diet roughly corresponding to 5 mg/kg bw/day) from embryonic day 7.5 (E7.5) to E18.5, Whitehead *et al.*, 2016, observed that BPA altered the differentiation program of pancreatic cells resulting in enhancement of the glucagon expressing cells and a decrease of the insulin pancreatic β- cells. There was also a change in the localisation of the α-cells, normally located in the periphery in rodents, whereas they were spread throughout the entire islet after BPA treatment. Pancreas dysfunction of β -cells was also observed in the study from García-Arévalo *et al.*, 2014. The authors showed that BPA-treated male OF-1 mice had enhanced body weight whether fed a control or a high fat diet (HFD) together with fasting hyperglycaemia, glucose intolerance and high levels of non-esterified fatty acids (NEFA) in plasma. Glucose-induced insulin secretion from isolated pancreatic islets was disrupted,

particularly in the HFD-BPA group. It was concluded that male offspring from BPA-treated mothers presented diabetes associated with obesity. To determine the cascade of the metabolic events, the group of Nadal and colleagues (Garcia-Arévalo *et al.*, 2016) further demonstrated using the same experimental model that exposure to BPA (subcutaneous exposure of pregnant OF-1 mice to BPA (10 and 100 µg/kg bw/day, BPA10 and BPA100) between gestation day (GD) 9 and GD16 during the embryonic phase of pancreas development resulted in alteration of insulin secretion in the BPA10 male offspring with no change in the BPA100-exposed males at postnatal day (PND) 30. This was associated with an increase in pancreatic β-cell mass at PND0, PND21, and PND30 together with increased β-cell proliferation and decreased apoptosis. Transcriptomic analysis confirmed the differential expression of genes related to cell cycle and apoptosis. Importantly, treatment of pregnant mice with E2 (10 µg/kg bw/day) between GD9 and GD16 also resulted in enhanced β-cell mass in the male offspring (PND30) as compared to controls, although it resulted from decreased apoptosis and not from changes in cell proliferation. Thus, it was proposed that modifications of the β-cell mass in the offspring as a consequence of estrogen signaling mechanisms initiated in fetal life and leading to an excess of insulin signaling during early life may contribute to the impaired glucose tolerance observed during adulthood and described in Garcia-Arévalo *et al.*, 2014. However, intolerance to glucose is not systemically described together with enhanced weight gain in adult male mice exposed to BPA during early life. For example, Liu et al (2013) reported reduced, enhanced or no change of weight gain depending on whether BPA exposure encompassed gestation, lactation or both, respectively also using subcutaneous injections and similar dosage than in Garcia-Arévalo and colleagues (2014, 2016). Wether this difference finds its origin in the strain used, C57bl6 *versus* OF-1 is presently unknown. Interestingly, in Liu et al (2013), BPA exposure during gestation was the most critical window for glucose intolerance. Of note, exposure during that period of time resulted in reduced weight gain in their study. Further, impairment of glucose tolerance was a very long-lasting effect observed up to 8 months of age in exposed males. It was also observed that males were more susceptible to BPA effects than females. In addition, glucose intolerance occurred despite higher fasting levels of insulin and normal glycaemia in males, indicating insulin resistance that was confirmed using insulin sensitivity metabolic tests. Thorough analysis of the pancreas using both islet morphometry and functional analysis indicated that β-cell dysfunction was more a functional issue than alteration of β -cell mass. Thus, the authors concluded that the inability of insulin secretion to compensate for a decrease in insulin sensitivity resulted in glucose intolerance in adult mice exposed to BPA during early life.

In vivo data on adult animals

In addition to investigating the metabolic status of the male offspring of pregnant OF-1 mice treated with BPA (Garcia-Arévalo *et al.*, 2014, 2016), the group of Nadal has also investigated the metabolic status of the mothers several months after delivery (Alonso-Magdalena *et al.* 2015). The authors observed that these female mice exhibited profound glucose intolerance and altered insulin sensitivity as well as increased body weight. Importantly, no effect was observed with non-pregnant mice. The authors also described reduced pancreatic β-cell mass as a consequence of decreased proliferation and increased apoptosis. Taken together, these data suggest that BPA exposure during gestation has long-term implications in glucose metabolism not only for the offspring but also for the mother. The pancreas was also targeted in males treated with BPA during their adult life (0.5 and 2 mg/kg bw/day for 4 weeks) in the study of Moghaddam *et al.* (2015). These authors reported enhanced oxidative stress through measurement of several markers including malondialdehyde levels glutathione (GSH) levels and the activities of super oxide dismutase (SOD) and catalase CAT with the strongest effects in mice treated with the highest dose. They concluded that the enhanced body weight, hyperglycaemia and hyperlipidemia reported in the BPA-treated mice may be associated with the oxidative stress described in the pancreas.

In the study of Jayashree *et al.*, 2013, it was found that following orally exposure to BPA (20 or 200 mg/kg bw/day), adult Wistar rat males exhibited one month later enhanced serum

insulin but no change in fasting blood glucose levels. These data indicated that the endocrine pancreas could still adapt its response to maintain glycaemia level. However, glucose oxidation and glycogen content were found to be decreased in the liver with both doses of BPA. In addition, there was impaired insulin response in the liver with decreased Akt phosphorylation, indicative of a lower response to insulin and thus of hepatic insulin resistance.

The origin of glucose intolerance did not either result from any detrimental changes in the islet area or morphology or the insulin content of β cells as shown in the study from Moon *et al.* (2015). In this study, the protocol consisted in an orally gavage of 50 µg/kg bw/day of BPA for 12 weeks of 4 to 6-week-old C57BL/6 male mice fed with HFD. Although body weight, percentage of white adipose tissue, and percentage of body fat did not differ between the BPA-treated and the control group, long-term oral exposure to BPA along with an HFD for 12 weeks induced glucose intolerance in the growing male mice. Interestingly the authors observed decreased phosphorylation of AKT and GSK3β in skeletal muscle indicative of a lower response to insulin and thus of insulin resistance in the muscle which might be one mechanism by which BPA induces glucose intolerance. Indeed, the skeletal muscle is the predominant site for insulin-mediated glucose uptake.

2.1.2. Mode of Action: insulin biosynthesis and secretion, and β-cell survival

Estrogen signaling occurs *via* at least the α- and β-estrogen receptors and *via* GPR30/GPER1, a membrane-bound estrogen receptor, resulting in distinct effects depending on the receptor activated. Activation of ERα enhances glucose-stimulated insulin biosynthesis, promotes β-survival from apoptotic stimuli and prevents lipotoxicity. Activation of ERβ enhances glucose-stimulated insulin secretion (GSIS). Activation of GPER1 protects from apoptosis and enhances GSIS without affecting its biosynthesis (Tiano and Mauvais-Jarvis, 2012). For example, using β-cells and islets of Langerhans recovered from wild type (WT) and ERβ-/- mice, it was shown that ERβ was involved in the BPA-mediated rapid regulation of KATP channel activity, potentiation of glucose induced-[Ca2+]i signals and insulin release (Soriano *et al.*, 2012).

Involvement of estrogenic mechanisms in the adverse effects of BPA on pancreas was investigated by Song *et al.* (2012). These authors isolated pancreatic islets of male Sprague Dawley rats and islet morphology and β-cell function were assessed after exposure to BPA and to different estrogenic compounds including E2 and diethylstilbestrol (DES). It was shown that BPA, E2 and DES impacted cell viability as well as the β-cell insulin content, the number of insulin granules, and the area and density of mitochondria in these cells. GSIS and expression levels of genes involved in β-cell function were analysed by qPCR (quantitative polymerase chain reaction). All data were converging at demonstrating impairment of both β-cell morphology and function after exposure to all three molecules although through distinct mechanisms, i.e. BPA is not mimicking all effects induced by E2 or DES. Importantly, the relationship between the doses and β-cell alteration was an inverted U-shape for BPA while dependent on the dose for E2 and DES. It is suggested that mitochondrial dysfunction could be an early event in the BPA-induced impairment of β-cells. It was also shown that BPA exposure could lead to disruption of insulin synthesis through enhanced oxidative stress and cell apoptosis. Using primary cultures of pancreatic islets recovered from C57BL/6 male mice, Carchia *et al.* (2015) described mitochondrial dysfunction and alteration of cell *via*bility in response to low dose of BPA (1×10^{-9} M). The authors showed that mechanisms were involving oxidative stress and enhanced cell apoptosis. Interestingly, the *in vitro* results were confirmed *in vivo* in diabetic mice transplanted with pancreatic islets previously treated with BPA. Indeed, the transplant with BPA-treated islets was unable to restore normal glycemic level neither in BPA treated nor in normal water administered mice at any time. Interestingly, it was described using a human pancreatic cell line (Menale *et al.* 2015) that BPA could decrease the expression of PCSK1, a gene involved in insulin production, opposing the effect induced by E2.

Finally, another study from Gong *et al.* (2013) explored the hypothesis that BPA could impair

β-cell function through misfolding islet amyloid polypeptide (IAPP) into toxic oligomers causing apoptosis of β-cells. IAPP is a peptidic hormone co-secreted with insulin as pro-peptides and involved in glycemic control. Using an artificial micelle system, and INS-1 cells as an *in vitro* culture system of β-cells, it was demonstrated that BPA increases the INS-1 cell apoptosis caused by exogenous addition of IAPP. BPA treatment also resulted in enhanced levels of reactive oxygen species and cell apoptosis. Effects were dose-dependent from 5 to 50 μM with first effects seen at 10 μM. This study is of interest because it enlarges the targets of BPA in the pancreas by showing that IAPP which is a hormone secreted by the pancreas could be impacted.

Epigenetic data

Mao *et al.* (2015) investigated epigenetic changes following BPA exposure (*via* oral administration) of Sprague Dawley rats. BPA exposure during early life results in generational transmission of glucose intolerance and β-cell dysfunction in the offspring through male germ line, which is associated with hypermethylation of the IGF-2 gene in islets. The changes of epigenetics in germ cells may contribute to this generational transmission. This study is one of the few investigating epigenetic changes with BPA exposure. However, more studies are required before conclusions can be made on the BPA-induced epigenetic changes.

Overall, it is suggested that the pancreas is a target of BPA exposure but that mechanisms of actions differ depending on whether exposure occurs during fetal life or in adulthood. Fetal differentiation of the pancreas appears highly sensitive to BPA exposure based on the outcomes surveyed e.g. β-cell proliferation and apoptosis. Limited data exist on the impact of BPA on α-cells and glucagon secretion. Conclusions point to BPA as a disruptor for endocrine pancreas morphology and function during fetal life and adulthood resulting in alterations of insulin synthesis and/or release.

2.2. Effects of BPA on insulin resistance

2.2.1. Adverse effect of BPA on insulin-sensitive organs including liver and adipose tissue

This section focuses on the effect of BPA on insulin resistance, focusing on new publications from 2013 to May 2016. These papers are presented successively and summarized in Table 2.

In the study from Angle *et al.* (2013) significant metabolic disturbances consistent with insulin resistance were observed in adult CD-1 male mice exposed *in utero* to BPA from 5 to 50,000 μg/kg bw/day. These disturbances included an age-related change in food intake, an increase of body weight and liver weight, of abdominal adipocyte mass (enhanced number and volume of adipocytes), and in serum leptin and insulin, together with a decrease in serum adiponectin and in glucose tolerance. The strength of the study stems from the multiple doses of BPA tested, the multiple outcomes surveyed the inclusion of a positive control (DES) as well as the high number of animals tested. They were 9-14 pregnant mice and 13-17 male offspring per group. Interestingly, a 0.1 μg/kg bw/day dose of DES resulted in some but not all low-dose BPA outcomes (eg. food consumption, renal and gonadal fat pad weight, more adipocytes and impaired glucose tolerance) indicative of estrogenic-dependent mechanisms initiated upon BPA exposure. For most of these outcomes inverted U-shape dose-response curves were reported by the authors although a more refined statistical analysis had not confirmed these observations (Beausoleil *et al.*, 2016). In the study of Biasiotto *et al.* (2016)

also, pregnant (C57BL/6) mice were treated with different doses of BPA (0.5, 5, 5, 500 µg/kg bw/day) except that the daily gavages continued on F1 males until the age of 140 days with an interruption from birth to weaning. Interestingly, the authors observed enhancement of fat mass and body weight starting from day 90 only in male mice dosed with BPA at 5 µg/kg bw/day. Consistently, perigonadal fat pads were enhanced at this dosage. Although the authors did not measure insulin and blood glucose, they found enhanced expression of genes encoding PPARγ (Peroxisome proliferator-activated receptor), ATGL (Adipose triglyceride lipase), HSL (Hormone sensitive lipase) and LPL (Lipoprotein lipase) both in fat pads and the liver, indicative of altered lipid metabolism. More recently, Rubin et al. (2017) treated pregnant CD-1 mice with either vehicle or BPA (0.25, 2.5, 25 or 250 µg/kg/day) from day 6 of pregnancy until postnatal day 21 (Perinatal exposure) *via* subcutaneous osmotic minipumps. At weaning, 2 males and 2 females from each litter received additional BPA exposure *via* the drinking water at doses comparable to those delivered by the pumps from PND 21-35 (Perinatal + Peripubertal exposure = P+P). In this study, early exposure to BPA resulted in alteration in body weight and body composition (% fat mass) in a dose specific and sex specific manner that varied with the precise window of BPA exposure. Glucose homeostasis was impaired at 40 weeks in P+P females (but not in males) exposed to BPA at the doses of 2.5 or 25µg/kg/day, as assessed *via* handling insulin tolerance test. Glucose tolerance tests did not reveal differences among the vehicle and the BPA exposed groups. Serum leptin levels were dose dependently increased in P females and at the dose of 25 µg/kg/day in P+P females at week 43 (sacrifice). Regarding triglycerides, levels were significantly enhanced in the liver of BPA exposed female mice for the two highest doses (25 and 250 µg/kg/day) and plasma levels tended to be higher in BPA than in vehicle exposed females. Leptin and triglycerides levels in male mice were not reported. In conclusion, in this study, the P+P females showed evidence of impaired glucose/insulin homeostasis consistent with hyperinsulinemia and the development of insulin resistance.

The metabolic effect of prenatal exposure to BPA was also the aim of the study from Veiga-Lopez *et al.* (2016) performed on sheeps. The endpoints studied included insulin resistance, adipose tissue distribution, adipocyte morphometry, and expression of inflammatory markers in adipose tissue. The second aim of the study was to assess whether postnatal overfeeding would exacerbate the deleterious metabolic effects as shown in rat by Wei *et al.*, 2011. Groups of 6-9 female sheep were thus daily injected subcutaneously with BPA (0.05, 0.5, 5 mg/kg/day from day 30 to day 90 of gestation, thus in the middle of gestation length since term occurs by day 147). In study 1, metabolic tests were made in pre- and post-pubertal F1 sheep at the age of 17 months. The authors described intolerance to glucose and reduced insulin sensitivity in post-pubertal F1 but no effect in prepubertal sheep. In study 2, F1 sheep were fed a HFD starting at the age of 14 weeks ending by 19 months of age. However, if the authors observed glucose intolerance and insulin resistance in the high-fat fed animals, they found that BPA did not impact these parameters. Worthy of note, there was a shift in response to BPA towards more hypertrophic adipocytes, also evidenced as expected in response to the HFD. Indeed, usually there is a bimodal repartition within adipocytes with a population of small adipocytes and a population of large adipocytes full of lipids. With obesity, the repartition of adipocytes is altered with a larger number of highly inflammatory and large adipocytes. However, there was no further aggravation in sheeps exposed to BPA and fed the HFD, which indicates that both challenges (a pollutant and high-fat diet) lead to similar defects as reported elsewhere (Labaronne *et al.*, 2017).

However, glucose intolerance and insulin resistance were not reported by Van Esterik *et al.* (2014)**.** Instead, they described other metabolic outcomes which were dependent on sex in the male and female offspring of BPA exposed C57BL/6 dam mice to 8 doses ranging from 0 to 3 mg/kg bw/day. Specifically, the authors aim to investigate if BPA could alter the metabolic program and observed male and female offspring for 20 weeks without further exposure to BPA. The authors observed sex-dependent effects with body weight increases in males possibly related to an increased overall body size rather than an increased fat mass and altered energy balance as evidenced by a dose-dependent decrease of circulating glucagon. However, there was an over-representation of small litters with the highest doses of BPA introducing a bias. In

addition, plasma levels of insulin and blood glucose did not change and food consumption could not be reliably evaluated because of spillages. In females, because no body weight changes were detected up to the age of 17 weeks, the authors have challenged all female mice with a high-fat diet for 6 weeks. In contrast to males, the authors observed in females decreased body weight with BPA, decreased leptin levels, decreased fatty acids and triglycerides which are consistent with the reported increase in energy expenditure (increased locomotor activity and of Ucp1 expression in brown adipose tissue). Overall, they concluded that *"Although these results suggest that BPA can program for an altered metabolic phenotype, the consistency within the complex of observed metabolic effects suggests that upstream key element(s) in energy homeostasis are modified and that sex-dependent factors contribute to the final phenotypic outcome."*

Insulin resistance could stem its origin from the muscle as well as described in the study of Moon *et al.* (2015) already cited above in which the authors observed glucose intolerance that they proposed to be associated with the decreased phosphorylation of AKT and GSK3β in skeletal muscle that is indicative of muscle insulin resistance. It could as well stem from a defect in the liver as shown in Moghaddam *et al.* (2015). Indeed, the authors observed decreased glucose oxidation and glycogen content in the liver of BPA-treated rats together with enhanced serum insulin but no change in fasting blood glucose levels. In addition and the most obvious evidence for hepatic insulin resistance was the impaired insulin response with decreased AKT phosphorylation in the liver. Adipose tissue was also demonstrated to be targeted by BPA exposure during adult life although sensitivity to insulin was not directly tested in the study of Yang *et al.*, 2016. The authors showed for both sexes that exposure to BPA for 30 days (starting with 5-week old animals) resulted in increased circulating inflammatory factors and local inflammation in the WAT. These mice had no alteration of glucose tolerance but enhanced body weight and fat mass when fed a chow diet. There were as well enhanced leptin plasma levels consistent with enhanced fat mass. Importantly, BPA effects were not dependent on doses which ranged from 5 to 5000 µg/kg bw/day and they were not either observed when animals were fed a high fat diet.

2.2.2. Mode of action

Literature search analysis of the *in vitro* experiments conducted from 2013 to November 2016 was subdivided considering, on the one hand, studies using the 3T3 L1 cell line, which is a mouse cell line very commonly used for assessing adipocyte differentiation and human mesenchymal cells or cultured cells from human adipose explants on the other hand. These studies are described below and reported in Table 3a and 3b.

In vitro studies using mainly murine 3T3-L1 cells

The murine 3T3-L1 adipocyte cell line has proven to be extremely valuable in elucidating mechanisms of adipocyte differentiation which cover the successive phases of undifferentiated growth, hormonal induction and terminal differentiation. This cascade of events is timely coordinated through activation of specific transcription factors including the members of the C/EBP and PPAR families (Rosen ED, 2005). It has been well reported that E2 stimulates the proliferative phase of 3T3-L1 but inhibits adipogenic differentiation induced by treatment with rosiglitazone (ROSI), a PPARγ agonist. Addition of E2 suppresses as well triglyceride accumulation induced by exposure to insulin which enhances lipogenesis in fat cells (Pedram et al 2016).These actions of E2 are consistent with the phenotype of obesity and insulin resistance in models of ERα-deficiency (Hevener et al Mol Cell Endocrinol 2015).

Interestingly, in Biasiotto *et al.* (2016) the authors found that BPA could activate ERα in undifferentiated cells although to a lesser extent than the pure estrogen receptor agonist namely DES. However, while DES reduced activation of both ERα and ERβ in differentiated adipocytes, BPA slightly activated ERβ in differentiated cells with no effect on ERα activation. These events were inhibited by the use of ICI-182790, a specific antagonist to both ERs.These data indicate that BPA could reproduce part but not all of the estrogen effects depending on the differentiation status of the adipocytes and probably on the receptor considered. Using a different murine cell line (C3H/10T1/2), the authors in Biemann *et al.* (2012) showed that BPA (10 µM) had an inhibitory effect during the undifferentiated growth but no effect during the subsequent phases of clonal expansion and terminal differentiation resulting in a lower number of differentiated cells in BPA-treated cultures. The authors concluded that BPA exerted an antiadipogenic effect in accordance with the literature indicating that obesity is associated with reduced estrogen signaling.

Contrasting with the few studies on the impact of BPA on the growth phase, a large number of experiments have focused on whether BPA could alter terminal differentiation of adipocytes. End-points included measurement of PPARγ activation to determine if BPA could be obesogenic or through the survey of adipogenesis markers such as FABP4 (also known as adipsin 2), and triglyceride (TG) accumulation by counting lipid droplets following red oil staining. Measurement of adiponectin and leptin which levels inversely and positively correlate with fat expansion, respectively allowed documenting on insulin sensitivity as described above together with the measurement of inflammatory cytokines. For example, in the study of Chamorro-Garcia *et al.* (2012), murine 3T3-L1 cells were used to study the adipogenic capacity of BPA (1, 10, 100, 1000, 10000 nM) by lipid accumulation measurement (oil red O staining and FABP4 mRNA and protein levels) and it was found that BPA enhanced significantly lipid accumulation and FABP4 at the 3 highest doses in 3T3-L1 cells. BPA was also shown to enhance FABP4 expression in a dose-dependent manner (Atlas et al 2014) and lipid accumulation (Atlas et al 2014**,** Ahmed and Atlas 2016). In this latter study, and using transcriptional assays, BPA was found to modestly activate PPARγ using a PPRE (PPARγ response element)-dependent luciferase construct. Co-treatment of cells with the selective PPARγ antagonist GW9662 inhibits BPA-, ROSI (rosiglitazone, a PPARγ agonist) but not DEX (dexamethasone, a glucocorticoid agonist)-dependent adipogenic differentiation, indicative that BPA requires PPARγ to induce adipogenesis but did not act through the glucocorticoid pathway. Activation of PPARγ and enhanced TG accumulation in BPA-treated 3T3-L1 cells by BPA was also reported in Biasiotto *et al.* (2016). PPARγ specificity was assessed using a specific antagonist (T0070907). Interestingly, the pure estrogen receptor agonist DES displayed an opposite action to that of BPA inhibiting PPARγ activity in adipocytes. A comprehensive analysis was also undertaken by Pereira-Fernandes *et al.* (2013). These authors have engineered a screening system for obesogenic compounds in the 3T3-L1 model (Pereira Fernandes *et al.*, 2014). The aim was to develop a reproducible and standardised protocol for the adipocyte differentiation assay to use as an *in vitro* tool for obesogenic compounds screening. It was based on PPARγ transactivation and antagonist studies considering that PPARγ signaling is a major regulator of differentiation. Outcomes included a lipid accumulation fluorescent test and the development of a PPARγ CALUX cell line. Positive controls included the reference compounds ROSI and Tributyltin (TBT) which are PPARγ agonists and T0070907, a PPARγ antagonist. The authors observed enhanced lipid accumulation but to a much less degree than after addition of ROSI consistent with a weak PPARγ activity. Interestingly, the authors have compared the transcriptomic profile of cells exposed to various compounds including ROSI, TBT and BPA within the 3T3-L1 model. They concluded that BPA had obesogenic properties in the 3T3-L1 model but that mechanisms of action were distinct from the frequently observed PPAR mediated obesogenicity. Enhanced lipid accumulation on confluent and differentiated 3T3-L1 cells together with a weak activity of PPARγ was also reported by Héliès-Toussaint *et al.* (2014) using a large dose range of BPA doses. The authors also observed increased lipolysis, a very light effect on Srebp1C (a transcription factor that stimulated lipogenesis) and FABP4 mRNA levels as well as enhancement of ERRα and ERRγ mRNA levels but no effect on leptin and on glucose uptake. Based on data yielded using either DES (enhancing lipid accumulation) or ROSI (enhancing

lipid accumulation, lipolysis, leptin levels and decreasing glucose uptake), the authors concluded that BPA could specifically activate adipocyte differentiation through binding to either estrogen-related receptor (ERR)α or ERRγ, effects which were not reported with DES or ROSI. Also and depending on the end-point, BPA was found to display estrogenic or PPAR activities to enhance lipid accumulation. In addition to enhancing lipid accumulation, several studies showed that BPA could decrease insulin sensitivity. For example, in the study of Valentino *et al.* (2013) the authors found that while markers of differentiation (Glut4, PPARγ) did not change with BPA exposure, glucose utilization was reduced and insulin signaling measured by phosphorylation levels of Insulin Receptor (IR), AKT/PKB and phosphor ERK), and leptin mRNA levels were decreased indicative of impairment of insulin action in BPA treated 3T3-L1 cells. Adipocyte metabolic dysfunction was also described in the study of Ariemma *et al.* (2016). Specifically, it was observed enhanced expression mRNA levels of PPARγ and C/EBPα and of leptin and IL6 indicative of enhanced inflammation in addition to enhanced FABP4 mRNA and protein. Decreased insulin sensitivity was also established with decreased glucose utilisation and insulin signaling.

There has been one study (Boucher *et al*., 2015) investigating if the main BPA metabolite, BPA ß-D-glucuronide has any biological activity and if such biological activity was distinct from estrogenic activity. To that purpose, 3T3-L1 cells were treated with BPA-G and lipid accumulation and expression markers of adiposity were assessed (both mRNAs and proteins). BPA-G was found active in inducing differention of adipocytes which were expressing higher amount of adipogenesis markers (FABP4, adipsin) than unstimulated cells. However, Pparγ remained unmodified. Furthermore, while ICI reverses the BPA-G induced increase of lipid accumulation, luciferase assay revealed no ER transcriptional activity. GR transcriptional activity was neither modified. However, the authors did not bring the absolute proof that deconjugation of BPA-G did not occur in the in vitro models. In addition, it should be considered that the concentration of efficient BPA-G, i.e., 10 µM is several orders of magnitude higher than what would be expected in the general population. Thus, the residual BPA occurring by deconjugation of the BPA-G may well trigger the effects reported but this question has not yet been answered.

In vitro studies using murine primary cells culture

In the study of Yang *et al.* (2016) primary cultures of adipocyte progenitors were prepared from the stromal vascular fraction recovered from the white adipose tissue of C57bl6 male mice. Interestingly and consistent with most studies performed on 3T3-L1 cells and described above, addition of BPA resulted in a stimulation of C/EBPα, PPARγ, and FABP4. However and contrasting with data reported by Atlas et al (2014) and Ahmed and Atlas (2016), it was indicated that mechanisms may involve the glucocorticoid receptor (GR) as shown through using RU486, a GR antagonist.

In vitro studies using human cells (either cell lines or cultured from explants)

Human cells (either cell lines or cultured explants) have also largely been used to explore the effects of BPA.

Apart from the study of Chamorro-Garcia *et al.* (2012) not reporting any adipogenic effect of BPA using human multipotent mesenchymal stromal stem cells (MSCs), most studies performed on human adipocytes recovered from biopsies have described that BPA was interfering with adipocyte physiology although with distinct mechanisms of actions. The involvement of an ER-mediated pathway was suggested by Ohlstein and colleagues (Ohlstein *et al.,* 2014). Using human adipose stromal cells recovered from subcutaneous abdominal adipose tissue of 3 normoweight caucasian females, the authors demonstrated that BPA

enhanced adipogenesis with a maximal response observed at 1 μM BPA. Using identical culture conditions, the addition of E2 (10 nM) elicited a slightly higher response than BPA. In addition, adipogenic effects of BPA were reversed by co-incubating cells with the ER antagonist ICI indicating the involvement of an ER-mediated pathway. The authors also analyzed the gene expression pattern of ERs and key adipogenic genes by qPCR–in cells treated with 1 μM BPA. They observed gene alteration consistent with the differentiating program of adipocytes from the preadipocyte stage to the full differentiated adipocyte expressing PPARγ and Lpl. However, the authors do not discuss their data in line with the reported antiadipogenic actions of estrogens although opposite functions of the receptors ERα and ERβ on fat metabolism have been reported. Indeed, while genetic invalidation of ERα results in obesity and insulin resistance, absence of ERβ improves metabolic performances of the mice with enhanced insulin sensitivity and reduced fat mass suggesting a pro-diabetogenic effect of ERβ (Barros and Gustaffson, 2011). These invalidation experiments have proven to be useful to demonstrate that metabolic actions of estrogen receptor beta (ERβ) are mediated by a negative cross-talk with PPARγ, resulting in improved insulin sensitivity in male mice knockout for ERβ, because of enhanced activation of PPARγ (Foryst-Ludwig *et al.*, 2008). It may be of interest to analyse such a ratio in Ohlstein *et al*. (2014) when considering that an imbalance in the ratio of the ERs (ERα/ERβ ratio) in adipose tissue may have implications for the development of metabolic diseases (Barros and Gustaffson, 2011),

In the study of Wang *et al.* (2013), enhanced PPARγ and LPL mRNA levels were also described in BPA-treated human adipocytes. However, the authors demonstrated involvement of the glucocorticoid pathway. Specifically, the impact of BPA (10 nM, 1 μM, 80 μM) was investigated in adipocytes recovered from omental biopsies from children undergoing abdominal surgery, boys and girls neither overweight nor obese. Together with enhanced PPARγ and LPL mRNA levels in BPA-treated adipocytes there were enhanced 11-bHSD1 mRNA and activity in adipocytes. Of note, the enzyme 11-bHSD1 converts the inactive cortisone into the active hormone cortisol (corticosterone in rodents) in adipose tissues and promotes adipogenesis. To further determine the mechanisms of action of BPA, the authors used HPA-V which is a human cell line isolated from human visceral fat tissue. They demonstrated promotion of adipogenesis (lipid accumulation) and enhanced 11-bHSD1, PPARγ and LPL mRNA levels. Addition of CBX, the 11-bHSD1 inhibitor or of RU486 to inhibit glucocorticoid signaling prevented partially the BPA-induced effects on 11-bHSD1 mRNA levels, thus confirming the involvement of the glucocorticoid pathway in the BPA mechanism of action. Involvement of the glucocorticoid pathway was not demonstrated in the study of Boucher *et al.* (2014). These authors have treated primary human pre-adipocytes with either BPA or DEX for 48 hours and gene expression microarray analysis was performed. Interestingly, transcriptomic profiling showed enrichment in genes involved in adipogenesis associated with the SREBF1 (encoding the sterol regulatory element binding protein 1c) but also genes associated with mTOR (mammalian target of rapamycin*)* and Thyroid receptor/RXR signaling but not glucocorticoid signaling as seen with the DEX-treated cells. Gene profiling was also performed in the study of Menale *et al.* (2015). In this study, adipocytes were prepared from subcutaneous explants recovered from children undergoing orchidopexy surgery, and treated with either BPA or E2. The analysis of deregulated genes in response to BPA allowed the identification of a small group of genes that are expressed in an opposite manner from that of adipocytes treated with E2. In particular, BPA increases, whereas E2 decreases the expression of pro-inflammatory cytokines and the expression of FABP4 and CD36, two genes involved in lipid metabolism. It indicated enhanced insulin resistance with BPA exposure. The authors (Menale *et al.* 2016) further evaluated the effect of BPA on insulin sensitivity using adipocytes prepared from subcutaneous explants recovered from children undergoing orchidopexy surgery. Specifically, they measured adiponectin and resistin by RT-qPCR. They demonstrated a significant down-regulation of adiponectin while resistin could only be quantified in BPA-treated cells. These findings are indicative of a reduced sensitivity to insulin. Alteration of insulin signaling was also described in the study of Valentino *et al.* (2013). The authors investigated the impact of nM doses of BPA (1 and 10 nM) on human adipocytes prepared from a biopsy of subcutaneous WAT. While markers of differentiation (Glut4, Pparγ) did not change with BPA exposure, the authors described

reduced glucose utilisation and insulin signaling measured by phosphorylation levels of IR, AKT/PKB and phospho ERK, and leptin mRNA levels, all converging to showing that BPA is impairing insulin action.

In summary, down-regulation of adiponectin release, a marker of insulin sensitivity, was observed in human cells by Menale *et al.* (2016) using adipocytes from subcutaneous explants recovered from children undergoing orchidopexy surgery. Alteration of glucocorticoid signaling was evidenced with adipocytes recovered from omental biopsies from normal weight children undergoing abdominal surgery (Wang *et al.*, 2013) as well from explants of C57bl mice treated *in vivo* with BPA (Yang *et al.,* 2016). Reduced glucose utilisation coupled to alteration of insulin signaling was observed in human subcutaneous adipocytes (Valentino *et al.*, 2013). A transcriptomic analysis pointed also to alteration of the thyroid receptor signaling pathway (Boucher *et al.*, 2014).

However, mechanisms of action of BPA have mostly been examined using the 3T3-L1 cells. For example, it has been demonstrated that exposure to BPA reduced estrogen signaling (Biemann *et al.*, 2012), reduced glucose utilisation and insulin signaling (indicative of resistance to insulin) (Valentino *et al.*, 2013), potentiated the transcriptional complex containing GR and C/EBP at the promoter of FABP4 (indicative of enhanced adipogenesis) (Atlas *et al.*, 2014), promoted adipocyte differentiation through the specific activation of the estrogen receptors α or ß (Biasiotto *et al.*, 2016). In other studies, BPA has been shown to decrease insulin sensitivity as a consequence of enhanced inflammation (Ariemma *et al.*, 2016). All these papers indicate an alteration of endocrine activity with reduced insulin sensitivity upon exposure to BPA. It remains debated whether BPA activates PPARγ which is a master transcription factor of adipogenesis. For example, it has been shown that BPA could weakly activate the PPARγ receptor (Pereira-Fernandes *et al.,* 2013; 2014); others demonstrated that BPA required PPARγ to induce adipogenesis (Ahmed & Atlas, 2016) or initiated adipocyte differentiation through binding to ERRα or ERRγ (Héliès-Toussaint *et al.*, 2014); but with no indication of insulin sensitivity.

In conclusion, the murine and human *in vitro* studies on adipocyte differentiation and function point to an alteration of endocrine pathway (e.g., adiponectin release, insulin signaling cascade effectors). It is not clear whether BPA activates PPARγ and/or other nuclear receptors. The importance of cross-talk between nuclear receptors must be further investigated.

3. Human data

In the previous risk assessments (ANSES, 2014, EFSA 2015; ECHA, 2015), it was concluded that based on available epidemiological studies, an association between BPA exposure and metabolic outcomes in humans cannot be established. Most of the studies were cross-sectional including those from the National Health and Nutrition Examination Survey (NHANES) coupled with the Centers for Disease Control and Prevention's National Biomonitoring Program. Lakind *et al.* (2012) conducted a re-analysis of the associations between BPA exposure and chronic disease, including diabetes, using four available NHANES data sets (2003–2004, 2005–2006, 2007–2008, and 2009–2010). Scientifically and clinically supportable exclusion criteria and outcome definitions were applied. All analyses were adjusted for creatinine, age, gender, race/ethnicity, education, income, smoking, heavy drinking, BMI, waist circumference, calorie intake, family history of heart attack, hypertension, sedentary time, and total cholesterol. When the a-priori selected methods were used to address the research question, no associations were found between urinary BPA and diabetes. The authors concluded that the

discrepancy between their findings with regard to diabetes and those reported previously (Lang *et al.,* 2008; Melzer *et al*., 2010) was largely explained by the choice of case definition. The Lakind *et al*., 2012 study did not support the associations and causal inferences that were suggested in the previous studies, and highlighted that data from cross sectional studies like NHANES surveys are inappropriate for drawing conclusions about relations between short-lived environmental chemicals and chronic diseases.

Since the publication of the previous risk assessment reports, several human studies investigating association between BPA exposure and metabolic outcomes were published. A systematic search of the scientific literature published in 2013, 2014 and 2015 was performed to check if the conclusions, which were reported in these reports, are still valid.

The studies were reviewed in a weight of evidence approach to assess a causal link between BPA exposure and metabolic outcomes and according the following criteria:

- Type of study: Analytical cross-sectional studies are sometimes carried out to investigate associations between risk factors and the outcome of interest. However this type of study is limited in its ability to draw valid conclusions about any association or possible causality because the presence of risk factors and outcomes are measured simultaneously is impossible to infer causality. Although these studies may suggest new hypothesis only analytical retrospective (i.e.: cases-controls) or, even better, prospective studies (i.e. cohort studies) may be useful to discuss a causal link.
- Validity of BPA exposure estimation: most of the studies used one or two spot urines to determine BPA concentration. The episodic nature of BPA exposure (mostlty throught diet), its short biological hal-life (< 6 H) and the substantial within-person variation, may lead BPA exposure misclassification accurate exposure assessement in epidemiological studies is challenging. Therefore this criteria was not used to exclude studies although it should be considered when analysing studies.
- Relevance of outcomes: some studies consider biological parameters (blood concentrations of leptin, adiponectin), other considered diseases (hypertension, diabetes) or athropometric characteristics such as BMI and body weight. Some of these variables may be considered as adverse outcomes whereas others are only indicative of biological characteristic without any direct link with a disease.
- Size of the samples and representativity of the study population compared to the general population.

Accordingly to these criteria, the following studies were not further considered. The study of Leclerc *et al.* (2014), because to the small sample size and lack of representativity of the population study; The studies from Aekplakorn *et al.* (2012, 2015), Ko *et al.* (2014), Choi *et al.* (2014), Khalil *et al.* (2014), Beydoun *et al.* (2014), Ronn *et al.* (2014), Xiong *et al.* (2015), Xue *et al.* (2015), Lee *et al.* (2015), Lin *et al.* (2015) and Savastano *et al.* (2015), because their cross-sectional designs; The case-control study from Ahmadkhaniha *et al.* (2014) because the small sample size and limited information on the control group and their health status.

Six original publications based on prospective cohort designs were considered.

In the MIREC birth cohort study in Canada, total BPA was measured in singles urines samples during early pregnancy (n = 1274) (Shapiro *et al.* 2015). No statistically significant associations were observed between BPA exposure and gestational impaired glucose tolerance or gestational diabetes mellitus.

Using nested case-control design within two adult cohorts in USA, the Nurses' Health Study (NHS) and the Nurses' Health Study II (NHSII), the association between urine BPA levels and

diabetes mellitus (DM) was investigated (Sun *et al.* 2014). In the NHS cohort, female nurses, between 30 to 55 years of age, were enrolled in 1976 and provided first-morning-void urine samples in 2000-2002 when they were aged 53 – 79 years. In the NHSII cohorts, female nurses, 25 to 42 years of age, where enrolled in 1989 and provided a urine samples between 1996 and 2001. Through 2007 and 2008, identified and confirmed diabetes mellitus cases were assessed. Conclusions diverged according to the cohorts and to the logistic regression models. In the nested case-control from the NHS cohort (394 cases and 393 controls), no associations were observed in three different adjusted models. By contrast, in the nested case-control) from the NHSII cohort (577 cases and 577 controls), a significant association was observed for the highest quartile of exposure compared to the lowest quartile in two of the three adjusted models, particularly when BMI was introduced (Odds ratio: 2.08, 95 % CI: 1.17 – 3.69, P-Trend: 0.02).

A small (n = 121) prospective study of patients with prevalent diabetes mellitus reported that BPA exposure evaluated by a single blood measurement at baseline was associated to decreased glomerular filtration rate (Hu *et al.* 2015). At baseline, the inclusion criteria was to have an estimated glomerular filtration (eGFR) above 60 mL/min/1.73m². Although this is an acceptable criteria in clinical settings, the US National Kidney Foundation defined a normal eGFR for adults as greater than 90 mL/min/1.73m^2. The mean +/- SD eGFR of patients at baseline was 87 +/- 15.6, suggesting that some of these subjects may have kidneay damage with mild loss of kidney function. As a consequence, as as also raised by the authors, a reverse causation ca not be exluded.

The association between prenatal exposure to BPA and child weigh status at 7 years of age (n = 470) was investigated in the INMA mother-birth cohort in Spain (Agay-Shay *et al.,* 2015). BPA concentrations were measured in two spot-urine samples during the first and third trimester of pregnancy. Exposure to BPA was not associated to weight change evaluated by the BMI-z score in children at age 7.

In the NHS and NHSII cohorts above mentioned, the association between exposure to BPA in adults and further weight change was investigated in a sample grouping female controls (n = 977) from the two nested case-control studies (Song *et al.* 2014). Body weights were self-reported at the time of urine collection and every 2 years thereafter for 10 years. In an adjusted linear regression model, women in the highest quartile of BPA urine concentrations had 0.23 kg per year (95 % CI, 0.07 – 0.38) greater weight gain when compared with women in the lowest quartile.

In 188 mother-child pairs from the CHAMACOS prospective study in USA (Volberg *et al.*, 2013), BPA was measured in two urinary spot samples during early and late pregnancy and in 9-year-old children. Maternal urine BPA concentrations during late pregnancy were associated with increased plasma lectin levels in boys whereas maternal urine BPA concentrations during early pregnancy were associated with plasma adiponectin in girls. Any significant associations were found between concurrent urine BPA concentrations and 9-year child adiponection or leptin. Overall, these results are difficult to put into perspective with weight changes observed in the same children where increasing BPA concentrations in mothers during pregnancy were associated with decreased BMI among their daughters at 9 years of age and where a positive association was observed between concurrent urine BPA concentration and BMI at 9 years of age (Harley *et al.*, 2013).

In conclusion, well designed epidemiological studies are needed to confirm that observed effets in experimental studies may be transposed to humans. Although some of these epidemiological studies here considered follow high quality standards, they are faced to the limitation of exposure measurements as above mentioned. One must keep in mind that few spot measurement of a substance with a very short half-live in the body and large day-to-day within-person variability, results in poorly estimates average levels over long periods. This results in exposure assessments that can not be reliable for health outcomes that require a long period of latency.

Beyond this aspect, recent prospectives studies do not converged in the direction of associations nor confirmed assumptions raised in previous studies, including cross-sectionals. In some studies, when statistical analysis varied slightly particularly when potential confounding factors varied, the same data produced different results. Consequently, and in full agreement with a systematic review of epidemiologic research no firm conclusions can be drawn concerning the relationship between BPA exposure and metabolic outcomes such as diabetes mellitus and obesity.

4. General conclusion on metabolism and obesity

In conclusion, based on animal studies (rodents and non-rodents) after prenatal and/or perinatal or adult exposure and considering that:

1- alterations in insulin secretion by pancreatic β-cells, or alterations of insulin action (signaling mechanisms) upon the insulin-sensitive organs (such as those leading to variations in the expression levels of hepatic or adipose tissue markers known to reflect a state of insulin resistance) are hallmarks of endocrine disruption mechanisms, especially if there is a combination of effects, (each leading to insulin resistance within the different insulin-sensitive tissues).
2- the endocrine pancreas is targeted by BPA exposure with mechanisms possibly different depending on whether exposure occurs during fetal life or in adulthood. Indeed, Fetal differentiation of the pancreas appears highly sensitive to BPA exposure based on the outcomes surveyed e.g. β-cell proliferation and apoptosis. In particular, BPA has been shown to alter insulin secretion and/or release by pancreatic β-cells,
3- BPA alters insulin (signaling within insulin-sensitive organs (i.e., liver, muscle, adipose tissues). This resulted in variations in the expression levels of hepatic or adipose tissue biological markers which are indicative of a state of insulin resistance.
4- energy homeostasis has long been demonstrated to be dependent on sex. While most studies were performed on males, a few studies have also examined the impact of BPA either on both sexes or on females and point to–sex-specificity of the metabolic impact of BPA. This constitutes another argument pleading in favor of an endocrine mechanism of action.

there is now evidence that BPA may increase the occurrence of type-2 diabetes *via* an ED MoA through the involvement of ERα, ERβ or GPR30 pathways as shown in recent experimental *in vivo* and *in vitro* studies. Other hormones such as leptin and adiponectin, which are involved in resistance to insulin and lipogenesis, are also modified following BPA exposure. This shows that BPA could interfere in the balanced interplay between insulin secretion and insulin action that controls glycaemia.

Even if available epidemiological studies are still inconclusive, these effects are considered relevant for humans because similarities exist in homeostatic regulation of insulin production and sensitivity between animals and humans and because of *in vitro* experimental data using human cells or tissue.

Table 2: *In vivo* experimental study on effects of BPA on glycaemia and insulin synthesis.

Ref.	Species Strain Model	Routes	Dose Exposure period	Group size	Outcomes reported				Conclusions of the authors	Comments
Angle *et al.*, 2013	Pregnant CD1 mice	Oral (administration with a pipette not by gavage)	5, 50, 500, 5000, 50000 BPA. 0.1 DES ug/kg/d **From GD9 to GD18. 30 µl of BPA**	9-14 pregnant mice and 13-17 male offspring	age-related change in food intake, an increase in bw, liver weight and abdominal adipocyte mass (nb, volume),	increase in serum leptin and insulin; decreased in serum adiponectin and in glucose tolerance	non-monotonic dose-response curves with no effect of the highest dose	not all effects were seen with DES; statistic tests should be looked at	Metabolic disruption in male mice due to fetal exposure to low but not high doses of BPA with effects on bw, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation.	convincing study because of the multiple doses (TDI up to 10-fold the NOAEL), multiple outcomes, positive control (DES), 9-14 dams, 13-17 males/ group
Jayashree *et al.*, 2013	Wistar rat	oral	Control and BPA 20 mg/kg and 200 mg/kg 4 weeks	n= 6/group	↗ blood insulin with BPA dose dependently No effect on glucose but ↓ in glucose oxidation ↓ in glycogen content ↓ testosterone with BPA		↓ insulin receptor and Akt mRNA and protein kinase B with BPA 200 mg/kg ↗GLUT2 mRNA and its protein with BPA 20 and 200		The authors conclude that BPA impairs hepatic glucose oxidation and glycogen content through defective signal transduction	There is no indication of the BWof the animals and the doses used are extremely high
Liu *et al.*, 2013	Pregnant C57bl6 mice	Sc BPA (100 µg/kg/d) or vehicle	- **GD1-GD6,** (preimplantation exposure); - **GD 6 - PND0** (fetal exposure); - **PND0–PND21** (neonatal exposure); and - **GD6–PND21**, fetal and neonatal exposure)	N = 15-30 mice/ group	**Glucose homeostasis and Insulin release** ***gluc tolerance in F1 females - PND0–PND21: no effect of BPA **-** GD6–PND21: no effect of BPA -GD6-PND0: gluc intolerance at 3 and 6 months - GD1-GD6: impaired gluc tolerance at 6 m but not at 3m - no difference in gluc tolerance at 8m in all groups * gluc tolerance in F1 males - GD1-GD6: no effect of BPA - GD6–PND21: gluc intol at 3m - GD6-PND0: gluc intol at 3m, 6m and 8m - PND0–PND21: gluc intol at 3m * insulin release in F1 females:		**Insulin sensitivity (IpITT)** * in females: **-**GD1-GD6: no effect **-** PND0–PND21: no effect - GD6–PND21: no effect - GD6-PND0: ↓ insulin sensitivity at 3m and 6m * in males: **-** GD1 –GD6: no effect - GD6-PND21: ↓ insulin sensitivity at 3m **-** PND0–PND21: idem - GD6–PND0: idem and until 8m **Insulin secretion after glucose stimulation *in vitro***	Glucose homeostasis was impaired in GD6-PND0 mice from 3 to 6m of age, and until 8m in males. In PND0-PND21 and GD6-PND21, BPA-treated group, only the 3m old male F1 developed the glucose intolerance. Moreover at the age of 3m, perinatal exposure to BPA resulted in the ↗ in β-cells mass mainly due to the coordinate changes in cell replication, neogenesis and apoptosis.	Glucose homeostasis was impaired in males more than in females as assessed through handling glucose tolerance metabolic tests. After exposure to BPA, the b-cell mass ↗ while insulin secretion was either ↓or remained invariable. It suggested that beta cells were less functional in BPA-

					- GD6-PND0: ↓insul release at 3m but improved at 6m and normal at 8m - GD1-GD6: ↓insul release at 6m - PND0–PND21: no effect - GD6–PND21: no effect <u>* insulin release in F1 males:</u> - GD6-PND0: ↓insul release at 3m and 6m - PND0–PND21: ↓insul release at 3m but ↗ at 6m - GD6–PND21: ↓ insul release at 3m but ↗ at 6m - GD1-GD6: no effects Normal at 8 m in all groups	↗ in GD1-GD6 females ↓ in GD6-PNDO females and males and in PND0-PND21 and GD6-PND21 males No effects at 8m in all groups **Islet morphologic analysis** <u>β-cells mass:</u> - GD1-GD6: no effect in m and f -GD6-PND21:↗ 'β-cells mass in m and f - GD6-PND0: :↗ β-cells mass in m only - PND0–PND21:↗ β-cells mass in m only <u>β-cells turnover</u>: - GD6-PND0 ↓ in females - PNDO-PND21 ↓ in males - GD6-PND21: idem		exposed mice. The alterations of insulin secretion and insulin sensitivity, rather than b-cell mass, were consistent with the development of glucose intolerance. Data also indicated that the fetal development stage may be a critical window of susceptibility to BPA exposure.
Van Esterik *et al.*, 2014	C57bl6 mice	In diet (fed a BPA-containing CD)	3, 10, 30, 100, 300, 1000, 3000 µg/kg/d During gestation and lactation	**(4 females /group); groups were made of 8 mice per sex (4-10)**	<u>in males</u>: increased body size (not fat mass) with altered energy balance (glucagon increase) (but overrepresentation of small litters)	<u>in females</u>: no effect, so the authors decided to give a High Fat diet to all mice by week 17 and for 6 weeks. It resulted in decreased bw, leptin levels, fatty acids and triglycerides consistent with increased locomotor activity and of Ucp1 in the BAT	the authors conclude "Although these results suggest that BPA can program for an altered metabolic phenotype, the sexual dimorphism of effects and diversity of outcomes ... do not mark BPA as a specific obesogen. The consistency within the complex of observed metabolic effects suggests that upstream key element(s) in energy homeostasis are modified. Sex-dependent factors contribute to the final phenotypic outcome. »	conclusions consistent with the data

Garcia-Arévalo *et al*., 2014	Pregnant OF1-mice	sc	Vehicle or BPA 10 µg/kg/d From E9 to E16	n= 8 mice/group 4 groups: males exposed to CD and CD-BPA or to HFD and HFD-BPA during 13 or 24 weeks	**Effects of BPA on weight, food intake, gonadal and retroperitoneal fat pad weight and plasma NEFA** At birth: - ↓ bw in BPA rats - slight↗ bw in BPA mice compared to CD 17 weeks: HFD-BPA ↓ perigonadal and retroperitoneal fat pad weight compared to HFD 28 weeks: CD-BPA ↗perigonadal fat pad weight compared ton control No effect on food consumption in ND groups but ↗in HFD-BPA compared to HFD **Glucose tolerance and insulin sensitivity** Fasting hyperglycemia, glucose intolerance and high levels of NEFA in CD-BPA, HFD and HFD-BPA mice compared to control no effect in ipITT	**Effect of BPA on glucose stimulated secretion (GSIS) and insulin content** Disruption of glucose stimulated insulin release, particularly in HFD-BPA group At 17 weeks: ↗islet insulin content in BPA and HFD –BPA mice compared to control but not at 28 weeks **Effects of BPA on gene expression in white adipose tissue, liver and skeletal muscle** - ↓mRNA expression of genes involved in fatty acid metabolism in white adipose tissue, comparable to HFD; -upregulation of *Pparγ* and *Prkaa1* genes in the liver; - ↓expression of *Cd36*	Impaired tolerance to glucose in BPA treated mice compared to control. The phenotype of the BPA group resembles that of HFD mice. Male offspring from BPA-treated mothers presented a form of diabetes which typically develops in later life and is associated with obesity	The experiment is well conducted. Only males but not females were studied. Mechanisms of action are not described nor did the authors used a positive control or inhibitor to explore the possible involvement of estrogeno-mimetic actions of BPA.
Moon *et al.*, 2015	Growing male C57 BL/6 mice	Oral during 12 week High Fat Diet (HFD)	BPA 50 µg/kg/d	4 groups: CD with or without BPA and HFD with or without BPA: CD; CD BPA HFD; HFD BPA n= 5 per group and experiments repeated 3 times	BW, % of WAT and % of body fat did not differ between BPA and control group. ↗glucose intolerance in HFD BPA mice (IpGTT)	Long-term exposure to BPA impairs insulin signaling: ↓Akt and GSK3β phosphorylation in skeletal muscle from BPA mice but not in hepatic of adipose tissue No changes in islet area or morphology or insulin content of β-cells.	Long-term oral exposure to BPA along with HFD for 12 weeks induced glucose intolerance and insulin resistance in growing male mice.	The study is well conducted. The effects are mostly subtle with the exception of the effects describing impairment of insulin signaling in the skeletal muscle.

Alonso-Magdalena *et al.*, 2015	OF-1 mice	sc	Pregnant mice: Vehicle (control<0) or BPA, 10 (BPA10) and 100 µg/kg/d (BPA100) From E9 to E16 Nonpregnant mice:	n= 10-30/ group depending on the tests and ages	BPA 10 and 100 in BPA **pregnant** mice: **PND 3 months**: no ≠ in glucose homeostasie (no effect on ipGTT) – no effect on ipITT **PND 4 months**: effects on glycemia homeostasie, slight effect on ipITT in BPA 100 **PND 5 months:** ↗effects on glycemia homeostasie; effect on ipITT in BPA 100 **PND 6 months**: strong effects on glycemia homeostasie; effect on ipITT in BPA 10 and 100 ↗ BW and perigonadal fat pad weight **PND 7 months**: ↓ plasma insulin levels in BPA10 and BPA 100 compared to control ←↓ glucose – stimulated insulin secretion ↓ β-cells mass in BPA 10 and BPA 100 ←↓ proliferation ; ↓ expression of *Ccnd2* (in BPA100) but no effect on Cdk4 ↗expression of *p16* , no ≠in *p53* gene expression, ↓ expression of cyclin D2 and CDK-4 proteins in BPA10 mice ↗expression of p16 and p53 proteins	BPA 10 and 100 in BPA treated **in non pregnant** mice: **PND 3, 4, 5 or months**: no effect on ipGTT – no effect on ipITT No change in glucose-stimulated insulin secretion	Exposure to low doses of BPA during pregnancy - is associated with maternal alterations of glucose homeostasis and insulin sensitivity in the long term (glucose intolerance and insulin resistance) - disrupts pancreatic β-cells function 7 months after delivery - has impact on β-cell mass, proliferation and cell death	The study is well conducted with a high number of animals and long expertise on the domain. The study is highly indicative that BPA could be considered as pancreas toxic during pregnancy
Moghaddam *et al.*, 2015	Adult Male mice	ip.	Control and BPA 0.5 and 2 mg/kg 4 weeks	n= 6/group	↗ BW with BPA ↗ blood glucose with BPA dose dependently ↗triglycerides, total cholesterol, LDL-C and ↓ HDL-C with BPA dose dependently	**Oxidative stress parameters:** - serum: ↗malondialdehyde ↓ GSH with BPA dose dependently - pancreas: ↗malondialdehyde ↓ GSH, TAS and SOD/CAT activities with BPA dose dependently No effect in proteins content	These results suggest that BPA exposure might induce hyperglycemia and its complications in adult male mice by induction of oxidative stress	Strain of mice not specified; composition of food not given; some authors not referenced.

Garcia-Arévalo *et al.*, 2016	OF-1 mice	sc	Vehicle (control <0); E2 10 µg/kg·d (control >0) or BPA, 10 (BPA10) and 100 µg/kg/d (BPA100), From E9 to E16	Control: n =73; BPA 10: n= 63; BPA 100: n= 56; E2: n= 18 (10 control + 8 treated)	BPA: lower birth weight, ↓ insulin secretion at P30 in the male offspring exposed to BPA10 but not BPA100 P0, P21, and P30 ! ↗ β-cell mass ↗ β-cell proliferation , ↓ apoptosis E2: Increase in pancreatic β-cell mass at P30 ← ↓ apoptosis and not ←proliferation		**Transcriptomic analysis**: differential expression of genes related to cell cycle and apoptosis		modifications of the beta cell mass in the offspring as a consequence of estrogen signaling mechanisms initiated in fetal life at a wrong timing and leading to an excess of insulin signaling during early life which may contribute to impaired glucose tolerance during adulthood	This study is of good quality with a high number of animals. It was performed in a group with well recognised expertise in the pancreas and BPA.
Whitehead *et al.*, 2016	mice	Diet	BPA: 25 mg/kg/d E7.5 to E18.5		At E18.5 : ↗ number of islet-cell clusters (ICCs) in fetal pancreas		**Immunohistochemical analysis**: BPA↗ glucagon expression and nb of glucagon-expressing islet cells		BPA promotes islet differentiation or delays conversion of ICCs into mature cells , indicating alterations in glucagon expression in islets and ICCs	These data indicate that BPA can alter the differentiation program of the pancreas.
Veiga-Lopez *et al.*, 2016	Female sheep		0.05, 0.5, 5 mg/kg/d D30-D90 (term 147d)	(groups 6-9)	<u>study 1</u>: metabolic tests in pre and post-pubertal F1 17 months; no effect in prepubertal sheep but intolerance to glucose and reduced insulin sensitivity in post-pubertal F1		<u>study 2</u>: overfeeding at 14 weeks till 19 months; HFD: glucose intolerance and insulin resistance; BPA: adipocytes are hypertrophic in response to BPA and to the HFD but no interaction		the authors conclude that exposure to BPA during fetal life at levels found in humans can program metabolic outcomes that lead to insulin resistance, a forerunner of type 2 diabetes, with postnatal obesity failing to manifest any interaction with prenatal BPA relative to insulin resistance and adipocyte hypertrophy.	the study is of good quality and the conclusions of the authors are fully consistent with the data yielded
Yang *et al.*, 2016	C57 bl6 mice (5-week old)	In diet fed for 30 days with CD or HFD containing BPA so	50, 500, 5000, 50000 µg/d	(9-12/group)	10% increases in bw of both males and females; no dose-effect	increases of fat mass, inguinal WAT and epididymal WAT with higher number	almost no effect is seen in the groups of mice fed the high-fat diet	in vitro data and a human study complete the publication	The authors demonstrated the non monotonic dose effects of BPA on adiposity and chronic inflammation in 5-week-old mice	study of good quality

		that exposure is of 50, 500, 5000, 50000 µg/d				of large adipocytes vs small adipocytes; no effect on GTT; increases of C/EBPα, PPARγ, FABP4, Scd1, Srebp1c (dpdt on doses) in WAT				
Biasiotto *et al.*, 2016	Pregnant C57bl6 mice	Gavage (daily)	doses: 0.5, 5, 5, 500 µg/kg/day from the 2nd wk of pregnancy; continued until 140 pnd except from birth to weaning	Groups ranged from 16 to 19**.**	Mice were fed a standard diet. The number of pregnant females is not given but it can be estimated to be around 5-6.	Fat mass and BW enhanced starting from day 90 in mice dosed with BPA 5 microg/kg/day probably linked to the enhancement in the epididymal weight.	little although significant increases of Pparg, ATGL, HSL and LPL in epididymal fat and liver	This study encompasses different protocols which together provide evidence that BPA acts as an EDC acting through a nuclear receptor mediated mechanism		Study of good quality. Only males were studied.
Rubin et al., 2017	Pregnant CD-1 mice	**Perinatal exposure:** Osmotic subcutaneous pump from GD8 to PND21 **Peripubertal exposure:** drinking water from PND21 to PND35	Doses: 0,0.25,2.5,25,250 µg/kg/day	Groups ranged from 6 to 13 , except for body weight : prior to weaning: n = 44-56, after weaning: n = 20-28	**In the perinatal exposed group:** • Effects of BPA on body weight (m+f) No difference in body weight by exposure level prior weaning in males and females No difference in body weight by exposure level after weaning in males After weaning increased body weight in females depending on the dose and time of measurements • Effects on glucose homeostasis (f only)		**In the perinatal + peripubertal exposed group:** • Effects of BPA on body weight (m+f) No difference in body weight by exposure level prior weaning in males and females No difference in body weight by exposure level after weaning in males After weaning increased body weight in females depending on the dose (more pronounced in the 2.5 and 25 µg BPA P+P) and time of measurements • Effects on glucose homeostasis (tested	Both perinatal exposure alone and perinatal plus peripubertal exposure to environmentally relevant levels of BPA resulted in lasting effects on body weight and body composition. The effects were dose specific and sex specific and were influenced by the precise window of BPA exposure. The addition of peri-pubertal BPA exposure following the initial peri-natal exposure exacerbated adverse effects in the females but appeared to reduce differences in body weight and body	Study of good quality. So called "extremely hyperactive females" were excluded from the data analysis if their percent fat at the time of expected heightened hyperactivity was greater than 2 standard deviations below the mean of the remainder of their treatment group. Serum leptin, lipid accumulation in

					No effects reported on glucose or insulin levels following 6h fast No effects following Insulin Tolerance Test (ITT) or Glucose Tolerance Test (GTT) • Serum measurements (f only) Dose dependant increase of serum leptin in BPA tretated females No significant effect on triglycerides	only in the females 2.5 and 25 µg BPA) (f only) Increased glucose levels at 34 weeks after 6h fasting (2.5 µg BPA but not 25) Increased insulin levels at 28 and 34 weeks after 6h fasting (2.5 µg BPA) Significant decrease of insulin sensitivity in the BPA exposed P+P mice (2.5 and 25 µg) • Serum measurements (f only) Dose dependant increase of serum leptin in BPA tretated females No significant effect on plasma triglyceride levels (but a tendency) Significant increase in hepatic triglyceride levels at the two highest doses of BPA (25 and 250 µg/kg/day	composition between control and BPA exposed males. Some effects of BPA on body weight and body composition showed a non-linear dose response. Deleterious metabolic effects in females include hyperinsulinemia and development of insulin resistance.	liver, insulin/glucose homeostasis, were only measured in females , as well as for insulin sensitivity and glucose tolerance tests only performed in females

Table 3a: Summary of the available recent *in vitro* studies on bisphenol A on adipocytes insulin action

Ref.	Model	BPA doses	TG accumulation	Glucose uptake	GLUT 4	FABP4 (aP2)	Adiponectin / leptin	Possible MoA (MOA)				Insulin sensitivity (IS)	Comments of the authors	Expert's comments
	3T3-L1 cells or MSCs							ER	Pparg	GR	PI3K			
Biemann *et al.*, 2012	mesenchymal stem cells (MSCs)	10 µM	↘ if treatment covered the proliferation phase	no effect when BPA was added at confluence or during the differentiation process									BPA is an EDC interfering with estrogenic action and reducing the commitment of MSCs into adipocytes	the study is of good quality
Pereira-Fernandes *et al.*, 2013 2014,	3T3-L1 cells	12.5, 25, 50 µM	enhanced (x1.5) and x2 if combined to insulin *versus* x5 with ROSI 1 microM						very weak activity *versus* ROSI 1µM				BPA has a very weak PPARγ activity	the study is convincing
Valentino *et al.*, 2013	3T3-L1 cells	1 and 10 nM		↘	not ↗		↘ of Leptin				↘ (WB Phospho)	↘ IS	↘ of IS	BPA is reducing insulin sensitivity
Atlas *et al.*, 2014	3T3-L1 cells	0.1nM, 1 nM, 10 nM	enhanced in the absence of DEX (presence of Ins)			dose-dpdt ↗			Not directly				BPA can interact with the transcriptional machinery at the promoter of FABP4	BPA is astimulating FABP4 but the weak activation of Pparg by BPA cannot explain the FABP4 increase
Hélies-Toussaint *et al.*, 2014	3T3-L1 cells	1fM, 1pM, 1nM, 1µM	↗ with 1fM, 1pM, 1nM	no effect	not ↗ but Glut1 is increased	1pM ↗ srebp1c, pparg, aP2	no effect on leptin	ERRa (not ERRg) ↗ at 1 pM, 1nM					BPA could activate adipocyte differentiation through binding to ERRα or ERRγ.	It is not possible to determine if BPA modulated insulin sensitivity (no effect on glucose uptake).
Ariemma	3T3-L1	1nM	↗ TG	↘	not ↗	↗	no		↗	GR not	↘ (WB	↘ of IS	Glucose	BPA is reducing

***et al.*, 2016**	cells	added during all phases	accumulation				change in adipoQ; increase of leptin			regarded; ↗ of C/EBPα	Phospho)		utilisation and insulin signaling were reduced. All data converge to show adipocyte metabolic dysfunction and inflammation; and decreased insulin sensitivity in 3T3-L1 cells.	insulin sensitivity. However, inconsistency with regards to leptin (same lab as in Valentino 2013) .
Biasiotto *et al.*, 2016	3T3-L1 cells either cultured with BPA in basal medium (BM) or BPA + MDI	10, 50, 80 µM	↗ at 50 and 80 µM			↗ in MDI		↗ ERa in BM, Erb in MDI (ICI 182,780)	↗ in MDI (TO)				specific activation of ERα in undifferentiated cells and ERβ in differentiated cells. BPA also activated PPARγ; opposite action of DES	not classical *in vitro* protocol. Initial plating of cells is very low with 103 cells per 12-well culture plate (instead of 50,000 cells).
Ahmed, and Atlas, 2016	3T3-L1 cells	0.01 to 25 µM	↗ lipid accumulation (2.5>25 µM)			↗			↗; further ↗with ROSI				BPA requires PPARγ to induce adipogenesis	the use of GW9662 to demonstrate the involvement of PPARg in BPA induced effects is convincing (RT-qPCR and Western-blot analysis)

Table 3b: Summary of the available recent *in vitro* studies on bisphenol A glucuronide on adipocytes insulin action

Boucher et al 2015	3T3-L1 cells	0.01 to 10Mm of BPA glucuronide (BPA-G)	Increased TG (a significant 3 fold increase	Enhanced mRNA expression of LPL and Srebpf1 at	Enhanced protein expression of LPL, aP2 and			Use of the ER antagonist ICI and of 1 nM E2	No effect on Pparg mRNA	Dexamethasone is used as a positive control and RU486 as an antagonist			BPA-G is not an inactive metabolite BPA-induced adipogenesis	The concentration of 10 µM is several orders of

			at the highest dose of BPA)	the highest dose of BPA with no change in Pparg, CEBPa, aP2, adipsin, perilipin)	adipsin with 10 µM BPA-G			ICI inhibits the BPA-G induced increase of lipid accumulation and of aP2, LPL and adipsin protein expression No effect of BPA-G on ER transciptional activity (luciferase assay)	levels	of GR No effect of BPA-G on GR transcriptional activity (luciferase assay)			is neither mediated via the ER or the GR Effects on adipogenesis may be indirectly related to the ER pathway explaining the inhibitory effects of ICI	magnitude higher than what would be expected in the general population The authors did not bring the absolute proof that deconjugation of BPA-G did not occur in the in vitro models
Boucher et al., 2015	Primary human preadipocytes	0.05 to 0.25 µM BPA-G			Enhanced protein expression of aP2 with 0.05 and 0.25 µM BPA-G								BPA-G can induce differentiation of preadipocytes in a primary human cell model	

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