

**Substance Name: 4,4'-isopropylidenediphenol
(BPA, Bisphenol A)**

EC Number: 201-245-8

CAS Number: 80-05-7

MEMBER STATE COMMITTEE

SUPPORT DOCUMENT FOR IDENTIFICATION OF

**4,4'-ISOPROPYLIDENEDIPHENOL (BPA,
BISPHENOL A) AS A SUBSTANCE OF VERY HIGH
CONCERN BECAUSE OF ITS ENDOCRINE
DISRUPTING PROPERTIES WHICH CAUSE
PROBABLE SERIOUS EFFECTS TO HUMAN HEALTH
WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF
CONCERN TO THOSE OF CMR¹ AND PBT/vPvB²
SUBSTANCES**

Adopted on 14 June 2017

¹ CMR means carcinogenic, mutagenic or toxic for reproduction

² PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

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List of abbreviations

11-bHSD1: 11 β -hydroxysteroid dehydrogenase type 1
3D: Three-dimensional
3T2-L1: cell line derived from mouse cells, having a fibroblast-like morphology and under appropriate conditions, differentiate into an adipocyte-like phenotype
4-OHT: 4-hydroxy tamoxifen, active metabolite of tamoxifen.
5-HIAA: 5-Hydroxyindoleacetic acid (main metabolite of serotonin)
5-HT: 5-hydroxytryptamine or serotonin
5 α -R: 5 α -reductase
AB: Alveolar bud
Abl1: v-abl Abelson murine leukemia viral oncogene homolog 1
ABS: acrylonitrile-butadiene-styrene
ACC: acetyl-CoA carboxylase
AChE: acetylcholinesterase
ADH: Atypical Ductal Hyperplasia
ADHD: attention deficit hyperactivity disorder
AFSSA: French food safety agency (became ANSES in 2010)
AhR: aromatic hydrocarbon receptor
AIST: Japanese National Institute of Advanced Industrial Science and Technology
Akt: protein kinase B (PKB) mRNA
AMPA receptor: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANIA: french association for food industry
ANSES: Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail (French Agency for Food, Environmental and Occupational Health & Safety)
AOP: adverse outcome pathway
AR: androgen receptor
ARC: arcuate nucleus
AREG: amphiregulin
ART: assisted reproductive technology
ATGL: adipose triglyceride lipase
ATP: Adaptation to Technical Progress
AUC: area under the curve
AVPV: anteroventral periventricular nucleus
BADGE: bisphenol A diglycidyl ether
BAT: brown adipose tissue
BBP: Butyl benzyl phthalate
BDNF: brain-derived neurotrophic factor
BDP: bisphenol A bis (diphenyl phosphate)
Bis-DMA: bisphenol A demethacrylate
BM: basal medium
BMI: body mass index
BMP-15: bone morphogenic protein 15
BPA: bisphenol A
BrdU: Bromodeoxyuridine
BSEF: international bromine production organisation
bw: body weight
C/EBP α : CCAAT/enhancer binding proteins *alpha*, a protein shown to bind to the promoter and modulate the expression of the gene encoding leptin
CA: cornu ammonis (area of the hippocampus)
CAF: cancer-associated fibroblasts
CALUX: Chemical Activated LUCiferase gene eXpression
CaM: calmodulin
CamKI: calcium/calmodulin-dependent protein kinase type I

CamKIV: calcium/calmodulin-dependent protein kinase type IV
CaMKK: calcium/calmodulin kinase kinase
CARACAL: Competent Authorities for REACH and CLP
CAT: catalase
CBP: CREB-binding protein
CBX: carbenoxolone, inhibitor of 11 β -HSD
CD: normal Chow Diet
Ccnd2: cyclin D (cell cycle activator)
CDK: cyclin dependent kinase; Cdk4 binds Ccnd2 (cell cycle activator)
CETIM: technical centre for mechanical industries (France)
CHAMACOS: Center for the Health Assessment of Mothers and Children of Salinas study
CHDM: cyclohexanedimethanol
CI: confidence interval
CIS: Carcinoma in-situ
CKD: chronic kidney disease
CL: corpora lutea
CLH: harmonised classification and labelling
CLP: Classification, Labelling and Packaging
CMR: Carcinogen, Mutagen, Reprotoxic
CNS: central nervous system
COMIDENT: coordinating committee of dental activities (France)
CREB: cAMP response element-binding protein
Crtc1: CREB regulated transcription coactivator 1
CSR: chemical safety report
CYP45017a: 17 α -hydroxylase-17,20-desmolase (also named CYP17A1)
CYP450arom: cytochrome P450 aromatase (also named CYP19A1)
CYP450scc: P450 cholesterol side chain cleavage enzyme (also named CYP11A1)
DA: dopamine
DAT: dopamine transporter
dBPA: deuterated BPA
DCIS: Ductal Carcinoma in situ
DES: diethylstilbestrol
DEX: dexamethasone
DG: dendate gyrus
DHEA: dehydroepiandrosterone
DiE-1: diestrus-1
DIN: Ductal Intra-Epithelial Neoplasia
DMBA: dimethylbenzanthracene
DMSO: dimethyl sulfoxide
DMT: dimethyl terephthalate
DNA: deoxyribonucleic acid
DNEL: Derived no effect level
DNMT: DNA methyl transferase
DOHaD: developmental origin of health and disease
DOPAC: 3,4-Dihydroxyphenylacetic acid (metabolite of dopamine)
DPN: diarylpropionitrile (ER β antagonist)
DS: dossier submitter
E2: 17- β estradiol
EB: estradiol benzoate
EC: European Commission
ECHA: European Chemical Agency
ECM: Extracellular matrix
ECN: embryo cell number
ED: endocrine disruptor
EDC: endocrine disruptor chemical
EE2: ethinylestradiol

EFS: embryo fragmentation score
 EFSA: European Food Safety Agency
 EGF: Epidermal Growth Factor
 EGPF: enhanced green fluorescent protein
 ELF-5: E74-like factor 5
 ELoC: equivalent level of concern
 EMT: epithelia-mesenchymal transition
 ENU: N-ethyl-N-nitrosourea
 EPA: eicosapentaenoic acid
 ER: estrogen receptor
 ErbB4: v-erb-a erythroblastic leukemia viral oncogene homolog 4
 EREs: estrogen response elements
 ERK1: extracellular-signal-regulated kinase 1
 ERK/MAPK: extracellular signal-regulated kinase/ mitogen-activated protein kinase
 ERR: estrogen related receptor
 ERR γ or ERR γ : Estrogen-related receptor gamma
 Era or ER α : estrogen receptor α
 ER β or ER β : estrogen receptor beta
 ERT: estrogen replacement therapy
 Ets: v-ets erythroblastosis virus E26 oncogene
 EU: European Union
 EU28: European Union with its 28 member states (since 2013)
 EuPC: European plastic converters
 EZH2: Enhancer of Zeste Homolog 2, a histone methyltransferase that has been linked to breast cancer risk and epigenetic regulation of tumorigenesis.
 EZH2: Enhancer of Zeste Homolog 2
 FABP4: fatty acid binding protein 4
 FAO: food and agriculture organization of the United Nations
 FAS: Fatty acid synthase
 FDA: US Food and Drug Administration
 FN: fibronectin
 FOPH: Swiss federal health authority
 FSH: follicle stimulating hormone
 FVB: Friend leukemia virus B
 G15: specific GPER inhibitor
 GAA1: glycosyl phosphatidyl-inositol anchor attachment protein
 GABA: gamma-aminobutyric acid, main inhibitory neurotransmitter in the central nervous system
 GAS: g-interferon activation sequence
 GATA4: a transcription factor characterised by its ability to bind to the DNA sequence "GATA"
 GD: gestation day
 GDF-9: growth/differentiation factor 9
 GDM: gestational diabetes mellitus
 GH: Growth hormone
 Glu: glutamate
 GLUT4: Glucose transporter type 4
 GnRH: gonadotrophin-releasing hormone
 GPER or GPR30: G protein-coupled estrogen receptor or G protein-coupled receptor 30, the membranous form of estrogen receptor
 GPER-1: G protein-coupled estrogen receptor-1³ .
 GR: glucocorticoid receptor (NR3C1)
 GRF: guanine nucleotide-releasing factor

³ coordinates fibronectin (FN) matrix assembly and release of heparin-bound epidermal growth factor (HB-EGF). This mechanism of action results in the recruitment of FN-engaged integrin $\alpha 5 \beta 1$ to fibrillar adhesions and the formation of integrin $\alpha 5 \beta 1$ -Shc adaptor protein complexes

GSH: Glutathione
 GSIS: Glucose-Stimulated Insulin Secretion
 GSK2beta: glycogen synthase kinase 3 beta
 GTT: glucose tolerance test
 GW9662: selective PPAR γ antagonist
 HBEC: human breast epithelial cell
 HB-EGF: heparan binding epidermal growth factor
 HDL-C: high density lipoprotein – cholesterol
 HEAL: Health and Environment Alliance (NGO)
 hESC: Human embryonic stem cells
 HFD: High Fat Diet
 HGFL: hepatocyte growth factor-like protein
 HMEC: Human mammary epithelial cells
 HOMA: homeostatic model assessment of insulin resistance
 HOTAIR: HOX transcript antisense RNA
 HOX genes: a subset of homeotic genes that control the body plan of an embryo
 HPG: hypothalamic–pituitary–gonadal axis
 HRG1: heregulin 1
 HSD3 β : 3 β -hydroxysteroid dehydrogenase
 HSL: hormone-sensitive lipase
 IAPP: Islet Amyloid PolyPeptide
 ICC: islet cells cluster
 ICI 182,780: specific inhibitor of ER α / β ⁴
 ICR: Institute of Cancer Research
 ICSI: intra-cytoplasmic sperm injection (ICSI)
 ICV: intracerebroventricular
 ID2: inhibitor of DNA binding 2
 iDMEC: induced differentiated mammary epithelial cell
 IGF-1: insulin-like growth factor-1
 IGF-2: insulin-like growth factor 2
 IGT: impaired glucose tolerance
 IKK α : Inhibitor of kappa B kinase alpha
 INERIS: French institute for environment and industrial risks
 INS-1 Cells: pancreatic beta cells line
 IOELV: indicative occupational exposure limit values
 ipGTT: intraperitoneal glucose tolerance tests
 ipITT: intraperitoneal insulin tolerance tests
 IQ: intelligence quotient
 IR: insulin receptor
 IRS-1: insulin receptor substrate 1
 IS: insulin sensitivity
 IVF: in vitro fertilisation
 JAK2: janus 2 kinase
 JRC: Joint Research Center, the European Commission’s in-house science service
 KATP: ATP-sensitive potassium channel (or KATP channel)
 KCC2: potassium chloride cotransporter 2
 KEWI-WISC: Korean Educational Development Institute’s Weschler Intelligence Scales for Children
 KGN: human cell line from ovarian granulosa cell tumor
 Kiss1R: kisspeptin receptor
 LBD: ligand binding domain
 LDES: Learning Disability Evaluation Scale
 LDL-C: low density lipoprotein -cholesterol

⁴ called also faslodex (Wakeling, 1992) acts in an anti-estrogenic manner in all tissues analysed (Dowsett *et al.*, 1995; Sibonga *et al.*, 1998).

Ldlr: Low density lipoprotein receptor
 LEF1: transcription factor of the HMG-box family⁵
 LH: luteinising hormone
 LIN: Lobular Intra-Epithelial Neoplasia
 LMO4: LIM domain only 4
 LN: lymph node
 LOAEL: lowest observed adverse effect level
 LPL: lipoprotein lipase
 LRH1: liver receptor homolog 1
 LTD: long-term depression
 MAPK: mitogen-activated protein kinase
 MAZE test: an appetite-motivated maze test
 MBP : methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene
 MCF: human breast epithelial cell lines
 MDA: Malondialdehyde
 MDI: induction medium containing methylisobutylxanthine, dexamethasone, insulin; used in protocols to induce differentiation of 3T3-L1 cells into adipocyte-like cells
 MEK: mitogen-activated protein kinase
 mGluR: metabotropic glutamate receptor
 MMTV: mouse mammary tumor virus
 MoA: mode of action
 MoE: margin of exposure
 mPFC: median prefrontal cortex (area of the hippocampus)
 MR: mineralocorticoid receptor (NR3C2)
 mRNA: messenger ribonucleic acid
 MSC: member state committee
 MSCs: Mesenchymal Stromal Cells
 MSCA: McCarthy scales of children's abilities
 MWM: Morris water maze
 NAC: N-acetylcystein
 NCTR: US National Center for Toxicological Research
 ND: no data or not determined
 NE: norepinephrine
 NEFA: Non-Esterified Fatty Acids
 NF- κ B: nuclear factor κ B
 NGO: non-profit organisation
 NHANES: US National Health and Nutrition Examination Survey
 NHS: nurses' health study
 NHSII: nurses' health study II
 Nlgn3: neuroligin 3
 NMDA: N-methyl-D-aspartate
 NMDAR: NMDA receptor
 NMU: N-nitroso N-methylurea
 NOAEL: no observed adverse effect level
 NR: mineralocorticoid receptor
 Nrnx1: neurexin 1
 NTP: US National Toxicology Program
 Nur77: nuclear receptor of the Nur family that act as transcription factors in neuron development and maintenance; also known as nerve growth factor IB (NGFIB)
 OEL: occupational exposure limit
 OP: object placement
 OR: object recognition
 ORa: odds ratio

⁵ it is activated early in all ectodermal rudiments (including hair, whiskers, mammary glands, and teeth). In its absence, those organs fail to develop.

OVLT: organum vasculosum, lamina terminalis
 OVX: ovariectomised
 P16: sequesters Cdk4 and prevent its interactions with D cyclins acting as an effector of cycle arrest and cellular senescence
 PCNA: proliferating cell nuclear antigen
 PC: polycarbonate
 PCOS: polycystic ovary syndrome
 PCSK1: gene encoding for proprotein convertase 1, an enzyme acting in the process of proinsulin and proglucagon in pancreatic islets
 PD 98059: inhibitor of ERK1/2.
 PE: proestrus
 PET: polyethylene terephthalate
 PFC: prefrontal cortex
 PGF2 α : prostaglandin F2-alpha
 PI3K: phosphoinositide 3-kinase
 PKA: cAMP-dependent protein kinase
 PKB: protein kinase B
 PL: prolactin
 PND: postnatal day
 PNW: postnatal week
 PPAR γ : Peroxisome Proliferator Activated Receptor gamma
 PPRE: PPAR γ Response Element
 PPT: propyl pyrazoletriol (ER α agonist)
 PPT-A: preprotachykinin A⁶
 PR: progesterone receptor
 PRC: polycomb repressive complex
 PRL: prolactin
 PrIR: prolactin receptor
 PSD: post synaptic density
 PTHrP: Parathyroid hormone-related peptide⁷
 PVC: polyvinyl chloride
 PWD: Post Week Day
 PXR: pregnane X receptor
 RAC: Risk Assessment Committee
 RAL: raloxifene⁸
 RANKL: receptor activator of NF κ B ligand
 RCR: risk characterisation ratio
 REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals
 RFRP-3: RFamide-related peptide-3
 RiME: Risk Management Expert meeting
 RISS: Japanese research Institute of Science for Safety and Sustainability
 RIVM: Netherlands National Institute for Public Health and the Environment
 RMM: risk management measures
 ROS: Reactive Oxygen Species
 ROSI: Rosiglitazone
 RP3V: rostral periventricular area of the third ventricle
 RT-qPCR: reverse transcription quantitative polymerase chain reaction
 RU-486: mifepristone, used as an antagonist to glucocorticoid

⁶ PPT-A gene products lead to Substance P and neurokinin A that are members of the mammalian tachykinin peptide family, currently referred to as neurokinins (1456). The physiology of these peptides are linked to neurotransmission.

⁷ Inactivation of either the PTHrP peptide or the receptor prevents formation communication of androgen receptors and outgrowth of the primary sprout at E16 while it does not affect early bud formation.

⁸ Raloxifene acts in an agonistic manner on the bone (Turner *et al.*, 1994), but as an antagonist in the mammary gland (Buelke-Sam *et al.*, 1998).

RXR: retinoid X receptor
 SC: subcutaneous route
 SCD-1: stearoyl-CoA desaturase
 SCENIHR: scientific committee on emerging and newly identified health risks
 SCOEL: Scientific Committee on Occupational Exposure Limits
 SD: Sprague Dawley
 SDN: sexual dimorphic nucleus
 SE: standard error
 SEAC: committee for socio-economic analysis
 SERM: Selective ER modulator (Dhingra, 1999).
 SF-1: steroidogenic factor 1 (transcription factor)
 SMA: smooth muscle actin (marker of myoepithelial cells)
 SNFBM: national association of manufacturers of packaging boxes and metal capping (France)
 SNITEM: national association of medical technology industry (France)
 SOCS: suppressor of cytokine signaling proteins
 SOD: superoxide dismutase
 SRC3 (AIB1): coactivator which plays a key role in transcriptional activation of several estrogen-regulated genes⁹
 SREBP-1C: sterol regulatory element binding protein-1C
 SRp20: splicing factor arginine/serine-rich 3
 ss: statistically significant
 StAR: steroid acute regulatory protein
 STAT: signal transducer and activator of transcription
 STOT RE: specific target organ toxicity after repeated exposure (hazard class from CLP)
 SVF: Stromal Vascular Fraction
 SVHC: substance of very high concern
 T0070907: a potent PPAR γ antagonist
 Tamoxifen: anti-estrogen¹⁰
 TAS: total antioxidant status
 TBBPA: tetrabromobisphenol A
 TBT: PPAR γ agonists
 TD: Terminal Duct
 TD2M: Type-2 diabetes mellitus
 TDI: tolerable daily intake
 TDLU: Terminal Ductal Lobular Unit
 TEB: Terminal End Bud
TFF1 (or *PS2*): estrogen-responsive gene trefoil factor
 TG: triglyceride
 TH: tyrosine hydroxylase
 TMCD: 2,2,4,4-tetramethyl-1,3-cyclobutanediol
 Tnc: tenascin
 TNF α : Tumor Necrosis Factor alpha
 TO: T0070907, used as a PPAR γ antagonist
 ToxRTool: Toxicological data Reliability assessment Tool
 TUNEL: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling
 Ucp1: a marker of energy expenditure through thermogenesis
 UDMA: urethane dimethacrylate
 US EPA: US Environmental Protection Agency
 VO: vaginal opening

⁹ including PS2 gene (Shao *et al.*, 2004; Labhart *et al.*, 2005), followed the similar pattern as the ER α

¹⁰ Tamoxifen is widely used in the therapy of breast cancer (Gradishar et Jordan, 1997; Wakeling *et al.*, 1991), displays agonistic activity on the endometrium (Jordan et Morrow, 1994), but antagonistic activity on the mammary gland (Jaiyesimi *et al.*, 1995).

WAP: whey acidic protein

WAT: White Adipose Tissue

WB: Western blott

WHO: World Health Organization

WT: Wild Type

XIAP: x-linked inhibitor of apoptosis protein

IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

Substance Name: 4,4'-isopropylidenediphenol (Bisphenol A, BPA)

EC Number: 201-245-8

CAS number: 80-05-7

- The substance is identified as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of the REACH Regulation.

Summary of how the substance meets the criteria set out in Article 57 of the REACH Regulation

Bisphenol A is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with *endocrine disrupting properties* for which there is scientific evidence of probable serious effects to human health which gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

Bisphenol A has a harmonised classification for the hazard class Reproductive toxicity category 1B (H360F 'May damage fertility') based on effects on reproductive function. BPA has been identified recently as SVHC according to Article 57(c) of REACH and included in the Candidate List by decision of ECHA ED/01/2017 of 4 January 2017.

BPA has been shown to affect the reproductive function, mammary gland development, cognitive function and metabolism through pathways that commonly involve disruption of estrogenic regulation. The effects on female reproductive function include the induction, after both developmental and adult exposures, of cystic ovaries, changes in the uterus morphology, alteration of fertility parameters as well as estrous cycle disturbance. The estrous cycle is a perfectly synchronised and sequenced event that relies on a permanent endocrine dialogue between the ovary and the hypothalamo-pituitary system. Those pathways differentiate during fetal life and are largely influenced by numerous factors and in particular the steroid environment of the foetus. BPA at the adult stage alters the endocrine steroidogenic function of the ovary and more specifically the production of estrogens by the follicle, leading to disturbance in the estrous cycle. At the neuroendocrine level, BPA can also act during the perinatal/postnatal organisation or adult activation of the hypothalamus-pituitary system through changes in kisspeptin, gonadotrophin-releasing hormone (GnRH) expression, activity or liberation and sex steroid receptor expression that impact estrous cyclicity.

The effects on the mammary gland, depending on the period of exposure, include: modifications in the mammary tissue such as an increased number of terminal end buds (TEBs) relative to the ductal area, fewer apoptotic TEB cells, increased lateral branching and ductal hyperplasia, increased cell proliferation and decreased apoptosis in the glandular epithelium, ductal (and occasionally lobuloalveolar) and intraductal hyperplasia - ultimately increasing its susceptibility to chemical carcinogens. These effects were observed in rodent or in non-human primate following prenatal and/or post-natal exposure to BPA. Available data also support the plausibility that BPA, through interaction with the nuclear estrogen receptors (ERs), or G protein-coupled estrogen receptor (GPER) and indirectly with the progesterone receptor (PR), modulates estrogen and progestin agonist

activities. Emerging epigenetic studies have suggested changes related to estrogen-dependent genes (such as EZH2 and HOTAIR), as well as HOX genes (involved in embryogenesis and post-natal development) which could be associated with BPA induced abnormal development and increased cancer susceptibility of the mammary gland.

BPA has been reported to alter memory and learning after developmental, pubertal or adult exposure, based on multiple converging experimental studies reporting this functional effect as well as molecular and cellular changes in the brain (reduced expression of NMDAR, altered synaptogenesis). These effects involve disturbance of estrogenic pathways as evidenced by the reversal of the functional, cellular and molecular effects of BPA by an ER antagonist and interference of BPA with estradiol-induced effects on behavior and spine density/neurogenesis.

The effects of BPA on metabolism in rodent and non-rodent after prenatal and/or perinatal or adult exposures include alteration of insulin secretion and/or release by β -pancreatic cell, or of insulin signalisation (signaling mechanisms) within insulin-sensitive organs (i.e., liver, muscle, adipose tissues) leading to variations in the expression levels of hepatic or adipose tissue markers which are indicative of a state of insulin resistance. It is therefore considered that BPA may increase the incidence of type-2 diabetes. Additionally, *in vivo* and *in vitro* experimental studies indicate that these effects may involve ER α , ER β or GPR30 pathways. 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. Most of the *in vitro* studies showing adverse effects of BPA on adipocyte differentiation and function point to alteration of endocrine mechanisms (e.g., adiponectin release, insulin signaling cascade effectors). Overall, it is suggested that the pancreas is targeted by BPA, that the mechanisms could differ depending on the period of exposure (fetal life or adulthood) and that an ED MoA is involved. Lastly, mainly based on similarities in homeostatic regulation of insulin production and sensitivity between animals and humans, these effects are considered relevant for humans.

The steps of the respective mechanisms of action are specific for each effect. The complexity of the toxic response to BPA suggests multiple MoA that may interact but **most importantly, the available evidence shows that disruption of the estrogenic pathway is central and consistently involved in each of the four effects.**

In conclusion, on the basis of evidence available in relation to alteration of reproductive function, mammary gland development, cognitive function and metabolism, BPA can be considered an endocrine disruptor for human health.

It is not excluded that BPA may also alter other physiological functions, e.g. the immune function, through a similar ED MoA but the level of evidence is considered insufficient at the moment for this effect to be presented.

The range of experimental effects induced by BPA in relation to its ED MoA is predictive of serious health outcomes. All these ED-related effects are characteristically (but not only) observed after developmental exposure to BPA, with consequences that are observed later in life. As they appear a long time after the exposure, they are indeed considered permanent and irreversible. In addition, the effects of BPA are associated with conditions that may lead to a reduced quality of life. In particular breast cancers, neurobehavioural disorders and diabetes are observed with high prevalence and increasing trends during the last decades in Europe and raise indisputable societal concern, also in relation to their potential economic burden on the health systems. Finally, for each of the four effects, the database shows important uncertainties in establishing a quantitative dose-response as well as safe levels, with some studies identifying effects at doses below the point of departure used by RAC for DNEL derivation and on-going discussions on the shape of the dose-response relationship and the parameters impacting the dose-response (period of

exposure and concomitant presence of estrogen in particular). Overall, based on the WoE presented, BPA is identified as an SVHC according to Article 57(f) for probable serious effects on human health, due to its endocrine disrupting properties, which are of ELoC.

Registration dossiers submitted for the substance: Yes

Justification

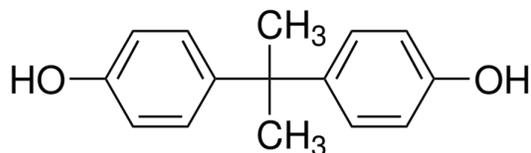
1. Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-245-8
EC name:	4,4'-isopropylidenediphenol
CAS number (in the EC inventory):	80-05-7
Deleted CAS numbers:	27360-89-0; 28106-82-3; 37808-08-5; 137885-53-1; 146479-75-6; 1429425-26-2
CAS name:	phenol, 4,4'-(1-methylethylidene)bis-
IUPAC name:	4,4'-propane-2,2-diylidiphenol
Index number in Annex VI of the CLP Regulation	604-030-00-0
Molecular formula:	C ₁₅ H ₁₆ O ₂
Molecular weight range:	228.29 g/mol
Synonyms:	Bisphenol A BPA 2,2-bis(4-hydroxyphenyl)propane

Structural formula:



1.2 Composition of the substance

Name: 4,4'-isopropylidenediphenol (Bisphenol A)

Description: 80-100%

Substance type: mono-constituent

Table 2: Constituents other than impurities/additives

Constituents	Typical concentration	Concentration range	Remarks
4,4'-isopropylidenediphenol (<i>bisphenol A</i>) (EC 201-245-8)	80-100%	No information	

Table 3: Impurities

Impurities	Typical concentration	Concentration range	Remarks
<i>None relevant for SVHC identification</i>			

Table 4: Additives

Additives	Typical concentration	Concentration range	Remarks
<i>None relevant for SVHC identification</i>			

1.3 Identity and composition of degradation products/metabolites relevant for the SVHC assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) REACH.

1.4 Identity and composition of structurally related substances (used in a grouping or read-across approach)

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) REACH.

1.5 Physicochemical properties

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) REACH.

2. Harmonised classification and labelling

Bisphenol A is covered by Index number 640-030-00-0 in part 3 of Annex VI to the CLP Regulation as follows, as amended by Commission Regulation (EU) 2016/1179 (9th ATP)¹¹:

Table 5: Classification according to Annex VI, Table 3.1 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008 (as amended by Commission Regulation (EU) 2016/1179)

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Spec. Conc. Limits, M-factors	Notes
				Hazard Class and Category code(s)	Hazard statement code(s)	Pictogram, Signal Word code(s)	Hazard statement code(s)	Suppl. Hazard statement code(s)		
604-030-00-0	bisphenol A; 4,4'-isopropylidenediphenol	201-245-8	80-05-7	Repr. 1B STOT SE 3 Eye Dam. 1 Skin Sens. 1	H360F H335 H318 H317	GHS08 GHS07 GHS05 Dgr	H360F H335 H318 H317			

3. Environmental fate properties

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) REACH.

4. Human health hazard assessment

Context

As summarised by Kortenkamp *et al.* (2016), the endocrine disrupting properties of Bisphenol A (BPA) have been discovered through screening exercises back in the 1930's. Chemists synthesised the chemical BPA in the laboratory in 1891. In the 1930's, scientists were searching for synthetic chemicals that could replace the expensive natural estrogen in pharmacological applications. They identified BPA as a weak functional estrogen. Its use as a pharmaceutical hormone was precluded by the synthesis of another chemical, diethylstilbestrol (DES), with even more potent estrogenic properties (Dodds and Lawson, 1938). DES was subsequently used as a pharmaceutical but showed severe side effects (Meyers, 1983).

General approach

The WHO/IPCS (2002) definition of an endocrine disruptor (ED) is widely accepted:

"An endocrine disruptor is an exogenous substance or mixture that

- alters function(s) of the endocrine system
- and consequently causes
- adverse health effects in an intact organism, or its progeny, or (sub)populations."¹²

¹¹COMMISSION REGULATION (EU) 2016/1179 of 19 July 2016 amending, for the purposes of its adaptation to technical and scientific progress, Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures

¹² Exact text quoted from WHO/IPCS (2002) definition but formatting of the text using bullet points added to

The European Commission's Endocrine Disrupters Expert Advisory group agreed in 2013 *"that the elements for identification of an endocrine disrupter were demonstration of an adverse effect for which there was convincing evidence of a biologically plausible causal link to an endocrine disrupting mode of action and for which disruption of the endocrine system was not a secondary consequence of other non-endocrine-mediated systemic toxicity. Relevance of the data to humans should be assumed in the absence of appropriate data demonstrating non-relevance."* (JRC 2013)

It is assumed in this report that a substance should fulfill the recommendations from the European Commission's Endocrine Disrupters Expert Advisory group outlined above in order to be identified as an endocrine disruptor, and available information has accordingly been assessed based on:

- Adverse health effects
- Endocrine mode of action (MoA)
- Plausible link between adverse effects and endocrine MoA
- Human relevance

As discussed in Kortenkamp *et al.* (2012) there is a clear distinction between the terms MoA and mechanism of action. The mechanism of action is typically defined as the totality of mechanistic steps, whereas the MoA refers to a less detailed sequence of key events. MoA is not intended to build a comprehensive model of a chemical mechanism of action. This is also in line with definitions proposed in the OECD guidance document on adverse outcome pathways (AOP) (OECD, 2013): "MoA differs from mechanism, in that the MoA requires a less detailed understanding of the molecular basis of the toxic effect".

For the purpose of the present analysis, the following definition is retained for MoA: "A sequence of key cellular and biochemical events with measurable parameters that result in a toxic effect."

BPA has been shown to trigger various adverse effects on health, as previously discussed at the EU level in the regulatory context of CLP (CLH dossier on fertility, ANSES 2013a) and REACH (restriction proposal of BPA in thermal paper, ANSES 2014). An ED MoA is considered to play a substantial role for several of these effects and it is important to consider and analyse in this report the scope of effects that are at stake when considering the ED properties and potential health impacts of BPA.

The adverse effects of BPA to be reviewed in the present dossier have been selected on the basis of the following criteria:

- The adverse effect is identified with a sufficient level of evidence in previous regulatory EU discussions, i.e. acknowledged by either a harmonised classification or by consideration of the effect in the process of risk assessment by RAC in the context of restriction (ECHA, 2015).
- There is relevant evidence of an ED MoA.

These criteria were considered to be fulfilled for the following adverse effects of BPA in relation to their ED MoA:

- Effect on the reproductive function
- Alteration of mammary gland development
- Alteration of brain development and cognitive function

- Metabolic alterations

It is noted that the three last effects are not acknowledged by a harmonised classification. Indeed, with respect to regulatory priorities in harmonisation as emphasised in Article 36(3) of CLP and for the sake of regulatory efficiency, the harmonisation procedure of BPA in the classification dossier (ANSES, 2013a) focused only on effects on fertility. However, these additional effects were included in a previous restriction analysis and already discussed by RAC. Harmonisation of the identification of these additional effects *via* harmonised classification is not expected to result in any major additional regulatory impacts and is therefore not foreseen.

Each of these effects will be presented in a separated section. Depending on the complexity and level of evidence for each effect, a more specific adverse effect may be chosen, described and analysed for its ED-related MoA in more detail for the sake of producing a clear and detailed analysis representative of BPA's ED MoA.

Other effects of BPA on human health e.g. immunotoxicity, may involve an ED MoA. However, the adversity of these effects and/or the ED MoA may not yet be clearly demonstrated with a sufficient level of evidence. They are shortly discussed in the general conclusion to ensure a comprehensive picture of the substance effects. Moreover, effects of BPA on the environment in relation to an ED MoA are not addressed in this dossier.

Considerations related to the relevance of data

The presentation of the specific adverse effects selected and the analysis of their ED MoA are generally based on literature searches up to May 2016.

Many studies have investigated the effects of BPA by oral route of exposure as well as by subcutaneous route of administration. As discussed in detail in a previous report (ANSES, 2014), when BPA is administered by subcutaneous route, the daily dose can be controlled with greater accuracy. However, subcutaneous administration bypasses the digestive barrier, intestinal and/or skin metabolism and the enterohepatic first-pass effect. The data collected in rodents show significantly higher proportions of unconjugated BPA after subcutaneous and intraperitoneal administrations, than in the case of an oral administration. It is generally well accepted that unconjugated BPA is the biologically active form. In humans, after oral exposure, BPA is known to undergo a high hepatic first-pass effect resulting in a short half-life (< 6 hours) and a low systemic availability of unconjugated BPA. In rats, BPA is also predominantly glucuronidated, but the BPA-glucuronide formed undergoes enterohepatic recirculation resulting in slower elimination of BPA (half-lives between 20 and 80 hours). The oral administration of an equivalent dose may result in higher plasma levels of unconjugated BPA in rats compared to humans. However, in rodents and to a lesser extent in humans, the hepatic metabolism capacity in newborns is limited, resulting in a reduced hepatic first-pass effect. A study in non-human primates (VandeVoort et al 2016) indicates that rapid maternal metabolism of BPA by oral route does not alleviate exposure to the developing fetus. Therefore, the effects observed after subcutaneous exposure are considered directly relevant when the exposure has taken place during the developmental period.

In addition, other non-artificial routes of exposure such as the dermal route or inhalation also bypass hepatic first pass. Although dose correction would be required in the context of risk assessment, the subcutaneous route of exposure is therefore considered relevant for the purpose of the present hazard identification and MoA considerations.

The other routes of administration used (intracerebral, intraperitoneal) are anecdotal and the corresponding studies were considered only in relation to the analysis of the MoA.

The selection of the studies have not been restricted to specific levels of doses and both

“low doses” as well as “standard doses” for regulatory testing have been considered as relevant for the identification of adverse effects and the understanding of the MoA. It is, however, recognised that the MoA may have a different pattern and modulations across the whole range of doses.

Finally, although not considered as relevant for the identification of an adverse effect, studies performed in non-intact animals (i.e. ovariectomised animals) were included for the understanding of the MoA.

Systematic rating of studies using Klimisch scores was not considered relevant for the present analysis. Klimisch scores are intended to provide a score in comparison to standard regulatory guidelines. However, several of the specific adverse effects examined in the present analysis (e.g. alteration of the mammary gland development, alteration of neural structures and alteration of insulin production and insulin sensitivity) are not addressed in any specific guideline protocol. In addition, the analysis was focused on studies that preferentially investigate mechanistic aspects and not only the adverse effect. Standard regulatory guideline studies are for this purpose neither required nor generally conducted for practical reason as very complex protocols would be needed to additionally dig into specific parameters. Finally, for most adverse effects, a very large database of studies is available and not all studies provide similar results. Therefore it was considered most relevant to follow a weight of evidence (WoE) approach for the present analysis. As defined in ECHA’s Practical Guide: How to use alternatives to animal testing (ECHA 2016) “The weight of evidence approach commonly refers to combining evidence from multiple sources to assess a property under consideration”. As discussed in the guide, the WoE approach is beneficial when the information from each source individually may be regarded as not sufficient and when several available studies give conflicting results. It also emphasizes that “Expert judgement is vital in the construction and appraisal of the WoE package, namely when considering the reliability, relevance and adequacy, integrating and comparing different pieces of information and assigning a weight to each piece of data.”

The present analysis was performed in collaboration with the ANSES’ Thematic Working group on Endocrine Disruptors¹³. Scientific studies considered irrelevant due to major deficiencies in their design and/or reporting were not included in the analysis and are not presented in the report. The studies were considered on the basis of their relevance, reliability and adequacy for the analysis and were qualitatively weighted based on expert judgement to produce a conclusion on the selected adverse effects and their ED MoA. Human data were analysed together. Experimental data were compared to each other with specific consideration given to the periods of exposure in particular. The conclusion of the WoE for each effect was based on the combination of human and experimental *in vivo* and *in vitro* data.

In order to provide further support and transparency to the robustness of the conclusions, ToxRtool was used to assess the reliability of the experimental studies that were considered as the most informative to reach the conclusions. ToxRtool¹⁴ was developed by the European Commission's Joint Research Center in 2009 (Segal *et al.*, 2015) and builds on Klimisch categories by providing additional criteria and guidance for assessing the reliability of toxicological studies. It is applicable to various types of experimental data, endpoints and studies (study reports, peer-reviewed publications).

The rating of the most informative studies to reach the conclusions resulted in a reliability score of 1 (reliable without restriction) or 2 (reliable with restriction) according to ToxRtool. They are identified by a grey shade in the summary tables in each section. Subcutaneous route was considered a relevant route of exposure as discussed above. In some cases, the use of a single dose level in a study was not systematically considered as a substantial flaw

¹³ <https://www.anses.fr/en/content/endocrine-disruptors>

¹⁴ <https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/toxrtool>

but its score was downgraded of one category in the rating in order to take into account this weakness. This final scoring reflects the overall quality of the study itself and its context. For example, studies conducted with one dose level by a same research group having previously performed studies with multiple dose levels, were considered as reliable.

4.1 Binding of BPA to hormonal receptors

With two hydroxyphenyl rings, BPA has structural features (Figure 1: Chemical structure of 17 β -estradiol (E2), diethylstilbestrol (DES) and Bisphenol A (BPA)) that confer the ability to bind to the two nuclear estrogen receptors α and β (ER α and ER β) (INSERM, 2011).

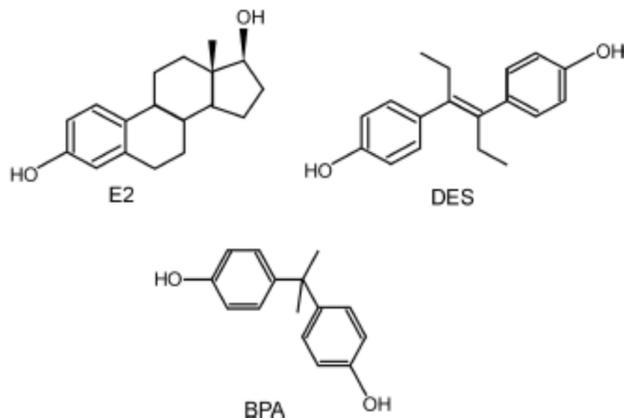


Figure 1: Chemical structure of 17 β -estradiol (E2), diethylstilbestrol (DES) and Bisphenol A (BPA). From INSERM 2011.

Kuiper *et al.* (1998) have measured the binding affinity of BPA to ER β , which is 10,000-fold lower than 17 β -estradiol. Similar results were obtained for ER α by Lee *et al.* (2012). On this basis, BPA is generally considered as a weak agonist of ER α and ER β . Examining the ER conformation, Acconcia *et al.* (2015) however reported that BPA may act as an ER α agonist while BPA does not allow the ER β ligand-binding domain to assume the right conformation, thus acting as an antagonist.

In addition, several studies indicate that BPA may exert effects at lower concentrations, compared to ER-binding effective doses, and very quickly through the involvement of extranuclear receptors. Studies by Watson *et al.* (2007 and 2010) have identified quick (< 1 minute) responses with low doses (1 picomolar) of BPA mediated through ER that are localised in the plasma membrane.

BPA may also interact with the transmembrane G protein-coupled estrogen receptor 1 (GPER or GPR30) (Wozniak *et al.*, 2005). GPER is largely recognised as the mediator of 'rapid nongenomic' effects by modulating second messengers and kinase pathways, which may also regulate gene expression (Filardo *et al.*, 2000; Prossnitz and Maggiolini 2009).

BPA was also identified as an estrogen-related receptor γ (ERR γ) ligand (Takanayagi *et al.*, 2006; Abad *et al.*, 2008; Okada *et al.*, 2008b), of which the specific physiological functions are unknown. The ERR γ does not bind to estrogens, however ERR γ can bind to estrogen response elements. The ERR γ is highly expressed in the mammalian brain during development as well as in the brain, lung and other tissues in adults (Acconcia *et al.*, 2015). ERR γ is a constitutively active receptor and the affinity of BPA to ERR γ is in the order of magnitude of the nanomolar.

As reported in INSERM (2011), several studies (e.g. Sohoni *et al.*, 1998; Li *et al.*, 2010) have shown that BPA binds to the androgen (AR) nuclear receptor. BPA is an antagonist

for AR and its affinity is in the order of the micromolar causing a moderate anti-androgenic effect. Structural binding characterization of BPA and of its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) using molecular docking simulation approaches revealed novel interactions of MBP with AR and the progesterone receptor (PR) and of BPA with PR (Rehan *et al.*, 2015).

Direct binding of BPA to the glucocorticoid receptor is also observed (EC₅₀ of 18.82 µM) by Zhang *et al.* (2017).

Halogenated BPA-derivates, but not BPA, have been shown to bind to thyroid hormones receptors (Kitamura *et al.*, 2002).

Finally, BPA may also activate other cellular receptors although no direct binding has been established. BPA is able to induce the expression of the nuclear receptor involved in the proliferation of PPAR γ (Kwintkiewicz *et al.*, 2010). Some studies report a modification of the aromatic hydrocarbon receptor (AhR) expression (Nishizawa *et al.*, 2005; Bonefeld-Jørgensen *et al.*, 2007). BPA is able to activate retinoid X receptors (RXR) in a reporter gene assay with metabolism (Li *et al.*, 2008), to act as a potent agonist for human (but not mouse) pregnane X receptor (PXR) (Sui *et al.*, 2012) and to increase the expression of nuclear receptor Nur77 (Song *et al.*, 2002; Ahn *et al.*, 2008) involved in steroidogenesis.

4.2 Effect on the reproductive function

4.2.1 Overview of previous evaluation of BPA's effect on reproductive function

Overview

Several recent experimental studies have investigated the effects of BPA on the reproductive system and reported a broad range of effects. Some of the parameters related to the reproductive function were also investigated in epidemiological studies. The main outcomes of these studies are synthesised below to give an overview of the toxicity of BPA on the reproductive function.

In particular in females, the following effects are reported:

- **Ovarian toxicity:** experimentally, an increased incidence of cystic ovaries has been consistently reported in several studies in mice and rats (Newbold *et al.*, 2009; Signorile *et al.*, 2010; Delclos *et al.*, 2014; Newbold *et al.*, 2007; Adewale *et al.*, 2009; Fernandez *et al.*, 2009). A reduced ovarian weight or small ovaries, depletion of corpora lutea (Nikaido *et al.*, 2004; Takagi *et al.*, 2004; Newbold *et al.*, 2007; Adewale *et al.*, 2009; Nikaido *et al.*, 2005) and of antral follicles (Delclos *et al.*, 2014; Fernandez *et al.*, 2009) as well as decreases in the number of primordial follicles (Wang *et al.*, 2014a; Rodriguez *et al.*, 2010) are often, although not systematically, observed. In some studies investigating the reproductive tract in older animals, more severe lesions were also found, including progressive proliferative lesions of the oviduct (Newbold *et al.*, 2009). Concerning the oocytes development, meiotic abnormalities leading to aneuploidy were shown in several studies using different exposure scenarios. Although rodent ovarian cysts are not the "exact" model for polycystic ovary syndrome (PCOS), it is interesting to note that in humans a cross-sectional epidemiological study with limitations showed that women with polycystic ovaries compared to controls have higher serum BPA concentrations (Kandaraki *et al.*, 2011).
- **Alterations of uterus morphology:** in experimental studies, BPA also induces changes in the uterus morphology in several studies, mainly in the form of hyperplasia of the endometrium. Benign lesions like endometrial hyperplasia or atypical hyperplasia, which is a precursor lesion to adenocarcinoma, were reported in several studies in mice

(Newbold *et al.*, 2009 and 2007; Signorile *et al.*, 2010; Hiyama *et al.*, 2011). Malignant invasions (squamous metaplasia or polyps) were also described in 18-month-old mice (Newbold *et al.*, 2009 and 2007). An increased thickness of uterine epithelia and stroma and an increased incidence in cystic, hyperplastic and metaplastic endometrium are also reported in some studies in rats (Mendoza-Rodriguez *et al.*, 2011; Delclos *et al.*, 2014). Epidemiological studies reported contradictory associations between the endometrial hyperplasia and BPA concentration (Cobellis *et al.*, 2009; Itoh *et al.*, 2007; Hiroi *et al.*, 2004). As they presented some methodological limitations, they were not considered to provide conclusive elements.

- Age at puberty: in rodents, the effects of BPA on puberty timing are still not clear. An acceleration of puberty is observed in several studies in mice (Howdeshell *et al.*, 1999, Honma *et al.*, 2002; Nikaido *et al.*, 2004; Naulé *et al.*, 2014; Nah *et al.*, 2011; Tyl *et al.*, 2008). It is also observed in rats in some studies (Patisaul *et al.*, 2014; Adewale *et al.*, 2009; Fernandez *et al.*, 2009), but an absence of effect (Kwon *et al.*, 2000; Rubin *et al.*, 2001, Takagi *et al.*, 2004; Yoshida *et al.*, 2004; Ryan *et al.*, 2010a; Delclos *et al.*, 2014; Laws *et al.*, 2000) or even a delayed pubertal timing was reported in other studies (Tinwell *et al.*, 2002, Tyl *et al.*, 2002). The diverging results may, in part, be explained by differences in sensitivity in terms of exposure period, strain of rats and/or BPA doses but are not fully understood. In humans, two recent studies performed in a large number of girls do not identify a relationship between the BPA urinary concentration and the onset of their puberty (Wolff *et al.*, 2008 and 2010).
- Estrous cycle disturbance: consistent results of an adverse effect of BPA on the estrous cycle are reported in several different experimental studies, including irregular and prolonged cycles in mice (Honma *et al.*, 2002; Nikaido *et al.*, 2004; Wang *et al.*, 2014a; Nah *et al.*, 2011; Tyl *et al.*, 2008) as well as in most studies in rats (Rubin *et al.*, 2001, Mendoza *et al.*, 2011; Patisaul *et al.*, 2014; Delclos *et al.*, 2014; Adewale *et al.*, 2009; Fernandez *et al.*, 2009; Zaid *et al.*, 2014; Lee *et al.*, 2013; Laws *et al.*, 2000). Some studies failed to reveal significant differences in estrous cycle patterns after prenatal (among others Betancourt *et al.*, 2010), perinatal exposure to high levels of BPA (Kwon *et al.*, 2000), after a prepubertal exposure (Nikaido *et al.*, 2005), or of multi-generation studies (Tyl *et al.*, 2002 or Ema *et al.*, 2001). The strains and windows of exposure might play a role in the sensitivity to estrogen, and therefore to effects of BPA on estrous cycle. One epidemiological study investigated the link between BPA and the characteristics of menstrual cycles. An association with shorter luteal phases was observed even if no robust conclusion in humans can be drawn on the basis of this single study (Jukic *et al.*, 2015).
- Fertility parameters: a decline in reproductive capacity, i.e. a decrease in the number of pregnancies and/or a decrease in the number of pups born (decreased litter size), was observed in several studies when exposure of dams occurs *in utero* or during the first days of life in mice and rats (Wang *et al.*, 2014a; Cabaton *et al.*, 2011; Fernandez *et al.*, 2009; Varayoud *et al.*, 2011). Exposure to BPA during adulthood consistently results in a decrease in the number of pregnancies and implantations in several studies (Moore-Ambritz *et al.*, 2015; Berger *et al.*, 2008 and 2010; Al Hiyasat *et al.*, 2004; NTP, 1985). In humans, high levels of BPA were associated with implantation failures in women undergoing medically-assisted procreation (Ehrlich *et al.*, 2012) or with consecutive spontaneous miscarriages (Sugiura-Ogasawara *et al.*, 2005). Decreased ovarian function as part of an *in vitro* fertilisation (IVF) was also reported with higher BPA exposure in two studies (Fujimoto *et al.*, 2011; Mok-Lin *et al.*, 2010).

Regarding the male reproductive function and fertility, despite the fact that some studies observe limited or no effects, there is a convergence in several studies in the effects observed after BPA exposure. Sperm production is decreased and several studies report effects on one or several male reproductive organs such as effects on the weight of the testes and the seminal vesicles, the ventral prostate or the epididymides (Tinwell *et al.*,

2002; Ida *et al.*, 2002; Kabuto *et al.*, 2004; Okada 2008a; Salian *et al.*, 2009b and 2009c; Aikawa *et al.*, 2004; Nakamura *et al.*, 2010; Tan *et al.*, 2003; Takahashi *et al.*, 2003; Chitra *et al.*, 2003; Toyama *et al.*, 2004a, b; Tyl *et al.*, 2008).

In humans, occupational exposure to BPA was associated with a greater risk of sexual dysfunction, a declining sexual function or a decrease in sperm concentration, motility and vitality compared to unexposed workers (Li *et al.*, 2010a, 2010b; Li *et al.*, 2011). No association between BPA exposure and sperm parameters were identified in a population of fertile men (Mendiola *et al.*, 2010), but a link between BPA and impaired sperm quality was established in men consulting an infertility clinic and between BPA and higher embryo fragmentation score (EFS) and a reduced embryo cell number (ECN) in men involved in IVF (Meeker *et al.*, 2010b; Bloom *et al.*, 2011).

Many effects listed above seem to be highly sensitive to many factors across studies, including the level of doses, period of exposure, species and strain differences. Due to variations in the combination of these factors in the different experimental studies available, as well as to variations in the endpoints investigated and sometimes to experimental limitations (limited group size), it is very difficult to capture the specific influence of each parameter on the effect. However, considering the respective limitations and strengths of the different studies available, as well as the reproducibility of effects for many specific endpoints (ovarian toxicity, alteration of uterus morphology, estrous cycle disturbance, impairment of fertility parameters and impairment of sperm production), the toxicity of BPA on the reproductive function has been established with a sufficient weight of evidence.

Assessment of RAC under the CLH process

In its opinion of March 2014 (ECHA, 2014), RAC adopted the following conclusions in support of classification of BPA as Repr. 1B – H360F as quoted in the text box hereafter:

"Effects on female reproductive capacity:

*In one of the supplementary studies (Cabaton *et al.*, 2011), using a forced breeding design enabling the identification of effects that became apparent with time, a reduction in the cumulative number of pups from F1 females exposed in utero to BPA in the highest dose group was observed. This finding was evident in the absence of systemic toxicity in the exposed F0 dams and the F1 generation. The effect on the number of pups was therefore not considered to be a secondary non-specific consequence of other toxic effects. The effect reported in the Cabaton *et al.* (2011) study supported the effects on fertility reported in the NTP (1985) continuous breeding study and in the multi-generation study by Tyl *et al.* (2002). There were also other subcutaneous non-guideline supplementary studies with higher doses but with shorter exposure periods than in the study Cabaton *et al.* (2011), providing some support to the fertility effects in the NTP (1985) and Tyl *et al.* (2002) studies.*

Effects on female reproductive organs:

*RAC concludes that BPA exerts its toxic effects on the ovaries, either due to direct effects on the ovaries or indirectly via effects on the HPO-axis. Regarding the effects on uterus morphology, the majority of the studies reported hyperplasia of the endometrium or no effects. The effect of BPA on the onset of puberty seemed to vary according to experimental design such as exposure period, species, strain and dose. This variation in the onset of puberty was also seen in animals in the positive control group, e.g. orally administered 17 α -ethinylestradiol (EE2) markedly delayed the onset of puberty in SD rats in the NCTR (2013) study, whereas it accelerated it in Long Evans rats in the study by Ryan *et al.* (2010). The guideline studies did not report any significant effects on the oestrous cyclicity. However, in the NCTR (2013) study and in most of the studies using subcutaneous dosing,*

BPA induced irregularities in the oestrus cycle (Mendoza-Rodríguez et al., 2011, Kato et al., 2003; Fernandez et al., 2009).

Effects on male reproductive organs:

Several non-guideline supplementary studies included in the CLH report demonstrated effects of BPA on male reproductive function. The original studies had variable shortcomings e.g. small sample sizes, a few or single dose groups, non-oral routes of administration and/or lack of details on the methodologies used, which in some cases limited the usefulness of the findings. RAC notes that differences in strains, doses, routes of exposure or windows of exposure made a direct comparison between the studies sometimes difficult. Despite the limitations in these studies, they were considered acceptable to be used in a weight of evidence approach. More than 2/3 of the supplementary oral route studies included in the CLH report reported effects on male sexual parameters (either on sperm quality, spermatogenesis, sex hormones, or on sexual function). In several studies, exposure to BPA (at various doses or periods of exposure) led to a decrease in the serum testosterone level, to some effects in reproductive organs, and/or to a decrease in sperm production.

RAC concluded that these findings supported the observations reported in the test-guideline studies (Tyl et al., 2002; Tyl et al., 2008; NTP 1985b) and in the recent GLP-compliant NCTR 2013 study.

[...]

Effects on the male reproductive tract, evident as an impaired sperm production following BPA exposure, were observed in several studies. The decrease in sperm production was accompanied by lower testosterone levels. RAC noted that the observed effects on the testosterone levels may have been the cause of the decreased sperm production."

Identification of the ED MoA of BPA on the alteration of the reproductive function: focus on a specific effect on the reproductive function

Considering that the effects of BPA on the reproductive function have been presented and discussed in an extensive way at EU level under the previous classification and restriction regulatory processes, it was decided not to further present nor discuss the whole database in the main part of the present dossier.

For the purpose of demonstrating that BPA exerts its effects on reproduction through endocrine disruption and, for the sake of clarity considering the extent of the database, the analysis will focus on a more specific well established effect and for which the ED MoA is the most obvious.

With this aim, the following section will focus on the ability of BPA to affect cyclicity of the female reproductive system and how this alteration is exerted through hormonal changes.

The summary of the effects of BPA on toxicity for reproduction presented above is mainly based on the literature collected for the classification and restriction dossiers. However, recent studies investigating alterations of the estrous cyclicity have been collected until May 2016 to produce the detailed analysis presented hereafter.

4.2.2 Adverse effect: alteration of estrous cyclicity

4.2.2.1 Non-human information

Experimental studies investigating the effects of exposure to BPA on estrous cyclicity are summarised in Table 8 presented in section 4.2.5.

Reliable results were reported in several experimental studies showing an adverse effect of BPA on the estrous cycle, including irregular and prolonged cycles.

In mice, this effect was observed following subcutaneous or oral administration during prenatal (Nikaido *et al.*, 2004; Honma *et al.*, 2002; Wang *et al.*, 2014a) or postnatal exposure (Nah *et al.*, 2011). No effect was observed further to the prepubertal exposure (Nikaido *et al.*, 2005) or adult exposure (Moore-Ambritz *et al.*, 2015). Although an effect was not reported in the two-generation study by NTP (NTP 1985), an increased incidence of females in estrus was identified at the high dose in the two-generation study by Tyl *et al.* (2008) in mice by oral route.

In rats, similar effects were consistently observed in Wistar and Long Evans rats after perinatal exposure by oral route (Mendoza-Rodriguez *et al.*, 2011), postnatal exposure by subcutaneous route (Adewale *et al.*, 2009; Monje *et al.*, 2010) and adult exposure by oral route (Laws *et al.*, 2000). The results were inconsistent with Sprague-Dawley rats, a strain considered sensitive to estrogens. Several studies fail to reveal an effect of BPA on the estrous cycle (Tinwell *et al.*, 2002; Kwon *et al.*, 2000; Takagi *et al.*, 2004; Yoshida *et al.*, 2004; Ferguson *et al.*, 2014; Tyl *et al.*, 2002) while others report alterations of cycles after perinatal (Rubin *et al.*, 2001; Delclos *et al.*, 2014), postnatal (Fernandez *et al.*, 2009), peripubertal (Zaid *et al.*, 2014) or adult exposure (Lee *et al.*, 2013). These data show that the reproducibility of this effect is strain dependant.

In its opinion of March 2014 (ECHA, 2014) in support of classification of BPA as Repr. 1B – H360F, RAC produced the following conclusions on estrous cycle disturbances in experimental studies:

"Three of the guideline studies (NTP, 1985, Tyl et al., 2002, 2008, Ema et al., 2001) did not report any significant effects on the oestrous cyclicity. However, in Tyl et al., 2008, a higher percentage of the high-dose females were in oestrus as compared to controls. Furthermore, in the NCTR¹⁵ (2013) study, in which SD rats were exposed during GD6-PND90, 63% of the animals in the high-dose group had an asynchronous oestrous cyclicity versus 12% in the vehicle control. It was noted by RAC that the control vehicle group was also affected as compared to the naïve controls (0% asynchronous estrous cyclicity). Based on vaginal cytology, disruption of the oestrous cycle at the highest BPA dose was reported on PND69-90 and at the two highest doses on PND150-170 in a similar manner as for the positive control (EE2). The increase of the proportion of animals showing asynchronous estrous cycle on PND150-170 was statistically significant at 100 mg/kg bw/day (n=14), but not at 300 mg/kg bw/day (n=7). Maternal toxicity in this study included a significant reduction in body weight gain (6-13% with an average at 10%) at PND4 and beyond in the two BPA high dose groups. No effect on body weight gain was observed in low dose groups.

Several of the remaining non-guideline studies reported BPA-induced irregularities in the oestrus cycle (Mendoza-Rodríguez et al., 2011, Kato et al., 2003; Fernandez et al., 2009).

In contrast, in the study by Kwon et al. (2000), in which SD rats were exposed via oral gavage to 3.2, 32 or 320 mg BPA/kg bw/day between GD11 and PND20, no effects were reported on the oestrous cycle.

RAC concluded that BPA-treated F0 females were twice more in estrus as compared to controls at 600 mg/kg in Tyl et al. (2008), and that BPA induced irregularities in the oestrus cycle also in the NCTR (2013) study and in most of the studies using subcutaneous dosing (Mendoza-Rodríguez 2011, Kato et al. 2003; Fernandez et al. 2009)."

4.2.2.2 Human information

The link between BPA human exposure and cycle parameters in women has been studied

¹⁵ In the present report the NCTR study (2013) is referenced as Delclos *et al.*, 2014

in a single recent study.

In the mid-80's, 221 women with no fertility disorders were enrolled at the time they stopped contraceptive method to become pregnant and followed for up to 6 months as a population-based cohort (Jukic *et al.*, 2015). From these women, daily first-void morning urine samples were collected for hormonal measurements, the ovulation day recorded; the plausible conception day and early pregnancy loss were calculated. BPA was measured using one urine sample per week pooled over one cycle.

When looking for associations between the urinary concentration of BPA in a cycle and the length of the follicular phase of the menstrual cycle, the risk of early pregnancy loss, the duration for becoming pregnant and the duration of the luteal phase, BPA concentration of a given cycle was associated with a reduced length of the luteal phase. When looking for associations with BPA concentration in the previous cycle, no association was found with the length of the follicular or luteal phases.

The study and its design are considered as of overall good quality although it should be noted that it is common that women experience instability in the first cycles after cessation of contraception. An imprecision in the determination of the ovulation day through daily hormonal measurement may also influence the outcome of the study.

Based on this single study, no robust conclusion can be drawn on the effect of BPA on the estrous cycle in women.

4.2.2.3 Summary and discussion of alteration of estrous cyclicity

An alteration of estrous cycles by BPA is identified in many experimental studies in rats and mice after exposure during different periods of exposure.

In particular, an effect was observed either:

- After exposure of adult females (Tyl *et al.*, 2008; Laws *et al.*, 2000; Lee *et al.*, 2013)
- After exposure during the developmental phase of the reproductive system, i.e. *in utero* (Honma *et al.*, 2002; Nikaido *et al.*, 2004; Wang *et al.*, 2014a), perinatal (Rubin *et al.*, 2001; Mendoza *et al.*, 2011; Patisaul *et al.*, 2014; Delclos *et al.*, 2014), postnatal (Nah *et al.*, 2011; Adewale *et al.*, 2009; Fernandez *et al.*, 2009) or prepubertal exposure (Zaid *et al.*, 2014)

This effect was recognised by RAC in its opinion in support of classification of BPA as Repr. 1B – H360F as summarised in the RAC opinion on restriction of BPA (ECHA 2015): "RAC's opinion (RAC 2014) was based on adverse effects, such as disturbances in the oestrous cycle, at a dose of 600 mg/kg bw/day (Tyl *et al.*, 2008) and at a dose of 100 mg/kg bw/day (Delclos *et al.*, 2014)."

Proper cyclicity is considered essential to reach successful ovulation. An alteration of cyclicity may therefore directly induce at least subfertility through disturbed (delayed or absent) ovulation. The hormonal regulation of the cycle also influences the maturation process of the ovarian follicles. Different studies have linked estrous cyclicity modification with impaired follicles such as in Zaid *et al.* (2014) in which BPA treatment induced a persistent diestrous (5/8 animals), an increase of the number of large antral-like follicles that did not reach ovulation and of atretic cystic-like follicles and a decrease in the number of preantral follicles and *corpus luteum*. These data show that BPA delays the development of preovulatory follicles and their ovulation. Modifications in the length and/or hormonal environment of the different phases may impact the quality of oocytes and the quality of embryos. An association with implantation failure and spontaneous miscarriages may also occur. Therefore, the effect on cyclicity needs to be considered in relation to the alteration of fertility observed (decreased implantation and litter size).

As synthesised by Kortenkamp *et al.* (2012), an association between menstrual cycle characteristics and sub-fecundity and spontaneous abortion has been observed in humans and lifelong menstrual patterns have been associated with chronic diseases, including breast and ovarian cancer, uterine fibroids, diabetes and cardiovascular disease. Chronic anovulation is a well-established cause of female infertility. The few studies of menstrual cycle characteristics and fecundity have found that shorter cycles were less likely to be followed by conception, while both shorter and longer cycles were more likely to be followed by spontaneous abortion. Cycles with up to 4 days menstrual bleeding had lower fecundity, and spontaneous abortion was less likely after cycles with more than 5 days of menstrual bleeding (Small *et al.*, 2006). Alteration of cyclicity is therefore considered as an effect fulfilling fully the criteria of adversity.

The specific pathways at stake during development or in adulthood are expected to be largely mediated through modification of the hormonal regulation of the cycle. They will be discussed in relation to the age at exposure in the next section on endocrine disruptive MoA.

4.2.3 Endocrine disruption in relation to the alteration of the estrous cyclicity

4.2.3.1 Adult exposure

4.2.3.1.1 Background on the regulation of the estrous cycle in rodents

In rat and mouse, the estrous cycle is characterised by the following sequential stages, called estrus, diestrus-1 (also named metestrus), diestrus-2 (also called diestrus) and proestrus; each of which lasting about one day. At the end of the follicle growth, ovulation occurs on estrus stage at 02:00hrs. During the night between proestrus and estrus, females are receptive for mating. After the oocyte expulsion, the follicle becomes the corpus luteum, which predominantly secretes progesterone.

The time-related changes in hormonal secretions by the ovary and pituitary gland during the estrous cycle are well-known processes (Figure 2).

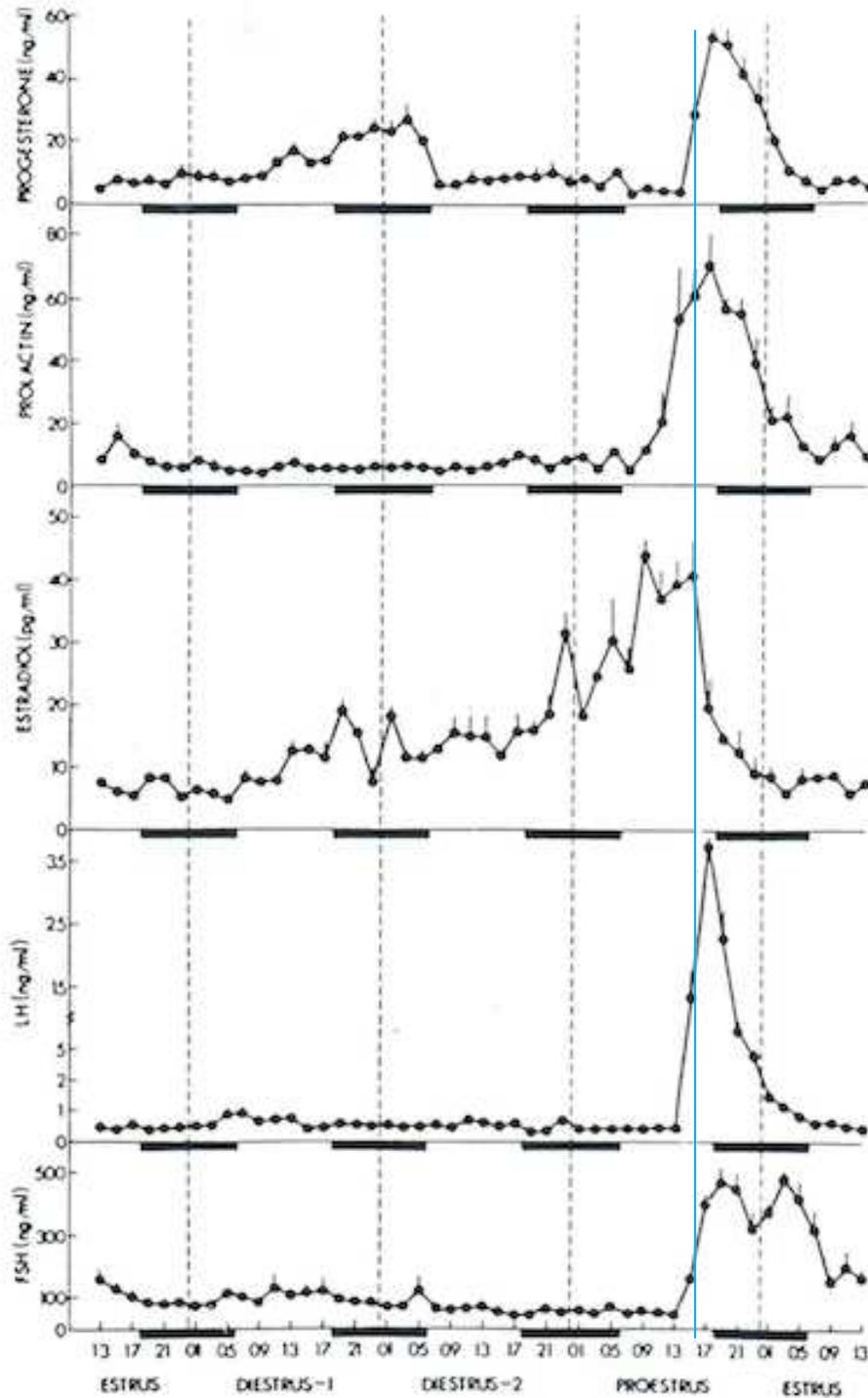


Figure 2: Concentrations of progesterone, prolactin, estradiol, LH and FSH in the peripheral plasma of the 4-days estrous cycle of the rat.

Mean values \pm SE of 5-6 rats are represented. Horizontal black bars represent the dark period in the animal room (18:00-06:00) of the 24-hr clock. Note that estradiol concentrations are expressed with pg/mL unlike all other hormonal concentrations which are expressed as μ g/mL. The blue line indicates 15h00 at proestrus (from Smith *et al.*, 1975).

The sequence of regulation during the estrous cycle is presented in Figure 3.

The diestrus 1, diestrus 2 and proestrus before 15h00 correspond to the ovarian phase during which the pool of recruited preantral and antral follicles are growing and produce estrogens. Estrogen production results from a collaborative work inside the ovary: the theca-interstitial cells synthesise androgens from cholesterol and the granulosa cells, convert the androgens produced by theca-interstitial cells into estrogens as they specifically express *cyp19a1* (also called *cyp19a1*) encoding the aromatase, catalysing this conversion.

During this period, the follicles grow, the production of estrogens increases and the plasma estradiol concentration shows a peak during a few hours before 15h00 of the proestrus stage. These processes are stimulated by both FSH (stimulating granulosa cells) and LH (mainly stimulating theca-interstitial cells, and granulosa cells incidentally). Importantly, the preovulatory surge of estrogens is also largely due to the self-stimulation as the rise in estrogens further stimulates their own production (Figure 4). The estrogens stimulate follicle growth and protect the follicle from atresia, and consequently more and more cells produce estrogens. Furthermore, in theca-interstitial cells, estrogens act in a paracrine mode to up-regulate the stimulatory effect of LH on androgen production. Lastly, in granulosa cells, estrogens up-regulate in an autocrine fashion, via binding to ER β , the stimulatory effect of FSH and of LH on the expression of the *cyp19a1*.

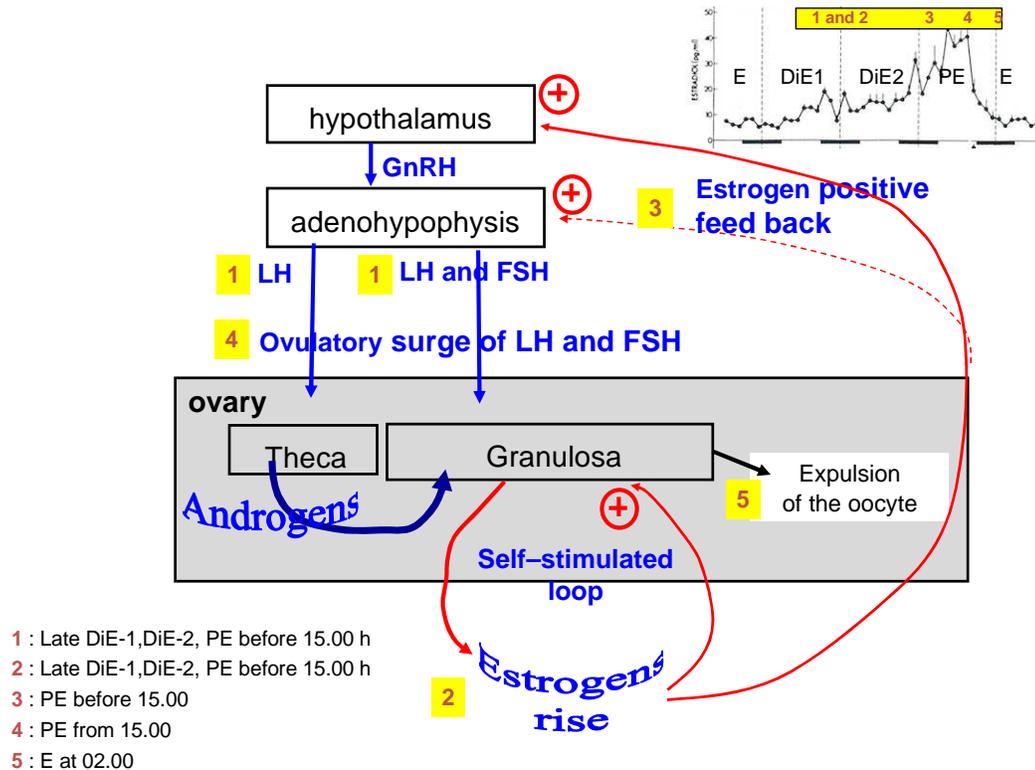


Figure 3: Temporal sequence of the main endocrine controls of the final follicle growth and ovulation in the rat

Step 1: From late Diestrus-1 (DiE-1) until proestrus (PE) 15h00 LH stimulates the production of androgens by theca cells and FSH (and incidentally LH) stimulates the conversion of these androgens into estrogens.

Step 2: Concomitantly, estrogens stimulate their own production via a self-stimulated loop as detailed in Fig 2. Thus the estrogen production increases.

Step 3: High levels of estrogens act positively on the hypothalamo-hypophysis system and trigger a surge of LH and FSH from PE 15:00hrs (step 4) that induces ovulation at the estrous stage (step 5).

The side insert into fig 3 represents the level of estradiol-17 β in the peripheral plasma during the estrous cycle (from Smith *et al.*, 1975).

The self-amplifying feedback mechanism of estrogens on their own production is mediated

by intra-follicular factors. Among them, IGF-1 produced by theca-interstitial cells and granulosa cells plays an important role by enhancing the action of gonadotrophins. IGF-2, SF-1, BMP-15, GDF-9 and GATA4 are also involved. Lastly, in the granulosa cells of the preovulatory follicle, transcription factors such as PPAR- γ inhibit CYP450arom transcription, whereas others such as LRH-1, SF-1, CREB and GATA4 activate this transcription. The ovary-specific proximal PII promoter of CYP450arom contains response elements for these transcription factors.

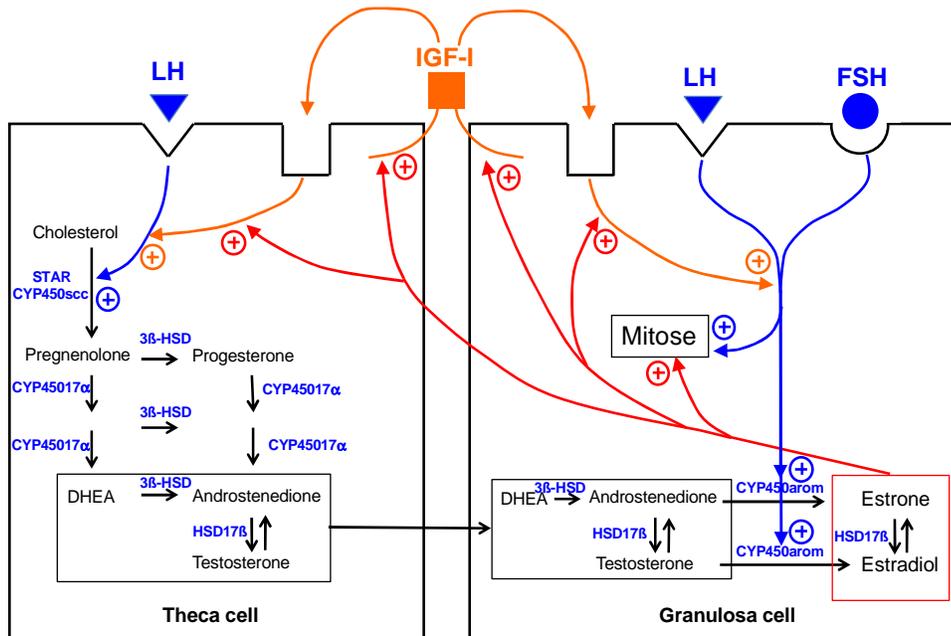


Figure 4: Main mechanisms of the self-stimulated estrogen synthesis in the preovulatory follicle. Estrogens (estrone and estradiol-17 β) stimulate the proliferation of the granulosa cells, and the secretion and action of IGF-I in the granulosa cells (autocrine action), and in thecal cells (paracrine action). In each cell type, IGF-I potentiates the positive effect of the gonadotrophins (LH in theca cells, and FSH (incidentally LH) in the granulosa cells) on specific steps in the steroid hormone biosynthetic pathway. STAR, steroid acute regulatory protein; CYP450scc: P450 cholesterol side chain cleavage enzyme (also named CYP11A1); CYP45017a: 17 α -hydroxylase-17,20-desmolase (also named CYP17A1); CYP450arom: cytochrome P450 aromatase (also named CYP19A1); HSD3 β : 3 β -hydroxysteroid dehydrogenase; HSD17 β : 17 β -hydroxysteroid dehydrogenase; IGF-I: insulin-like growth factor I.

Aromatase is an enzyme responsible of the production of estradiol. It is expressed in multiple organs such as gonads, placenta, brain, adipose tissue, blood vessels, skin, bones and uterine mucosa (Simpson *et al.*, 1994). In the non-pregnant female, ovaries are by far the major source of circulating estrogens in vertebrates. Consequently, the increase in plasma estrogen concentration is due to an increase in their production by follicles.

During proestrus, when plasma estrogens reach a threshold, they act positively on the hypothalamo-pituitary system to provoke the ovulatory peak of LH and FSH. This is known as the positive feedback of estrogens on the hypothalamo-pituitary system. This mechanism is common to all cycling mammals.

More precisely, the ovarian estradiol acts in the hypothalamic preoptic area to trigger GnRH

liberation, which in turn stimulates LH increase in the pituitary. This gonadal feedback does not act directly on GnRH neurons but involves a neuronal cell type expressing kisspeptin. This hypothalamic neuropeptide coded by *Kiss1* gene acts upstream of GnRH. Kisspeptin neurons located in the rostral periventricular area of the third ventricle (RP3V; including the anteroventral periventricular, caudal and rostral periventricular nuclei) of the preoptic area send projections to GnRH soma cells, which express the kisspeptin receptor (Kiss1R). Kisspeptin is a crucial regulator of the onset of puberty, sex hormone-mediated secretion of gonadotrophins, and control of fertility. During the proestrous phase, estradiol targets kisspeptin neurons, which express ER α and therefore integrate the positive signal of estradiol necessary to trigger the ovulatory surge of LH, through GnRH liberation.

Furthermore, in rodents, the anteroventral periventricular nucleus (AVPV), where kisspeptin neurons are involved in the positive feedback of estradiol, receives afferent fibers from the suprachiasmatic nucleus. The circadian clock located in the latter nucleus coordinates and provides precise timing for the LH surge which starts at 15h00.

It may be noted that, in rodents, the ovulatory surge of LH triggers progesterone surge (peak following estradiol surge (Figure 2), which is required for female receptivity. During this stage, progesterone acts in hypothalamic areas through progesterone receptors, which are up-regulated by estradiol, thereby leading to the induction of female sexual behaviour.

In conclusion, the estrous cycle appears as a process basically controlled by sequential endocrine/paracrine and autocrine regulations. The key event is the endocrine dialogue between the hypothalamo-pituitary system and the ovarian follicles *via* the levels of estrogens that trigger the ovulatory surge of LH.

4.2.3.1.2 *In vitro* information indicative of endocrine activity of BPA

A number of *in vitro* studies show that BPA can alter the activity of ovarian cells. This is very likely to result in the disturbance of the estrous cycle, in particular when the steroidogenic activity of the ovaries is concerned (see above the biological process which controls the estrous cycle). The *in vitro* data are described below and summarised in Table 9 presented in section 4.2.5.

Theca-interstitial cells

Using isolated antral follicles from adult cycling female FVB mice, Peretz *et al.* (2011) observed dose-dependent and time-dependent reductions in estradiol-17 β , estrone, testosterone, androstenedione and DHEA-S synthesis after exposure to 100 and 10 mg/L (440 and 44 μ M) BPA. Using addition of steroid substrates and RT-PCR analyses, they demonstrated that BPA acts by reducing the activity and/or expression of STAR and CYP450scc, but not that of 3 β -HSD and CYP45017- α . Moreover, these effects of BPA are reversible once BPA is removed from the culture media (Peretz and Flaws, 2013).

In contrast, using theca-interstitial cells isolated from immature (30 days old) female Sprague-Dawley rats (previously daily injected with 1mg 17 β -estradiol from 28 to 30 days of age to stimulate ovarian development), Zhou *et al.* (2008) observed that BPA in the culture medium at high concentrations increases the expression of STAR (from 10 to 100 μ M i.e 2 to 23 μ g/mL) and CYP450scc (from 0.1 to 100 μ M i.e from 0.2 to 23 μ g/mL).

Granulosa cells

Rodent and porcine granulosa cells

Zhou *et al.* (2008) used a granulosa cells culture isolated from mature Sprague-Dawley rats and observed that an exposure range between 1 to 100 μ M BPA for 48 hrs reduced the estradiol production and CYP450arom mRNA level in a dose-dependent fashion though the expression of various genes increase at the same time (P450scc, StAR) and production

of progesterone increases.

Peretz *et al.* (2011) showed that the mouse antral follicle cultured for 120 hours in the presence of 44 μM BPA contained 4 times lower CYP450arom than the controls, but the difference was not statistically significant. The negative effect of 44 μM BPA on estradiol and estrone productions by the mouse antral follicle in culture for 4 days cannot result from a cytotoxic effect since the follicular growth throughout the culture period is not affected by BPA.

Mlynarcikova *et al.* (2005) reported that 1 to 100 μM BPA inhibits FSH-induced estradiol-17 β synthesis in cultured granulosa cells isolated from antral porcine follicle. In the meantime, the production of progesterone was not affected, which shows that the effect on estradiol is not due to cytotoxicity.

Human granulosa cells

Importantly, the negative effect of BPA on aromatase expression and activity was observed using human cells. Watanabe *et al.* (2012) used a KGN cells line (a human ovarian granulosa-like tumor cell line), and exposed them to BPA between 5 to 100 μM . They observed a dose-dependent reduction of the mRNA levels and activity of CYP450arom.

Kwintkiewicz *et al.* (2010) used KGN cell line, and exposed them to BPA in a range between 40-100 μM . They observed a dose-dependent reduction of FSH-induced aromatase expression and estradiol secretion, and a reduction of the FSH-induced IGF-1 expression. mRNA levels of transcription factors SF-1 and GATA4 were decreased after BPA treatment. In contrast, both mRNA and protein levels of PPAR γ were significantly up-regulated by BPA in a dose-dependent manner and the authors suggests that the inhibitory effect of BPA on the expression of aromatase is mediated via PPAR γ since overexpression of PPAR γ in KGN cells also provokes a decrease in the expression of aromatase and IGF-1. Metabolic cell activity is assessed and does not favour the hypothesis that the effects observed are due to general toxicity.

Recently, Mansur *et al.* (2016) assessed the effects of BPA on a human granulosa cells culture obtained from patients undergoing IVF. The cells were exposed for 48 hrs to 8.8 nM, 88 nM, 880 nM, 8.8 μM or at 88 μM of BPA. The progesterone secretion was reduced for 8.8 and 88 μM of BPA but not at lower doses. The highest BPA concentration showed a decrease of the estradiol production. The BPA at 8.8 and 88 μM significantly reduced the mRNA levels of 3 β -HSD, CYP450scc and CYP450arom and at lower concentrations (8.8 nM to 0.88 μM) no change was observed. The BPA exposures concentration did not affect the STAR and CYP17 α mRNA levels. Lastly, 3 β -HSD, CYP450scc and CYP450arom protein levels were reduced by 88 μM of BPA.

Note

It can be noted that the inhibitory effect of BPA on the aromatase activity and/or expression was also observed *in vitro* in other cell types such as placental cells (Nativelle-Serpentini *et al.*, 2003; Benachour *et al.*, 2007; Huang & Leung, 2009) and Leydig cells (Akingbemi *et al.*, 2004a, b). During public consultation, it was raised that an alternative mechanism for the reduction in aromatase activity reported at supra-physiological (μM) concentrations in the *in vitro* studies could be due to cytotoxicity of BPA at these high concentrations. An analysis of the studies mentioned above showed that high doses of BPA decrease the expression and/or the activity of aromatase in a specific manner in the granulosa cells although the authors of the different papers used various *in vitro* models. The assumption that this inhibition could result from the cytotoxicity of high doses of BPA is invalidated by the results, thus the effects described on aromatase are specific.

Summary and conclusion

In conclusion, the *in vitro* effect of BPA on the theca-interstitial cells steroidogenesis seems to depend on the species and/or the experimental procedure. In contrast, data dealing with the *in vitro* effect of BPA on the granulosa cells steroidogenesis are all converging to show that **BPA reduces the estrogen production by this cell type by reducing the aromatase expression in all species studied including in humans.**

4.2.3.1.3 *In vivo* evidence with regard to an endocrine MoA

There are currently few data reporting *in vivo* effects of adult exposure to BPA together with investigation of the MoA. These studies are summarised in Table 10 presented in section 4.2.5.

Lee *et al.* (2013) developed a key study using adult female Sprague-Dawley rats (PND 56) treated by oral gavage with 1 or 100 µg/kg/day of BPA for 90 days. Estradiol benzoate (EB, 1µg/kg/day) was used as positive control. It is a well-conducted study (n=18 rats/group, sacrifice of all the animals at the same stage of the estrous cycle, the day of estrous). Both BPA doses and EB lengthened the estrous phase, decreased plasma estradiol and testosterone concentrations, and increased apoptosis in follicle and corpus luteum. They decreased the protein levels of StAR but not those of P450SCC and 3β-HSD in theca-interstitial cells. The magnitude of these effects is important: plasma estradiol-17β concentration was 2 times lower in rats treated by 1 µg/kg/day as compared with controls. No change in the levels of FSH in the plasma and the pituitary gland were observed. Both doses of BPA but not EB:

- decreased aromatase levels in the granulosa cells (with a stronger effect with 1 than with 100 µg/kg/d BPA),
- decreased estrogen-induced proteins (PCNA calbindin-D9k) and collagen contents of the uterus,
- increased plasma LH concentration and pituitary LH content. The authors interpret this increase in LH levels as the following cascade: BPA primarily acts on the ovaries to reduce their estrogen production; this provokes a partial removal of the inhibitory negative feedback that is exerted by the circulating estrogens on the hypothalamo-pituitary system at this period of the estrous cycle, and, consequently, an increase in LH secretion.

This work is in accordance with *in vitro* data described above. It shows that **one clear-cut primary target of BPA is the reduction of the expression of aromatase in granulosa cells.** BPA first decreases estradiol levels by disturbing P450arom protein expression. Then, it is likely that the prolonged status of reduced estradiol subsequently provokes decreased feedback regulation of LH, lengthening of the estrous cycle as well as ovarian cell apoptosis.

Wang *et al.* (2014b) reported that adult exposure to BPA for 6 hours during proestrous, but not during estrous or diestrous, increases *Kiss1* and *GnRH* expression as well as levels of LH, FSH and estradiol. The assessment of LH surge showed an increased baseline before the LH surge, but no changes in the timing and level of this surge.

In a recent study, Kurian *et al.* (2015) using a microdialysis method, examined the effects of BPA (0.1, 1, and 10nM) directly infused to the stalk-median eminence on the release of GnRH and kisspeptin in mid to late pubertal ovarian intact female rhesus monkeys. They observed that the highest level of BPA exposure (10 nM i.e about 2 ng/ml which is a relevant concentration as far as human exposure is concerned) suppressed both GnRH and kisspeptin release suggesting that persistent exposures to BPA could impair female reproductive function by directly influencing the hypothalamic neuroendocrine function.

Although the potential effects of such changes were not assessed on the estrous cycle due to the short time of exposure, these studies suggest that BPA can also affect the expression of two key neuropeptides involved in the ovulatory surge of LH process in rodents and non-human primates and that a neuroendocrine mechanism can also contribute to BPA-reprotoxicity (see detailed presentation of neuroendocrine regulation in the next sections).

4.2.3.1.4 Summary of the plausible link between adverse effects and endocrine MoA regarding adult exposure

So far, there are indications of direct effects of BPA exposure during adulthood on the neuroendocrine system controlling the estrous cycle in rodents and non-human primates. However, it remains difficult to establish a clear link between the data on BPA-induced changes of this system and alterations of the estrous cycle based on the few data available. Indeed, although BPA-induced changes in the neuroendocrine expression of kisspeptin and GnRH were reported, data are far too limited to propose a succession of key events linking directly neuronal changes to ovarian cycle disruption.

On the contrary, convergent data explain how the effects of BPA on the ovary lead to alteration of the estrous cycle. The negative effect of BPA on ovarian estrogen production is clearly demonstrated: in rodents, domestic animals using both *in vitro* and *in vivo* studies and in human cells *in vitro*. Whereas the effect of BPA on theca-interstitial cells depends on the model and the protocol, BPA consistently reduces the conversion of androgens into estrogens in granulosa cells. This reduction is, at least in part, a consequence of a decreased transcription of CYP450arom either *via* a direct effect in the granulosa cells or *via* changes in intrafollicular signaling factors that regulate follicular growth and endocrine activity. Given the regulatory scheme of the estrous cycle, such an alteration in the preovulatory follicle steroidogenic activity is very likely to be associated with a disruption of the estrous cycle (Figure 5) as shown in the Lee *et al.* study. **These results demonstrate a clear endocrine mode of action, namely the alteration of the ovarian steroidogenic activity, underlying estrous cycle disruption in adult rodents.**

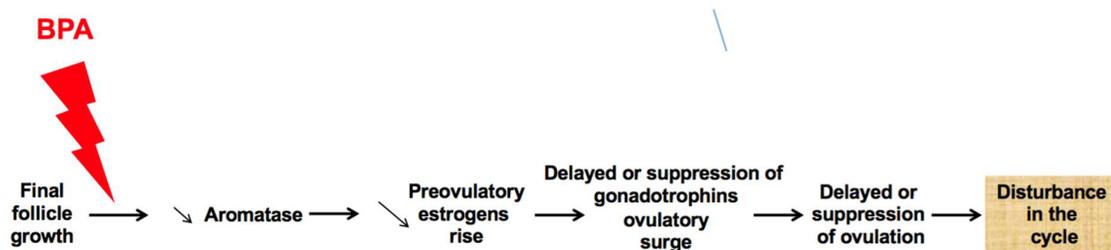


Figure 5: Sequential cascade from the endocrine effect of BPA to its adverse effects.

A clearly demonstrated target of BPA is aromatase, in the preovulatory follicle. The BPA-induced reduction in the expression of this steroidogenic enzyme induces a reduction in the synthesis of estrogens. Thus, the preovulatory rise of estrogens is attenuated. Consequently, the estrogen-induced gonadotrophins ovulatory surge, is delayed or suppressed, and this induces disturbances in the cycle.

4.2.3.2 Developmental exposure (*in utero*, perinatal, postnatal and/or prepubertal)

4.2.3.2.1 Background on neuroendocrine fetal programming of estrous cyclicity

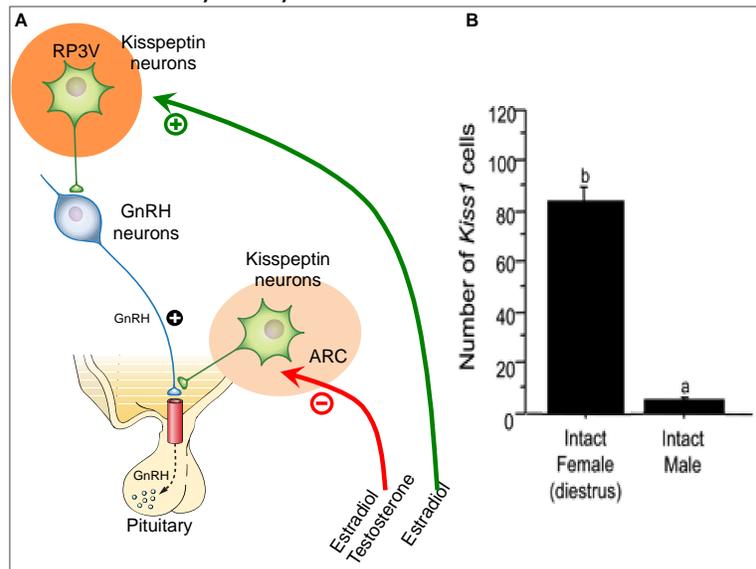


Figure 6: The neuroendocrine system involved in the control of the estrous cycle is regulated in a sexual dimorphic manner.

A. Kisspeptin neurons located in the hypothalamic arcuate nucleus (ARC) integrate the negative feedback exerted by estradiol or testosterone and progesterone in both males and females. By contrast, kisspeptin neurons of the RP3V nucleus integrate the positive feedback exerted by estradiol in females. (From Naulé *et al.*, 2016)

B. This positive regulation does not exist in males since the RP3V nucleus contains very few kisspeptin neurons in males by comparison to females (From Kauffman *et al.*, 2007). The RP3V nucleus is located in the preoptic area of the hypothalamus and includes the AVPV nuclei as well as periventricular nuclei. Kisspeptin neurons were first localised in AVPV nuclei but were later also detected in other parts of RP3V (periventricular neurons).

The neuroendocrine pathways underlying the gonadotropic function are regulated by sex steroids in a sexually dimorphic manner (Figure 6). Indeed, the positive feedback exerted by estradiol to trigger GnRH/LH preovulatory surge is specific to females. As developed above, ovarian estradiol exerts a positive control during the proestrous phase. It also exerts an inhibitory feedback during the other phases in females. In males, testosterone exerts only a negative feedback. Both positive and negative feedback exerted by sex steroids involve kisspeptin neurons. Two hypothalamic neuronal populations of kisspeptin are differentially involved in the integration of these positive and negative signals. Kisspeptin neurons of the RP3V nucleus are targeted by estradiol during the proestrous phase in females, while kisspeptin cells of the hypothalamic ARC mediate the negative control of sex steroids in both males and females. Kisspeptin neurons of the ARC send also projections to GnRH neurons and co-express two neuropeptides neurokinin B and dynorphin, which are suggested to play also a role in the regulation of the gonadotropic axis. At the neuroanatomical levels, the RP3V contains more kisspeptin neurons in females than in males.

This sexual dimorphism is programmed as early as the perinatal period and is under the control of sex steroids (Figure 7). In males, the perinatal surge of testicular testosterone masculinizes the RP3V region. In females, brain regions are not impacted by sex steroids during the perinatal period since the ovaries are inactive and the neural structures involved in female reproduction are protected from maternal and sibling sex steroids. Exogenous administration of testosterone to female neonates masculinises the preoptic area, in terms of kisspeptin neuronal number, thereby suppressing the ovulatory surge of LH during adulthood. Importantly, the same effect is obtained by administration of estradiol. Indeed, in males, perinatal testosterone is converted in the nervous system into estradiol, which

masculinises brain areas including the preoptic area.

At birth, the expression level of kisspeptin is low in the female RP3V. It increases progressively during the postnatal period under the control of ovarian estradiol. Indeed, the ovarian production of estrogens, which starts around postnatal day 7, promotes *Kiss1* expression in this hypothalamic region. A maximal increase is observed during the prepubertal period and will be necessary for pubertal activation of GnRH/LH axis and initiation of estrous cyclicity and female reproduction.

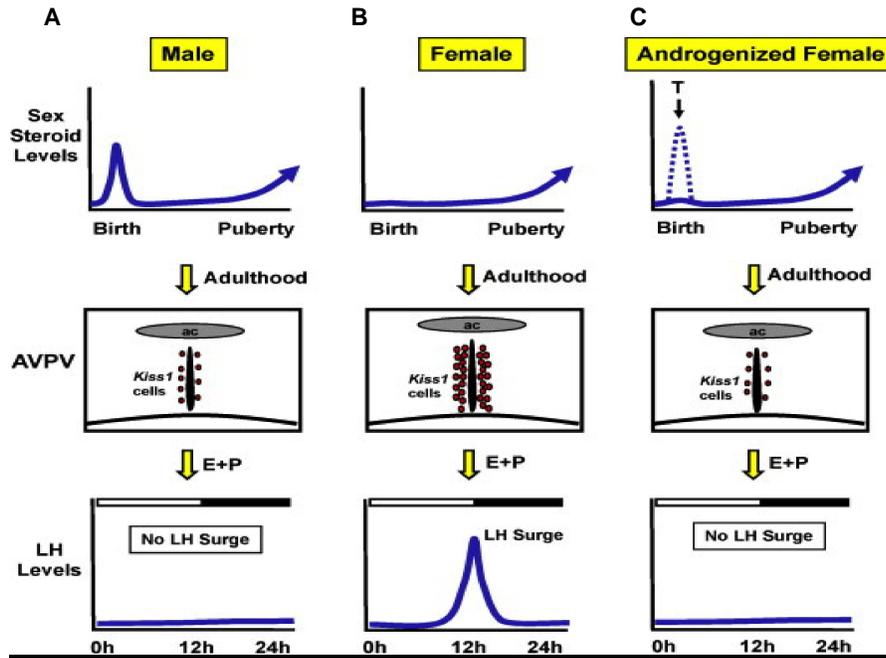


Figure 7 : Perinatal organisation of the gonadotropic axis required for normal GnRH/LH surge and ovulation to occur (From Kauffman, 2009)

A. In males, gonadal testosterone masculinises the AVPV, thereby leading to a reduced number of kisspeptin neurons in this area and the absence of the ovulatory surge of LH in response to estradiol/progesterone stimulation. **B.** In females, in the absence of ovarian steroids, this area develops normally and the ovulatory surge of LH occurs at adulthood. **C.** Perinatal exposure of females to androgens triggers a male phenotype with low kisspeptin cell number and suppression of the ovulatory surge of LH.

4.2.3.2.2 *In vitro* information indicative of endocrine activity at the neuroendocrine level

One *in vitro* study (Klenke *et al.*, 2016) investigated the action of BPA on the neuroendocrine components of regulation of the estrous cycle and is summarised in Table 11 presented in section 4.2.5. In this study, BPA reduces the frequency of oscillations in GnRH neurons from embryonic nasal explants collected after emergence of GnRH cells and other neuronal cell types from the plasma codes.

4.2.3.2.3 *In vivo* evidence with regard to an endocrine MoA after developmental exposure

As presented above, the perinatal and postnatal periods of exposure are sensitive to hormonal changes. Changes are required for the permanent programming of the female

neuroendocrine system. Exposure to exogenous factors exhibiting hormone-mimetic activities such as BPA could then interfere with these processes and induce long-term effects on the integrity of the gonadotropic axis and the estrous cyclicity.

Studies associating estrous cyclicity alteration as well as an ED MoA of BPA

Several studies provide evidence of alteration of the estrous cycle as well as indications about the MoA. They are summarised below and in

Table 12 presented in section 4.2.5.

In the study from Wang *et al.* (2014a), effects on estrous cyclicity were observed at low doses. However, the only evidence that these alterations might be due to endocrine-mediated mechanisms arises from the observation that part, but not all, of these effects are reproduced in the DES positive controls.

In the study from Delclos *et al.* (2014) issued from the BPA-clarity initiative (Heindel *et al.*, 2015), it is noted that groups administered doses of BPA up to 80 µg/kg/d had the same unconjugated BPA serum levels as the vehicle negative controls, which the FDA scientists attributed to inadvertent contamination of the negative controls (Churchwell *et al.*, 2014). Contaminated negative controls would make it impossible to find effects in this low-dose range. Nevertheless, effects on estrous cyclicity were conclusive in the higher dose group after periconceptional and all life-long exposure. This effect was seen in the positive EE2-treated animals as well.

In addition, the similarity of effects between BPA and positive estrogenic controls or ER alpha antagonist was also observed in Nikaido *et al.* (2004) and Adewale *et al.* (2009), respectively.

This shows that BPA displays an estrogen-like effect.

In another study (Rubin *et al.*, 2001), results in some female offspring exposed perinatally to the highest dose of BPA (1.2 mg/kg bw/d) revealed intermittent extended periods of diestrus, whereas other females exhibited extended periods of proestrus and/or estrus. Beside altered patterns of estrous cycle in approximately 80% of 4-month and 6-month old females, the offsprings of the high-dose BPA females also revealed decreased levels of plasma luteinising hormone (LH) (-18%) in adulthood after ovariectomy. Decreased LH secretion in BPA-treated ovariectomised animals showed an alteration of the endocrine function of the hypothalamo-pituitary gonadotropic axis. However, it should be noted that LH was not evaluated in the ovary of intact animals, thus direct correlation to the estrous cycle disturbances could not be made.

More convincing evidence toward involvement of hormonal disruption was provided by the concomitant observation of estrous cycle disturbance together with modification of LH and/or GnRH release (Monje *et al.*, 2010; Fernandez *et al.*, 2009).

In addition to the studies presented above, two studies were reviewed but could not be considered in the analysis due to major methodological limitations and/or lack of details regarding the methodology, in particular inappropriate schedule of observation to properly monitor estrous cyclicity (Patisaul *et al.*, 2014) and lack of information on the stage at which samples for hormonal monitoring were collected (Zaïd *et al.*, 2014).

Finally, one additional study did not identify effects on cyclicity but provides relevant indications of a disruption of its hormonal regulation. In the study from Veiga-Lopez *et al.* (2014), sheep were exposed to BPA during gestation from GD30 to GD90 at doses of 0.05-0.5 or 5 mg/kg/d. This exposure was not associated with major alterations of the amplitude and/or the timing of the estradiol and LH surges during the preovulatory phase of the estrous cycle. However, the time interval between estradiol and LH peaks (equivalent of a proestrous phase in rodents) appeared to be decreased in BPA-exposed animals as compared to vehicle ones. The impact of such modifications on the overall estrous cycle

was not determined in this study. Several other studies in sheep indicate that BPA developmental exposure (either *in utero* or neonatally) can alter the follicle dynamic (Rivera *et al.*, 2011; Veiga-Lopez *et al.*, 2014) or the ovarian response to FSH (follicular growth, FSH-induced estradiol secretion) in prepubertal animals (Rivera *et al.*, 2015).

Evidence for a neuroendocrine basis to BPA-induced disruption of estrous cyclicity following developmental exposure

Ten studies addressing the effects of developmental exposure to BPA on kisspeptin and GnRH expression or liberation are summarised in Table 13 presented in section 4.2.5. Among these studies, 8/10 report changes in these processes, suggesting a potential long-term effect of BPA exposure at the neuroendocrine level. The limited number of studies and differences in doses and analyses do not allow to conclude whether developmental exposure to BPA inhibits or increases neuropeptide expression. It seems, however, that neonatal and early postnatal exposure diminishes, while a longer exposure time starting from gestation until weaning increases, kisspeptin expression.

Recent neuroanatomical studies described modifications in the expression levels of estrogen receptors in brain areas underlying female reproduction, such as the preoptic area and AVPV subregion or the mediobasal hypothalamus and ARC, following developmental exposure to BPA (Monje *et al.*, 2009; Monje *et al.*, 2010; Rebuli *et al.*, 2014; Cao *et al.*, 2014; Yu *et al.*, 2015). These data clearly indicate that BPA developmental exposure can be associated in animal models with an altered development of the neuroendocrine component of the gonadal axis. From a physiological point of view, it is legitimate to assume that most of these alterations can possibly lead to disruption of estrous cyclicity later in life. However, the majority of these studies did not monitor cyclicity, which precludes any definitive conclusion regarding a potential functional link between these developmental neuroendocrine alterations and perturbation of estrous cyclicity in adults.

4.2.3.2.4 Summary of the plausible link between adverse effects and endocrine MoA regarding developmental exposure

Not all studies provide clear indications of a direct link between a disruption of estrous cyclicity due to BPA exposure and endocrine or neuroendocrine mechanisms. In particular, the delay between the expression of the neuroendocrine mode of action evidenced during developmental stages (evaluation of tissue expression of genes/ proteins such as kisspeptin) and the effect on estrous cyclicity that can be evidenced only in fully mature animals render their observation within the same study/animal almost impossible. Nevertheless, many studies show that the basic (neuro) endocrine mechanisms implicated in the finely tuned regulation of the gonadotropic function underlying the estrous cycle can be altered in response to BPA exposure, in particular after developmental exposure. BPA has been shown to affect the hypothalamic expression of kisspeptin, a key neuropeptide in the regulation of the hypothalamic–pituitary–gonad (HPG) axis to later achieve the release of hormones at the appropriate time and concentrations during the cycle. In particular, studies by Monje *et al.* (2010) and Fernandez *et al.* (2009) provide a link between neuroendocrine changes and alteration of the cycles through concomitant observation of an alteration of hormones of the HPG axis and a cycle disturbance. In addition, the affected targets are similar to a large extent to targets affected by either estrogen agonist or estrogenic positive controls. Thus, animal and *in vitro* data support the hypothesis of an endocrine-related MoA of BPA to induce perturbation of estrous cyclicity after developmental exposure.

It is noteworthy however, that based on available data it is sometimes difficult to state whether those endocrine alterations are the primary mode of action or just consequences

of a non-endocrine related mechanism such as meiotic alteration or epigenetic modifications in the oocytes and/or other follicular cell types. This is typical of the regulatory loop systems that are the basis of endocrinology. As long as an endocrine-related modification can be evidenced for at least one step of these regulatory loops, it can be considered that the substance is acting as an endocrine disruptor.

4.2.3.3 Human relevance

Most of the evidence comes from rodent studies. Peculiarities of the reproductive physiology in those species as potential sources of uncertainties on the relevance of the results for humans are discussed hereafter together with commonalities across species.

4.2.3.3.1 Circadian synchronisation of estrous cycle is specific for rodents

The preovulatory LH surge, which characterises the proestrus depends on neural hypothalamic signals tightly coupled to the 24 hrs light-dark cycle in rodents. The disruption of this signal, through pentobarbital administration during mid-proestrus for example, leads to a delayed ovulation by exactly 24 hours. In rat, the synchronisation of the estrous cycle is related to the expression of an endogenous circadian rhythm. In women, the spontaneous initiation of the preovulatory LH surge generally occurs in the morning in association with high cortisol levels, suggesting a role for the hypothalamus in timing human ovulation. However, the most recent evidence suggests that this neural component of the control system timing the LH surge in women translates diurnal changes in environmental cues rather than an endogenous circadian rhythm.

It appears therefore that in rodents, the modification of the duration of the different phases of the estrous cycle might in some cases reflect disruption of the circadian synchronisation of the GnRH/LH preovulatory surge and that this is likely not the case in humans.

The picture is quite different when the observed parameter is the percentage of females exhibiting regular estrous cycles. This type of modification is more likely to signal a profound alteration of the basic mechanisms underlying the cross talk between the ovaries, the pituitary and the brain, which are well preserved among animal species.

Conclusion

There is some degree of uncertainty regarding the relevance to humans of rodent data on the estrous cycle disturbances when they relate to the duration of each phase of the cycle. However, when the results are expressed in terms of percentage of females exhibiting irregular estrous cycles as seen in several studies with BPA it is very likely that these effects can be considered as relevant to humans. In addition, alterations of the ovarian steroidogenic activity and/or of the neuroendocrine pathways mediating sexual steroid feedback are evidenced with BPA and provide support for human relevance since these are basic mechanisms underlying the estrous cycle that are common to most mammal species.

4.2.3.3.2 Differences in the timing of the ontogeny of the neuroendocrine axis and/or the gonads

The sequential events and regulations during development of the gonads and neuroendocrine reproductive axis is common to all mammals including human. However, the duration of each period is highly variable as shown in Figure 8, Thus, as an example, an exposure to BPA at birth will act on ovaries at different degrees of maturation in rodents (meiosis period) and in humans (meiosis ended).

Conclusion

Although the critical periods of exposure may differ in humans, it does not affect the general relevance of the effect/MoA.

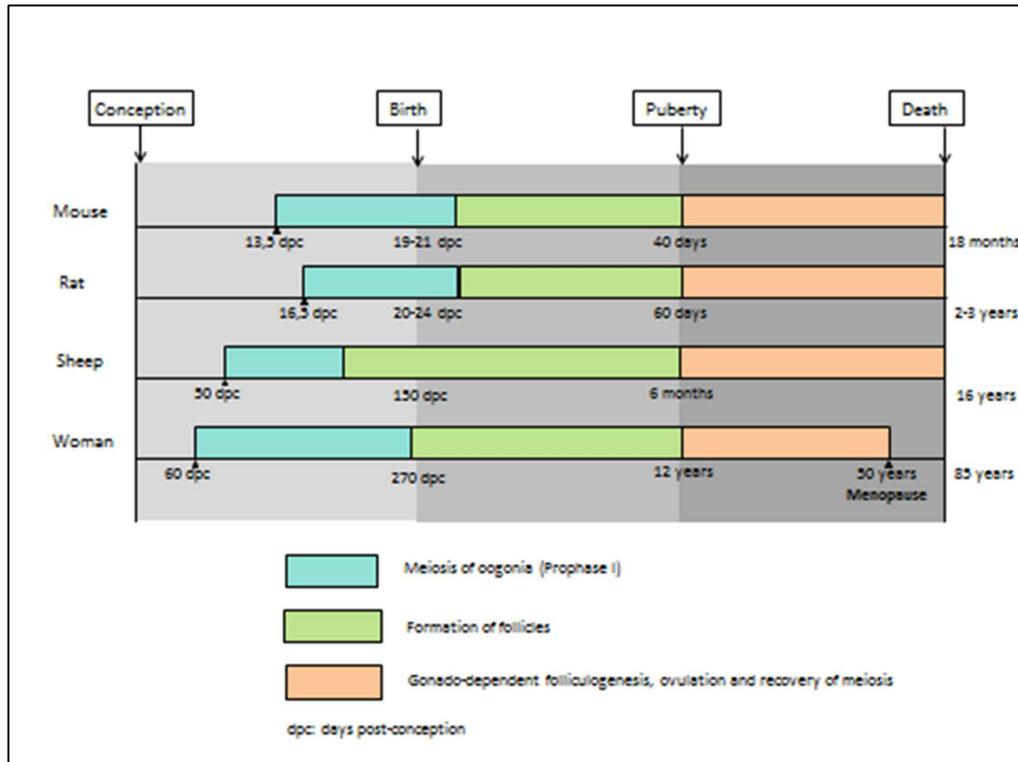


Figure 8: Ontogeny of the ovarian function in different species of mammals.

4.2.3.3.3 Differences and commonalities in the endocrine and neuroendocrine control of the ovarian cycle in adults

Ovarian control of the ovarian cycle

The cycle in humans differs from that in rat and mice in its duration (28 days on average), in a clear separation between follicular, and lutea phases, and a uterine cycle characterised by menstruations.

Furthermore, as explained hereabove, the follicular phase of the estral cycle in rodents is characterised by a peak of progesterone induced by the ovulatory surge of LH since the *corpus lutea* of the previous cycle is still functional at this time in rodents. This peak of progesterone does not impact the running of the cycle and is important to synchronise ovulation and female receptivity to male mounting in rodents. Indeed, liberated progesterone induces female receptivity, which is restricted to this period. This peak of progesterone does not occur in women, since progesterone is secreted during the luteal phase of the estral cycle only.

Furthermore, in humans there is a larger variability in the duration of the cycle from one cycle to another in the same woman and from one woman to another. It results from variability in the duration of the terminal growth of the follicle, leading to variability in the delay to reach the estrogens threshold that will trigger the pituitary gonadotrophins surge. Thus, the duration of the follicular phase is variable. Conversely, the duration of the luteal phase is relatively constant.

Another difference is the control of the *corpus luteum* regression, which is exerted by the *corpus lutea* in primates and by the uterus in rodents, but both mechanisms involving the same hormonal control involving PGF2 α .

In conclusion, there are some differences in the endocrine control of the cycle between rodents and humans. Nevertheless, the key regulatory endocrine mechanisms of the cycle are the same. Importantly, in all cycling mammalian species including primates, it is the progressive increase in estrogens secretion at the end of the follicular phase that triggers the release of LH and FSH ovulatory surges. In all cycling species, the experimental suppression of the production or the action of estrogens during the end of the follicular growing phase suppresses the ovulatory peak of gonadotrophins. Consequently, the BPA-induced reduction of the aromatase expression in the follicle described above is expected to trigger disturbances in the menstrual cycle in humans as well as in rodents.

Neuroendocrine control in humans

In humans, the importance of kisspeptin was first demonstrated by the hypogonadotropic hypogonadism of patients carrying a mutation of the KISS1R (de Roux *et al.*, 2003; Seminara *et al.*, 2003). More recent studies show that kisspeptin acts also upstream of GnRH neurones to coordinate GnRH and LH pulsatility (reviewed in Skorupskaite *et al.*, 2014). It stimulates the secretion of both LH and FSH, with a preferential stimulation of the former. Kisspeptin has also been shown to mediate both negative and positive feedback of sex steroids. In women, it seems therefore that sex steroid feedback involves both the hypothalamus and the pituitary gland.

At the neuroanatomical level, kisspeptin neurons extend from the preoptic area through to the infundibular nucleus (homologous to the ARC in rodents), as for GnRH neurons (Figure 9). In the infundibular region, kisspeptin neurons express also neurokinin B and dynorphin. By contrast to rodents where the RP3V and ARC respond to positive and negative sex steroid feedback respectively, the human infundibular nucleus relays signalling of both. It is, however, possible that the two processes are mediated by different neuronal populations.

The kisspeptin system seems also sexually dimorphic, although the critical period and origin of this dimorphism are still unknown. More kisspeptin fibres were detected in the infundibular nucleus and ventral periventricular area in women than in men (Hrabovszky *et al.*, 2010). Sex differences were also reported in the number and expression of kisspeptin cell bodies, which are present in the rostral periventricular zone of the female only.

In addition, the recent study by Kurian *et al.* (2015) on mid to late pubertal ovarian intact female rhesus monkeys suggests that persistent exposures to BPA could impair the female reproductive function by directly influencing the hypothalamic neuroendocrine function as evidenced by an alteration of kisspeptin release and GnRH pulsatility.

The role of kisspeptin in the neuroendocrine control of the HPG axis is relevant to humans. Therefore, it can be considered that BPA-induced alterations of the hypothalamic kisspeptin/GnRH system are also relevant in humans.

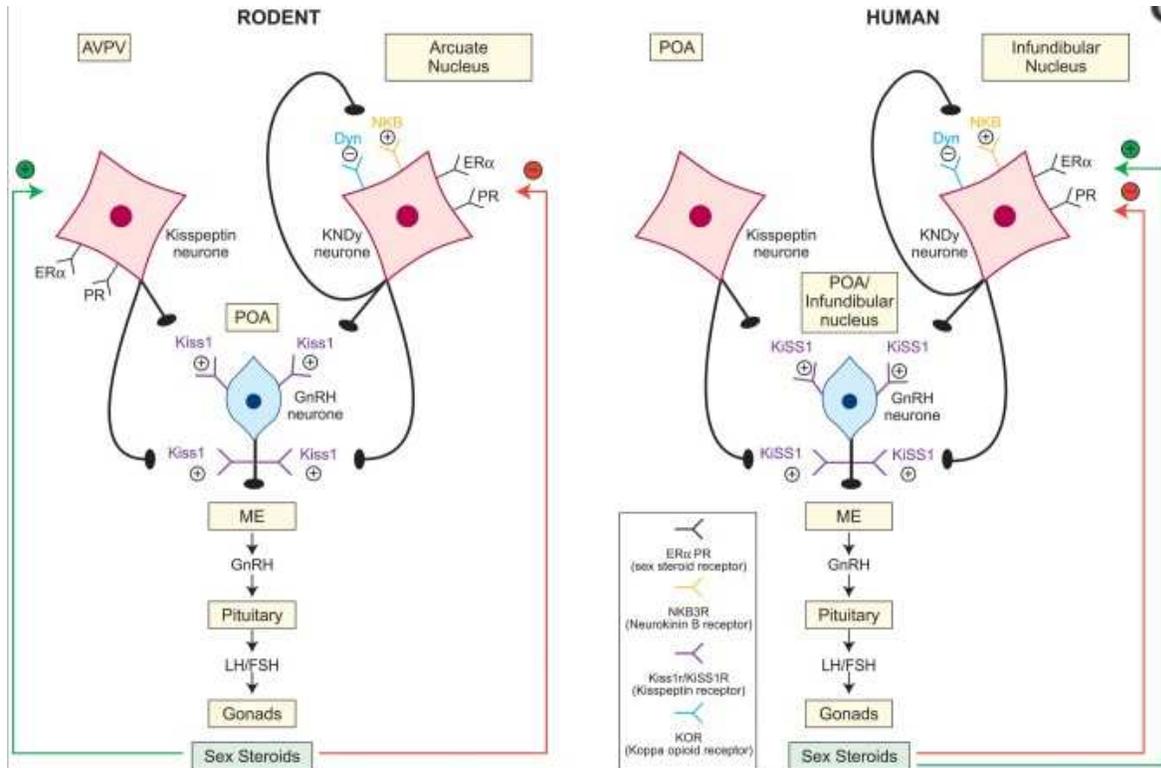


Figure 9: Comparison of kisspeptin neuronal distribution and regulation by sex steroids between rodents (left) and humans (right). Reproduced from Karolina Shorupskaitė *et al.* The kisspeptin-GnRH pathway in human reproductive health and disease. *Human Reproduction Update* (2014) 20 (4): 485-500. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology (ESHRE) online at: <https://academic.oup.com/humupd/article-lookup/doi/10.1093/humupd/dmu009>

Evidence from human data of an ED MoA

The association of BPA with altered hormonal levels in women has been investigated in a few epidemiological studies of good quality.

In a prospective study by Mok-Lin *et al.* (2010), which included women (n=84) following an ovarian stimulation protocol as part of an *in vitro* fertilisation, the authors indicated that there was a negative correlation between urinary levels of BPA (n=203 urine samples; 2 samples during 91 cycles and one sample during 21 cycles of IVF) and ovarian response in terms of number of oocytes collected as well as amplitude of the preovulatory estradiol peak. A mean decrease of 12% in the number of oocytes recovered per cycle and of 213 pg/mL from the estradiol peak was observed for each log unit increase of urinary SG-BPA (BPA specific gravity, i.e., the BPA concentration corrected by the urine specific gravity). The BPA levels found were compared to urinary BPA concentrations observed in the general population in the NHANES 2003-2008 cohort. The concentration of urinary BPA found reflects BPA exposure at the time of collection, but not during the period of follicular maturation several months earlier. In addition, it is noted that it may be difficult to extrapolate the results observed in a sample of infertile women undergoing an *in vitro* fertilisation to the general population.

Nevertheless, the results were consistent with those observed in another recent study.

Ehrlich *et al.* (2012) studied the association between urinary BPA concentrations and early reproductive outcomes among 174 women aged 18-45 years representing a total of 237 IVF cycles at a fertility center in Boston, USA. The study was a follow up of Bloom *et al.* (2011), who previously reported an association between urinary BPA and decreased

ovarian response (peak serum estradiol (E_2) and oocyte count at the time of retrieval) in women undergoing IVF. After adjustment for age and other confounding parameters (Day 3 serum FSH, smoking, BMI), there was a linear dose-response association between increased urinary BPA concentrations and decreased number of oocytes (overall and mature), decreased number of normally fertilised oocytes and decreased E_2 levels (mean decreases of 40, 253 and 471 pg/ml for urinary BPA quartiles 2, 3 and 4, when compared with the lowest quartile, respectively; p-value for trend=0.001). Women with urinary BPA above the lowest quartile had decreased blastocyst formation (trend test P-value=0.08). The results from this extended study, using IVF as a model to study early reproductive health outcomes in humans, indicate a negative dose-response association between urinary BPA concentrations and serum peak E_2 and oocyte yield.

Despite the fact that these studies were limited to a specific group of women with fertility disorders and despite the inherent limitations of epidemiological studies investigating a substance with short half-life, these studies provide some indications supporting the ability of BPA to alter hormonal regulation.

Overall conclusion on differences and commonalities

Differences between rodents and humans in the regulation of cycles are identified in relation to the role of circadian synchronisation and to differences in the timing of ontogeny of the neuroendocrine axis. In contrast the key principles of endocrine mechanisms of regulation of the cycle are the same between rodents and humans. Overall, these elements therefore bring support to the conclusion that the effects of BPA on disruption of cycles are relevant for humans.

In particular, both components that are shown to be involved in the endocrine MoA of BPA on cycle disturbance, i.e. the role of aromatase in estrogen production as well as the role of kisspeptin neurons in the ontogeny of the HPG axis are known to be relevant for humans.

4.2.4 Summary and discussion

In both primates and non-primate mammals, follicle selection, growth, and maturation, as well as ovulation, oocyte quality, and subsequent *corpus luteum* function, all depend on subtle sequential actions of gonadotropins and intraovarian regulators. Furthermore, the ovary and the hypothalamo-pituitary system are in permanent endocrine dialogue with each other. Consequently, any disturbances in the endo/para/autocrine activities of the ovary and/or the hypothalamus-pituitary system lead to cycle disturbance.

In addition, the estrous cycle is a perfectly synchronised and timely event that relies on specific neuroendocrine circuitries. Those pathways differentiate during fetal life and are largely influenced by numerous factors and in particular the steroid environment of the foetus. Thus fetal exposure to steroidogenic compounds is very likely to result in estrous cycle disturbances after puberty.

This review clearly shows that exposure to BPA at the adult stage alters the endocrine steroidogenic function of the ovary and more specifically the production of estrogens by the follicle, potentially leading to disturbance in the estrous cycle. Although most of the reported evidence relies on rodent studies there are *in vitro* data showing the same negative effect of BPA on the estrogen production in the human follicle cells. Furthermore, an indication of a negative association between the ability of the follicle to produce estrogens and exposure to BPA was observed in women. Lastly, the role of estrogens in the maintenance of the cycle is similar in rodents and humans. Thus, we conclude that it is quite likely that BPA may alter the ovarian cycle in humans through the disruption of the endocrine activity of the ovarian follicle.

At the neuroendocrine level, BPA can also act during the perinatal/postnatal organisation

or adult activation of the hypothalamus-pituitary system in rodents or primates. Because of the similarities in sex-steroid-induced regulation of this axis between humans and rodents, it is possible that the changes in kisspeptin, GnRH expression, activity or liberation and sex steroid receptor expression induced by developmental or adult exposure to BPA occur also in humans and therefore impact estrous cyclicity.

Table 6 below summarises the documentation supporting the ED-mediated MoA of BPA proposed for each of the two different periods of exposure. It is likely that both MoA may simultaneously contribute to the effect observed during both periods of exposure but the present analysis has been focused on available lines of evidence.

Table 7 provides an overview of the critical elements in the identification of an endocrine disruptor and how they are fulfilled for alteration of estrous cycle by BPA.

Table 6: Summary table of proposed ED-mediated MoA of BPA on alteration of estrous cyclicity and its documentation

Mode of action documented for the respective periods of exposure	Underlying cellular/molecular events		Alteration of organ/function
Alteration of sex steroid ovarian steroidogenesis during adult exposure	Reduced expression and activity of aromatase - <i>In vitro</i> : Watanabe 2012, Kwintkiewitz 2010, Mansur, 2016 - <i>In vivo</i> : Lee <i>et al.</i> , 2013	Decreased estradiol levels - <i>In vitro</i> : Zhou 2008, Peretz 2011, Mlynarcikova 2005, Kwintkiewitz 2010, Mansur 2016 - <i>In vivo</i> : Lee <i>et al.</i> , 2013	Alteration of estrous cyclicity - <i>In vivo</i> : Lee 2013, Laws 2000, Tyl 2008
Alteration of neuroendocrine regulation/ development of the reproductive function during developmental exposure	Alteration of kisspeptin expression - <i>In vitro</i> : Klenke 2016 - <i>In vivo</i> : Cao 2012, Navarro 2009, Xi 2011, Naulé 2014, Kurian 2015	Alteration in GnRH and/or LH secretion - <i>In vivo</i> : Monje 2010, Fernandez 2009, Viega-Lopez 2014, Kurian, 2015	Alteration of estrous cyclicity - <i>In vivo</i> : Honma 2002, Nikaido 2004, Wang 2014a, Rubin 2001, Mendoza 2011, Patisaul 2014, Delclos 2014, Nah 2011, Adewale 2009, Fernandez 2009, Zaid 2014

Table 7: Overview of the elements supporting the identification of an alteration of estrous cyclicity as an ED-mediated effect of BPA

	Adverse effect	Plausible ED MoA	Human relevance
Adult exposure	Key study of Lee 2013*	Direct link established with reduction of estradiol production through reduced aromatase activity	Hormonal regulation of cycles highly conserved in mammals Support from <i>in vitro</i> data in human cells

			Consistent with indications from human data
Developmental exposure	Identified in several studies*: Honma 2002, Nikaido 2004, Wang 2014a, Rubin 2001, Mendoza 2011, Patisaul 2014, Delclos 2014, Nah 2011, Adewale 2009, Fernandez 2009, Zaid 2014	Physiologically-based high plausibility of alteration of kisspeptin expression involved in neuroendocrine control	Role of kisspeptin identified in humans Support from non-human primate data on BPA-MoA

* Acknowledged by RAC

It is well recognised that the effects of BPA on the reproductive function are more diverse in their expression than alteration of estrous cycles as described in the introduction of this section (section 4.3.1). However, the female cyclicity is highly dependent upon hormonal fine regulation and as so it represents a good indicator for the identification of the nature of BPA endocrine MoA on the reproductive function.

Finally, it is noted that RAC in its opinion of March 2014 on classification (ECHA, 2014) provided an analysis of the MoA in the context of analysis of relevance for humans and reached the following conclusion:

"BPA was shown to influence the female reproductive tract. The associated alterations in pituitary signalling, serum hormone concentrations and reproductive organ morphology were likely causes of the reduced female fertility effects reported in the NTP (1985b) and Tyl et al. (2002) oral multi-generation studies and in several non-guideline research studies. Both oestrogenic and anti-oestrogenic effects of BPA were described and not all expected oestrogenic effects were observed. However, RAC concluded that the observed pattern of effects on the female reproductive tract suggest an overall oestrogen-like response in vivo.

Effects on the male reproductive tract, evident as impaired sperm production following BPA exposure, were observed in several studies. The decrease in sperm production was accompanied by lower testosterone levels. The effects observed on the testosterone levels may be the cause of the decreased sperm production.

RAC concluded that the classification of BPA for adverse effects on sexual function and fertility should be based mainly on the results from rodent studies. Disruption of oestrogenic signalling was considered to be the main MoA for the effects of BPA on fertility, based on current knowledge. The hormonal systems are well conserved between mammalian species, and the effects observed in rodents are therefore also relevant for humans. Detection of the active form of BPA (aglycone/unconjugated BPA) has been reported in humans (serum, cord blood and in placenta), but the credibility of these low-concentration measurements has been questioned due to the analytical techniques applied and potential contamination of the samples. However, after oral administration of low doses of stable isotope-labelled BPA (to exclude confounding sample contamination) and using sensitive and specific methodology, low systemic concentrations of aglycone/unconjugated BPA have been reported in rodents and in non-human primates, suggesting that unconjugated BPA becomes bioavailable in primates and rodents after oral exposure. Additionally, RAC noted that other routes of exposure such as sublingual, buccal, dermal and inhalation exposure are potential routes of human exposure and they bypass the extensive first-pass hepatic metabolism (first-pass effect). Taken together, RAC considered the MoA to be relevant to humans."

In conclusion, it is generally considered that the effects of BPA result from complex mechanisms that are most probably interacting together. Not all of them are fully understood. However, the overall database shows that an alteration of the regulation of estrogens is an essential pattern of the MoA.

4.2.5 Summary tables of studies

In the Table 8 below, studies are grouped by periods of exposure and sub-grouped by tested species (mouse/rat/other species), then chronological order of publication.

Table 8: Summary table of studies investigating the effects of BPA on the estrous cycle in female animals

Reference	Species	Routes	Dose Exposure period	Effect on estrous cycle
Gestational exposure				
Honma <i>et al.</i> , 2002	ICR Jcl mouse	Subcutaneous	0, 2, 20 µg/kg bw/d GD11- GD17	↗ the length of the estrous cycle of 1 day at both dose ($p \leq 0.05$).
Nikaido <i>et al.</i> , 2004	CD-1 mouse	Subcutaneous	0, 0.5, 10 mg/kg bw/d GD15 – GD19	Increased cycle length of 3 days in both groups with increased time of diestrus ($p < 0.01$)
Wang <i>et al.</i> , 2014a	FVB mouse	Oral (gavage)	0, 0.5, 20 or 50 µg/kg bw/d GD11-PND0	↗ time in diestrus and metestrus and ↘ in proestrus and estrus at 0.5 µg/kg ↘ in estrus at 20 µg/kg No significant effect at 50 µg/kg (↗ time in metestrus and ↘ in proestrus with DES)
Tinwell <i>et al.</i> , 2002	Sprague Dawley and Alderley park (derived from Wistar) rat	Oral (gavage)	0, 0.02, 0.1 or 50 mg/kg/d GD6-GD21	No difference in the stage of the estrous cycles at PND90.
Savabieas fahani <i>et al.</i> , 2006	Suffolk sheep	Subcutaneous	0 or 5 mg/kg bw/d GD30 – GD90	No effect on the length of progestogenic cycles during first breeding season but longer breeding season of 1 month ($p < 0.05$).
Perinatal exposure				
Naulé <i>et al.</i> , 2014	C57BL/6J mouse	Oral (gavage)	0, 0.05 or 5 mg/kg/d GD15 to PND21	Normal cycle analysed after PND60
Kwon <i>et al.</i> , 2000	Sprague Dawley rat	Oral (gavage)	0, 3.2, 32 or 320 mg/kg/d GD 11 – PND 20	No effect in 4-month F1 (irregular estrous cycle with DES)
Rubin <i>et al.</i> , 2001	Sprague Dawley rat	Oral (drinking water)	0, 0.1, 1.2 mg/kg bw/d GD6 – end of lactation period	Irregular cycles in 79% (4-week old) and 77% (6-month old) F1 at the high dose (significant). No significant effect at low dose. Intermittent extended period of diestrus, or extended period of proestrus and/or estrus.
Takagi <i>et al.</i> , 2004	Sprague Dawley rat	Oral	0, 7, 70 or 300 mg/kg bw/d (approx.) GD15 – PND10	No effect on estrous cyclicity (some animals with extended diestrus in the low dose group but not significant)
Yoshida <i>et al.</i> , 2004	Donryu rat	Oral gavage	0, 0.006 or 6 mg/kg bw/d GD2 – PND21	No effect on estrous cyclicity

Mendoza-Rodriguez <i>et al.</i> , 2011	Wistar rat	Oral (drinking water)	1.2 mg/kg bw/d GD6 – PND21	Observations in adult females (3 months) 79% with irregular cycles vs 6% in controls (determined on 4 consecutive weeks) characterised mainly by several continuous estrus days and in few animals persistent diestrus.
Delclos <i>et al.</i> , 2014	Sprague-Dawley rat	Oral gavage (aqueous solution)	2.5, 8, 25, 80, 260, 840, 2700 µg/kg bw/d and 100 and 300 mg/kg bw/d GD6- PND90	Observation between PND 69 to 90: At 300 mg/kg, ↑ incidence of animals with abnormal cycles primarily due to extended estrus and extended estrus/diestrus. Between PND150 and 170, ↑ incidence of abnormal cycles from 100 mg/kg. Effect similar to EE2. Similar serum level of unconjugated BPA between dose groups up to 80 µg/kg/d and negative controls is reported due to possible contamination. Identification of effects at lower doses of BPA is therefore impossible. (Churchwell <i>et al.</i> , 2014)
Ferguson <i>et al.</i> , 2014	Sprague Dawley rat	Oral (gavage)	0, 2.5 or 25 µg/kg GD6 to PND21	No effect on the proportion of days spent in each phase (lower proportion of days in diestrus with EE2, extended estrus transitions)
Postnatal exposure				
Nah <i>et al.</i> , 2011	ICR mouse	Subcutaneous	0, 0.1, 1, 10, 100 mg/kg bw PND8	↘ number of estrus days at the high dose (examined during 9 days from PND20)
Kato <i>et al.</i> , 2003	Sprague-Dawley rats	Subcutaneous	0, 0.25, 1 or 4 mg/pups PND0 – PND9	Observation from PND 61 to 94: irregular estrous cycles in 4/6 females and persistent estrus in 2/6 at 4 mg/kg; (similar effects with 10 µg/kg E2)
Adewale <i>et al.</i> , 2009	Long-Evans rat	Subcutaneous	0, 50 µg/kg bw/d or 50 mg/kg bw/d PND1- PND3	At 50µg/kg: 14% of females were not cycling anymore by 15 weeks after vaginal opening. At 50mg/kg: 67% of females were not cycling anymore by 15 weeks after vaginal opening. (100% in controls)
Fernandez <i>et al.</i> , 2009	Sprague Dawley rat	Subcutaneous	0, 6,2/2,5, 62.5/25 mg/kg bw/d PND1- PND10	Irregular estrus cycle in adult Female at high dose with high prevalence of estrus after PND90 (p<0.05)
Monje <i>et al.</i> , 2010	Wistar rats	Subcutaneous	0, 0.05 or 20 mg/kg bw/d PND1-PND7	Observations of females for 2 weeks from PND85: ↑ in proestrous/estrous time (p<0.001) at 0.05 mg/kg (high dose not examined for cyclicity)
Franssen <i>et al.</i> , 2016	Wistar rats	Subcutaneous	0, 25 ng/kg or 5 mg/kg bw PND1 – PND5 or PND15	Observations from VO to PND 80: no effect on estrous cyclicity
Prepubertal exposure				
Nikaido <i>et al.</i> , 2005	ICR mouse	Subcutaneous	0 or 10 mg/kg bw/d PND 15 to 18	No effect on the estrus cycle during 5-8, 9-12 and 21-24 weeks of age.
Zaid <i>et al.</i> , 2014	Sprague-Dawley rats	Oral (gavage)	0 or 10 mg/kg/d For 42 days from PND28	Only 3/8 rats with normal cycles (p<0.05) and 5/8 rats with persistent diestrus (p<0.05)
Adult exposure				
Moore-Ambritz <i>et al.</i> , 2015	C57BL/6 mouse	Oral (gavage)	0 or 50 µg/kg/d For 12-15 days from day of 1 st estrus (approx. PND39)	No effect on duration of each estrous stage during the dosing period (lengthened cycle with DES).
Laws <i>et al.</i> , 2000	Long Evans rat	Oral (gavage)	0 or 100 mg/kg for 25 days in cycling	Reduced number of 4-5 day cycles (p<0.05). Extended diestrus in 6 animals, extended

			animals	estrus in 2 animals, normal cycles in 7 animals. (similar effects but more pronounced with E2)
Lee <i>et al.</i> , 2013	Sprague-Dawley rat	Oral (gavage)	0, 0.001 or 0.1 mg/kg bw/d 8-week animals exposed for 90 days	Observations of female for 30 days after the 90-day exposure: ↗ in duration of the estrus phase: p<0.001 at 0.001 mg/kg, p<0.01 at 0.1 mg/kg and p<0.05 with EB No animal in a persistent estrus phase.
Multi-generation studies				
NTP, 1985	CD-1 mouse	Oral (diet)	0, 300/350, 600/650 or 1200/1300 mg/kg/d (M/F) 1 week before mating until sacrifice (continuous breeding; 2 G)	No effect on estrous cycle
Tyl <i>et al.</i> , 2008	CD-1 mouse	Oral (gavage)	0.003, 0.03, 0.3, 5, 50 or 600 mg/kg/d 8 weeks before mating to adulthood (2 G)	F0 treated females were twice more in estrus as compared to controls at 600 mg/kg
Ema <i>et al.</i> , 2001	Sprague-Dawley rat	Oral (gavage until weaning (2 G)	0, 0.2, 2, 20 or 200 µg/kg/d 10 (M) or 2 (F) weeks pre-mating	No effect
Tyl <i>et al.</i> , 2002	Sprague-Dawley rat	Oral (diet)	0.001, 0.02, 0.3, 5, 50 or 500 mg/kg/d 10 weeks before mating until PND21 (3 G)	No effect on estrous cycle length.

Table 9: Summary of the *in vitro* studies showing an alteration of the ovarian steroidogenesis function likely to result in disturbance of estrous cyclicity if occurring *in vivo*.

Cell type	Reference	Tissue	Concentration	Results	Conclusion
Theca-interstitial cells	Peretz <i>et al.</i> , 2011	Mouse isolated antral follicles	100 and 10 mg/L (=440 and 44 µM) BPA	↓ Testo, ↓Androstenedione, ↓DHEA-S, ↓ STAR, ↓CYP450scc, → ¹⁶ 3 β-HSD, → CYP450 17apha	No clear-cut conclusion
	Zhou <i>et al.</i> , 2008	Theca-interstitial cells isolated from immature SD rats	10 to 100 µM BPA	↑ STAR, ↑CYP450scc	
Granulosa cells	Zhou <i>et al.</i> , 2008	Granulosa cells isolated from mature SD rats	1 to 100 µM BPA	↓ estradiol	BPA reduces CYP450arom expression and estrogen production in rodents, domestic animals and human granulosa cells in all studies.
	Peretz <i>et al.</i> , 2011	Mouse isolated antral follicles	100 and 10 mg/L (=440 and 44 µM) BPA	↓ estradiol	
	Mlynarcikova <i>et al.</i> , 2005	Porcine granulosa cells from antral follicles	1 to 100 µM BPA	↓ FSH-induced estradiol	
	Watanabe <i>et al.</i> , 2012	KGN (a human granulosa-like cell line)	5 to 100 µM BPA	↓ CYP450arom	
	Kwintkiewitz <i>et al.</i> , 2010	KGN and human granulosa cells	40 to 100 µM BPA	↓ FSH-induced CYP450arom, ↓ estradiol, ↓ IGF-I, ↓ GATA-4, ↓ SF-I, ↑ PPAR-gamma,	
	Mansur <i>et al.</i> , 2016	Human granulosa cells	8.8 to 88 µM BPA	↓ estradiol, ↓ progesterone ↓ Cyp arom (mRNA and protein)	

¹⁶ Where the symbol "→" is presented, this is intended to be read as "no significant effect".

Table 10: *in vivo* evidence for endocrine mechanisms potentially underlying alteration of estrous cyclicity as a consequence of adult exposure to BPA

Reference	Species Age	Route (dose) Duration	Effect	Evidence for ED MoA
Lee <i>et al.</i> , 2013	Rat SD PND 56	Gastric: - BPA: 1 and 100 µg/kg bw/d - EB: 1 µg/kg bw/d 90 days	Disruption of the estrous cycle with extended estrous phase of 2-7 days with both BPA doses and EB	Both BPA doses and EB: ↓plasma estradiol, ↓testosterone, ↓STAR, ↑apoptosis in follicles and corpus lutea. → FSH Both BPA doses but not EB: ↓aromatase in granulosa cells ↓ uterine estrogen-induced proteins (PCNA calbindin-D9k) ↓ uterine collagens ↑plasma and pituitary LH concentrations
Wang <i>et al.</i> , 2014b	Mouse ICR Adult in pro-estrous	Oral (20 µg/kg) or ICV (0, 0.02, 0.2, 2.0, 20.0, and 200.0 nM/3 ml) Single exposure Analysis 6 hours after exposure	↑ GnRH and Kiss 1 expression ↑LH, FSH, E2	Effects blocked by GPR54 and ERβ antagonists Estrous cyclicity not monitored but effects very likely to be related to disruption of estrous cyclicity
Kurian <i>et al.</i> , 2015	Rhesus monkey Pubertal female (approx. 38 months)	Infusion of 0, 0.1, 1 or 10 nM BPA into stalk-median eminence of the hypothalamus for 240 min Simultaneous collection of dialysate	↓ GnRH and kisspeptin release, ↑ pulse amplitude interval at 10 nM	Alteration of GnRH secretion

Table 11: Summary of the *in vitro* study showing an alteration of GnRH activity by BPA

Reference	Tissue and treatment period	Type of evaluation	Type of modification	Evidence for PED MoA
Klenke <i>et al.</i> , 2016	Nasal explants from mice embryos at embryonic day 11.5 (after emergence of GnRH cells and other neuronal cell types from the plasma codes) used after 6 days of culture – 50 µM	Calcium imaging	BPA reduces the frequency of oscillations in GnRH neurons	Direct effect of BPA on GnRH neurons

Table 12: Evidence for BPA-induced disturbance of estrous cyclicity in animal models and link with an endocrine disruptive MoA

Reference	Species Period	Route Doses	Type of evaluation	Type of modification	Evidence for ED MoA
Rubin <i>et al.</i> , 2001	Rat SD GD6- PND21	Oral (drinking water) 0.1-1.2 mg/kg/d	Daily vaginal cytology for 18 consecutive days at 4 and 6 months 8 female offspring/ group were ovariectomised and killed 3 months later to assess LH levels.	↓% of animals with regular estrous cycle ↑ duration of the cycle and ↓ nb of estrous cycles/animal with 1.2 mg/kg/d	↓LH secretion in BPA- treated OVX animals showing a BPA-induced alteration in the endocrine function of the hypothalomo- hypophysis gonadotropic axis. But LH was not evaluated in ovary intact animals
Nikaido <i>et al.</i> , 2004	Mouse CD1 GD15- GD19	Subcutan eous 0.5-10 mg/kg/d	Daily vaginal smears between 9 to 11 weeks	↑mean cycle length ↑time in diestrus	For diestrus: similar increase with DES
Adewale <i>et al.</i> , 2009	Rat Long- Evans PND0- PND3	Subcutan eous 50 - 50000 µg/kg/d sc	Daily 4 days vaginal lavage from 2 weeks after VO and every two weeks for 13 weeks	Time and Dose- dependent decrease of the number of cyclic females	Similar but more rapid and more sustained effect with PPT a selective agonist of ER α
Monje <i>et al.</i> , 2010	Rat Wistar PND1- PND7	Subcutan eous 0.5 or 20mg/kg /d	Daily vaginal smears from PND85 to PND 100 performed in the group exposed to 0.05 mg/kg	↑time spent in proestrus/ estrus at 0.05 mg/kg (not examined at 20 mg/kg)	Females at 20 mg/kg were incapable of producing the LH surge. Alteration of GNRH maturation process and ER α expression in the AVPV and ARC, and PR in the AVPV
Fernandez <i>et al.</i> , 2009	Rat SD PND1- PND10	Subcutan eous ~5-50 mg/kg/d	PND90	Irregular estrus cycles (persistent estrus)	↓pituitary sensitivity to GnRH in estrus in vivo and in vitro ↑GnRH pulse frequency in juvenile and adults
Wang <i>et al.</i> , 2014a	Mouse FVB GD11- PND0	Oral (gavage) 0.5-20- 50 µg/kg/d	From PND21-51 Daily Vaginal smears	0.5 µg/kg/d ↓proestrus and estrus ↑metestrus 20 µg/kg.d: ↑estrus Not with high dose 50µg/kg/d	For proestrus and metestrus: idem DES (ref Berger <i>et al.</i> , 2016 for PE mechanisms)

Delclos <i>et al.</i> , 2014	Rat SD From GD6	Oral (gavage) 2.5*, 8*, 25*, 80*, 260, 840, 2700, 100000, 300000 µg/kg/d	Daily vaginal cytology from PND 69 to 90 and from PND 150 to 170	↑% female with extended estrus (highest dose only)	EE2-like effect ↑E2 and PRL- ↓progesterone
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* Unconjugated BPA serum level of these groups are similar to negative control: Interpretation challenged by possible contamination of the negative controls.

Table 13: Studies addressing the effects of developmental exposure to BPA on the kisspeptin/GnRH system

Reference	Animal model	Doses (route)	Period	Effects on Kisspeptin	Effects on GnRH
Cao <i>et al.</i> (2012)	Long Evans rats	50 µg/kg – 50 mg/kg (SC)	PND0-PND2	Diminished Kiss1 expression (mRNAs) in the RP3V at PND10. No modification of Kiss1 in the ARC	
Adewale <i>et al.</i> (2009)	Long Evans rats	50 µg/kg – 50 mg/kg (SC)	PND0-PND3		No modification in GnRH expression in the OVLT
Fernandez <i>et al.</i> (2009)	Sprague-Dawley rats	50 µg/50µL – 500 µg/50µl (SC)	PND1-PND10		Increased infantile GnRH pulsatility. During adulthood, diminished GnRH-induced secretion of LH and disturbed pulsatility
Navarro <i>et al.</i> (2009)	Wistar rats	100 – 500 µg/rat (SC)	PND1-PND5	Diminished Kiss-1 mRNAs levels in the hypothalamus at PND30	
Patisaul <i>et al.</i> (2009)	Long Evans rats	50 µg/kg – 50 mg/kg (SC)	PND1-PND4	No effect of BPA exposure in the AVPV, decrease in the ARC at the higher dose. Diminished kisspeptin cell density in the AVPV and ARC by with ERalpha agonist.	
Monje <i>et al.</i> (2010)	Wistar rats	0.05, 20 mg/kg (SC)	PND1-PND7		mRNAs GnRH increased at BPA-0.05 mRNAs GnRH decreased at BPA-20
Losa-Ward <i>et al.</i> (2012)	Wistar rats	50µg/kg – 50mg/kg (SC)	PND0-PND3	No differences in kisspeptin cell density in the AVPV or kisspeptin-GnRH appositions. Diminished cell density and number of RFRP3 neurons, and RFRP3-GnRH appositions	
Franssen <i>et al.</i> (2016)	Wistar rat	25 ng, 25 mg, 5 mg/kg/d (SC)	PND1-PND15		GnRH IPI increased by 25 ng, reduced by 5 mg
Xi <i>et al.</i> (2011)	CD1-mice	12 – 25 - 50 mg/kg (oral)	G1-PND49 or PND21-PND49	G1-PND49: Increased Kiss1mRNA expression in the hypothalamus at BPA- 25 and 50	G1-PND49: increased GnRH expression levels in the hypothalamus at BPA-25 and 50. No modification in GnRH-R expression levels in the pituitary
Naulé <i>et al.</i> (2014)	C57BL6J mice	0,05-5 mg/kg/d (oral)	GD15-PND21	Increased kisspeptin number in the RP3V of adult females	

4.3 Alteration of mammary gland development

4.3.1 Overview of previous evaluation of BPA's effect on mammary gland

Effects of BPA on mammary gland have already been assessed in previous European reports (ANSES, 2014; EFSA, 2015; ECHA, 2015). The main outcomes of these evaluations are reported below.

The EFSA (2015) opinion concluded: *"The proliferative responses and possibly enhanced sensitivity to mammary gland carcinogens seen in animal studies might be of relevance for human health and are therefore included in the risk assessment."* and *"the CEF Panel concluded that BPA-induced effects on the mammary gland of rats, mice or monkeys exposed pre- or perinatally were "likely" effects".*

However, EFSA considered none of the available studies to be sufficiently robust in terms of methodology, or a consistent dose-response for deriving a health-based guidance value based on mammary gland effects.

See sections 3.9 and 4.3 of the EFSA (2015) opinion for more details."

In the restriction BPA dossier (ANSES, 2014), on thermal paper, ANSES considered that the effects of BPA on the mammary gland were "recognised" effects in animals and should be taken into account to assess the risk to human health. ANSES observed that EFSA's draft opinion also considered that the effects of BPA on mammary gland development are "likely" and that these effects are relevant to humans.

ANSES considered that it is important to take into account the possibility of increased cancer risk in the children of women who have a high level of endogenous estrogens or xeno-estrogens during pregnancy and are then exposed to tumour initiating agents. Based on the studies described later in the opinion of RAC (ECHA, 2015), RAC concluded that *"the Dossier Submitter considered ductal hyperplasia and effects on the architecture of the mammary gland, including effects on Terminal End Buds (TEB) as critical effects for the human risk assessment. For effects on these undifferentiated epithelial structures (Terminal Ducts (TD and TEB), an oral NOAEL of 25 µg/kg bw/day and a LOAEL of 250 µg/kg bw/day were proposed by the Dossier Submitter based on Moral et al. (2008).*

In its opinion of **June 2015 (ECHA, 2015)**, RAC adopted the following conclusions in relation to the analysis of the effect of BPA on mammary gland:

"The overall qualitative conclusion of RAC regarding the mammary gland changes is that BPA caused an acceleration of mammary gland maturation in experimental animals. There are slight indications of relevant intraductal hyperplasia from two studies with subcutaneous exposure (Murray et al., 2007 and Vandenberg et al., 2008).

Conclusion

RAC agrees that BPA has been shown to have a proliferative effect on mammary tissue at doses below the doses causing general toxicity (such as kidney weight changes). RAC in principle agrees with EFSA's conclusion on mammary gland effects. The effects on mammary gland development should be taken into account in hazard and risk assessment and in health impact assessment. In line with EFSA (2015), no individual study is however considered robust enough by RAC to serve as critical study for the identification of a starting point for DNEL derivation. Therefore the effects will be accounted for in the setting of Assessment Factors.

The Dossier Submitter derived a LOAEL of 0.26 mg/kg bw/day based on increased body weight and increased cholesterolemia in female mice in Miyawaki et al., 2007."

The evidence for the effects of BPA on mammary gland presented above and considered for the risk assessment included in the restriction dossier were supplemented with recent *in vivo* and epidemiological studies investigating "BPA and breast cancer risk" published until May 2016. The *in vitro* key experimental studies were also collected in order to substantiate the ED MoA and plausible link between adverse effects and the MoA of BPA.

In a first section, background information is provided. In the following sections, the available evidence on adverse effects on the mammary gland are described, together with the data showing that these effects are related to an ED MoA in a causal way.

4.3.2 Background information on mammary gland development

As indicated above, this review will address the effects of BPA during the mammary gland development following early life exposure as well as long lasting effects including hyperplastic lesions after fetal / perinatal BPA exposure. Experimental *in vitro* and *in vivo* findings including recent results from epigenetic studies generated since the evaluation quoted above, have been considered in this dossier. All this information is developed in the following sections.

The following information is given in order to facilitate comprehension of the experimental mechanistic studies available on BPA. The mammary gland develops in sequential steps that will be described below. The hormonal implication, mammary epithelial estradiol signaling, and progesterone (PR) and prolactin (PL) receptor involvement depending on the developmental period are presented. There is a summary of key mechanisms that have been described after β -estradiol as well as DES exposure.

Lastly, relevant background information on both epigenetic mechanisms and HOX genes that are key players in embryogenesis and post-natal development, and during the early stages of neoplastic process, are also reported in the section below.

4.3.2.1 Mammary gland structure and its sequential development

Critical events in breast development begin during fetal life with epithelial bud sprouting, whereas extensive branching morphogenesis continues into postnatal life.

Rapid mammary gland development occurs *in three distinctive life stages*: fetal, peri-pubertal, and pregnancy (Fenton, 2006). Development of the mammary glands is initiated in the embryo with a major part occurring in adult stage. While development in puberty and pregnancy is hormone-dependent, early postnatal development appears to progress without hormone activation.

- *Prenatal and early postnatal development:*

Epithelial-mesenchymal interactions are critical during development. Formation of the bud, the first appearance of hormone receptors, formation of the primary sprout and ductal elongation have been shown to be regulated by epithelial-mesenchymal signaling. The "primary mammary mesenchyme" is distinguished by the expression of specific genes among them those coding for steroids, namely androgen and estrogen receptors, and for PPT-A (Robinson *et al.*, 1999). Some of the signaling molecules that are required in these processes such as LEF1 or PTHrP have been identified through gene inactivation (Robinson *et al.*, 1999).

- *Peri-pubertal and adult period:*

A rudimentary ductal system present at birth begins to unfold during puberty and gains in complexity during adulthood with recurrent hormone stimulation during menstrual/estrous cycles. Ductal complexity increases further during pregnancy and finally secretory structures of saccular shape, called alveoli, bud all over the ductal system.

As an illustrative example the period around puberty in mice, i.e. between 20 and 30 days of age, is characterised by the reinitiation of ductal growth in the mammary glands with expansion of the epithelial tree into the surrounding stromal tissue (see Figure 10). Estrogens play a major role in this process (Topper and Freeman, 1980). Estrogen-induced cell proliferation during puberty concentrates at the ductal tips. As a result, they enlarge and form club-like structures that measure between 1.5 to 10 times the diameter of the subtending ducts and are called TEBs. These structures invade the stroma and mediate the longitudinal growth of the subtending ducts. When the ductal tree reaches the edge of the fat pad, the TEBs mature into a resting structure, the terminal ends, and ductal growth ceases. In adulthood, the ductal complexity increases through progesterone-induced side branching. Side branching is enhanced during early pregnancy. Alveoli develop later in pregnancy and will be fully distended by milk during lactation.

This morphological differentiation is sustained by high cellular activities. At puberty, as the TEBs become bulbous, they show both high proliferative and high apoptotic activity. A large number of apoptotic cells are observed in TEBs at PND30 in mice. Death of the body cells is essential for the formation of the lumen on the proximal side of the TEBs (Humphreys *et al.*, 1996) and for the growth of the subtending duct (Munoz de Torro *et al.*, 2005).

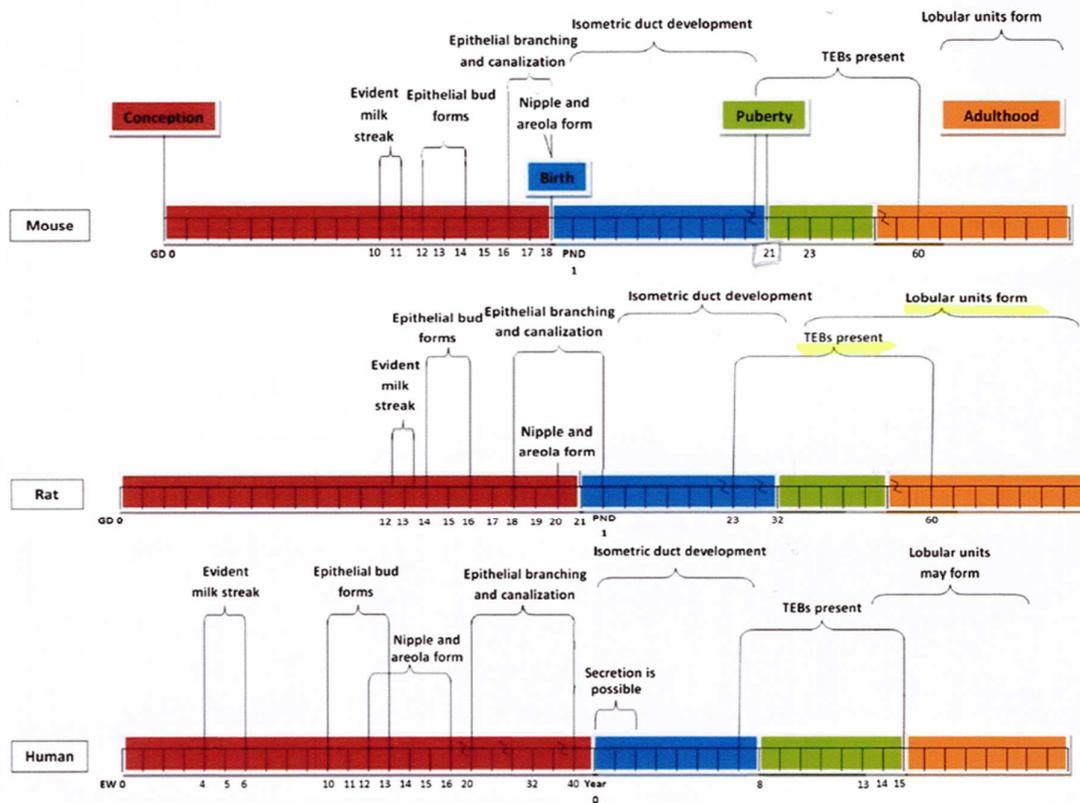


Figure 10: Mammary development in different species (mice, rat, humans) quoted from Davis and Fenton, 2013

Parenchyma: constituted of one or two major lactiferous ducts that grow from the nipple into the surrounding fat pad and of terminal epithelial structures such as Terminal End Bud (TEBs).

Stroma: constituted of conjunctive, adipose tissues and of blood vessels

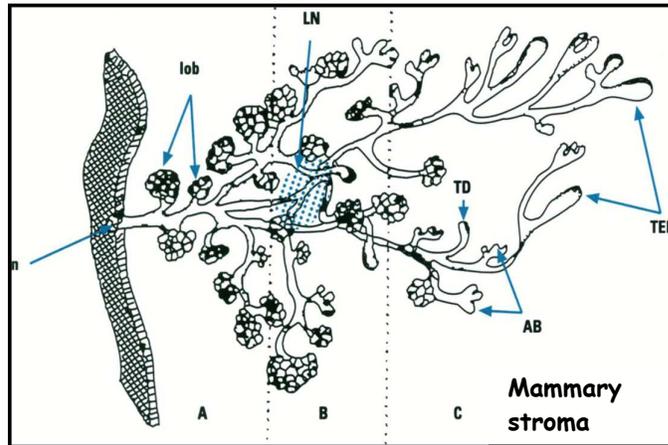
Legend:

The gland is divided into three areas:

- **Zone A:** proximal to the nipple (**n**), contains more numerous lobules

- **Zone B:** medial, encompasses the lymph node (**LN**).

- **Zone C:** distal to the nipple, contains the majority of actively growing terminal end buds



Schematic representation of the fourth (abdominal) mammary gland of a 55-day-old virgin female rat (according to Russo et Russo, 1980 and 1996)

Figure 11: Schematic representation of the fourth (abdominal) mammary gland of a 55-day-old virgin female rat (according to Russo and Russo, 1980 and 1996)

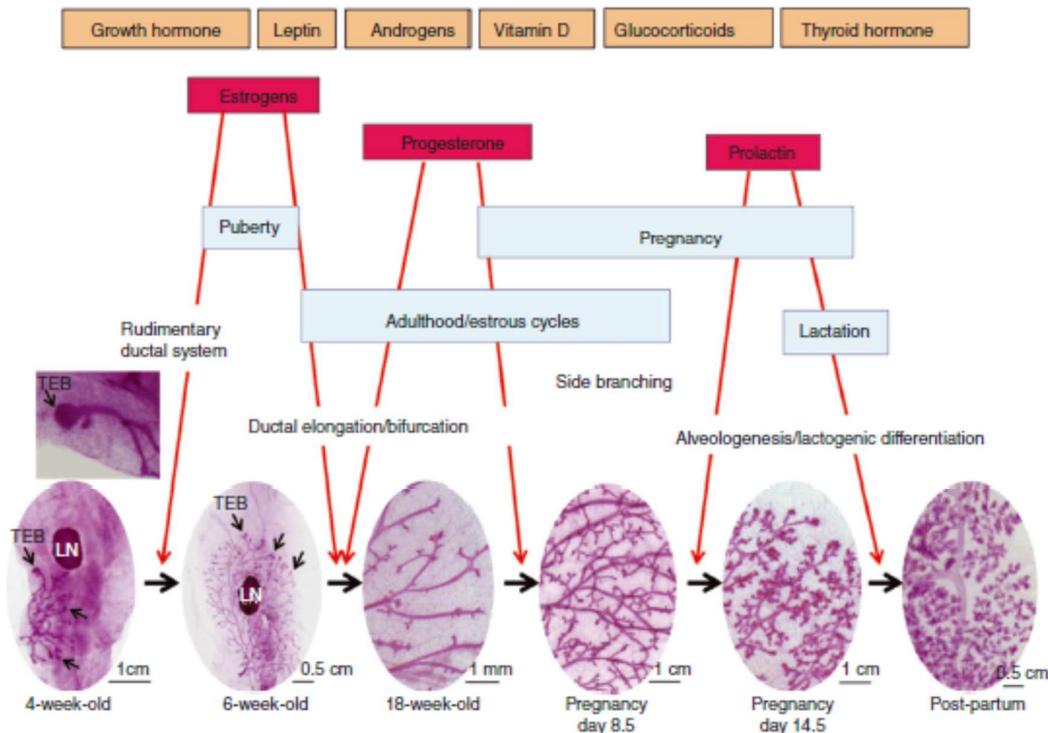


Figure 12: Hormonal control of mouse mammary gland development.

Legend: Whole-mount micrographs of inguinal mammary glands of C57Bl6x129Sv mice at different stages of mammary gland development illustrate critical stages in mammary gland development. Corresponding reproductive stages are

depicted by light blue boxes. In the red boxes the key female reproductive hormones. Arrows indicate at which stages the respective downstream receptor mediated signaling is limiting. The orange boxes at the top indicate other endocrine factors that have been implicated in mammary gland development but whose precise role remains to be determined. During puberty, the rudimentary ductal tree elongates under the influence of estrogen until the edges of the fat pad are reached and simple ductal system is established through bifurcations. Terminal end buds (TEBs) (arrows) are characteristic of the pubertal stage. In adulthood, the ductal complexity increases through progesterone-induced side branching. Side branching is enhanced during early pregnancy. Alveoli develop later in pregnancy; they will be fully distended by milk during lactation. LN, lymph node.

Cited from Brisken and Ataca, 2015.

4.3.2.2 Estrogen, progesterone and prolactin hormones

A large number of mouse mutant strains are available, and tissue recombination experiments allow the generation of epithelial specific mutants. This approach has revealed that mammary epithelial intrinsic ER α signaling is required for pubertal ductal elongation (Mallepell *et al.*, 2006).

The progesterone receptor (PR) is essential in the mammary epithelium for side branching and alveologenesis (Brisken *et al.*, 1999), whereas the epithelial prolactin receptor is required for alveologenesis and milk secretion (Brisken *et al.*, 1999) (see also Figure 12, from Brisken and Ataca, 2015). A small amount of budding of alveolar structures develops during each menstrual/estrus cycle due to the stimulus of ovarian hormones (Russo *et al.*, 2001). During pregnancy and lactation, the number of lobuloalveolar structures increases exponentially under the control of the prolactin pituitary hormone (Brisken *et al.*, 1999; Brisken and Rajaram, 2006).

The mammary epithelium responds differently to a hormonal stimulus depending on its developmental stage. Hormone ablation and replacement experiments have shown that 17- β -estradiol induces cell proliferation specifically in pubertal (Daniel *et al.*, 1987) but not in adult mammary glands. In the adult, i.e., more than 8-week-old, female mouse 17- β -estradiol pretreatment induces the expression of the progesterone receptor (Haslam and Shyamala, 1979), whereas subsequent stimulation with progesterone triggers cell proliferation (Grimm *et al.*, 2002). Hence, in the adult female, PR signaling is the major stimulus of cell proliferation. Lastly, Fenton (2006) emphasises that dose levels and timing (windows of sensitivities) to EDC exposures may affect the severity, or lack thereof, of an effect on mammary gland growth and consequently breast cancer risk.

4.3.2.3 Molecular changes recently suspected to be involved in the process of carcinogenesis

Epigenetics describes a range of DNA and histone modifications that influence levels of gene expression without modifications to the underlying coding sequence (Goldberg *et al.*, 2007). Such modifications include DNA methylation (DNMT), histone modifications (such as the polycomb PRC2) and non-coding RNA. Epigenetic changes have been implicated in the process of carcinogenesis (Esteller, 2007; Jones and Baylin, 2007). Recent studies have shown that abnormal epigenetic silencing of genes can frequently occur during the early stages of the neoplastic process, such as the preneoplastic stages of breast carcinogenesis (Feinberg *et al.*, 2006; Baylin and Ohm, 2006; Fernandez *et al.*, 2012; Faryna *et al.*, 2012).

The EZH2 gene has been shown to be upregulated by estradiol, in an *in vitro* and *in vivo* study conducted by the Mandal team on rat mammary glands (Bhan *et al.*, 2014a). Its expression is modulated through an epigenetic mechanism. EZH2, an enzyme histone methyltransferase, promotes proliferation and neoplastic transformation of breast epithelial cells (Kleer *et al.*, 2003, Collett *et al.*, 2006). EZH2 is linked to two Polycomb repressive complex, PRC2/3 (which are members of the EZH family of histone methyltransferase and initiate gene silencing), and PRC4 which is expressed in cancer and embryonic stem cells (Baylin and Ohm, 2006). The polycomb complex PRC2 controls cell fate, development and cancer (Sparman and van Lohuizen 2006; Margueron and Reinberg, 2011). EZH2 has been described as a molecular marker for a precancerous state in morphologically normal breast tissues and preneoplastic progression in

the breast (Ding and Kleer, 2006). Lastly, two important genes are targeted by EZH2 namely p57 (CDKN1C), a cyclin dependent kinase inhibitor, and E-cadherin which is important in cell-cell adhesion and migration. Repression of p57 and E-cadherin by EZH2 may lead to the increase of cell proliferation, or increased invasiveness, seen in some breast tumors.

HOTAIR (HOX antisense intergenic RNA) coordinates with chromatin modifying enzymes and regulates gene silencing. It is overexpressed in various carcinomas including breast cancer. HOTAIR is crucial for cell growth and viability and its knockdown induced apoptosis in breast cancer cells. Bhan *et al.* (2013) showed that HOTAIR is transcriptionally induced by estradiol (E2). Its promoter contains multiple functional estrogen response elements (EREs). Similar to protein-coding gene transcription, E2-induced transcription of antisense transcript HOTAIR is coordinated *via* ERs and ER coregulators, and HOTAIR overexpression is linked to invasiveness and metastasis in several cancers (Bhan *et al.*, 2013).

The HOX genes are an evolutionary conserved family of genes that are key players in embryogenesis and post-natal development (Mallo and Alonso, 2013). Several HOX genes control development of the mouse mammary gland in response to pregnancy (Chen and Capecchi, 1999). Among them, HOXC6 and HOXB9 are associated with mammary gland development. HOXC6 null female mice show complete absence of mammary epithelium in thoracic and dilated ducts in inguinal glands (Garcia-Gasca and Spyropoulos, 2000). HOXB9 is involved in cell proliferation, cell-cycle progression, and differentiation. The overexpression of HOXC6 or HOXB9 increases the expression of growth factors (Shrestha *et al.*, 2012). Many HOX genes also appear to play critical roles in tumor cell proliferation and metastasis (Friedman *et al.*, 1994; Chen and Sukumar, 2003). HOXB9 overexpression alters the tumor microenvironment and promotes breast tumor metastasis, associated with clinical outcome in patients (Hayashida *et al.*, 2010; Seki *et al.*, 2012).

4.3.3 Adverse effect

As indicated previously, exposure to an estrogen-like compounds (xenoestrogen) during critical stages of development can interfere with hormonal signaling and may result in persistent morphological abnormalities and altered gene expression (Fenton *et al.*, 2012; Reed and Fenton, 2013; Macon and Fenton, 2013).

Several findings prompted researchers to hypothesise that fetal exposure to xenoestrogens may play a role in the risk of contracting breast cancer observed in the last 50 years. First, a positive association was observed between increased intrauterine levels of estrogens (a phenomenon observed in twin births) and risk of breast cancer in daughters born from such pregnancies (Ekbohm *et al.*, 1992) which supports the link between perturbation in the fetal environment and breast cancer. Secondly, epidemiological data also revealed an increased incidence of breast cancer in women exposed *in utero* to DES (Hoover *et al.*, 2011; Palmer *et al.*, 2002 and 2006). DES has been shown to cause epigenetic changes in HOXA10 expression in the reproductive tract by altered DNA methylation (Bromer *et al.*, 2009 and Bromer *et al.*, 2010). Epigenetic changes as a result of exposure may predispose to malignancies in adulthood.

Fetal exposure to low doses of BPA alters the tissue organisation of fetal mammary gland in rodents and non-human primates, *i.e.*, during the period of exposure. These changes include increased ductal area, changes in the periductal stroma and maturation of the fat pad in female exposed fetuses (Vandenberg *et al.*, 2007; Tharp *et al.*, 2012), see Table 14. The non-human primates study from Tharp *et al.* assessed unconjugated BPA serum measurement which show internal doses similar to human systemic doses of unconjugated BPA. Age and dose- specific effects on mammary gland morphology were also observed in the mammary gland of male offspring after prenatal exposure with 0.25-250 µg/kg bw/day (Vandenberg *et al.*, 2013).

In addition to an immediate effect on mammary gland development during the period of exposure (fetal or fetal/perinatal exposure) at relevant doses of BPA (25 µg/kg bw/day, by oral or sub-cutaneous routes) in rodent models, BPA leads to effects throughout post-natal life

(ANSES, 2013b). These mammary gland changes were observed during peri-puberty in mice (PND30) (Markey *et al.*, 2001; Munoz de Toro *et al.*, 2005; Ayyanan *et al.*, 2011) and at 6 and 9 months of age (Markey *et al.*, 2003), and in different rat strains (PND50) (Murray *et al.*, 2007; Durando *et al.*, 2007; Moral *et al.*, 2008; Jenkins *et al.*, 2009). Mammary gland changes concern ductal elongation, an increased number of TEBs relative to the ductal area and fewer apoptotic TEB cells compared to the controls, increased lateral branching (Munoz de Toro *et al.*, 2005) and ductal hyperplasia. Increased cell proliferation and decreased apoptosis were observed in the glandular epithelium. Ductal (and occasionally lobuloalveolar) hyperplasia have also been described during both the peripubertal period or later on (Murray *et al.*, 2007; Acevedo *et al.*, 2013; Mandrup *et al.*, 2016), possibly associated with disorganisation of periglandular stroma (Maffini *et al.*, 2004; Durando *et al.*, 2007; Fischer *et al.*, 2016).

In utero and perinatal exposure of rats and mice to environmentally relevant doses of BPA has also been linked to the development of intraductal hyperplasia that appears during adulthood (over PND100) (Durando *et al.*, 2007; Vandenberg *et al.*, 2008; ANSES 2013b), as well as to an increased number of carcinogen-induced tumors (DCIS) in rats (Durando *et al.*, 2007; Jenkins *et al.*, 2009 and 2012; Betancourt *et al.*, 2010) (Table 16). Intraductal hyperplasia ('ductal beading' representing the merging of actively proliferating luminal epithelial cells) is considered to be linked with precursors (pre-neoplastic lesions) of breast carcinoma in rodents and humans (Russo *et al.*, 1990; Russo and Russo, 1996; Vandenberg *et al.*, 2007). The increased tumor response from carcinogen exposure early in life is attributed to the presence of proliferating and undifferentiated structures such as TEB, which are present during the pubertal mammary epithelial expansion.

A recent study from Gomez *et al.*, 2017 suggests an increased susceptibility of adult mammary gland to subsequent exposures to estrogen replacement therapy (ERT). Briefly, after 3 months of ERT, rats previously exposed to BPA during the prenatal and post-natal period, and ovariectomized at PND 360, had a higher incidence of ductal hyperplasia and atypical lobular hyperplasia than animals under ERT alone.

BPA increases the susceptibility of adult mammary gland to subsequent exposures to chemical carcinogens. Briefly, when BPA exposure is coupled with a genotoxic carcinogen, either DMBA or NMU, several studies reported a significantly decreased tumor latency and increased mammary tumor multiplicity (ANSES 2013b; for recent reviews see Seachrist *et al.*, 2016 and Romagnolo *et al.*, 2016). The most reported effects occurred upon exposure to high doses of BPA (250 µg/kg bw/day, orally or sub-cutaneously administered) either following *in utero* or pre-pubertal exposures (Jenkins *et al.*, 2009 and Betancourt *et al.*, 2010). Yet, an increase in tumor susceptibility was also reported with low dose BPA: 25 µg/kg bw/day (Acevedo *et al.*, 2013).

The effects of chronic exposure to BPA from gestation day 6 (GD6) on rat mammary glands were studied by Delclos *et al.* (2014) in juvenile and adult animals. Ductal hyperplasia, as defined by the authors, was observed in this study as well as one mammary adenocarcinoma at the lowest tested dose level only (2.5 µg/kg bw/day, oral route). Some methodological uncertainties (which, in particular, limit the sensitivity of this study) made it difficult to assess the doses from which BPA causes hyperplastic ductal lesions.

In conclusion, there is substantial evidence from rodent studies indicating that early-life BPA exposures lead to increased susceptibility to mammary cancer.

4.3.4 MoA (see Figure 13 and Figure 14)

4.3.4.1 Cellular effects: Epithelio-mesenchymal transition (EMT), proliferation and migration

Data presented above on adverse effect describes how fetal exposure to low doses of BPA alters the tissue organisation of fetal mammary gland in rodents and non-human primates, causing effects such as increased ductal area, and changes in the periductal stroma and maturation of

the fat pad in exposed fetuses (Vandenberg *et al.*, 2007; Tharp *et al.*, 2012).

- *Epithelio-mesenchymal transition (EMT)*

Studies using transcriptional analyses on the stromal and epithelial compartments isolated from the fetal female mammary gland (mouse) demonstrate that low dose BPA exposure in dams alters the mesenchymal and epithelial transcriptomes (Wadia *et al.*, 2013; see Table 14). Changes in gene expression in the BPA-fetal exposed mammary gland are related to proteins involved in apoptosis (increased expression of the anti-apoptotic gene, *Birc2*, *Abl1*), myoepithelial differentiation (increase of *Krt8*) and changes in the composition of extracellular matrix (ECM) and in adipogenesis (see Table 14). Several other signaling pathways are affected by exposure to BPA, such as genes involved in EMT (Betancourt *et al.*, 2014), apoptosis and immune function (Moral *et al.*, 2008); see Table 15). While E2 is known to modulate EMT through ER α , the estrogen-receptor involved in BPA-induced EMT is still unknown.

- *In vitro effects of BPA: proliferation, migration (see Table 16)*

The effect of BPA on cellular proliferation, senescence and migration was studied in human mammary epithelial cells (HMEC, a model system for studying early events in mammary tumorigenesis). Changes in cellular proliferation and senescence were observed in HMEC treated with BPA (and E2), and associated with dysregulation of proteins such as Bcl2, cyclin D1 and cyclin E (Lee *et al.*, 2012; Qin *et al.*, 2012).

BPA induced also an increase in proliferation using the normal-like human breast epithelial cell line MCF-10F. Activation of proliferation and increased level of estrogen-responsive gene *ps2* by BPA (10 μ M) showed the engagement of ER α and the co-activator SRC3 to a similar level as the positive control group (E2 was tested at 10 nM) (Sengupta *et al.*, 2013). Another study has reported that GPER is required for growth effects and migration in breast cancer cells (SKBR3) and cancer-associated fibroblasts (CAFs) that lack the classical ER (Pupo *et al.*, 2012).

- *Disruption of acini by BPA: an estrogenic effect demonstrated in an in vitro 3D model (EMT) (see Table 16)*

The effects of BPA on the morphogenesis/disruption of breast glandular structures was also investigated using *in vitro* 3D models for breast glandular structure development, using non-transformed breast epithelial MCF-10F and MCF-12A cells, cultured in a reconstituted basement membrane matrix (Matrigel) (Fernandez and Russo, 2010; Marchese and Silva, 2012). Unlike monolayer cultures, immortalised mammary epithelial cells grown in 3D recapitulate numerous features of the breast epithelium *in vivo*, including the formation of duct-like structures and growth arrested polarised acini with hollow lumen. These well-organised acinar structures reproduce important features of malignant transformation and breast development observed *in vivo*, i.e., the events that trigger disruption of these structures and potentially lead to breast cancer.

MCF-10F cells grown in normal conditions formed duct-like structures in collagen resembling the ducts of the mammary gland, but did not form colonies in agar model (3D culture); BPA altered the ductular pattern (wider and larger) in the collagen matrix. BPA decreased the formation of tubules and increased the formation of spherical masses in the collagen matrix, similarly to E2, compared to the control DMSO group. Interestingly, the number of solid masses after BPA treatment may even be significantly higher than in cells treated with E2 (Fernandez and Russo, 2010).

MCF-12A cells are estrogen receptors ER α , ER β and GPER competent, allowing the investigation of the effects of BPA on mammary gland formation and disruption (Marchese and Silva, 2012). Under normal conditions, MCF-12A cells form organised acini, with deposition of basement membranes and hollow lumen. However, treatment with BPA (10 μ M) or estradiol, resulted in deformed acini and filling of the acinar lumen, similarly to E2 (1 nM). When BPA treatment was combined with ER and GPER inhibitors, the deformed acini recovered normal features, such as a spheroid shape, proliferative arrest and luminal clearing, suggesting a role for ER and GPER in

the estrogenic disruption of acinar formation. Interestingly, a similar outcome was not observed in ER α negative MCF-10A cells treated with E2.

4.3.4.2 Changes in estrogen-dependent susceptibility: gene transcription following *in utero* and postnatal exposure (see Table 14 and Table 15)

Transcriptional effects of BPA were also compared with those of EE2, a potent estrogen. Hierarchical clustering analysis suggests that BPA and EE2 similarly affect the transcriptional response of epithelial cells, whereas the response to these agents was different in peri-ductal stromal cells (Wadia *et al.*, 2013). These observations support a model whereby BPA (and EE2) act directly on the stroma of the fetal gland, which expresses ER α , ER β ¹⁷ and GPER30 (at GD 18 observation time in Vandenberg *et al.*, 2007, at birth observation time in Wadia *et al.* 2013). In turn, stroma affects gene expression in the epithelium, even if ER α and ER β are below the level of detection at this stage of development. These results indicate that BPA alters gene expression in the BPA-exposed mammary gland during fetal development through estrogen receptor(s), acting as an estrogenic agent (Wadia *et al.*, 2013).

In addition to modifications observed at fetal stage, modifications on ER are also observed during peripuberty.

- Some studies indicate that mice and rats, after *in utero*/lactational exposure to BPA, have a significantly higher sensitivity to estradiol during peri-puberty, as indicated by histoarchitecture changes and by an increase of E2-dependent gene expression such as amphiregulin (AREG), rather than changes in the ER expression level (see Table 17) and Wadia *et al.* (2007).
- AREG is a ligand for epidermal growth factor receptor, the unique EGF family member to be transcriptionally induced by estrogen in the mammary glands at a time of exponential expansion of the ductal system.
- Other peripubertal change is associated with an increased number of estrogen-receptor ER α in the exposed animals (Murray *et al.*, 2007).

Among the four *in vivo* studies that have assessed the Progesterone Receptor (PR) expression, three of them reported an increase in the level of PR expression, an estrogeno-dependent gene, in rodents exposed to BPA when they were fetuses or juveniles (Munoz de Toro *et al.*, 2005; Jenkins *et al.*, 2009; Ayyanan *et al.*, 2011). Although Vandenberg *et al.* (2008) did not report an increased expression of PR at 9 months of age in the epithelium, they found increased expression of PR in epithelial cells in intraductal hyperplasia. Lastly, it should be noted that increased PR mRNA expression induced by BPA was similar to the positive control (DES) included in the Ayyanan *et al.* study.

The observed increased expression of PR in mice and rats may explain the increased ductal density and the increased lateral branching as PR is essential for side branching in the mammary epithelium (see Table 18 and Background section in 4.3.2).

Furthermore, exposure to BPA during fetal life provokes at adult stage an increase of the expression of both *RankL* (a critical connection between progesterone and epithelial cell proliferation) and *Wnt4* (involved in progesterone-induced side branching in early adult life), see respectively Jenkins *et al.* (2009) and Ayyanan *et al.* (2011) and Table 16.

¹⁷Note: ER α KO mice display multiple significant defects in reproduction and mammary gland development, ER β KO phenotypes are more limited, and GPER KO exhibit no reproductive deficits.

4.3.4.3 Molecular changes involved in the process of carcinogenesis

A series of studies have described a link between molecular and abnormal mammary gland changes in rodents exposed to BPA. Those studies addressed the effects of BPA on EZH2 and HOTAIR involved in the increase of cell proliferation, or increased invasiveness, seen in some breast tumors and which also contribute to breast cancer progression. HOTAIR and EZH2 were recently shown as being estrogen-regulated genes (Bhan *et al.*, 2013 and Bhan *et al.*, 2014a, respectively).

Doherty *et al.*, (2010) showed that *in utero* and lactational exposure of mice to BPA at 5 mg/kg bw/day, or with DES at 10 µg/kg bw/day, up-regulates the expression of EZH2 through an epigenetic mechanism. This EZH2 up-regulation by BPA has also been shown *in vitro* in breast cancer cells (Doherty *et al.*, 2010; Weng *et al.*, 2010; Knower *et al.*, 2014 (for review); Bhan *et al.*, 2014a).

Some studies from the group of Mandal (Bhan *et al.*, 2014a – 2014b; Hussain *et al.*, 2015 and Deb *et al.*, 2016) have described the induction of epigenetic marks in the BPA-exposed adult mammary gland.

Bhan *et al.* (2014a) confirmed the previous observation of Doherty *et al.* (2010) showing the induction of EZH2 with BPA. This induction of EZH2 (mRNA and protein) was observed in BPA-treated MCF7 cells as well as *in vivo* in the mammary glands from ovariectomised rats exposed to BPA with 25 µg/kg bw for 24h, estradiol (5 µg/kg bw) or DES (DES 5 µg/kg bw). Induction of HOTAIR expression was also described in both *in vivo* and *in vitro* models. HOTAIR is a key player in survival and maintenance of breast cancer cells (Bhan *et al.*, 2014b) and is transcriptionally regulated by estradiol (see above and in background section). Moreover, Bhan *et al.* (2013) showed that knockdown of ERs down-regulated the BPA- and DES-induced HOTAIR expression.

Studies from this group (Bhan *et al.*, 2013) analysed molecular mechanisms involved in the regulation of EZH2 and HOTAIR by BPA in breast-cultured cells (MCF-7). EZH2 and HOTAIR miRNA interacts with gene silencing machinery, through recruitment of PRC2 and LSD1 complexes into the target promoter, which leads to target gene silencing. The complexes bind to the promoters of EZH2 and HOTAIR, and modify chromatin (histone methylation and acetylation) for recent review see also Romagnolo *et al.*, 2016.

Altogether, these observations suggest that BPA exposure (at least in the conditions cited above) alters the epigenetic programming of the promoter of these genes. These genes have been shown to be EE2 dependent, their epigenetic modulation by BPA may contribute to their endocrine disruption *in vitro* and *in vivo* in an EE2-like manner.

An initial study from Markey *et al.*, (2001) suggested that the mechanisms by which BPA affects the morphology and secretory function of the mouse mammary gland a long time after the period of exposure, could be mediated through misexpression of HOX genes. Using the same protocol as described above, the group of Mandal showed that the HOXB9 and HOXC6 expressions are transcriptionally regulated by estradiol (E2) and also by BPA in cultured human breast cancer cells (MCF7) as well as *in vivo* in the mammary glands of ovariectomised (OVX) rats (Deb *et al.*, 2016 and Hussain *et al.*, 2015, respectively).

Dhimolea *et al.* (2014) have shown that fetal exposure to BPA at 250 µg/kg bw/day (*via* subcutaneous administration) triggers changes in the post-natal mammary gland epigenome (rats). In particular, methylation of lactalbumin gene (promoter)¹⁸ was increased (*via* pro-activation histone H3K4 trimethylation) at PND4 concomitantly with enhancing mRNA expression of the gene. The majority of differentially methylated genomic DNA segments between BPA- and

¹⁸ Hormone-dependent marker of differentiation.

vehicle control animals were observed at PND21. However, no link between methylation status and gene expression was observed at PND21 and PND50 at these two periods.

Altered methylation of genes involved in DNA repair (*BRCA1*), cell cycle regulation and apoptosis (*CCNA1* and *CDNK2A/p16*), has been observed with low concentration of BPA (100 nM). This altered gene methylation was observed in cells such as human breast normal-like cells, human normal mammary epithelial cells (HMEC) and MCF-10F cells (Fernandez *et al.*, 2010b and 2012; Qin *et al.*, 2012), or in other ER positive human breast epithelial cell lines. Rounded ductal colonies (similar to spheres) treated with BPA showed increased methylation levels of key genes associated with tumor development such as *BRCA1*, *CCNA1*, *CDKN2A*, indicating that BPA alters the epigenome in a manner that promotes proliferation, senescence and tumour development at 10^{-8} M – 10^{-7} M (Qin *et al.*, 2012). Additional cell line studies have demonstrated aberrant methylation of the genome resulting in downregulated pro-apoptotic genes (Fernandez *et al.*, 2012).

Hypermethylation of promoter CpG islands is often associated with tumor suppressor genes (*BRCA1*, *E-cad*, *CDNK2A/p16*) and is recognised as one of the key events leading to tumor initiation and progression (Faryna *et al.*, 2012; Knowler *et al.*, 2014). Silencing of *CDKN2A/p16* could allow BPA-exposed epithelial cells from the mammary gland to escape senescence.

4.3.5 Summary of the plausible link between adverse effects and endocrine MoA: Effects of BPA on mammary gland

Because of temporality issues, it is rather difficult, if not impossible to demonstrate all the mechanisms or MoA together with the delayed effects they are leading to (increased susceptibility to carcinogens or modification of morphology) in a standalone study. What one can achieve at best is to demonstrate key events known to be involved in specific MoA which lead ultimately to adverse effect, with each point when animals need to be culled being demonstrated in independent studies.

There is substantial evidence from experimental studies indicating that BPA fetal exposure alters the tissue organisation of fetal mammary gland in rodents and non-human primates, causing effects such as increased ductal area, and changes in the periductal stroma and maturation of the fat pad in exposed fetuses. The experimental data have also shown that early-life BPA exposure may lead to increased susceptibility to mammary cancer.

Some studies using transcriptional analyses on the stromal and epithelial compartments isolated from the fetal mammary gland (mouse) demonstrate that BPA exposure in dams alters the mesenchymal and epithelial transcriptomes (Wadia *et al.*, 2013). Changes in gene expression in the BPA fetal exposed mammary gland were related to proteins involved in apoptosis (increased expression of the anti-apoptotic gene, *Birc2*, *Abi1*), myoepithelial differentiation, changes in the composition of ECM and in adipogenesis.

BPA MoA involves estrogen receptor and/or ER co-regulators. In human breast cells, activation of ER and GPER were shown after BPA exposure leading to disruption of acini formation. Involvement of ER dependent mechanisms was highlighted using positive controls, 17 β -estradiol or DES. When BPA or 17 β -estradiol were combined with ER and GPER inhibitors (ICI 182 780 and G15, respectively), these effects were reversed (Marchese and Silva, 2012). Alterations of the ductular pattern, described after BPA exposure, were similar to those formed by the cells treated with positive control (E2) as shown by Fernandez and Russo (2010). BPA induced increased cellular proliferation in human mammary epithelial cells (Qin *et al.*, 2012; Sengupta *et al.*, 2013) similar to the positive control 17 β -estradiol in Lee *et al.* (2012). Additionally, activation of proliferation and increased level of estrogen-responsive gene *ps2* by BPA showed the engagement of ER α and the co-activator SRC3 to a similar level as 17 β -estradiol (Sengupta *et al.*, 2013). Lastly, the BPA-induced increase in the expression of HOTAIR, a procarcinogenic gene, was suppressed after ER invalidation and the same observation was made for DES. Besides ERs, BPA may act via co-factors. GPER is known to be required for growth effects and

migration in cancer (SKBR3) cells and CAFs that lack the classical ER. As these proliferative effects were cancelled when GPER expression was silenced by shGPER, it can be concluded that BPA induces stimulatory effects as a GPER agonist in these breast cancer cells and CAFs (Pupo *et al.*, 2012).

It can be noted that a series of studies described how BPA alters the epigenetic programming of the promoter of HOTAIR and EZH2. These genes have been shown to be EE2 dependent and have been shown to be modulated by BPA. They are involved in the increase of cell proliferation, or increased invasiveness, seen in some breast tumors and also contributing to breast cancer progression.

It is shown that rodents and non-human primate mammary gland and human breast cells are targeted by BPA exposure and that mechanisms could involve different key events as indicated above. The schemes presented below aim to link those different key events in a dynamic way up to the adverse effect.

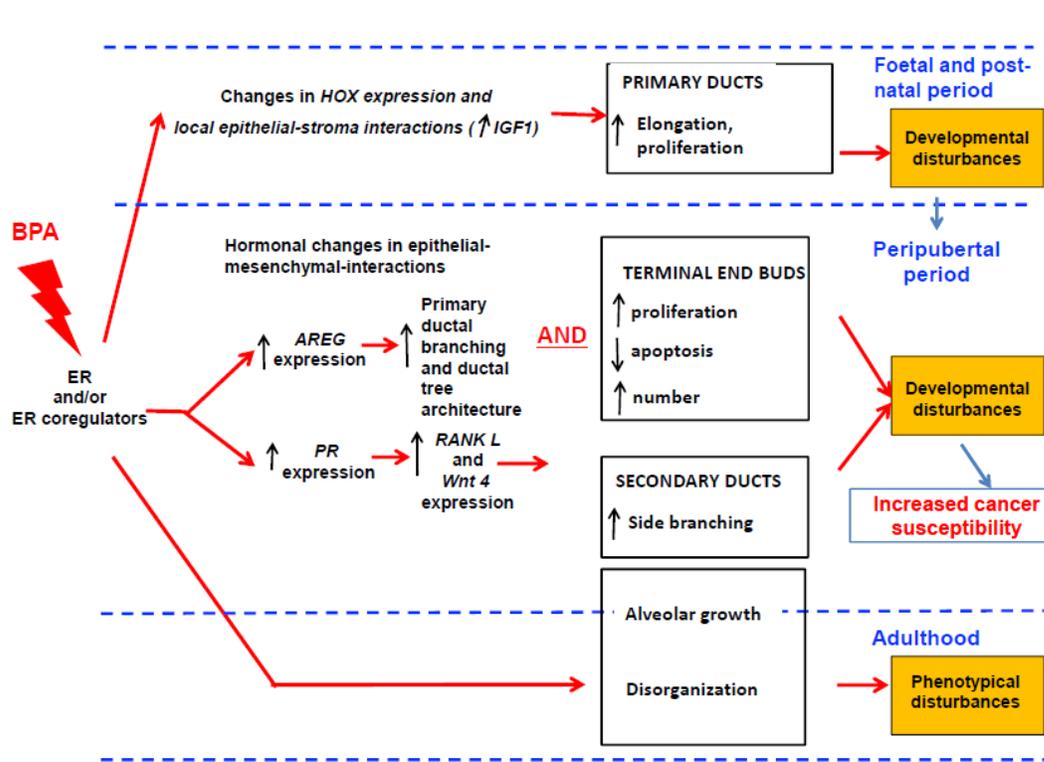


Figure 13: Some cascades of events from BPA action on ER or on its coregulators to the developmental and phenotypical disturbances of the mammary gland.

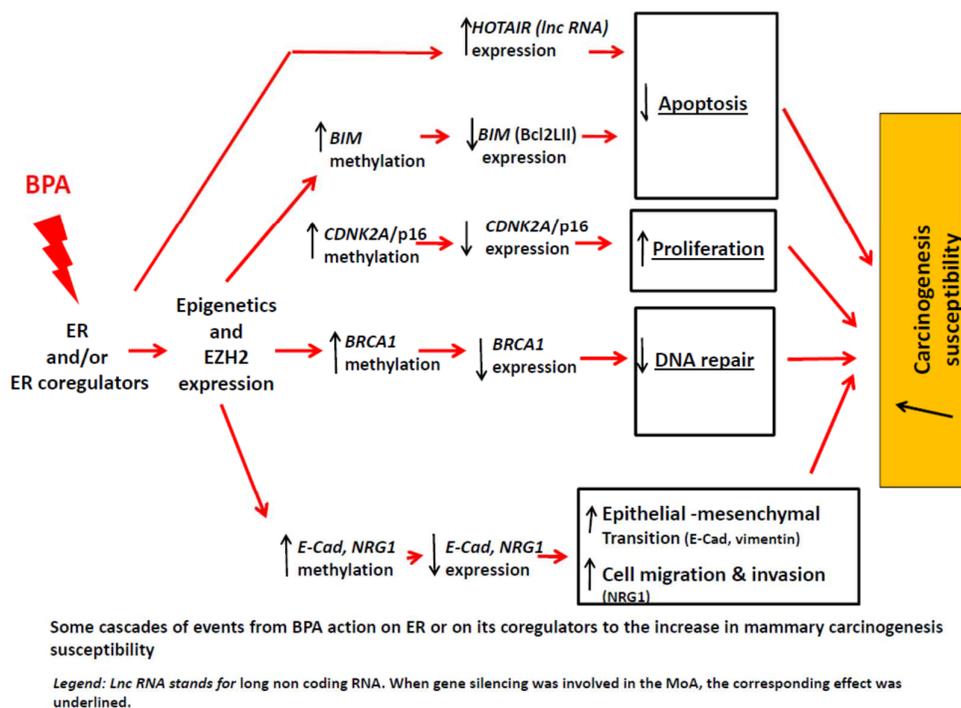


Figure 14: Some cascades of events from BPA action on ER or on its coregulators to the increase in mammary carcinogenesis susceptibility.

4.3.6 Human information

Very few epidemiological studies are available.

Aschengrau *et al.* (1998), in a case-control study found no association between occupational exposure to BPA and breast cancer. Another study determined blood BPA level in women with and without breast cancer (Yang *et al.*, 2009). There were no significant differences in blood BPA level between the cases and controls. In those studies, a link between BPA exposure and human breast cancer could not be determined.

More recently, Sprague *et al.* (2013) conducted a cross-sectional study in 264 postmenopausal women for whom a mammography was performed. This mammography was used to measure breast density (an important breast cancer risk factor) and was reported as a ratio of breast density (percentage of high density areas to the total breast area). BPA was measured on a single spot blood sample at the time of mammography. The authors observed that compared with women with non-detectable¹⁹ BPA values ($n = 193$), those who had BPA values, below ($n = 35$) or above ($n = 34$) the median of detectable values had a higher mammary density ratio (P trend = 0.01). However, this cross-sectional study coupled with a single measure of exposure, with a very low detection rate of the molecule and focusing on a risk factor (breast density) and not to the occurrence of the disease (breast cancer), does not provide evidence of a causal link.

Trabert *et al.* (2014) conducted a population-based case-control study in Poland. 575 incident cases of post-menopausal breast cancer (which represents the majority of cases in the Caucasian population) were compared to 575 women without breast cancer and matched on age (± 5

¹⁹ * below the analytical detection limit

years) and region of residence (Warsaw, Lodz). All control women were also menopausal. There are some aspects that deserve attention such as large population size, data analysis that takes into account many confounding factors and fairly compelling sensitivity analyses. Exposure to BPA was estimated on the basis of a urine assay on samples collected for 12 hours at night. This is a more reliable measure than a simple spot, but unfortunately it cannot account for the exposure in any previous critical window or for a long period before the occurrence of a disease. Again, measurement of exposure is the major limitation. Considering this limitation, the authors found no significant association between BPA exposure and the risk of developing breast cancer.

A more biologically relevant study design would be longitudinal epidemiological studies measuring BPA *in utero*, as breast cancer most likely takes years to develop and even be established in the womb (Soto *et al.*, 2008). Indeed, it should be noted that the existing studies evaluate BPA exposure on the date of the study when the effects looked at are long-term effects appearing more than 40 years after exposure.

4.3.7 Transposition to humans: interpretation issues

4.3.7.1 Comparison of the pre- and postnatal development of the human breast to the mouse mammary gland

The anatomy of the human breast is more complex than the mouse mammary gland which contains 15–25 ducts where each gives rise to a lobe containing multiple terminal ductal lobular units and 2 distinct stromal compartments, the intralobular and interlobular stroma, which has a single stem ductal tree embedded in a homogeneous fatty stroma. Nevertheless, terminal ductal lobular units (TDLU) present in humans are structurally similar to TEBs in rats and mice during the same life stage. These structures are undifferentiated and highly proliferative, and as such they are sensitive to the effects of carcinogens and other chemicals. In terms of hormonal regulation, there seem to be substantial similarities across species. In most mammals, the ovaries first secrete estrogens in response to increased secretion of gonadotropins, and sexual maturity coincides with the establishment of cyclic peaks of ovarian progesterone secretion. Progesterone levels increase after ovulation when the body anticipates pregnancy, and continue to rise when pregnancy is established.

Pathologists observe proliferative activity in the breast epithelium during the luteal phase, when progesterone levels peak (Masters *et al.*, 1977; Longacre and Bartow, 1986), suggesting that mouse and human mammary epithelia may indeed be similarly regulated, at least with regards to hormonal control of cell proliferation. Recently developed *ex vivo* models of the human breast have shown that progesterone elicits cell proliferation (Tanos *et al.*, 2013; Wang *et al.*, 2013). Notably, the dog, a species with a particularly long luteal phase, is especially prone to mammary carcinoma (Sleeckx *et al.*, 2011).

The flowchart hereabove in section 4.3.2.1 (see Figure 10) summarises the different developmental steps in species such as mice, rat and also human (quoted from Davis and Fenton, 2013).

4.3.7.2 Comparison of precancerous/ cancerous phenomenon between species

Precancerous lesions of the breast are atypical epithelial proliferations which develop within the lactiferous duct tree and are of two types: ductal and lobular. These two types differ not only in their location but also in the type of their constituent cells. Histological diagnosis of precancerous lesions is difficult and inter-pathologist reproducibility is poor as shown by a number of studies. The classification of precancerous lesions in humans is divided into the terms ductal (DIN) or lobular (LIN) intraepithelial neoplasia. Ductal carcinoma *in situ* (DCIS) is a preinvasive cancerous lesion. In the United States, DCIS accounts for almost 20% of the cancers picked up in screening (1 case of DCIS per 1300 screening mammographies) (Ernster *et al.*, 2002).

When left in place, a preneoplastic or precancerous lesion can turn into a preinvasive carcinoma or an *in situ* carcinoma which can itself turn into an invasive carcinoma. The theory about the existence of a continuum between the normal mammary gland and invasive breast cancer, even if it may appear too simplistic, is based on direct and indirect arguments (Antoine *et al.*, 2010). Recent epidemiological studies have shown that women with a history of benign breast lesions had an increased risk of breast cancer.

Similarly, after 10 years' follow-up in women who had undergone a diagnostic biopsy of low-grade DCIS without any other treatment than biopsy excision, 32% of these women had a diagnosis of invasive cancer in the same breast (Page *et al.*, 1995). The natural development of high-grade DCIS or of clinically palpable DCIS, on the other hand, is not well characterised since, in most cases, the tumour is removed in its entirety by surgery which is also the case with atypical ductal hyperplastic (ADH) lesions.

The substantial increase in the number of biopsies performed on the basis of infra-clinical images and recent data provided by molecular study of the lesions have shed new light on the risk of hyperplastic lesions becoming cancerous. Molecular markers of tumoral transformation in the breast such as the estrogen receptor, expressed by normal epithelial breast cells, are expressed by more than 70% of DCIS and the proto-oncogene HER2/neu is overexpressed in half the cases of DCIS but not in atypical hyperplasias (Allred *et al.*, 1992).

Rodents, i.e. rats and mice, have been widely used to study mammary carcinogenesis, in models of either spontaneous or induced tumours. The main advantage of the rat model is that the carcinoma most resembles human breast cancer; breast cancer in mice is often of viral and hormone-dependent origin (Cardiff *et al.*, 2000; Gould, 1995). In CD-1 mice, spontaneous non-neoplastic and neoplastic lesions are not very common (less than 5%: (Gad, 2007)).

The different strains of rats used have shown different sensitivities in developing neoplasms induced by chemicals or by radiation, Sprague-Dawley or Wistar being more susceptible than the Fisher rat. In Sprague-Dawley rats, the incidence of spontaneous tumours is close to 50% in chronic studies (example, historical data (NTP, 2010)). Certain strains, such as Wistar-Furth, show increased susceptibility to mammary carcinogenesis *via* chemical carcinogens (Gould, 1995).

The factors of mammary gland susceptibility include, in addition to genetic factors, the degree of differentiation of the breast tissue at the time of exposure, physiological and hormonal status, and diet. Susceptibility is increased in prepubertal females during the mammary development period: the ducts end in TEBs which will progressively differentiate into alveolar buds (AB) and alveolar lobules. The greatest number of tumours was induced in female SD rats at between 40 and 46 days, the period of most active differentiation of the TEBs regarded as the target of chemical carcinogens (Russo and Russo, 1996). Breast carcinomas were induced in rats by chemical agents or ionising radiation. The most commonly used chemical carcinogens include the polycyclic aromatic hydrocarbon dimethyl-benzanthracene (DMBA) or the alkylating agents N-ethyl-N-nitrosourea (ENU) and N-methyl-N-nitrosourea (NMU). Carcinogen-induced tumors in rodents are characterised using the same criteria as used for human breast DCIS. Studies in human and rodent models demonstrate that hormonal factors that affect mammary gland development also influence susceptibility to carcinogens.

These similarities include the development in a multistage process. Most of the cancers induced by DMBA (or NMU) are hormone-dependent with a similar morphological pattern i.e. hyperplasia, intraductal hyperplasia regarded as preneoplastic, adenomas/ adenocarcinomas. DCIS are regarded as a morphological progression towards breast carcinoma from intraductal proliferative lesions.

4.3.8 Summary and conclusion

There is evidence from rodents and non-human primate studies that prenatal and post-natal exposure to BPA causes endocrine modifications in the mammary tissue, ultimately increasing its susceptibility to chemical carcinogens, as previously reported (ANSES, 2013b and review by Soto *et al.*, 2013). All data presented here support the possibility that BPA, through interaction with the nuclear ERs, or GPER (which may also play a role, see Filardo *et al.*, 2006), and indirectly with PR, modulates estrogenic- and progestin agonist activities. Emerging epigenetic studies have suggested changes related to estrogen-dependent genes (such as EZH2 and HOTAIR), as well as HOX genes (involved in embryogenesis and post-natal development) which could be associated with the BPA induced abnormal development and cancer increased susceptibility of mammary gland.

4.3.9 Summary tables of studies

The *in vivo* and *in vitro* experimental data are reported in the following tables from Table 14 to Table 18.

Table 14: Summary table of *in vivo* experimental studies investigating the effects of BPA on mammary gland following foetal exposure with a mammary gland evaluation around birth

Literature reference	Species	Route of exposure	Pre-natal exposure	Endpoint: fetal mammary gland development	BPA and ER expression	BPA effects
Vandenberg et al., 2007	CD1 Mice	Alzet osmotic pump	GD8 to GD18 BPA 250 ng/kg bw/day (in DMSO) Observation: GD18	Advanced maturation of the fat pad in the stroma and changes in the extracellular matrix, - altered growth, decrease in cell size and delayed lumen formation in the epithelium.	No statistically significant differences in ER α or ER β mRNA expression between BPA-exposed animals versus controls or by immunohistochemistry quantification. ERα et ERβ transcripts present at GD 18. ER α -positive cells present in the loose connective tissue and fat pad compartments of the developing stroma and blood vessels.	
Wadia et al., 2013	C57Bl6 (ER+/-) Mice	Alzet osmotic pump	GD8 to GD19 - BPA: 250 ng/kg bw/day (in DMSO) EE2: 10 ng/kg bw/day Epithelial and stromal dissection in female fetuses at GD 19.		Estrogen receptors : ER α (only stroma), and GPER (stroma periductal), ERR γ (not present). Other nuclear receptors: GR, TR α (epithelium and stroma), AR (stroma).	Transcriptome analysis results : BPA acts on ductal epithelium and periductal stroma 1-BPA directly affects the periductal stoma which correlates with morphological changes: altered expression of genes involved in the focal adhesion pathway (down-regulation of Tenascin (Tnc)), and in adipogenesis pathways (upregulation of PPAR γ , a master gene of adipogenesis, as well as other adipogenic genes such as low density lipoprotein receptor (Ldlr), G protein-coupled receptor 81(GPR81), and Fabp4)). 2-Significant similarities in the transcriptional changes induced by BPA and EE2 in the exposed

						epithelium compartment: - changed expression of genes regulating the apoptosis pathway, with up-regulation of anti-apoptotic genes baculoviral IAP repeat-containing protein 2 (Birc2) and v-abl Abelson murine leukemia viral oncogene homolog 1 (Abl1). - up-regulation of the hepatocyte growth factor receptor/Met proto-oncogene (Met), implicated in branching of the mammary gland.
Tharp et al., 2012	Rhesus macaques (M. mulatta)	Oral	From GD 100 to GD 165 400 µg/kg bw/d. Period corresponding roughly to the third trimester of human gestation. Unconjugated seric BPA concentration of 0.68 ± 0.31 ng/ml. The internal doses used in that study are similar to human systemic doses of unconjugated BPA.	Significantly increased density of mammary buds (increased number of buds per ductal area). More advanced overall mammary gland development : -increased number of buds (i.e. incipient branches), terminal ends, branching points, bifurcating ends, total mammary gland area, ductal area, and number of ductal units (namely, the number of lactiferous ducts that define the number of lobes), as compared with controls. -prenatal differentiation of myoepithelial cell layer (compared to mice, postnatally).	Both ER α and ER β are expressed in epithelial cells at birth. No differences in the expression of ERs are observed between BPA-treated animals and controls.	

Table 15: Summary table of mice *in vivo* experimental studies investigating the effects of BPA on mammary gland following a pre and/or postnatal exposure with a post-natal or adult mammary gland evaluation

Literature reference	Species	Route of exposure	Perinatal exposure Gestation or gestation and lactation	Endpoints : Developmental alterations (peripubertal and at adult age)	BPA and hormonal expression	BPA effects
	Mice			Epithelial density alteration Canalair hyperplasia, stroma changes		
Markey <i>et al.</i>, 2001	CD-1 Mice	Alzet osmotic pump	GD9 to GD20 BPA: 25 and 250 µg/kg bw/day (in DMSO) Observation: PND 10, PND 30 (puberty) and PND 180 (mice killed on the afternoon of proestrus).	Dramatic expansion of the ductal network at 1 month (25 µg/kg bw/day). Most apparent changes in the histo-architecture of the mammary gland at 6 month of age (25 and 250 µg/kg bw/day) : - significant increase in all epithelial (ductal and alveolar) structures relative to the control group, including a significant increase in the relative area of AB (300%), of terminal ducts (TD, resting structures) and terminal end buds (TEB); - increased DNA synthesis in the stroma at 6 months of age; - increased presence of secretory product in alveoli resembling early gestation period.	ND	These histoarchitecture changes coupled with an increased presence of secretory product within alveoli, resemble those of early pregnancy, and may suggest a disruption of the hypothalamic-pituitary-ovarian axis and/or misexpression of developmental genes (such as homeobox genes: HOXA9, HOXB9, and HOXD9 see Chen et Capecci, 1999).
Markey <i>et al.</i>, 2003	CD-1 Mice	Alzet osmotic pump	GD9 to GD20 BPA: 25 and 250 µg/kg bw/day (in DMSO) Observation: 1 (puberty), 3, 4, 6, 9 and 12 months of age (mice killed on the afternoon of proestrus).	Changes in epithelial structures using the same methodology described in Markey <i>et al.</i> , 2001.		This manuscript provides a follow-up to the animals evaluated in the 2001 study. Here, animals exposed during the perinatal period were maintained until 9 months of age, and a number of organs including the mammary glands were evaluated. Increased lobular structures (AB+ lobuloalveoli) were significantly increased at 6 and 9 months, extending previous results (Markey 2001). Mice exposed to BPA (25 µg/kg bw/day) exhibits a

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						trend of an increase in AB+ lobuloalveoli structures in the mammary gland at 4 months, compared to the control group; however, this BPA-induced change became significant at 6 months. In the 250 µg/kg BPA group, the increase in AB + lobuloalveoli structures became significant at 6 and 9 months.
Munoz-de-Toro et al., 2005 (A. Soto's team)	CD-1 Mice	Alzet osmotic pump	GD9 to PND4 25 and 250 ng/kg bw/day (in DMSO) Observation at PND20, PND30 and PND120 (mice at PND120 were killed on the afternoon of proestrus at PND120).	-Increased number and area of TEBs relative to the ductal area at PND30 in the BPA-exposed animals indicating that ductal growth might be impaired. -Increased epithelial density. -Decreased apoptotic activity from 25 ng/kg bw/day, -Increased number of lateral branches at PND 120 in BPA-exposed mammary glands (25 ng/kg bw/day).	Expression of ERα in both the epithelial and stromal compartments of mammary glands at PND30. No effect of BPA on the expression of ERα (either in stroma or in epithelial ducts) at PND 30. Expression of PR only observed in the epithelium at PND30. Increased expression of PR in the epithelium with BPA from 25 ng/kg bw/day. Clusters of PR positive cells are believed to be indications of future branching points (see Seagroves et al., 1998).	The morphological changes found in 30-d-old animals exposed perinatally to BPA could be attributed at least in part to an increased sensitivity to estrogens. Of note, BPA enhances mammary glands sensitivity to estradiol in ovariectomised CD-1 mice. Progesterone is the main mediator of lateral branching and alveolar growth Increased expression of Wnt4 (mediator of lateral branching, downstream mediator of progesterone action) at PND30 in BPA-exposed animals at 250 ng/kg bw versus control.
Vandenberg et al., 2008 (A. Soto's team)	Mice CD-1	Alzet osmotic pump	GD8 to PND16 BPA: 0.25-2.5-25 µg/kg bw/day (in DMSO) Observation at 3, 9, 12, 15 months.	- Significant increase of the volume fraction of AB, but not terminal ducts, using Whole Mount analysis, both at 3 months of age in the 0.25 µg/kg bw/day BPA group, and at 6 months of age in the 0.25 and 2.5 µg/kg bw/day BPA group. - At 9 and 12-15 months of age, presence of ducts with a beaded appearance classified as intraductal hyperplasia (see also Murray et al., 2007). Of note, however, a trend to the lack of beaded ducts in the 12-15-month-old in the 25 µg/kg bw/day BPA group (due to	No effect of BPA on the expression of ERα, PR or Ki67 (marker of proliferating cells) at 9 months of age. Increased expression of PR and proliferation index	Intraductal hyperplasia/ increased proliferative index (cf also Murray et al., 2007 in rat and mice Bern, 1983) which suggest an increased estrogen-sensitivity.

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				either a too small sample size or to regression of beaded ducts in this group during later life).	(Ki67) in epithelial cells in intraductal hyperplasias. Because PR expression in mammary epithelium is thought to be dependent on estrogen exposure, the expression of PR in these beaded ducts suggests that they are estrogen-sensitive.	
Ayyanan et al., 2011	Mice C57Bl6	Oral route (Drinking water)	<i>in utero</i> until PND24 0.6-1200 µg /kg/j (n=18-animals per dose levels) Observation: PND30, 3 and 6 months DES (0.12 or 1.2 µg/kg-bw/d) another estrogen receptor (ERα) agonist as a positive control.	- At PND30: significant increase in adjusted number of TEBs at a dose of 3 µg/kg-bw (non-monotonic dose-response) - At 3 months of age: increased cell number with BPA and DES. Increased proliferation/ apoptosis ratio (at 6 µg/kg/d) - Up to more than 1 year, no palpable mammary carcinoma is detected. Of note C57Bl/6 mouse strain has no predisposition to mammary carcinogenesis	- At PND30, *ERα mRNA expression is not affected by BPA nor by DES treatment. *Increased PR mRNA expression similar with DES and BPA treatment. Decreased SLP1 (secretory leuko-protease inhibitor) similar with DES and BPA treatment at PND30. - At 6 and 12 month of age, pronounced increase of PR-positive cells within the luminal epithelial cells in the 6 µg/kg bw/day BPA group.	At PND30: non-monotonic dose response trend in AREG mRNA expression with BPA. At peri-puberty, BPA increases mRNA expression of well-characterised estrogen -regulated genes (<i>PR</i> , <i>AREG</i>) which are linked with the increased TEB number and proliferation. In adult females, BPA increases Wnt-4 and RANKL mRNA levels (6µg/kg bw/day). Wnt4 and RANKL are important downstream mediators of progesterone function, both of which are implicated in the control of stem cell proliferation; their downstream signaling pathways are deregulated in mammary carcinogenesis Epigenetic changes as a mechanism underlying the effects observed in adult females is suggested
Fischer et al., 2016	Mice CD-1	Osmotic infusion mini-pump	GD9-GD21 Dose: 5 mg/kg bw/day (sesame oil) Female pups ovariectomised six weeks after birth,		-Decreased expression of ERα (:2.5) -Increased expression of ERβ (x3).	<u>Gene expression analysis:</u> BPA exposure caused high gene expression of ER β, likely leading to subsequent decrease in the expression of several chemokines (CXCL12, CXCL4, CXCL14, CCL20), some interleukins (IL1β),

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			then treated at 8 weeks with estradiol (E2 300 ng) or with vehicle. Observation : 6 hours after treatment			interferons (Irg1 and Irf9) and of leukocyte marker (inflammation and immunity mediators).
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Table 16: Summary table of rat *in vivo* experimental studies investigating the effects of BPA on mammary gland following a pre and/or postnatal exposure with a post-natal or adult mammary gland evaluation

Literature reference	Species	Route of exposure	Gestation / gestation and lactation exposure	Endpoints : Evaluation of mammary gland developmental alterations at peripubertal and at adult age	BPA and ER (and PR) expression	BPA effects
	Rats					
Murray et al., 2007 (A. Soto's team)	Wistar-Furt Rat	Alzet osmotic pump	GD9-PND1 (<i>in utero</i>) 2.5-25-250 and 1000 µg/kg bw/day in DMSO (n=4) Observation: PND50 (peripubertal period) and at PND95.	At PND50 and PND95: -increased (intra)ductal hyperplasia (3-4 fold ↗) and increased epithelial and stromal proliferation (see Durando <i>et al.</i> , 2007). -intraepithelial neoplasia from 250 and 1000 µg/kg bw/day, described as cribriform like structures and classified as CIS 'carcinoma in situ'	Increased ER α and Ki67 expression at PND50 and PND95 in hyperplastic lesions versus normal ductal structures.	Increased estrogenic activity.
Durando et al., 2007 (A. Soto's team)	Wistar rat	Miniature osmotic pump	GD 8 to GD23 (<i>in utero</i>) BPA: 25 µg/kg bw/day in DMSO with or without administration of N-nitroso N-methylurea (NMU) at a sub-carcinogenic dose of 25 mg/kg at PND50 with observation at PND110 and PND180. Observation of female offspring at PND30 (pre-puberty)-PND50 (puberty) and PND110-PND180 (adulthood).	-At PND50 : increased proliferative/apoptotic ratio (stroma and parenchyma) -At PND110 : increased ductal hyperplasia -At PND 50, 110, 180 : stromal desmoplastic reaction (including mastocytes, fibroblasts, inflammation) - At PND110 and PND180 : increased sensitivity to NMU (ductal hyperplasia); -At PND180 : ductal carcinoma with BPA and NMU versus negative control.	Note: ER α and β are only expressed in the stroma and the epithelium at the end of gestation. Note: NMU is a direct acting carcinogen which generates tumors that closely mimic the human disease in terms of tumor histology and ovarian hormone dependence (Russo 1996, Thompson 2000, Murray and Rosso 2009)	- In peripubertal period: proliferative/apoptotic ratio modified increased sensitivity to E2 (cf Munoz de Toro <i>et al.</i> , 2005) - At adulthood, ductal hyperplasia (pre-neoplastic lesion) associated with increased stromal density (modification of the interactions between the epithelium-stroma, mastocytes).
Delclos et al., 2014	Sprague-Dawley rat	Oral (gavage)	2.5, 8, 25, 80, 260, 840, 2700, 100 000, 300 000 µg BPA/kg bw/day	At PND 21 : significant elevated incidences of mammary gland duct hyperplasia of minimal severity in the female groups at 2 700 and 100 000	Not determined	Significantly higher plasma levels of estradiol and prolactin in the female BPA groups at 100 000 and 300 000 µg/kg bw/day whereas the

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			<p>Negative controls: naive and vehicle Positive control: EE2 0.5 and 5 µg/kg bw/day F0: females exposed from GD6 up to labour onset Pups from PND 1 until tissue harvesting, up to PND 90 GLP study. (Mod. OECD TG 408)</p>	<p>µg/kg bw/day, but not at 300 000 µg/kg bw/day. At PND 90: minimal severity of mammary gland duct hyperplasia also reported in the high dose female BPA groups, increase was statistically significant at 300 000 µg/kg bw/day group (Poly-k test) and 2700, 100 000 and 300 000 µg/kg bw/day (JT/SW or RTE statistical tests). BPA did not cause duct hyperplasia in the mammary glands of male rats, while conversely the reference estrogen EE2 induced hyperplasia in the male but not the female mammary gland. One mammary adenocarcinoma observed at the lowest tested dose level only (2.5 µg/kg bw/day) Similar serum level of unconjugated BPA between dose groups below 80 µg/kg/d and negative controls due to possible contamination challenging interpretation of results at lower doses. (Churchwell et al., 2014)</p>		<p>EE2 values (positive controls) were only mildly elevated in comparison to negative controls.</p>
<p>Acevedo et al., 2013 (A. Soto's team)</p>	<p>Sprague-Dawley rat</p>	<p>Alzet osmotic pump</p>	<p>GD 9 to GD21 and GD9 to PND21 ; BPA: 0.25-2.5-25 and 250 µg /kg bw/day (without co-carcinogen administration); Observation at: PND50-PND90-PND140 and PND180 BPA quantification (detectable at 250µg/kg bw/day</p>	<p>Neoplastic lesions (including DCIS) observed postnatally and at adulthood (see Murray et al., 2007). Adenocarcinoma were observed at PND90, 140 and 200, although not statistically significant (5 animals in total in different BPA exposure groups). However, these carcinomas are extremely rare in this rat strain and were only observed in BPA-treated groups.</p>	<p>ND</p>	<p>How BPA contributes to the initiation and progression of neoplasia, mechanisms are discussed (change in the hypothalamic-pituitary-ovarian axis, increased sensitivity to ovarian hormone: BPA acting as an estrogen).</p>
<p>Moral et al., 2008 (Russo's team)</p>	<p>Sprague-Dawley rat</p>	<p>Oral Gavage</p>	<p>GD9-PND1 (<i>in utero</i>) BPA: 25 and 250 µg/kg bw/day (in sesame oil) Observation at PND21-PND35-</p>	<p>At PND21: increased number of TEB (Terminal End Buds) and lobular structures (at 250 µg/kg bw/day) without major influence on proliferative index.</p>	<p>ND</p>	<p>Very large transcriptomic analysis at PND21, PND35, PND50 and PND100: -Few changes at PND21 and PND35. -At PND50: increased number of up-modulated genes related to:</p>

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			PND50 and PND100	At PND100: increased number of undifferentiated structures by this age as TDs (Tubular Ducts at 250 µg/kg bw/day).		apoptosis, immune function and response to stress (Cd3d, cathepsin E, Ctse, Cd5, and Sod2) at low dose and related to differentiation at high dose. - At PND100 , important cluster of upregulated genes related to immune response (Cd3d, Ctse, Cd5, Ltb, Cxcl10, Ccl5, Mefv, Cd2, A2m, and Il1b) at high dose.
Jenkins et al., 2009 (Russo-Lamartinière-Jenkins' team)	Sprague-Dawley rat	Oral Gavage	PND2 - PND20 BPA: 25 and 250 µg/kg bw/day (in sesame oil), administrated to lactating mothers 5 days/week (~ to 15 adm./mother). Observation at PND 50 DMBA administrated in F1 females at PND50 (dose: 30 mg/kg).	At PND50: Without DMBA: increased cell proliferation (Ki67) and decreased apoptosis in TEBs on PND50 at 250 µg/kg/ bw/day With DMBA: dose-dependent increase in number of mammary tumors, with reduced tumour latency	No change for ER α or PR-B expression. Increased expression for PR-A (PR-A and co-activators). Note: PR-A is the predominant isoform at puberty.	At PND50: Increased expression of PR-A, co-regulator SRC proteins (SRC-1, SRC-2, and SRC-3), up-regulation of Akt expression/activation plausibly linked to a greater incidence of side branching and alveologenesis, increased susceptibility to carcinogenesis.
Betancourt et al., 2010 (Russo-Lamartinière-Jenkins' team)	Sprague-Dawley rat	Oral Gavage	GD10-GD21 BPA: 25 and 250 µg/kg bw/day (sesame oil) from GD10-GD21 followed with DMBA administration in prenatally exposed females on PND50 (dose: 30 mg/kg bw) with observation at PND 50. BPA: 250 µg/kg bw/day (sesame oil) from GD10-GD21	At PND50: no significant increased tumour incidence. At PND100: increased tumour incidence indicating an increased susceptibility to carcinogenesis. Increased cell proliferation (Ki67) at PND100 in epithelial cells but not in the stroma. <u>Note:</u> In studies of cancer causation or chemo-prevention, the standard protocol for administering DMBA is at day 50, because this is within the period (days 40–60) of high mitotic index in the terminal ductal structures	- Down regulation of ERα at PND50 and up-regulation at PND100. - Upregulation of co-regulator SRC proteins (SRC-1, SRC-2, and SRC-3) at PND100 and of SRC-3 at PND50.	Proteome analysis (Maldi-Tof et LC-MS/MS): At PND50: proteins and major pathways altered by prenatal BPA exposure : up-regulation of vimentin (epithelial to mesenchymal transition), down-regulation of SPARC and TGFβ (cell proliferation and differentiation, regulation with the extracellular matrix), up-regulation of 14-3-3 (signaling pathway and proliferation), members of the Raf and ERK families (key signal transduction proteins known to be involved in tumorigenesis)

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			followed with DMBA administration in prenatally exposed females on PND100 (dose: 30 mg/kg bw) with observation until - PND300	of rats (Russo <i>et al.</i> , 1983; Welsch 1985).		
Ibrahim et al., 2016	Albino rat	Oral Gavage	Adult female rat BPA: 5mg/kg bw/day (in corn oil) for 8 weeks.	At adult age , increase in the number of the ducts and acini of the mammary gland with hyperplasia of their lining epithelium, increase in both amount and distribution of collagen fibers .	No significant effect on ER α expression.	NB. Adult exposure
Mandrup et al., 2016	Wistar rat	Oral Gavage	Adult gestant female rat: BPA: 0.025-0.25-5-50 mg/kg bw/day (in corn oil) from GD7 to GD21 and from the day after birth until pup day (PD) 22. Obs : PND22, 3-4 months (PD 100) and 13 months (PD 400)	At PD 400 , statistically significant increase in intraductal hyperplasia observed in females treated with 0.25 mg/kg bw/day but not on PD 100. Changes in lobular morphology in females; hypertrophy and tubuloalveolar morphology (males).	ND	The severity of the lesions (more than 30% of the mammary tissue) was increased in the groups exposed to 0.025 and 0.25 mgBPA/kg/d (low doses), but not in the high doses. This study strengthens the case that perinatal exposure to BPA induces effects that do not follow a classical monotonic dose response relationship In males at PND100 : trend to increased frequency of female-like morphology with the highest dose groups

Table 17: Summary table of *in vitro* experimental studies investigating the effects of BPA on mammary gland

Literature reference	Human cells	Cell characteristics	Condition of exposure	Evaluated endpoints:	Results:
Diel et al., 2002	MCF-7	MCF-7: Human breast cancer cell line (immortalised cells, ER α positive)	BPA: 10 ⁻⁸ M-10 ⁻⁷ M - 10 ⁻⁶ M- 10 ⁻⁵ M, ICI 182 780 (anti-estrogen) RAL (Raloxifene) E2 Selective ER modulator (SERM).	Cell proliferation. Apoptosis Expression of: progesterone receptor (PR); AR; ER α protein. Gene expression such as estrogen dependent.	BPA: strongly inhibited apoptosis, slightly increased cell proliferation at 10 ⁻⁵ M, BPA did not effect the expression of PR mRNA, AR mRNA, ER α protein versus positive controls.
Qin et al., 2012	HMEC	HMEC: Human normal mammary epithelial cells	BPA: 10 ⁻⁸ M – 10 ⁻⁷ M Exposure to BPA at passage 8 for 1 week. E2	Cell proliferation. Senescence.	BPA at 10 ⁻⁷ M increased significantly the proliferation, sphere size and senescence (increased number of human heterochromatin protein-1 γ positive cells, protein levels of both p16 and cyclin E (transition G1/S)) of HMEC, in a quite similar manner as E2. BPA increased DNA methylation of genes related to development of most or all tumor types, such as BRCA1, CCNA1, CDKN2A.
Fernandez and Russo, 2010 (review)	MCF-10F	MCF-10F: spontaneously immortalised breast epithelial cell line, considered as "normal stage" (ER α negative, and ER β positive cells).	BPA: 10 ⁻⁶ M-10 ⁻⁵ M -10 ⁻⁴ M-10 ⁻³ M BBP (Butyl benzyl phthalate) Treatment during two weeks in DMSO or media. E2: 0.007 nM - 70 nM -3.6 μ M	Ductules formation in collagen (3D model). In-vitro neoplastic transformation.	BPA increased cell proliferation, but significantly decreases ductules formation in collagen vs control (3D cultures) at 10 ⁻⁶ M -10 ⁻⁵ M BPA increased the percentage of spheric solid masses vs control in a statistically significant manner at 10 ⁻⁶ M. BPA was toxic at 10 ⁻⁴ M and 10 ⁻³ M. No statistically significant increase in the invasive capacity with BPA treatment.
Fernandez and Russo 2010 (Russo's team)	Transformed, invasive and fully malignant-derived MCF-10F cancer cell types	<i>In vitro</i> model of transformed and tumorigenic cells generated from MCF-10F : transformed by E2 (trMCF), invasive (bs MCF), and obtained from tumors generated on SCID mice (CaMCF),		Analysis of epigenetic and transcription changes	Analysis of epigenetic changes induced by estradiol during the neoplastic process. Data showed that the methylation pattern of different genes (such as neuroregulin NRG1) related to ductulogenesis, branching pattern, and during the invasion and tumor stages are involved in 'early and late stages of breast cancer'. Other processes such as proliferation, apoptosis (BIM), and estrogen metabolism were also altered.

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		compared to MCF10F		Human breast tissue samples	Methylation of NRG1, and associated down-regulation of NRG1, (gene and protein), were observed in bsMCF10 as compared to original MCF10F) . NRG1 was also partially methylated in invasive breast carcinoma as compared to normal breast tissue
Fernandez et al., 2012 (Russo's team)	MCF-10F	MCF-10F: (see Fernandez et al., 2010 and Fernandez & Russo, 2010)	BPA: 10 ⁻⁶ M–10 ⁻⁵ M during two weeks in DMSO or media.	Gene expression and DNA methylation analyses.	Increased expression of genes at 10 ⁻⁶ M involved in DNA repair, (such as BRCA1, BRCA2), and down-regulation at 10 ⁻⁶ M–10 ⁻⁵ M of several genes involved in apoptosis (such as BCL2L11, also known as BIM). Hypermethylation of several genes was shown (in particular BCL2L11 or BIM, IGF1R and Twist). Results also indicate that breast tissue of women with BRCA1 or BRCA2 mutations could be more susceptible to the effects of BPA.
Marchese and Silva, 2012	MCF-12A MCF-10A	MCF-12A: non-transformed breast epithelial cells (Era, ERβ and GPER competent); they form spheroid acini and hollow lumen in matrigel matrix. MCF-10A : Immortalised non-malignant breast cells (GPER competent; ERα negative/ and ERβ very low); they do not form acini.	BPA 10 μM; E2 1 pM-1 nM ICI 182 780 (anti-estrogen) G15: GPER inhibitor Two weeks of treatment.	Acini development in Matrigel (in-vitro3D model) including evaluation of: -cell proliferation, -ECM -differentiation, -central lumen formation in MCF-12A.	BPA and 17β-estradiol treatment resulted in misshaped acini and filling of the acinar lumen in MCF-12A. When these chemicals were combined with ER and GPER inhibitors (ICI 182 780 and G15, respectively), these effects were reversed. No effect of BPA on MCF-10A vs control. <u>Note :</u> The correct functioning and organisation of 3D acini depends on a fine balance between cell proliferation and cell death (apoptosis). An overstimulation or perturbation of these processes will lead to aberrations in glandular structure, such as formation of large misshapen acini with filled lumen and no apico-basal polarity (see Haenssen <i>et al.</i> , 2010; Debnath <i>et al.</i> , 2003; Reginato <i>et al.</i> , 2005; Yanochko <i>et al.</i> , 2006 and Dimri <i>et al.</i> , 2005)

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Lee et al., 2012	MCF7	MCF-7: Human breast cancer cell line (ER positive)	BPA 10 ⁻⁷ M - 10 ⁻⁶ M E2 and DES (10nM) ER α agonist (propyl pyrazoletriol, PPT) ER β antagonist (DPN, diarylpropionitrile) ER antagonist (ICI 182,780)	Cell proliferation. Gene expression Senescence	BPA induced proliferation of MCF-7 cells in a dose-dependent manner, similarly to E2. BPA induced cyclin D1 expression and reduced p21 expression associated with the G1/S transition, resulting in the proliferation of MCF-7 cells. These effects were inhibited by the ER antagonist suggesting that the effects of BPA on cancer cells occur through ER signaling and mainly through ER α based on the results with ER α and ER β agonist).
Filardo et al., 2000	SKBr3	SKBR3: breast cancer cells (ER α and ER β negative, but GPER/GPR30 positive). GPR30 is an orphan receptor unrelated to nuclear estrogen receptors	<u>E2:</u> 1nM	Proliferation. Mechanism of action : E2 via GPER	The group of Filardo described for the first time the presence of the completed unrelated transmembrane receptor GPR30 which can mediate estrogen responsiveness in ER negative breast cancer cells (SKBr3). 17 β -estradiol activates the mitogen-activated protein kinases, Erk-1 and Erk-2, via the expression of GPR30 and not via known estrogen receptors. 17 β -estradiol also induces the release of HB-EGF (heparan binding epidermal growth factor) and activation of the EGF receptor, suggesting a novel signaling pathway with potential significance for breast cancer (see also Filardo, 2002).
Pupo et al., 2012	SKBr3 CAF	Both SKBR3 and CAF cells are ER α and ER β negative, GPER/GPR30 positive	BPA from 100 nM to 1 μ M (in DMSO)	Proliferation and migration of cancerous cells.	Note: Pandey <i>et al.</i> (2009) have reported that, in SKBr3, the activation of GPER/GPR30 signaling by E2 and tamoxifen (an ER antagonist but GPER agonist) induces a transcription factor network, which resembles that induced by serum in fibroblasts. The most strongly induced gene, CTGF, promotes proliferation and cell migration. In both SKBR3 cells and CAFs after 5-day treatment at 1 μ M, BPA induced cell proliferation and migration through GPER. These proliferative effects being cancelled when GPER expression was silenced by shGPER. These findings indicate that BPA induces stimulatory effects as a GPER agonist in both ER-negative SKBR3 breast cancer cells and CAFs. The use of specific pharmacological inhibitors and

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					gene-silencing procedures indicates that BPA induces the expression of the GPER target genes CTGF, c-FOS and EGR-1, and the corresponding proteins through the GPER/EGFR/ERK transduction pathway in SKBR3 breast cancer and CAFs cells. Thus, GPER is required for growth effects and migration stimulated by BPA in both cancer (SKBr3) cell and CAF.
Magruder et al., 2014	SKBr3	<u>SKBR3</u> - breast cancer cells (ER α and ER β negative, GPER-1+) Murine 4 T1 cells	<u>BPA</u> : 10 nM 17 α estradiol 10nM 17 β estradiol 10nM ICI 182 780 : 1 μ M	Adhesion, Cell growth. Molecular mechanisms	GPER-1 stimulation of murine 4 T1 or human SKBR3 breast cancer cells with 17 β -estradiol (E2 β) promotes the formation of focal adhesions and actin stress fibers, resulting in increased cellular adhesion and haptotaxis on fibronectin (FN), but not collagen. These actions are also induced by BPA (data not shown), ICI 182 780 (ER antagonist), but not the inactive stereoisomer, 17 α -estradiol (E2 α).

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<p>Sengupta et al., 2013</p>	<p>MCF-7 and MCF-7 : 5</p>	<p><u>MCF-7</u>: (ER positive) <u>MCF-7</u>: 5C (estrogen deprived) MDA: MB-231 cells transfected with wild-type ERα (MC2 cells) or mutant ERα (JM6 cells, D351G).</p>	<p><u>BPA</u>: 10⁻¹³ to 10⁻⁵M, or at 10⁻⁶ and 10⁻⁵M for gene expression analysis E2: 10⁻⁹M RAL: Raloxifen 4-OHT: 4-hydroxy tamoxifen; dose dependence</p>	<p>Cell proliferation. Apoptosis. Analysis of E2-dependent gene (<i>TFF1</i> (or <i>PS2</i>)).</p>	<p>-Increased cell proliferation (max. at 10⁻⁶M) in MCF-7 cells with BPA, -Induced apoptosis with BPA and E2 in MCF-7: 5C cells. -Up-regulation of <i>TFF1</i> (<i>PS2</i>), an estrogen-regulated gene with BPA (at 1-10 μM). 4-OHT, an estrogen antagonist via ERα failed, as expected, to up-regulated PS2 expression. In vivo, transcriptional activation of PS2 by BPA in comparison to E2 and 4-OHT treatment: -Recruitment of ERα and SRC3 at the promoter region of PS2 gene which has a well-characterised functional estrogen-responsive element (ERE) in its promoter (Metivier <i>et al.</i>, 2002) with BPA at 10⁻⁶ M and 10⁻⁵M in a concentration-dependent manner, equivalent to results obtained with E2 treatment. -4OHT did not recruit, as expected, SRC3 and was comparable to vehicle treatment. Activation of TGFα gene in MDA -MB 231 cells stably transfected with wt ERα (MC2 cells) or mutant ERα (JM6 cells, D351G) with BPA, but at higher concentrations as compared to E2. Molecular docking of BPA to the ligand-binding domain (LBD) has shown that the binding mode predicted for the agonist conformation of ERα is more likely. BPA decreases the apoptosis via ERα. Note: The coactivator, SRC3 (AIB1) plays a key role in transcriptional activation of several estrogen-regulated genes, including PS2 gene (Shao <i>et al.</i>, 2004; Labhart <i>et al.</i>, 2005), similarly to ERα.</p>
<p>Yang et al., 2013</p>	<p>hESC</p>	<p><u>hECS</u>: derived from human Chinese blastocysts.</p>	<p><u>BPA</u>: 10⁻⁶ - 10⁻⁷- 10⁻⁸-10⁻⁹ M in DMSO E2: 10⁻⁹ M (positive control)</p>	<p>Three-dimensional (3D) culture system used for analysis of differentiation of</p>	<p>Low dose BPA and E2 could influence the mammosphere area of induced differentiated mammary epithelial cells (iDMECs) and upregulate the expression level of Oct4 and Nanog proteins,</p>

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			Treatment during the differentiation process.	progenitor cells into epithelium mammary cells. Expression of CK18, E-cadherin (usually considered as a marker of mammary epithelial cells), epithelial cell adhesion molecule (EpCAM). Expression of pluripotency molecular markers: Oct4, Sox2 and Nanog.	while only BPA could downregulate the expression of E-cadherin protein.
Song et al., 2015	MCF-7 SkBr3	<u>MCF-7</u> : (ER α positive, GPER+) <u>SkBr3</u> : (ER α and ER β negative, GPER+)	<u>BPA</u> : from 10 ⁻¹⁰ to 10 ⁻⁵ M at 6 dose levels in DMSO versus control group.	Mechanisms of the BPA- increased proliferation in MCF-7 and SkBr3.	<p>BPA upregulates the protein levels of cell nuclear antigen PCNA and Bcl2 in MCF-7 and SkBr3 cells.</p> <p>Since neither ICI 182,780 (a specific inhibitor of ERα/β) nor G15 (the specific inhibitor of GPER) abolished the BPA-induced proliferation of MCF-7 and SkBr3 cells, it suggests that the stimulation effects of BPA on the proliferation of breast cancer cells was independent of Era and GPER.</p> <p>Silencing of ERRγ (estrogen related receptor gamma) by its specific siRNA significantly abolished the BPA-induced proliferation of breast cancer cells (MCF-7 and SkBr3), while si-ERRα had no effect. BPA up regulated the mRNA and protein levels of ERRγ and triggered its nuclear translocation via a time dependent manner.</p> <p>These results indicate that BPA can trigger the proliferation of breast cancer cells via ERK1/2/ERRγ signals.</p> <p><u>Note</u> that the expression of ERRα and ERRγ are associated with an unfavorable and favorable prognosis of breast cancer, respectively (Ariazi et al., 2002). The nuclear immunoreactivity of ERRγ was detected in 79% of breast cancer patients and tended to correlate with the lymph node status (Ijichi et al., 2011) while exogenously transfected ERRγ increased MCF-7 cell proliferation (Ijichi et al.,</p>

					<p>2011). These results suggested that ERRγ plays an important role as a modulator of estrogen signaling in breast cancer cells.</p> <p>Considering that over expression of ERRγ can increase the risks of breast cancer (Dairkee <i>et al.</i>, 2008) and BPA has great binding affinity with ERRγ (Okada <i>et al.</i>, 2008), therefore we hypothesised that BPA can stimulate the proliferation of breast cancer cells. In the present study, we revealed that nanomolar BPA can significantly increase the proliferation of both ER positive and negative breast cancer cells, and ERRγ mediated this stimulation effect of BPA.</p>
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Table 18: Summary table of recent experimental studies (on homeogens and epigenetics) investigating the effects of BPA on mammary gland following pre and/or postnatal exposure

Literature reference	Species	Cell characteristics for the in vitro study	Period of exposure: Fetal, post-natal or adult	Endpoints	MoA: d'action : epigenetic mechanisms
Dhimolea et al., 2014 (A. Soto's team)	Rat Wistar Furth		GD9-PND1 (<i>fetal</i>) BPA 250 µ/kg/day (subcutaneous -osmotic pump) Obs: PND 4, 21, first estrus after PND 50	Not tested cf previous studies -Post-natal mammary gland development: significant increase in proliferation/apoptosis ratio -Ductal carcinoma in situ	Fetal BPA exposure triggers changes in the post-natal and adult mammary gland epigenome and alters gene expression pattern PND4: Higher levels of pro-activation histones H3K4 trimethylation of α-lactalbumin promotor in treated mammary gland compared to control, with concomitant increase (x2) in RNA expression of this gene. PND21: majority of methylated gDNA segments (Nimblegen ChIP array) PND50: majority of gene expression differences between BPA and vehicle-treated rats (transcriptional analysis)
Camacho et al., 2015	Sprague Dawley Rat		Oral (gavage) GD6-PND90 BPA: 7 'low doses' (2.5, 8, 25, 80, 260, 840, and 2700 µg/kg bw/day) and 2 'high doses: (100,000 and 300,000 µg/kg bw/day);	Mammary gland, prostate and uterus. Gene expression of five estrogen receptors: nuclear ERs(<i>Esr1</i> and <i>Esr2</i>), <i>GPER</i> and ERR (<i>Esrra</i> , <i>Esrrb</i> and <i>Essrg</i>) with QRT-PCR at	This study assesses the potential of low BPA doses to induce changes at the molecular level, epigenetic, estrogenic, and other molecular pathways in the female mammary gland (also in prostate), extending morphological changes previously described (see also Delclos <i>et al.</i> , 2014 in <i>Table 16</i>). This study assesses the potential of low BPA doses to

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			<p>two reference estrogen controls (EE2 0.5 and 5 µg/kg bw/day). Ten animals per dose group Obs : PND4 (time when circulating levels of free BPA was highest) or PND 90 ±5. GLP study. (Mod. OECD TG 408)</p>	<p>PND4 and 90. Gene expression analysis at PND4 on prostate and female mammary glands on 7 out of the 10 animals/ groups. Global genomic DNA methylation at PND90, The expression of four estrogen-responsive genes, coding complement component 3 (C3), progesterone receptor (Pgr), calbindin D9K (S100g), and vascular endothelial growth factor A (Vegfa), was assessed in the adult uterus. The expression of C3 was not affected by any dose of BPA or EE2. Both EE2 doses up-regulated Pgr and S100g, while Vegfa was significantly up-regulated by the high EE2. No significant differences were detected between the naïve or BPA dose groups and the vehicle control.</p>	<p>induce changes at the molecular level, epigenetic or gene expression, in the female mammary gland and prostate), to link these molecular changes with morphological changes previously described (see also Delclos <i>et al.</i>, 2014 in <i>Table 16</i>). Gene expression analysis of the nuclear ERs (Esr1 and Esr2), G-protein-coupled ER (Gper), and members of the ERR family (Esrra, Esrrb, and Esrrg): no BPA-induced change at PND4 as compared to control animals in female mammary glands. No change in any receptor expression was observed at PND90. Gene expression analysis and GO term enrichment analysis in PND4 mammary gland: the majority of changes in gene expression were observed with EE2 (associated with inflammation and immunity and calcium ion homeostasis). Few genes that were up- or down- regulated with low BPA doses (2.5 and 25 µg/kg/d) versus the tissue matched control, using microarray analysis, were not changed in qRT-PCR analysis. The effect of the high BPA dose level at the genome-wide gene expression overlapped to some extent with that of the low dose EE2, in particular in the female mammary gland. At PND90, there was no statistically significant global genomic DNA methylation change in either BPA- or EE2-treated animals <i>versus</i> the vehicle control.</p>
<p>Doherty et al., 2010</p>	<p>Mice CD1 <i>In vivo and in vitro</i></p>	<p><u>MCF-7:</u> Human breast cancer cell line (immortalised cells, ERα and Progesterone (PR) positive)</p>	<p><u>GD9-GD26</u> (<i>in utero</i>) BPA 5 mg/kg (i.p.); DES 10µg/kg (i.p.) Female offspring with <i>in utero</i> exposure were euthanized 6 weeks after birth. <u>in MCF-7</u> DES: 5 dose levels from 5.10⁻⁶; to 10⁻¹⁰ M BPA: 5 dose levels from 2.5. 10⁻⁴; to 2.5. 10⁻⁸M</p>	<p><i>Female offspring mammary tissue analysed for mRNA analysis and protein analysis.</i> Epigenetic mechanisms</p>	<p>BPA exposure <i>in utero</i> significantly alters EZH2 expression (protein, >2 fold) and activity (histone H3, tri-methyl K27) in adult mammary tissue when compared to control mice. DES increased also EZH2 expression (gene and protein) which is elevated in mammary gland of mice exposed <i>in utero</i>. EZH2 is a key epigenic regulator. Histone methylation by EZH2 is a known epigenic modifier in breast cancers. BPA exposure of (MCF-7) breast cancer cells increases functional activity of EZH2, i.e., histone H3 trimethylation. Similar results with DES. Discussion. Two important target genes of EZH2 are p57 (CDKN1C), a cyclin dependent kinase inhibitor, and E-</p>

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			for 48 h.		cadherin which is important in cell-cell adhesion and migration. Repression of p57 and E-cadherin by EZH2 may lead to the increase of cell proliferation, or increased invasiveness, seen in some breast tumors.
Bhan et al., 2014a (<i>J Steroid Biochem Mol Biol</i>)	Rat Sprague Dawley In vitro		Same protocol in vivo and in vitro as Bhan, 2014b: Ovariectomised adult rats. BPA sc: 25 µg/kg, 24h. Positive control: E2 and DES 5 µg/kg MCF-7 cells: BPA 100 nM Positive control: : E2 0.1 nM, and DES 10nM)	Epigenetic mechanisms	<i>-in vivo</i> , BPA increases HOTAIR expression (mRNA, 4-fold increase) in the mammary gland. This effect is also observed with DES (x 4.3) and E2 (x3.3). HOTAIR is a long non-coding RNA (which is not translated into protein), and is a key player in gene silencing and breast cancer. -in vitro, BPA (100 nM) increases HOTAIR expression (x6) in MCF-7 cells -molecular analysis of the HOTAIR promoter in BPA-treated cells (same protocol as for EZH2): the HOTAIR promoter contains functional estrogen-response elements (ERE2 and ERE3); ER (ER α and ER β are involved for BPA-induced HOTAIR expression). Along with ER, co-activators such as MLL (2 methyltransferases H3K4, MLL1 and MLL2) and CBP/p300 (histone acetylase) bind to the HOTAIR promoter and modify chromatin in the presence of BPA. These effects are inhibited when BPA-treated cells are knocked-out for ER α or ER β .
Bhan et al., 2014b (<i>J Mol Biol</i>)	Rat Sprague Dawley In vitro		Same protocol in vivo and in vitro as Bhan 2014a Ovariectomised adult rats BPA sc: 25 µg/kg, 24h (Positive control : E2 et DES 5 µg/kg) In vitro: BPA 100 nM Positive control: E2 and DES (10 nM) <i>MCF-7, T47D, MDA-MB-231</i>	Epigenetic mechanisms	<i>-in vivo</i> , BPA increases EZH2 expression (mRNA, 6-fold increase, and protein) in the mammary gland. This effect is also observed with DES (x6) and E2 (x4.8) <i>-in vitro</i> , BPA (100 nM) increases the expression of EZH2 (mRNA et protein) in MCF-7 and T47D cells, but not in MDA-MB-231 (devoid of nuclear ER receptors). Results indicate an ER-mediated estrogenic effect of BPA. -molecular analysis of the EZH2 promoter in BPA-treated cells - EZH2 promoter contains functional estrogen-response elements (ERE1 et ERE2) (<i>luciferase-based reporter construct</i>) - Both ER α and ER β are involved in the BPA-induced EZH2 expression (<i>ChIP assay, chromatin immunoprecipitation</i>). BPA increases the fixation of ER co-régulators such as MLLs (MLL2 et MLL3, key

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					<p><i>enzymes involved in methylation and acetylation of histones H3K4) and CPB/P300 (histone acetylase HAT). These effects are also observed with DES and E2</i></p> <ul style="list-style-type: none"> - BPA increases the functional activity of EZH2, <i>i.e.</i>, level of histone acetylation and H3K4 tri-methylation, at the EZH2 promoter, as well as polymerase II.
<p>Hussain et al., 2015 (BBA)</p>	<p>Rat Sprague Dawley</p> <p><i>In vitro</i></p>	<p>Same protocol in vivo and in vitro as Bhan, 2014a and b</p> <p>Ovariectomised adult rats. BPA sc: 25µg/kg, 24h. Positive control: E2 and DES 5 µg/kg</p> <p>Cells: MCF-7, T47D, MDA-MB-231 BPA (several doses) 4h</p>	Developmental genes	<p>BPA induced HOXC6 expression both in vivo and in vitro. HOXC6 is a homeobox-containing gene associated with mammary gland development and is overexpressed in a variety of cancers including breast cancer.</p> <ul style="list-style-type: none"> - <i>In vivo</i> BPA (25 µg/kg) increases HOXC6 gene expression (mRNA and protein in the mammary gland. These effects are also observed with E2 and E2+BPA -<i>In vitro</i> BPA (10 nM to 100nM) increases HOXC6 expression (x6) in ER-positive breast cancers cells (MCF-7 and T47D), but not in ER- cell line, indicating a potential regulation by ER. This effect is also observed with E2 (1 nM). -molecular analysis of the HOXC6 promoter in BPA-treated cells (same protocol as for EZH2 and HOTAIR). Along with ERα, co-activators such as MLL2 and MLL3 and CBP/p300 (histone acetylase) bind to an HOXC6 promoter ERE region upon treatment with BPA affecting its gene expression. This effect is also observed in E2-treated cells; reversed in ERα-knock-out BPA (or E2) treated cells 	
<p>Deb et al., 2016 (Gene)</p>	<p>Rat Sprague Dawley</p> <p><i>In vitro</i></p>	<p>Same protocol in vivo and in vitro as Bhan, 2014a and b</p> <p>Ovariectomised adult rats. BPA sc: 25µg/kg, 24h. Positive control: E2 5 µg/kg</p> <p><i>In vitro</i> MCF-7 Several doses 0-BPA 100 nM 6h (Ctrl: E2)</p>		<p>BPA induced HOXB9 expression both in vivo and in vitro. HOXB9 is a homeobox-containing gene that play a key role in mammary gland development and is associated with breast and other types of cancer. It is involved in several processes including cell proliferation, cell-cycle progression.</p> <ul style="list-style-type: none"> -<i>In vivo</i> BPA (25 µg/kg) increases HOXB9 expression (x4) in the mammary gland of ovariectomised rats. <i>In vitro</i> BPA (100nM) induces HOXB9 expression (x9.4) in MCF-7 cells. While E2 also increases HOXB9 expression, BPA counteracts the level of E2-induced HOXB9 in 	

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					<p>comparison to E2 alone.</p> <p>-HOXB9 promoter ERE is responsive to BPA. Molecular analysis of the HOXB9 promoter in BPA-treated cells (same protocol as for EZH2 and HOTAIR). Along with ER, ER-co-activators such as MLL3 and CBP/p300 (histone acetylase) bind to the HOXB9 promoter in the presence of BPA, modify chromatin and lead to its gene activation even in the absence of estradiol</p>
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4.4 Alteration of brain development and cognitive function

4.4.1 Overview of previous evaluation and focus on a specific effect

Overview of the effects of BPA on the brain and behaviour based on previous works

A summary of the toxic effects of BPA on the brain and behaviour is presented hereafter on the basis of the information collected in the restriction proposal of BPA in thermal paper (ANSES, 2014).

In animals, several studies investigated brain functionality and behaviour following pre-, post- and/or perinatal exposure to BPA.

The studies examining the effects of pre- or perinatal exposure to BPA **on anxiety** were conducted at a wide range of exposure levels, thereby making it difficult to establish a comparison between the results achieved. Indeed, BPA has been shown to have no effects (Stump, 2010), to increase the level of anxiety (Cox, 2010; Poimenova, 2010) or to reduce it (Tian, 2010). It is not known whether an 80-fold difference in the administered doses can explain the different effects induced by BPA. Anxiety-related behaviour can be modulated by various factors including the chemical properties of the compound, the behavioural task used and the experimental protocol. Therefore, no clear conclusion can be drawn. It is however noted that many studies have been recently published on BPA and anxiety but they were not included in the present overview.

Impairment of **memory** including spatial and avoidance abilities was observed in mice exposed to BPA (0.5-50 mg/kg/d) during gestation and lactation (Xu *et al.*, 2010b). In addition, spatial memory performance was also reduced in both male and female rats exposed to a lower dose of BPA (40 µg/kg/d) during the same periods (Poimenova *et al.*, 2010).

Suppression of **sexual dimorphism in the neural structures involved in sexual behaviour** was reported in offspring mice orally exposed *in utero* to 8 mg/kg/d (Cox *et al.*, 2010). However, it was not observed in two rat studies using respectively 2 to 200 µg/kg/d BPA by oral route during gestation and lactation and 50 µg or 50 mg/kg/d BPA by subcutaneous injections during PND0-3 (Ryan *et al.*, 2010a and Adewale *et al.*, 2009).

Suppression of **sex differences** was identified in rat offspring with regard to their locomotor activity and exploratory behaviour in the open-field (Kubo *et al.*, 2003; Rubin *et al.*, 2006; Palanza *et al.*, 2008), activity and avoidance memory (Kubo *et al.*, 2001), and social novelty behaviour (Cox *et al.*, 2010; Palanza *et al.*, 2008). In addition, decreased exploratory behaviour was observed only in females in Poimenova *et al.* (2010). These effects have been observed in experimental studies using a dose-range of 25 to 250 ng/kg/d BPA by subcutaneous route (Rubin *et al.*, 2006) and 10 µg to 8 mg/kg/d BPA by oral route (all other studies).

Changes in maternal behaviour related to pre- or postnatal exposure to BPA (10 µg/kg/d to dams by oral route) have also been reported (Palanza *et al.*, 2002).

Effects on **cerebral development** linked to pre- or perinatal exposure to BPA have been demonstrated in several studies including changes in neural differentiation (Funabashi *et al.*, 2004; Patisaul *et al.*, 2007; Rubin *et al.*, 2006), alterations of the glutamatergic NMDA receptors and aminergic systems (Tian *et al.*, 2010; Matsuda *et al.*, 2010; Xu *et al.*, 2010a and 2010b), changes in the expression of estrogen receptors (ER) α and ER β (Xu *et al.*, 2010b; Mahoney *et al.*, 2010), and changes in the number of neurons responsive to oxytocin and serotonin (Adewale *et al.*, 2009). These changes occurred in particular regions like the hypothalamus (more precisely in sexually dimorphic areas) and the

hippocampus, a region involved in cognitive processes, namely those linked to NMDA receptors.

These neural effects were identified in experimental studies following prenatal or neonatal exposure to 25 ng/kg/d to 50 mg/kg/d BPA by subcutaneous route and to 0.05 to 50 mg/kg/d BPA by oral route. Thus, they could explain and support, at least in part, the behavioural effects observed concomitantly.

Assessment by RAC under the restriction process

In its opinion of June 2015 (ECHA, 2015), RAC adopted conclusions on the analysis of BPA-induced effects on brain and behaviour as quoted in the text box hereafter:

"The Dossier Submitter considered the oral study by Xu *et al.* (2010b) in mice as the key study for neuro-developmental toxicity. The critical effects in this study were the alteration of memory and learning functions paralleled by a decrease in the expression of glutamate NMDA receptors.

The EFSA (2015) opinion concluded on neurological, neurodevelopmental and neuroendocrine effects as follows: "[...] *In summary, there are indications from prospective studies in humans that prenatal BPA exposure (BPA exposure during pregnancy) may be associated with altered child behaviour in a sex-dependent manner. However, the associations were not consistent across the studies and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations reported do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or childhood and neurodevelopmental effects in humans.*

A number of new studies report changes that may indicate effects of BPA on brain development (effect on neurogenesis and on gene expression, neuroendocrine effects, effects on the morphology of certain brain regions, etc.). Whether such changes are mechanistically related to the neurobehavioral responses reported following exposure is attempted addressed by some studies but with inconsistent results.

Several new animal studies investigated anxiety-like behaviour, learning and memory, social behaviour and sensory-motor function. Some studies report changes in anxiety-like behaviour after BPA exposure. Some, but not all, studies reported significant impairment of either learning and/or memory capacities. A few studies also report effects on social behaviour and sensory-motor function. However, the studies present methodological shortcomings, such as small sample size, lack of consideration of the litter effect, not properly controlled variability of exposure through diet and inadequate statistics. Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to neurological, neurodevelopmental and neuroendocrine effects of BPA. [Since the likelihood]²⁰ level for this endpoint is less than "likely" (see Appendix A), this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of uncertainty for hazard characterisation and risk characterisation".

See sections 3.4 and 4.3 of the EFSA (2015) opinion for more details.

RAC considers that the results from the Xu *et al.* (2010) study suggest that developmental exposure to BPA can interfere with learning and memory capacities in different learning tasks in rodents, including spatial learning and passive avoidance learning together with down-regulation of the NMDA receptors. However, the effects of BPA on learning and memory abilities of laboratory rodents are not fully consistent, as both positive and negative effects are reported in different studies.

²⁰ Included by RAC for clarification.

Two studies that were not included in the restriction report or in EFSA (2015) were submitted during public consultation (Elsworth *et al.*, 2013 and Ferguson *et al.*, 2014). Elsworth *et al.* (2013) showed effects on brain development (loss of midbrain TH-immunoreactive neurons and loss of hippocampal spine synapses) in non-human primates at low BPA doses. No alterations in sexually dimorphic behaviors in male and female Sprague-Dawley rats were observed by Ferguson *et al.* (2014).

Conclusion

RAC in principle agrees with EFSA's conclusion on effects on brain and behaviour. Since effects on brain and behaviour have been observed at and below the range where kidney effects occur, RAC considers it prudent to take them into account in hazard and risk assessment and in health impact assessment. RAC however acknowledges that the available information does not allow a quantification of the dose-response relationship, therefore this endpoint will be accounted for in the setting of Assessment Factors."

During the Public Consultation of the Annex XV dossier, a comment reported that recent epidemiological studies suggest an association between autism spectrum disorder and BPA exposure (Arbuckle *et al.*, 2016; Kardas *et al.*, 2016; Kondolot *et al.*, 2016; Stein *et al.*, 2015). In addition it was noted that although this is currently understudied, there are indications that BPA exposure has an effect on neurodegenerative processes both through an estrogenic pathway or disruption of TH action (Preciados *et al.*, 2016; Jiang *et al.*, 2016; Boxian *et al.*, 2014). These effects were however not further investigated because the level of evidence is considered insufficient at this point due to lack of appropriate experimental studies.

Focus on a specific effect of BPA on the brain based on previous assessments and recent publications

More recent studies have investigated the effects of exposure to BPA on behaviour. In particular, alteration of learning and memory was confirmed in several new studies and it appears as the endpoint with the most convincing evidence among the other central effects induced by exposure to BPA as well as the endpoint with the most specific indications of its link with endocrine disruption.

Therefore, also for the sake of clarity, considering the extent of the available toxicological data, the present section addresses in detail only alteration of learning and memory ability. This adverse effect which is now well established is the focus of the analysis below. The following section analyses i) the ability of BPA to affect learning and memory in detail and ii) the endocrine basis of these changes.

For this purpose, studies addressing BPA effects on learning/memory and its MoA were collected up to May 2016.

4.4.2 Adverse effects

Background on learning and memory and underlying cellular and molecular mechanisms

Learning and memory is the function by which individuals acquire knowledge about their environment and refers to the process by which the learned information is encoded, stored and later retrieved. Several brain areas are involved in learning and memory (cortex, hippocampus, amygdala...) and damage to a given region may cause the loss (partial or complete) of a specific function. The medial temporal system of high vertebrates includes the hippocampus, which is one of the most important areas involved in the processing of

memory. Neurological studies in human patients and in rodents established the central role of this brain region for contextual memory. The hippocampal formation was associated with a wide variety of cognitive functions such as spatial navigation and planning, memory encoding and retrieval, relational processing, and novelty detection. The rodent hippocampus was also shown to play a key role in both spatial and non-spatial memory (visual object recognition, temporal processing of information...).

At the cellular level, learning and memory involve various neuronal plasticity mechanisms, which include long-term potentiation, synaptogenesis, modulation of intrinsic excitability, adult neurogenesis in the dentate gyrus and modulation of the glutamatergic neurotransmitting system. At the molecular level, memory involves changes, which have been extensively studied in rodents, especially in glutamatergic N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The NMDAR is an ion channel protein composed of four subunits (including GluN1 and GluN2 subunits) and stimulated by the glutamate neurotransmitter. These receptors contribute to the induction of synaptic plasticity, are also involved certain types of brain neuronal plasticity and are necessary for the encoding of many types of memory. It has been shown that the hippocampus is essential for spatial learning and memory and is subjected to the induction of long-term potentiation involved in synaptic plasticity. The detailed steps of these processes are presented in Figure 15. Among the required kinases, phosphorylated ERK and CamKIV translocate into the nucleus to phosphorylate the transcription factor CREB. Phosphorylated CREB induces gene transcription and protein synthesis necessary for structural changes at existing synapses as well as synaptogenesis.

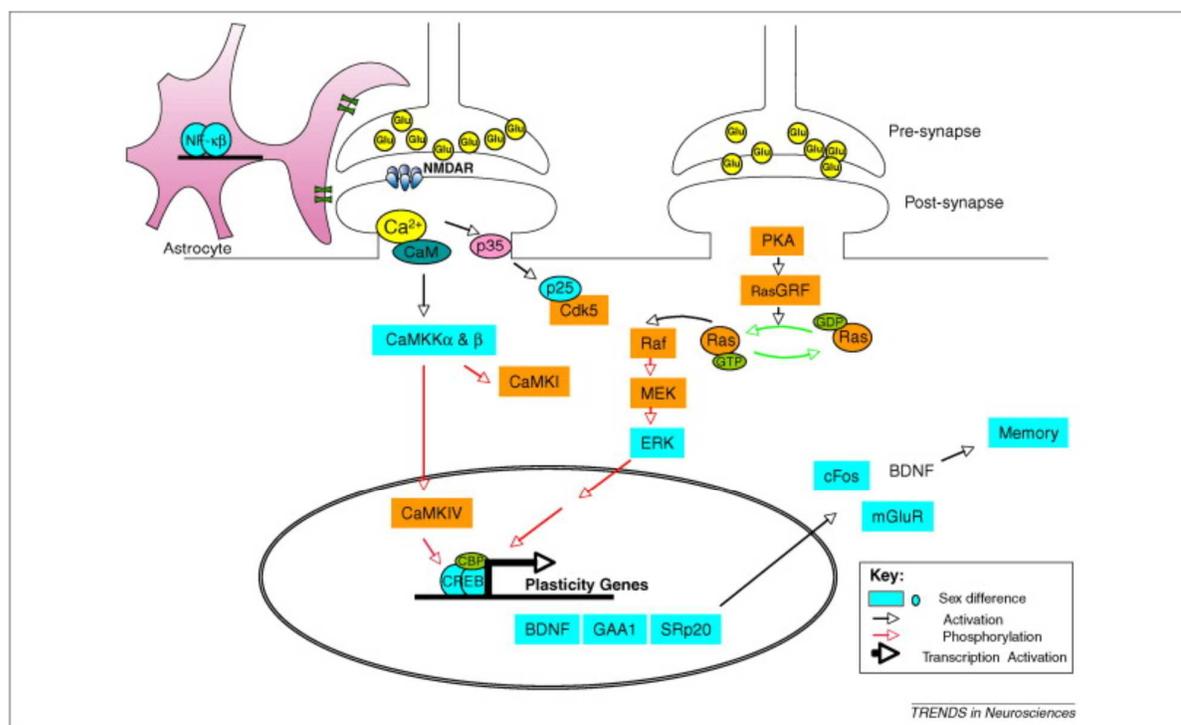


Figure 15: Steps involved in induction of long-term synaptic plasticity at various hippocampal synapses in rodents. From Mizuno & Giese, 2010

At glutamatergic synapses, activation of NMDA receptors induces Ca^{2+} influx into the postsynaptic cell. This triggers activation of various synaptic kinases and induction of gene transcription (C/EBPB, EPA, BDNF) by phosphorylated CREB followed by protein synthesis. Sex differences are highlighted in blue. Abbreviations- BDNF: brain-derived neurotrophic factor; CaM: calmodulin; CaMKI: Ca^{2+} /CaM kinase I; CaMKIV: Ca^{2+} /CaM kinase IV; CaMKK: Ca^{2+} /CaM kinase kinase; CBP: CREB-binding protein; Cdk5: cyclin-dependent kinase 5; CREB: cAMP-responsive element binding protein; ERK/MAPK: extracellular signal-regulated kinase/ mitogen-activated protein kinase; GAA1: glycosyl

phosphatidyl-inositol anchor attachment protein; Glu: glutamate; GRF: guanine nucleotide-releasing factor; MEK: mitogen-activated protein kinase kinase; NMDAR: NMDA receptor; NF- κ B: nuclear factor κ B; mGluR: metabotropic glutamate receptor; PKA: cAMP-dependent protein kinase; SRp20: splicing factor arginine/serine-rich 3.

Learning and memory processes are very sensitive to both endogenous (neurotransmitters, hormonal systems) and exogenous factors (e.g. stress, drug abuse). Gender-specific differences are observed in the execution of behavioural tasks. Sex modulations in the molecular pathways involved in memory processes are highlighted in blue in Figure 15.

4.4.2.1 Non-human information

Studies investigating the effects of exposure to BPA on learning and memory as well as potential related alterations in brain regions underlying these processes are summarised in Table 21 presented in section 4.4.5. They are discussed by type of exposure (developmental, prepubertal and pubertal, adult) and species studied (mice, rats, then other species).

Developmental exposure

Twenty-two studies addressed the developmental effects of exposure to BPA on learning and memory in rodents (8 in mice, 14 in rats).

- **Spatial memory** was assessed in 17 studies by using dedicated paradigms (Morris water maze, Barnes maze, Y maze, or radial maze). About 70% of these studies (12/17): 5 in mice (Xu *et al.*, 2010b; Jasarevich *et al.*, 2011, 2013; Jang *et al.*, 2012; Kumar *et al.*, 2014) and 7 in rats (Poimenova *et al.*, 2010; Goncalves *et al.*, 2010; Kuwahara *et al.*, 2013; Xu *et al.*, 2014b; Wang *et al.*, 2014b; Johnson *et al.*, 2016; Hass *et al.*, 2016) reported learning and memory disturbances in adult animals developmentally exposed to BPA, whereas no effects were observed in 5 studies: 2 in mice (Nakamura *et al.*, 2012; Viberg *et al.*, 2011) and 3 in rats (Stump *et al.*, 2010; Jones *et al.*, 2012; Ferguson *et al.*, 2012).

Among these studies, 8 analysed spatial memory in both males and females. Four studies (2 in mice, 2 in rats) reported sex differences in BPA-induced effects (Jasarevic *et al.* 2011; Jasarevic *et al.*, 2013; Goncalves *et al.*, 2010; Johnson *et al.*, 2016), while a similar memory deficit in both sexes was observed in 1 rat study (Poimenova *et al.*, 2010). No effects in either sex were reported in 4 analyses (1 in mice and 3 in rats) (Nakamura *et al.*, 2012; Stump *et al.*, 2010; Jones *et al.*, 2012; Ferguson *et al.*, 2012).

At the molecular level, the behavioural alterations induced by developmental exposure to BPA were associated with reduced hippocampal expression of NMDAR subunits (Xu *et al.*, 2010a and 2010b) and changes in hippocampal ER levels (Xu *et al.*, 2010b; Xu *et al.*, 2014b). Modifications in levels of corticosterone and their receptors (Poimenova *et al.*, 2010), as well as changes in hippocampal expression of two cell adhesion proteins (Nrxn1 and Nlgn3) involved in synaptic plasticity (Kumar *et al.*, 2014) were also reported.

- **Short-term or long-term memory** was analysed in 5 studies: 1 in mice (Xu *et al.*, 2010b) and 4 in rats (Kubo *et al.*, 2001; Goncalves *et al.*, 2010; Xu *et al.*, 2011a; Kuwahara *et al.*, 2013) by using the passive avoidance test. They all described memory impairment in males and females developmentally exposed to BPA, with one study reporting sex differences in BPA-induced effects (Kubo *et al.*, 2001). Reduced hippocampal expression of NMDAR subunits was found in the same study assessing long-term effect of early exposure to BPA (GD7-PND21) on both spatial memory and short-term/long-term memory (Xu *et al.*, 2010b). In another study investigating the effects of a single exposure to BPA (1 single injection at PND18 and males analysed 1h or 24h later), the levels of

NMDAR subunits were not affected, but their phosphorylated levels were increased (Xu *et al.*, 2011a). These differences in response in NMDAR expression are very likely to be related to the duration of exposure to BPA (single vs repeated exposure). Repeated exposure to BPA (GD6 to GD17) also triggered a decrease in adult neurogenesis in the dentate gyrus of developmentally exposed females, together with a decrease in hippocampal levels of phosphorylated ERK and CREB, and brain-derived neurotrophic factor (BDNF) (Jang *et al.*, 2012), three key actors of synaptic plasticity and memory processing.

- **Visual memory** recognition was analysed in two rat studies, which reported behavioural impairments in males and females in one study (Goncalves *et al.*, 2010), and only in males in the other (Wang *et al.*, 2016) following exposure to BPA. In Wang *et al.* (2016), the levels of Akt, MAPK and their phosphorylated forms were reduced, and phosphorylated levels of BDNF and CREB inhibited, indicating inhibition of processes involved in synaptic plasticity.

- **Fear memory** was enhanced in females following exposure to BPA from GD10 to PND20 (Matsuda *et al.*, 2013). Serotonin metabolite concentrations were increased in hippocampus, striatum, midbrain, pons and medulla oblongata of juvenile females but not in adult females or juvenile/adult males. The expression levels of Tph2, Slc6a4, monoamine oxydases (enzymes involved in serotonin synthesis, transport and metabolism) were also enhanced.

One study was performed on a non-human primate model (Rhesus macaque) with the objective to compare BPA effects during prenatal exposure or juvenile stage (Elsworth *et al.*, 2013). BPA during fetal development of Rhesus macaques reduced the number of dopaminergic neurons or of spine synapses in the hippocampal CA1 region, but not in the prefrontal cortex. No behavioural evaluation was conducted in the part of the study where animals were exposed developmentally.

Prepubertal and pubertal exposure

Spatial memory was tested in 6 rodent studies (4 in rats, 2 in mice) using the Morris water maze and object recognition placement test. Impaired memory was found in 4/6 studies (Kim *et al.*, 2011., Xu *et al.*, 2011b; Jain *et al.*, 2011; Diaz Weinstein *et al.*, 2013), with reported sex differences in the study analysing both sexes (Xu *et al.*, 2011b). Memory assessed by the passive avoidance and object recognition tests was found affected by prepubertal/pubertal exposure to BPA (Xu *et al.*, 2011b, Jain *et al.*; 2011; Bowman *et al.*, 2015; Diaz Weinstein *et al.*, 2013). One study described no effect on **fear memory** (Kawahara *et al.*, 2014).

Reduced adult neurogenesis in the dentate gyrus (Kim *et al.*, 2011) and spine density in the hippocampal CA1 area as well as an oxidative stress (Jain *et al.*, 2011) were reported in these studies.

The Elsworth study performed in juveniles of African green monkeys found no effect by using a cognitive task that tests working memory capacity (Elsworth *et al.*, 2013). The number of dopaminergic neurons and of spine synapses remained unaffected in the hippocampus, contrary to the decrease noted when BPA was administered during fetal development. It is, however, important to indicate that this study presents a limitation due to the analysis of animals without taking account of their sex and that sex-specific effects may hence be missed.

Adult exposure

Spatial memory was analysed in 5 rodent studies (2 in mice, 3 in rats) by using the Morris water maze and the object recognition placement test. Out of 5 studies, 4 showed

impaired memory following adult exposure to BPA (Xu *et al.*, 2013b; Eilam-Stock *et al.*, 2011; Inagaki *et al.*, 2012; Fan *et al.*, 2013).

Short-term and long-term memory, as assessed by the passive avoidance test, was also impaired in mice (Xu *et al.*, 2013b). One study in African vervet male monkeys using the two-choice spatial delayed response task showed impaired memory (Elsworth *et al.*, 2015). The number of spine synapses was diminished in the prefrontal cortex and CA1 region.

At the neuroanatomical level, these behavioural alterations were associated with disrupted synaptic density and histological alterations in the hippocampus and/or prefrontal cortex in rodents (Inagaki *et al.*, 2012; Xu *et al.*, 2013b; Eilam-Stock *et al.*, 2011) and primates (Elsworth *et al.*, 2015). At the molecular level, the expression level of NMDAR and AMPAR subunits and synaptic proteins were affected (Xu *et al.*, 2013b; Eilam-Stock *et al.*, 2011). Reduced acetylcholine esterase was also observed in the hippocampus (Fan *et al.*, 2013).

In one study analysing spatial (Morris water maze) and non-spatial memory (passive avoidance test) in ovariectomised female mice, BPA treatment suppresses the reduction in abilities observed in ovariectomised animals (Xu *et al.*, 2015b). An enhancement of capacities was observed in male mice in a fear-conditioning task (Zhang *et al.*, 2014). Changes in expression levels of NMDAR and synaptic proteins were observed in these studies.

4.4.2.2 Human information

Epidemiological studies

While several epidemiological studies investigated the association between perinatal exposure to BPA and child behaviour, only two studies investigated a link between exposure to BPA and the cognitive performance of children more specifically (Casas *et al.*, 2015; Hong *et al.*, 2013).

Casas *et al.* (2015) assessed prenatal exposure to BPA by urinary measurements in two spot samples collected in the first and third trimester of pregnancy in 438 pregnant women from the Spanish mother-child cohort INMA-Sabadell (median, 25th-75th percentiles; 1st-trimester sample: 2.1, 1.2-3.7 µg/L; 3rd-trimester sample: 1.8, 1.0-3.1 µg/L). Cognitive and psychomotor abilities were assessed by psychologists at both 1 and 4 years of age with standardised neuropsychological tests (Bayley Scales of Infant Development BSID at age 1, McCarthy Scales of Children's Abilities (MSCA) at age 4; expected mean (standard deviation)=100(15)). Numerous potential confounders, including socioeconomic status or other well-established neurotoxicants (PCBs and methylmercury during pregnancy), as well as strong determinants of children's neuropsychological abilities such as maternal intelligence score, were taken into account, some of them having been assessed prospectively. Multivariable models were conducted and non-linearity was considered. A statistically significant decrease of the psychomotor score at age 1 was observed among the highest-tertile urinary BPA concentrations, compared to the lowest-tertile concentrations (beta=-4.3, 95% CI:-8.1,-0.4), while no association was observed with the MSCA motor score at age 4. No association was observed between prenatal urinary BPA concentrations and the cognitive scores of children aged 1 or 4 years. No sex interaction was observed.

Hong *et al.* (2013) assessed childhood BPA exposure from urinary measurements in first-morning spot sample of 1008 children aged 8-11 years recruited from school lists of 5 different regions in Korea (median BPA level was 1.2 ug/L). The learning performance of the children was assessed using the parent-reported questionnaire (Learning Disability Evaluation Scale; LDES) and an abbreviated form of the Korean Educational Development

Institute's Wechsler Intelligence Scales for Children (KEDI-WISC) was administered. Several potential confounders, including socioeconomic status or other well-established or suspected neurotoxicants (lead during childhood, phthalates) were taken into account. Multivariable models were conducted and non-linearity was considered. Statistically significant adverse associations were observed between the urinary BPA concentrations and the parent-reported learning score (LDES). No association was observed between the urinary BPA concentrations and the intelligence scores of the children.

BPA has a short biological half-life and intra-day and inter-day variabilities of urinary BPA concentration are well-known. The findings of the existing literature are thus limited by the use of single spot urinary BPA measurement that might mostly reflect BPA exposure during the preceding hours or days. Note that some authors expect that large sample size, as observed in these two studies, might help to limit the noise and the potential bias induced by exposure measurement.

***In vitro* human study**

An *in vitro* study showed that exposure to BPA (100 nM) of primary human cortical neurons, from 16-21 week-old fetal brain, decreased mRNA levels of potassium chloride co-transporter 2 (KCC2) (Yeo *et al.*, 2013). This potassium chloride co-transporter is responsible for chloride extrusion from mature neurons. During CNS development, chloride concentration is of fundamental relevance for migration of cortical inhibitory precursor neurons to their proper final locations in the brain's cortex. KCC2 expression increases during the period preceding the chloride shift, which characterises mature neurons. Similar effects were observed for rat and mouse in the same study. The delayed perinatal chloride shift induced by BPA was mediated through an epigenetic modification of the *Kcc2* gene. Effects were more accentuated in females than in males.

4.4.2.3 Summary and discussion of effects on brain and behaviour

Epidemiological evidence on the potential role of exposure to BPA early in life on learning and memory performance is still insufficient. However, about 72% of rodent studies (total of 26/36) reported impaired spatial and non-spatial memory following exposure to BPA, regardless of the period of exposure. In the remaining 28% of studies, there was either no effect (7/36) or enhanced performance (3/36). The causes of these differences are difficult to address given the differences in the experimental conditions between studies (sex, period of exposure, BPA doses), but the overall evidence has recently substantially increased and altogether strongly points toward the conclusion that BPA alters memory in rodents. In addition, as the 72% of studies reporting impaired cognitive behaviours were also performed under various experimental conditions (various doses, routes and period of exposure and test species), this means that this impairment is a robust BPA-induced effect.

Another interesting finding which can be drawn from this analysis, is the sex-dependent effect observed in a number of studies. Furthermore, although not systematically assessed in all these studies, the neural mechanisms associated with the behavioural alterations consist of a reduction in the level of expression of NMDAR subunits, kinases, enzymes involved in neurotransmitter regulation, and synaptic proteins as well as decreased spine density or neurogenesis. Such molecular, cellular and structural changes are fully relevant and could underlie the impaired learning and memory performance observed in the same animals.

Finally, although there is a limited number of studies conducted in non-human primates, these studies have shown that BPA during the prenatal stage of development has detrimental effects on the midbrain DA system and on spine synapses in the hippocampus, while it has no effect when applied at a juvenile stage (Elsworth *et al.*, 2013). They also indicated in adult BPA-exposed monkeys a potential for significant cognitive impairment (Elsworth *et al.*, 2015).

It is also interesting to note that down-regulation of estradiol-induced increase in spine synapses in the hippocampus and prefrontal cortex in adult ovariectomised monkeys was also reported in another study of the same laboratory that is discussed in the next section 4.4.3.2 (Leranth *et al.*, 2008).

Overall, BPA has been demonstrated to alter memory and learning after developmental, pubertal or adult exposure, in multiple converging experimental studies reporting this functional effect as well as molecular and cellular changes in the brain in line with the functional changes observed.

4.4.3 Endocrine disruption

4.4.3.1 *In vitro* information indicative of an endocrine MoA

Few studies have investigated the potential MoA of BPA in neural development *in vitro* or *ex vivo*. The data are summarised in Table 22 (presented in section 4.4.5).

In cultured hippocampal neurons from 24-hr old rats, BPA (10 and 100 nM) promoted dendritic development (length of dendrites and mobility and density of dendritic filopodia) (Xu *et al.*, 2014a). The promoting action of BPA on dendritic development was completely blocked by the ER antagonist ICI 182,780. The effect of BPA could be exerted through enhancement of F-actin cytoskeleton (critical in morphological changes occurring in dendritic filopodia and spines during development and synaptic plasticity), and modifications in expression levels of Rho proteins (involved in intracellular actin regulation). These changes were shown to be mediated through ER- *via* ERK1/2 signaling pathways since they were suppressed by the ER antagonist.

In another study with rat cultured hippocampal neurons, BPA (10-1000 nM) rapidly increased the mobility and density of dendritic filopodia and phosphorylation of the NMDAR subunit N2B known to be primarily expressed in immature synapses of the hippocampus during postnatal development (Xu *et al.*, 2010c). The ER antagonist ICI 182,780 completely reversed BPA-induced effects on filopodia and GluN2B subunit activation.

An *ex vivo* study confirmed the action of BPA on ER observed *in vitro*. In cultured adult rat hippocampal slices, BPA (10 nM) enhanced long-term depression of synaptic transmission (Hasegawa *et al.*, 2013). This effect was suppressed by 4-OH-tamoxifen, an estrogen-related receptor gamma (ERR γ) antagonist, but not by ICI 182,780 (ER antagonist).

In conclusion, despite their limited number, *in vitro* studies provide evidence that BPA affects dendritic development and synaptogenesis in hippocampal rat neurons (Xu *et al.*, 2010c; Xu *et al.*, 2014a) through changes in ER-dependent pathways. Neural development and cerebral plasticity is based on a subtle balance between cell proliferation and apoptosis. The effects observed *in vitro* with BPA, if they occur *in vivo* may induce neural dysfunction. The *ex vivo* study performed by Hasegawa *et al.* (2013) extends the effects of BPA to other estrogen-receptor signaling pathways such as ERR- γ . This might suggest that estrogens act through multiple pathways, which can be impacted by BPA.

4.4.3.2 *In vivo* effects with regard to an endocrine MoA

Several of the studies that observed an effect of BPA in the alteration of learning and memory (presented also in Table 23 in section 4.4.5 as discussed before) were also considered to provide relevant information on a potential endocrine MoA and are summarised in more detail below.

Developmental exposure: In rats, Xu *et al.* (2014b) described a direct link between the behavioural effects induced by BPA and an estrogenic endocrine disruption. Male rats were

exposed to BPA from the last gestation day to PND21. BPA impaired spatial memory and increased exploratory activity. These effects were associated with decreased expression of ER α (mRNA and protein levels) at PND7 and 21 and increased expression at PND11 in the hippocampus of both hemispheres. All these effects were completely reversed by the ER antagonist ICI 182,780. Detailed analysis of the molecular mechanisms showed that BPA impaired ER α translocation to the nucleus and decreased the phosphorylation of nuclear and total ER α , which were also reversed by ICI 182,780.

In the hippocampus of mice, exposure to BPA decreased the expression of GluN1, GluN2A and GluN2B subunits of NMDAR and ER β . These effects were associated with impaired spatial and avoidance memory (Xu *et al.*, 2010b).

Postnatal exposure: In another study, Xu *et al.* (2011a) reported that single subcutaneous injection of 18-day-old male rats with BPA alone or EB alone increased the latency in retention test in the passive avoidance task at 1h but not 24h after exposure. These behavioural changes were associated with increased levels of phosphorylated NMDAR subunits. Interestingly, when males were exposed to both substances, no effect was observed as if there was a blockade of estradiol-induced responses by BPA. Pre-treatment with ER antagonist ICI 182,780 inhibited BPA- or EB-induced effect on phosphorylated NMDAR subunits.

Adult exposure: The behavioural disturbances induced by EB in ovariectomised mice were partially blocked by BPA (Xu *et al.*, 2015b). Co-treatment with BPA also blocked the estradiol-induced modifications of synaptic interface and expression of synaptic proteins in the hippocampus. In agreement with these data, BPA blocked estradiol-enhanced object placement memory in ovariectomised female rat, and object recognition memory performance during proestrus (high levels of estradiol) in intact females (Inagaki *et al.*, 2012). In the same study, BPA altered estradiol-induced increase in spine density of pyramidal neurons in the hippocampus in ovariectomised females. These rodent studies are consistent with the data published by Leranthe *et al.* (2008) showing that BPA blocked the estradiol-induced increase of spine synapses in the prefrontal cortex and hippocampus of ovariectomised female monkeys.

Altogether, these data point to a MoA for BPA targeting the estrogenic pathway with alterations of learning and memory as a consequence.

Other studies, that did not address the behavioural effects of exposure to BPA on memory, provided additional ED-related evidence of cerebral changes in brain areas underlying learning and memory. They are summarised below and in Table 23 (presented in section 4.4.5).

In the hippocampus of male rats, BPA reduced the expression of GluN1, GluN2A and GluN2B NMDAR subunits (Xu *et al.*, 2010a). These changes were associated with a decreased expression of ER β and a dose-dependent increase of aromatase cytochrome P450 (P450arom).

In the brain of mice orally exposed twice at GD6 and GD15 to BPA, expression of genes involved in neurodevelopment was decreased (*Xist*, *Gdi1*, *Nlgn3* and *Park3*, *Fmr1*) (Kumamoto & Oshio., 2013). These effects were associated with reduced expression of the AR gene and the mRNA levels of ER β , and the increased expression of ER α .

In several studies, exposure to BPA has been shown to modulate the expression of key steroidogenic enzymes in different cerebral structures. In the prefrontal cortex of adult rats orally exposed, BPA decreased the expression levels (mRNAs and protein) of 5 α -reductase 2 (5 α -R2) and 5 α -R3 (Castro *et al.*, 2015), two enzymes involved in testosterone metabolism. When exposed subcutaneously for 4 days during adulthood, the expression (mRNAs and protein levels) of 5 α -reductase 1 (5 α -R1) was reduced in females but not in

males (Castro *et al.*, 2013). BPA also increased the expression of the aromatase gene, involved in testosterone metabolism into neural estradiol, in males and females and the aromatase protein levels in males.

In adult mice (10-week-old) orally exposed during 12 weeks, BPA decreased the expression of ER β protein in males but not in females. These effects were associated with increased anxiety state level and decreased brain levels of testosterone in males. No effect on brain and serum levels of estradiol was observed in females (Xu *et al.*, 2015a).

An *in vivo* study was conducted on adult female African green monkeys, which were ovariectomised and implanted with the vehicle, EB alone, BPA (50 μ g/kg/d) alone or EB plus BPA for 20 days (Leranth *et al.*, 2008). Estradiol induced a synaptogenic effect, in hippocampal regions (CA1, CA3 and dentate gyrus (DG)) and prefrontal cortex, which was abolished by the continuous exposure to BPA.

4.4.3.3 Summary of the plausible link between adverse neural effects and endocrine MoA

BPA disrupts learning and memory processes. The cognitive effects induced by BPA are associated with the disruption of two important pathways in cerebral regions: i) NMDAR, their down-stream targets leading to gene transcription (ERK, CREB, BDNF...) and synaptic proteins involved in synaptic plasticity, and ii) estrogen-dependent pathways involving ER α or ER β .

The possibility that BPA alters the cellular and molecular pathways involved in learning and memory processes through disruption of estrogen-dependent pathways was first suggested in the study of Xu *et al.* (2010a and 2010b). In this study, decreased expression of ER β was also reported, but no demonstration was given of a potential link between BPA-induced effects on memory and estrogenic pathway alteration. In the study of Xu *et al.* (2014b), the link between BPA-induced effects on learning and memory processes and estrogenic pathway disruption was clearly established from the demonstration that the ER antagonist ICI 182,780 reversed both BPA-induced effects on ER α (modulation and regulation) and memory. Three other studies performed in adults indicated that BPA is also able to interfere with estradiol-induced effects on behaviour and spine density in rodents (Xu *et al.*, 2015b; Inagaki *et al.*, 2012) and on synaptogenesis in non-human primates (Leranth *et al.*, 2008). Additional evidence was provided by *in vitro* studies showing that BPA-induced effects on NMDAR signalling and synaptic proteins were reversed by the ER antagonist. In one *in vitro* study, BPA-induced disruption extended to other non classical estrogen receptors (ERR γ).

The modulatory effects on cognitive behaviours and processes by estrogens are now well established, although they were studied more extensively in females than in males (for reviews in females, see Galea *et al.*, 2013; Pawluski *et al.*, 2009). The importance of the estrogenic pathway in the regulation of cognitive behaviour and synaptic plasticity has also been reported in male rodents (Picot *et al.*, 2016). In the male nervous system, testosterone may act directly or through its non-aromatisable metabolite dihydrotestosterone to activate the AR. Testosterone can also be aromatised locally into neural estradiol, which stimulates ERs. In this context, it has been shown that performance in spatial learning and memory abilities, such as object recognition, fear conditioning and spatial memory tasks are decreased by castration and restored by testosterone replacement. The estrogenic component of this regulation in males is demonstrated in the following studies. Intra hippocampal injection of estradiol enhanced memory in a spatial water maze task, possibly through an interaction with muscarinic cholinergic systems (Packard *et al.*, 1996). Acute treatment using estrogens, estrogen receptor agonists or selective estrogen receptor modulators were shown to facilitate long-term potentiation in adult hippocampal slices, affecting the number and shape of dendritic spines in CA1 pyramidal neurons and decreasing thorn density of hippocampal CA3 neurons (Kramar *et*

et al., 2009). Estradiol through both ER α and ER β was also reported to regulate NMDAR-mediated transmission and thus synaptic plasticity in the dentate gyrus of juvenile males (Tanaka *et al.*, 2013). In mice, activation of hippocampal ER α after learning impaired memory formation in contextual fear conditioning tasks (Cho *et al.*, 2015). Detailed effects of adult estrogens on learning and memory and the mechanisms underlying these effects in both males and females are described in recent reviews (Frick *et al.*, 2015; Hamson *et al.*, 2016). Figure 16 illustrates potential molecular mechanisms underlying estradiol-induced regulation of NMDAR signaling.

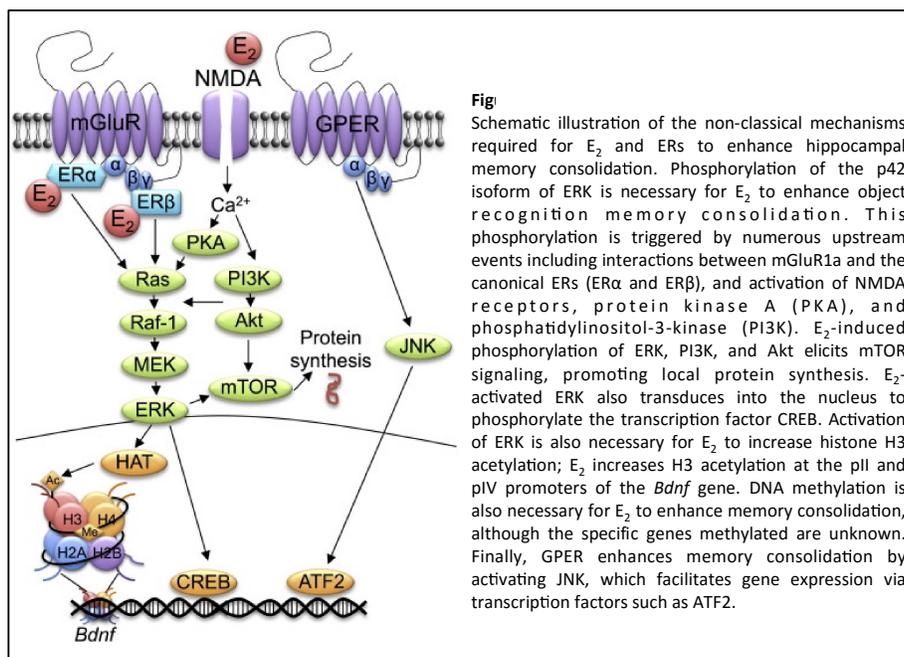


Fig Schematic illustration of the non-classical mechanisms required for E₂ and ERs to enhance hippocampal memory consolidation. Phosphorylation of the p42 isoform of ERK is necessary for E₂ to enhance object recognition memory consolidation. This phosphorylation is triggered by numerous upstream events including interactions between mGluR1a and the canonical ERs (ER α and ER β), and activation of NMDA receptors, protein kinase A (PKA), and phosphatidylinositol-3-kinase (PI3K). E₂-induced phosphorylation of ERK, PI3K, and Akt elicits mTOR signaling, promoting local protein synthesis. E₂-activated ERK also transduces into the nucleus to phosphorylate the transcription factor CREB. Activation of ERK is also necessary for E₂ to increase histone H3 acetylation; E₂ increases H3 acetylation at the pII and pIV promoters of the *Bdnf* gene. DNA methylation is also necessary for E₂ to enhance memory consolidation, although the specific genes methylated are unknown. Finally, GPER enhances memory consolidation by activating JNK, which facilitates gene expression via transcription factors such as ATF2.

Figure 16 : Schematic illustration of the non-classical mechanisms required for E2 and ERs to enhance hippocampal memory consolidation (From Frick, Hormones & Behavior 2015)

In comparison with the numerous data reporting the role of sex steroid hormones in adults, fewer studies have addressed their developmental effects on learning and memory processes. Roof and Havens (1992) showed that perinatal testosterone might act by increasing the size of the granule cell layer of the hippocampus, thereby improving rat performance on a spatial navigation task during adulthood. Receptors of sex steroid hormones, and in particular ERs, are highly expressed during the perinatal period in the male brain, which is sensitive to the perinatal surges of testosterone. ER β knockout males exhibited memory impairment in a hippocampus-mediated fear-conditioning paradigm. ER β activation improved performance in hippocampus-dependent memory tasks, enhanced long-term potentiation in hippocampal slices of wild-type but not ER β knockout mice and increased dendritic branching and density of mushroom-type spines. In females, the perinatal brain is protected from the potential masculinising effects of sex steroid hormones by the alpha-fetoprotein. It is, however, important to note that ovarian estradiol increases from postnatal day 7 and could hence act on the female brain to regulate cerebral functions.

These sex differences in hormonal impregnation during the critical periods of development, and also during adulthood, may explain the sex differences observed in the expression of cognitive behaviours and their alteration by BPA.

Altogether, data therefore provide evidence that disturbance of, in particular, estrogenic pathways is involved in alteration of learning and memory by BPA.

4.4.3.4 Human relevance

Cognitive function in humans involves signaling pathways, which seem similar to those described above in rodents. The involvement of NMDAR signaling pathway in memory processes in healthy and diseased brain has been largely reviewed (e.g. in Gilmour *et al.*, 2012; Campos *et al.*, 2016; Arnsten *et al.*, 2017). The link between human cognitive functions and sex steroids, and the similarities and differences between humans and rodents are reviewed below.

Periods of sex steroid liberation in humans:

Sex steroids are synthesised from the developmental period in both humans and rodents. However, the periods of sensitivity to sex steroids seem to differ between mammalian species. Testosterone release by fetal testes peaks between gestational weeks 12 and 18 in humans whereas in rodents it occurs during the last days of gestation. Indeed, testosterone level rises again during the three first postnatal months in boys and in the first four hours after birth in the rat. In girls, unlike female rodents, the fetal ovaries are active and synthesise estrogens. It is, however, suggested that the female brain may also be protected from the masculinising effects of steroids by the alpha-fetoprotein. The following rise in sex steroids occurs during the pubertal period and secretion is maintained during adulthood in both sexes in humans and rodents.

Overall, there are some differences between humans and rodents in the timing of sex steroid secretion during brain development but in both species sex steroids are present during these critical developmental periods, in particular in males.

Modulation of human cognitive processes by sex steroid hormones:

Estrogens were shown to modulate hippocampus-dependent learning in women and non-human primates (Hampson, 1990; Lacreuse, 2006., and review by Hamson *et al.*, 2016). For instance, the excitability of the hippocampus is increased with increased endogenous levels of estradiol as in rodents, and this seems to be correlated with changes in synapse density, synaptic proteins, and long-term potentiation.

Testosterone also modulates cognitive functions in men. Hypogonadism affecting young adults, chemically castrated individuals or older men is associated with spatial, visual, verbal and episodic memory defects. Testosterone levels have been linked to performance in visual and episodic memory tasks, with hypogonadic and elderly men performing poorly in such tasks. Spatial abilities were shown to be reduced in men undergoing combined flutamide (anti-androgen) and leuprolide (GnRH agonist decreasing LH and thus testosterone secretions) treatment (Cherrier *et al.*, 2009). Whether gonadal testosterone is converted into neural estradiol, which then regulates learning and memory processes needs to be better studied in men. However, one study showed that inhibition of aromatase (blocking the conversion of testosterone to estradiol) prevented testosterone-induced improvement of verbal memory in older men (Cherrier *et al.*, 2005).

Given these similarities in the modulatory effects of sex steroids on cognitive functions between rodents and humans, it is likely that the MoA observed in rodents occurs in humans and has similar effects on these processes in humans to those in rodents.

Whether developmental exposure to BPA could affect these processes in humans is more difficult to answer given the lack of studies supporting potential effects of sex steroids in the developing human brain. One study addressing the prenatal vs juvenile exposure to BPA in non-human primates found no effect on working memory for the juvenile exposure, but no tests were performed in the group prenatally exposed to BPA. The significant impact of BPA on synaptic plasticity at the prenatal and adult stages and a deficit in working memory performance in adult monkeys suggest cognitive disruption in humans, given the

analogies between primates: monkeys and humans share some uniquely primate morphological, endocrine and cognitive traits (Lacreuse and Herdon, 2009). In support of this hypothesis, the *in vivo* study conducted on adult female African green monkeys showed that a subcutaneous exposure to BPA (50 µg/kg/d) counteracts the synaptogenic effect of estradiol in the hippocampus and prefrontal cortex (Leranth *et al.*, 2008). Finally, during adolescence, it has been shown, by using high-resolution structural MRI scans, that sex steroids are associated with cerebral gray matter morphology in a sex specific manner (Koolschijn *et al.*, 2014).

There are therefore indications that this MoA may be relevant to humans during developmental exposure.

4.4.4 Summary and discussion

On the basis of i) the significant amount of *in vivo* and *in vitro* animal data showing impairment of learning and memory by exposure to BPA and the potential alteration of cellular and molecular mechanisms underlying these processes through disturbance of the estrogenic pathway, ii) the similar types of signalling pathways underlying human cognition and iii) the numerous data showing sex steroid regulation of these behaviours, exposure to BPA could also alter human cognitive abilities.

The main evidence is summarised in Table 19 and Table 20 below.

Table 19: Summary table of the ED-mediated MoA of BPA on learning and memory

Level	Molecular	Cellular	Organ/function
Alteration and available evidence	Alteration of the expression level of NMDAR, kinases, enzymes, involved in neurotransmitter. Regulation through estrogenic pathways - <i>In vitro</i> : Xu <i>et al.</i> , 2010c - <i>In vivo</i> : Xu <i>et al.</i> , 2010b, 2011a; 2013b, 2014b, 2015b; Eilam-Stock <i>et al.</i> , 2011; Fan <i>et al.</i> , 2013; Jang <i>et al.</i> , 2012; Matsuda <i>et al.</i> , 2013; Wang <i>et al.</i> , 2016; Zhang <i>et al.</i> , 2014	Modification of spine density or neurogenesis through estrogenic pathways - <i>In vitro</i> : Xu <i>et al.</i> , 2014a; Xu <i>et al.</i> , 2010c - <i>In vivo</i> : Xu <i>et al.</i> , 2015a and b; Inagaki <i>et al.</i> , 2012; Leranthe <i>et al.</i> , 2008; Kumar <i>et al.</i> , 2014; Kim <i>et al.</i> , 2011	Alteration of learning/memory through estrogenic pathways - <i>In vivo</i> : Xu <i>et al.</i> , 2010b; Xu <i>et al.</i> , 2011a; Inagaki <i>et al.</i> , 2012; Xu <i>et al.</i> , 2014b; Xu <i>et al.</i> , 2015b

Table 20: Overview of the elements supporting identification of alteration of learning/memory as an ED-mediated effect of BPA

Adverse effect	ED MoA	Human relevance
Alteration of learning and memory associated with cerebral modifications consisting of reduced expression levels of receptors (NMDAR), kinases, enzymes involved in neurotransmitter regulation, and synaptic proteins as well as decreased spine density or neurogenesis	Main evidence provided by inhibition of the functional and structural effects with ER antagonist or the suppression of estrogen-induced effects in ovariectomised animals.	Similar signaling pathways (e.g. NMDAR) in cognitive function in humans and rodents. Sex steroids known to modulate cognitive function in humans and rodents. Some indications for the effect (Elsworth <i>et al.</i> 2013 and 2015) or the estrogenic MoA (Leranthe <i>et al.</i> , 2008) in primate studies.

4.4.5 Summary tables of studies

The following table is presented by type of exposure (developmental, prepubertal and pubertal, adult), species studied (mice, rats, then other species) and year of publication.

Table 21: *In vivo* studies investigating the effect of BPA on learning and memory

Reference	Study design	Route of exposure Dose	Effects on learning and memory	Investigation of brain tissues and brain area relevant to the behaviour
Developmental exposure (gestation and/or lactation)				
Mouse studies				
Xu <i>et al.</i> , 2010b	ICR mice GD7-PND21	Oral (gavage) 0.05, 0.5, 5, 50 mg/kg/d	<p>F1 male pups at PND 21/26 or 56/61</p> <p>Morris water maze test:</p> <ul style="list-style-type: none"> - longer distance in all BPA-exposed group to find the hidden platform in the 4 consecutive days (both ages) - average escape length extended by BPA at 5 or 50 mg/kg/d in PND21 mice and at 0.5, 5, or 50 mg/kg/d in PND 56 mice - \searrow percentage of time spent in the target quadrant at 0.5 or 5 mg/kg/d (both ages) <p>Step down passive avoidance test:</p> <ul style="list-style-type: none"> - Dose-dependent \nearrow in error frequency to step down from a platform after received footshock - Dose- dependent \searrow in the latency of the step down 24 h after the training - Significant for both effects from 5 mg/kg in PND21 offspring and 50 mg/kg in PND56 offspring) <p>Global effect: impairment of spatial memory and avoidance ability</p>	<p>Hippocampus</p> <ul style="list-style-type: none"> - Decrease of the expression of GluN1, GluN2A and GluN2B subunits of NMDA receptors. - Decrease in the expression of estrogen receptors ERβ.
Jasarevic <i>et al.</i> , 2011	Outbred deer mice 2 weeks prior mating to end of lactation	Oral (diet) 7 mg/kg bw/d or EE	<p>PND60 (Barnes maze), males and females</p> <ul style="list-style-type: none"> - Control males exhibited shorter latency to exit the maze at day 2 than BPA, EE or females in all diet groups, - Control males and EE-exposed females committed fewer errors than the BPA-group - Failure to acquire spatial-oriented strategy in males exposed to BPA or EE. - \searrow latency to exit the maze and more rapid acquisition of spatial oriented strategy in females typical of males with EE but not with BPA 	

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			Global effect: altered spatial learning abilities in males, female abilities become more like those of control males	
Viberg <i>et al.</i> , 2011	NMRI mice (10-day old) Single exposure	Oral (gavage) 0.32, 3.2 or 4.8 mg/kg bw	3 month old males - no effect in the Elevated plus- maze. 4 month old males - no effect in the Morris water maze.	
Nakamura <i>et al.</i> , 2012	ICR mice GD0 - PND21	SC 20 µg/kg/d	PNW 12/13, males and females no effect on spatial memory as tested in the Morris water maze.	
Jang <i>et al.</i> , 2012	C57BL6 mice GD 6 – GD 17	IP 0.1, 1 or 10 mg/kg bw/d	8-week old F2 mice (females) - No effect on latency after repeated trials or after reverse trial with modification of platform location in the Morris water maze - Impaired memory retention : ↓ cross-over latency times at 1 and 10 mg/kg in the passive avoidance test indicating that memory retention of electric shock is impaired	Hippocampus 8-week old F2 mice - ↓ BrdU-positive cells in the hippocampal DG 10 mg/kg - ↓ levels of phosphor-ERK, BDNF and phosphor-CREB. - ↑ DNA methylation of CREB regulated transcription coactivator 1 (Crtc1)
Jasarevic <i>et al.</i> , 2013	Outbred deer mice 2 weeks prior mating to end of lactation	Oral (diet) 0.05, 5 or 50 mg/kg of feed	Approx. PND60 (after sexual maturity), males and females - Males at the two highest doses exhibit impairment in spatial learning assessed in the Barnes maze, similarly to EE. - No effect in females in contrast to EE Global effect: spatial memory impaired in males	
Matsuda <i>et al.</i> , 2013	C57BL/6J mice GD 10-PND20	SC 250 ng/kg bw/d	PNW4 (males and females) - Enhanced fear memory in females using contextual fear conditioning PNW 9 (males and females) - No effect Global effect: enhancement of contextual memory of fear conditioning in females	- ↑ serotonin metabolites in the brain (hippocampus, striatum, midbrain, pons, medulla oblongata of juvenile females (PNW4) but not adult females (PNW9) or juvenile or adult males - ↑ expression of Tph2, Slc6a4 and Maoa mRNA in the hippocampus of juvenile females.
Kumar <i>et al.</i> , 2014	Swiss albino mice GD7- PND21	Oral (gavage) 50 µg/kg bw/d	F1 pups at PNW 8 (only male tested) - Effect on the escape latency, ↑ distance taken to find hidden platform on days of training, ↓ time spent in the target quadrant in the probe test after removal of the platform in the Morris water maze Global effect: impairment of spatial memory	Cerebral cortex and hippocampus F1 male pups at PNW 3 or 8 - Upregulation of Nrnx1 and Nlgn3 mRNA and protein level for both areas and ages - ↑ dendritic spine density for both areas and ages
Rat studies				
Kubo <i>et al.</i> , 2001	Wistar rats	Oral (water) 1.5 mg/kg bw/day	F1 pups at PNW 7 (males and females)	F1 pups at PNW 7

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	GD0 - PND21		- lower avoidance memory in females and suppression of sexual dimorphism (step-through passive avoidance test)	- larger locus coeruleus in exposed females than in control males
Poimenova <i>et al.</i> , 2010	Wistar rats GD1 - weaning (42 days)	Oral 40 µg/kg bw/day	F1 pups at PND 46 (males and females) - ↘ spatial memory in both sexes (Y-maze test)	F1 pups at PND 46 - ↗ levels of corticosterone and ↘ levels of GR in males in basal state and in the 2 sexes after stress - No effects on the MR receptor in normal conditions, but ↘ MR level in females in the 2 groups of females
Stump <i>et al.</i> , 2010	Sprague Dawley rats GD 0 - PND 21	Oral (diet) 0.15, 1.5, 75, 750, and 2250 ppm (mean target doses of 0.01, 0.1, 5, 50, and 150 mg/kg/d)	PND 22 and 62 (males and females) - No effect on learning and memory as tested using the Biel water maze	PNDs 21 and 72 - No effect on brain and nervous system neuropathology and brain morphometry.
Goncalves <i>et al.</i> , 2010	Wistar rats Gestation (20 d), lactation (21 d) or gestation + lactation (41 d)	Oral (gavage) 40 µg/kg bw/day	F1 pups at PNW 16 (males and females) - Alteration of both short-term and long-term conditioning memory in the inhibitory avoidance task in males and females treated during gestation and lactation and males treated during lactation only - Altered short-term and long-term memory by treatment for object recognition in both sexes - In the Morris water maze: ↗ latency time during training in animals exposed during lactation and ↘ duration on the platform in the retention test in animals exposed during gestation and lactation. Global effect: impairment of inhibitory avoidance, object recognition and spatial memory	
Xu <i>et al.</i> , 2011a	Sprague Dawley rats (18-day old) Single exposure 1 or 24 hours before behavioural testing	SC 50 µg/kg bw or 500 µg/kg bw	Only males were tested: - ↗ latency in retention test 1 hour after footshock in the passive avoidance task when BPA was administered 1 hour but not 24 hour before testing in male rats. - Similar effect with EB - Co-treatment of BPA with EB did not affect latency Global effect: impairment of the short-term avoidance memory	Hippocampus - No effect on levels of NMDA receptor GluN1 or GluN2B by BPA or EB - ↗ level of phosphorylated-GluN1 and phosphorylated GluN2B 1 hour after exposure to BPA or EB. - Co-administration of BPA and EB inhibited the effect. - ↗ in mitogen-activated extracellular signal-regulated kinase (ERK) by BPA or EB - Pretreatment with ER antagonist ICI 182,780 inhibited BPA or EB effect on phosphorylated GluN1 and GluN2B and ERK. - An ERK-activating kinase inhibitor U0126 reduced BPA- or EB-induced phosphorylation of GluN1, GluN2B and ERK within 1 h.
Jones <i>et al.</i> , 2012	Long Evans rats	Oral (drinking water)	F1 pups at PND 90 to 150 (males and females)	

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	GD7 – PND14	5, 50, 500 or 5000 µg/kg/d	- No effect on spatial memory in the Morris water maze (The data are not shown, only the statistics)	
Ferguson <i>et al.</i> , 2012	SD rats Exposure from GD 6 - PND 21	Oral (gavage) 2.5 or 25 µg/kg/d	Males and females Barnes maze performance at PND 47-50: - No effect on latency to locate the escape box in males or females (analysed separately) (↗ latency with low dose EE ₂ in males and females) Water maze performance at PND 75-79: - No effect on water maze latency box in males or females (analysed separately) (no effect with EE ₂)	
Kuwahara <i>et al.</i> , 2013	Sprague Dawley rats GD10 – PND14	Oral 50 or 500 µg/kg/d	Only males were tested: - ↗ in time to reach the reward in the MAZE test in male offspring of dams exposed to 50 µg/kg (female not tested) - No significant effect in time to reach escape in the MWM test Longer latency during training session for the step-through passive avoidance test at 50 µg/kg/d. No alteration in retention Global effect: impairment of spatial learning and memory.	
Xu <i>et al.</i> , 2014b	Sprague-Dawley rats Exposure: Last GD to PND21	Oral (drinking water) 0.1 mg/L (approx.. 2.5 mg/kg bw/d)	PNW9 (only males were tested) - Increase of latency (Morris water maze) - Decrease of the time spent in the platform quadrant. The ER antagonist ICI 182,780 abolished the effects of BPA on rat behaviour and memory performance Global effect: Impairment of spatial memory.	Hippocampus Expression of ER at PND7, 11 et 21 - ↓ of the expression of ERα at PND7 et PND21 - ↗ of the expression of ERα at PND11 - No difference between both hemispheres. The ER antagonist ICI 182,780 abolished the effects of BPA. The effects are observed at mRNA, protein (W Blot) and immuno-histochemistry levels - No effect observed on ERβ Translocation of ERα towards nucleus (co-labelling of ERα and NeuN, neuronal nuclear antigen) - Alteration of the translocation of ERα towards nucleus. - ↓ of the expression of ERα in total proteins at PND7 and PND21, and ↗ at PND11. - ↗ of ERα in the nuclear proteins at PND7 and 11 (not easily observable at PND21 because of the strong decrease in ERα in controls). - ↓ ratio Nuclear ERα/total ERα, at PND7 and 11, suggesting a failure in the translocation of ERα towards the nucleus. The ER antagonist ICI 182,780 abolished the effects of BPA. Phosphorylation of ERα

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				<ul style="list-style-type: none"> - ↓ phosphorylation of total and nuclear ERα at PND7 and PND11. - ↓ phosphorylation of total and nuclear ERα-Ser118 at PND7 and PND11. <p>The ER antagonist ICI 182,780 abolished the effects of BPA.</p>
Wang <i>et al.</i> , 2014b	Sprague Dawley rats GD9-GD20	Oral (gavage) 0.05, 0.5, 5 or 50 mg/kg bw/d	<p>F1 pups at PND 21 (males)</p> <ul style="list-style-type: none"> - ↑ in working memory errors at all BPA doses and in reference memory errors at all doses except 0.5 mg/kg in the radial arm maze 	<p>Hippocampus</p> <p>F1 pups at PND 21</p> <ul style="list-style-type: none"> - Synaptic junction dose-dependently altered by BPA: enlarged synaptic cleft and thinned PSD as observed by electron microscopy - ↓ mRNA and protein expressions of synaptophysin, PSD-95, spinophilin, GluR1 and NMDAR1 in male offspring
Wang <i>et al.</i> , 2016	Sprague Dawley rats GD9-GD20	Oral (gavage) 0.05, 0.5, 5 or 50 mg/kg bw/d	<p>F1 pups at PND 21 (males)</p> <ul style="list-style-type: none"> - ↑ in exploration of the familiar object 1.5h after training (significant at 50 mg/kg) - ↓ in short-term (1.5h, significant from 0.5 mg/kg) and long-term (24h, significant from 5 mg/kg) recognition indexes (object recognition task) <p>Global effect: impairment of object recognition</p>	<p>Hippocampus</p> <p>F1 pups at PND 21</p> <ul style="list-style-type: none"> - Inhibition of expression of protein levels Akt, phospho-Akt, p44/42 MAPK and phospho-p44/42 MAPK protein levels - Inhibition of phosphorylation levels of CREB and BDNF
Johnson <i>et al.</i> , 2016	Sprague Dawley rats GD6 – PND21	Oral (gavage) 2.5, 25 or 2500 µg/kg bw/d	<p>F1 pups at PND 90 to 104 (males and females)</p> <p>Performance in the Barnes maze:</p> <ul style="list-style-type: none"> - animals in the 2500 µg/kg group sniffed more incorrect holes (7th session) - females in the 2500 µg/kg group had an overall longer latency as evidenced by reduced likelihood of locating the escape box - in the 2.5 µg/kg group, males had a reduced latency and females a longer but not significant latency - No significant effect on search strategy <p>Global effect: impairment of spatial navigational learning and memory.</p>	
Hass <i>et al.</i> , 2016	Wistar rats GD7-GD21, then PND1-PND22	Oral (gavage) 0, 2.5, 25 µg/kg, 5 or 50 mg/kg bw/d	<p>F1 at the age of 4-6 months (males and females)</p> <p>Performance in Morris water maze:</p> <ul style="list-style-type: none"> - Shorter swim length and slower swim speed in females at 25 µg/kg making their behaviour resemble male performance - No effect on latency to reach the platform - Significant sex differences for the 3 endpoints (swim length, swim speed and latency) were observed in controls but not in animals exposed to 25 µg/kg and 5 mg/kg BPA. <p>Global effect: altered spatial learning pointing toward masculinisation of the female brain</p>	

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Non-human primate study				
Elsworth <i>et al.</i> , 2013	Rhesus macaques GD 100 - GD 155	Oral exposure to 400 µg/kg/d or subcutaneous exposure to dBPA	Females No behavioural or cognitive test performed	Oral exposure: - serum unconjugated dBPA levels of 0.68 ± 0.28 ng/ml. - ↓ in tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra and ventral tegmental area Subcutaneous exposure: - serum level of 0.91 ± 0.13 ng/ml of unconjugated dBPA ↓ in number of spine synapses in the hippocampal CA1 but not in the prefrontal cortex.
Pre-pubertal or pubertal exposure				
Mouse studies				
Kim <i>et al.</i> , 2011	Male C57BL mice (6-week old) Exposure for 2 weeks	Oral (gavage in corn oil) 1, 5 or 20 mg/kg bw/d	Males Morris water maze: ↑ latency time after repeated training to reach the hidden platform at 20 mg/kg/d.	Hippocampus - ↓ BrdU-positive cells in the hippocampal DG at 20 mg/kg - ↑ in neurogenesis at 1 mg/kg No effect on neuronal loss or astrocyte activation.
Xu <i>et al.</i> , 2011b	Male and females ICR mice (4-week old) Exposure for 8 weeks	Oral (gavage in sesame oil) 40 µg/kg/d or 400 µg/kg/d	Males and female Elevated plus maze test: - Both doses of BPA reduced the number of open arm entries and the time spent in open arms in males but increased them in females (dose effect) Morris water maze test: - ↑ average escape pathlength in males at 40 µg/kg ; no effect in females - No significant effect in the probe trial with hidden platform removed Step-down passive avoidance test: - ↓ latency to step down 24h after footshock in males at 40 µg/kg. Global effect: alteration of spatial learning and memory in males tending to abolish sex differences. Effect was more marked at the lowest dose.	
Rat studies				
Jain <i>et al.</i> , 2011	Male Wistar rats (6 to 8-week old) Exposure for 28 days	Oral (gavage in propylene glycol) 2 or 20 µg/kg bw/d	Males Passive avoidance test - ↓ mean initial step-down latency (both doses). ↑ mean retention latencies following training at both doses (reversed by co-administration with of antioxidant N-acetylcystein (NAC)) Morris water maze - ↑ mean acquisition latency and prolongation in retention latencies at both doses (reversed by NAC).	Brain tissues - ↑ in malonaldehyde (marker of lipid peroxidation) dose-dependent ↓ in GSH - Effects reversed by co-administration of NAC

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			Global effect: BPA affected behaviour by stress oxidation that was reversed by NAC (antioxidant)	
Diaz Weinstein <i>et al.</i> , 2013	Male and female sprague Dawley rats (7-week old) Exposure for 6 to 12 days Observation began D6 post-injection	SC 40 µg/kg/d	Males and females - ↓ spatial memory performance in the object placement test. Ability to discriminate between the old and the new locations appears disrupted. Global effect: spatial memory impaired	
Kuwahara <i>et al.</i> , 2014	Sprague Dawley rat (5-week old) Single exposure	Oral: 0.05, 1 or 10 mg/kg performed under light anaesthesia with halothane Microinjection into dorsal hippocampus	Only males were tested - No difference in latencies to reach the reward in the MAZE test - No effect on fear-motivated memory performance in the step through passive avoidance test Global effect: no impairment of spatial learning and memory.	
Bowman <i>et al.</i> , 2015	Males and females adolescent Sprague Dawley rats (6-week old) Exposure: one week Observation at adulthood (>11 wk)	SC 40 µg/kg bw/d	PNW 11 (males and females) - elevated plus maze test: ↓ number of total visit and visit in the closed arm in males but not females - object placement trial : ↓ in time spent exploring but no effect on percentage of time spent with the object in the new location - object recognition trial : ↓ percentage of time spent with the object in the new spatial location in males but not females leading to an accentuation of the sex dimorphism. Global effect: impairment of non-spatial memory, object recognition in males but not in females. No effect on spatial memory, object placement	Hippocampus PNW 13 - ↓ spine density of both apical and basal dendrites on pyramidal cells in CA1 (both sexes). - No effect in the mPFC areas of the hippocampus
Non-human primate study				
Elsworth <i>et al.</i> , 2013	Male and females St. Kitts African green juvenile monkeys (14–18 months old) Exposure for 30 days	Subcutaneous exposure to dBPA resulting in a plasma level measured to be 13.1 to 16.8 ng/ml across 3 measures in time.	Males and females - No effect on working memory as tested in the 2-well spatial delayed response task. Animals were used indifferently of their sex.	- plasma level of 13.1 ±1.4 ng/ml at day 30 after implantation - no effect on dopaminergic (DA) neurons (TH immunoreactivity) in the ventral mesencephalon, or on number of spine synapses in the hippocampus and prefrontal cortex (while BPA treatment during fetal development induced a decrease in midbrain DA neurons, see above Elsworth <i>et al.</i> , 2013)
Adult exposure				
Mouse studies				
Xu <i>et al.</i> ,	Male and female	Oral (gavage)	PNW22 (males, females)	Hippocampus

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2013b	ICR mice Exposure for 12 weeks	0.4, 4 or 40 mg/kg bw/d	<p>Morris water maze</p> <ul style="list-style-type: none"> - ⚡ average escape pathlength to the hidden platform in males but not in females <p>Step-down passive avoidance task</p> <ul style="list-style-type: none"> - ⚡ step-down latency 24h after footshock in males but not in females <p>Global effect: sex specific response of males, linear and nonlinear dose-response was observed according to the task</p>	<ul style="list-style-type: none"> - ⚡ numeric synaptic density in males - Negative effect on the structural parameters of synaptic interface including enlarged synaptic cleft, reduced length of active zone and PSD thickness in males - Down regulation of expression of synaptic proteins and synaptic NMDA receptor subunit GluN1 and AMPA receptor subunit GluR1 in males
Zhang <i>et al.</i> , 2014	Male ICR mice (10-week old), Exposure for 90 days	Oral 0.4, 4 or 40 mg/kg bw/d	<p>Only males were tested</p> <ul style="list-style-type: none"> - ⚡ in freezing time 1h and 24h after fear conditioning training at 4 and 40 mg/kg <p>Global effect: enhanced acquisition and retention of fear memory</p>	<p>Hippocampus</p> <ul style="list-style-type: none"> - ⚡ level of NMDA receptor subunit GluN1 and ⚡ expression of histone deacetylase 2 before fear conditioning training - Enhancement by BPA of changes in expression of GluN1, phosphorylated extracellular regulated protein kinases and histone acetylation induced by fear conditioning
Xu <i>et al.</i> , 2015b	Female ICR mice (7-week old) undergoing ovariectomy or sham operation Exposure for 8 weeks	SC 40 or 400 µg/kg/d	<p>Only females were tested</p> <ul style="list-style-type: none"> - No significant effect in the escape path length or in performance in the probe trial in the Morris water maze in sham female mice exposed to BPA - No effect on step-down latency in the step-down passive avoidance task in sham mice exposed to BPA - Both doses of BPA shortened the escape path length and increased performance in the probe test in OVX females and thereby eliminate or decrease the difference between the vehicle sham and the OVX mice. - Co-treatment of BPA with EB partially eliminated EB-induced shortening of the escape path length of OVX mice. - Both doses of BPA decreased the step-down latency in OVX females and thereby decrease the difference between the vehicle sham and the OVX mice. - Co-treatment of BPA with EB partially eliminated EB-induced increased in step-down latency of OVX mice. <p>Global effect: BPA suppresses the reduction in spatial and passive avoidance memory observed in OVX mice and inhibits the rescue effect of estrogen</p>	<p>Hippocampus</p> <ul style="list-style-type: none"> - No effect of BPA in numeric synaptic density and in structure of the synaptic interface of sham females - BPA decreased synaptic interface modification in OVX females and thereby decrease the difference noted between the vehicle sham and the OVX mice. - Co-treatment of BPA with EB inhibited EB-induced modification of synaptic interface in OVX mice - No effect of BPA in expression in proteins synapsin I and PSD-95 and NMDA receptor GluN2B in sham females - These proteins were inhibited by BPA in OVX females. - Co-treatment of BPA with EB inhibited EB-induced enhancement of the expression of these proteins
Rat studies				

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Eilam-Stock <i>et al.</i> , 2011	Male Sprague Dawley rats (approx. 10-week old) Single dose on the day of the test	SC 40 µg/kg	Only males were tested - ↓ memory performance in the object recognition test - ↓ performance in the object placement test Global effect: impairment of visual and spatial memory	Hippocampus and prefrontal cortex - ↓ dendritic spine density on pyramidal cells in CA1 and medial prefrontal cortex - ↓ level of PSD-95, a synaptic marker, in hippocampus and ↑ cytosolic pCREB, a transcription factor, in medial prefrontal cortex.
Inagaki <i>et al.</i> , 2012	Female Sprague Dawley rats (3-month old), ovariectomised or intact animals Single dose 30 min before or immediately after the sample trial of the test	SC Ovariectomised animals: 0, 0.4, 1, 4, 40 or 400 µg/kg/d BPA, alone or in combination with 17β or 17α-E ₂ Intact animals : 0 or 40 µg/kg/d BPA, alone or in combination with 17β-E ₂	Ovariectomised animals - BPA alone did not significantly affect object placement (OP) or object recognition (OR) memory performance - BPA inhibited E ₂ -induced memory enhancing effects from 1 µg/kg for OP memory and 40 µg/kg for OR memory Intact animals tested at the different phases of the estrous cycle - No influence of BPA on OP memory performance at any phase of the cycle - Inhibition of OR memory performance in females in proestrus (when E ₂ levels are the highest) Global effect: BPA blocked E ₂ -enhanced recognition memory and impacted OR memory performance during proestrus	Ovariectomised animals Pre-frontal cortex - No effect of BPA on E ₂ -induced increase in spine density of pyramidal neurons Hippocampus. - BPA altered E ₂ -induced increase in spine density of pyramidal neurons
Fan <i>et al.</i> , 2013	Male Wistar rats (60-days old) Exposure for 10 weeks pre-mating	Oral (diet, in corn oil) 50 µg/kg bw/d	F0 males (female not exposed or tested) - ↑ time to find hidden platform and ↓ platform contact accuracy when platform was removed in the Morris water maze Global effect: acquisition and retention of spatial memory affected in exposed adults (see above for result of paternal exposure on offspring)	Hippocampus F0 males - ↓ AChE activity - Mild but significant association between decreased hippocampal AChE and cognitive decline
Non-human primate study				
Elsworth <i>et al.</i> , 2015	Adult St. Kitts African vervet monkeys Exposure for 30 days	SC with osmotic minipump to achieve 50 µg/kg bw/d deuterium-labeled BPA	Only males were tested: deficit in working memory accuracy in the two-choice spatial delayed response task after 1 week of exposure but not after 4 weeks.	- ↓ number of spine synapses in the PFC and CA1 regions after 4 weeks of exposure, - partial recovery following BPA withdrawal

Table 22: *In vitro* and *ex vivo* studies investigating the BPA MoA in neural brain cells

Reference	Tissue and treatment period	Type of modification	Evidence for ED/other MoA
Xu <i>et al.</i> , 2014a	Cultured hippocampal neurons from postnatal 24-hr old SD rats After 6-day culture in standard medium, 24-hr culture with 1, 10 or 100 nM BPA In culture with ICI 182,780 (ER antagonist), U0126 (MEK antagonist) or MK-801 (non-competitive NMDA receptor antagonist), cultures were pretreated with BPA for 30 min. 17 b-E2 used as positive control	Morphological examination: dose dependent \nearrow total length of dendrite (ss from 10 nM), motility (ss from 10 nM) and density of dendritic filopodia (ss at 100 nM). Similar effect with 17 β -E2. ICI 182,780 completely eliminated the effect of BPA (or 17 β -E2) U0126 or MK-801 partly eliminated the effect of BPA (or 17 β -E2) \nearrow in amount of F-actin (cytoskeleton critical in morphological changes during synaptic plasticity) present in dendrites filopodia from 10 nM that is inhibited by co-treatment with ICI 182,780, U0126 or MK-801 (similar with 17 β -E2) Expression of proteins of the Rho family (involved in intracellular actin regulation): - \nearrow expression of Rac1/Cdc42 from 10 nM, blocked by co-treatment with ICI 182,780 or U0126 - \searrow expression of RhoA from 10 nM, partly blocked by co-treatment with ICI 182,780 or U0126 (similar with 17 β -E2)	BPA may affect dendritic development through modification of expression of Rho proteins and enhancement of F-actin cytoskeleton and involve ER- and ERK1/2 signaling pathways
Xu <i>et al.</i> , 2010c	Cultured hippocampal neurons from postnatal 24-hr old SD rats After 7-day culture in standard medium, 30-min exposure to 1, 10, 100 or 1000 nM BPA In some culture pre-treatment with ICI 182,780 for 30 min. 17 b-E2 used as positive control	Morphological examination: - dose dependent \nearrow filopodia motility and density (ss from 10 nM). - ICI 182,780 suppressed the effects of BPA On filopodia motility: - similar effect with 17 β -E2 - BPA partly suppressed (ss) the effect of 17 β -E2 Expression of NMDA receptors: - no effect of BPA after 30 min incubation on NMDA GluN1 and GluN2B expression - Similar absence of effect with 17 β -E2 and co-treatment BPA+17 β -E2. Phosphorylation of NMDA GluN2B (on Ser 1003): - \nearrow level of pGluN2B by BPA alone or 17 β -E2 alone - suppression of effect by co-treatment BPA+17 β -E2 - suppression of effect by ICI 182,780 (ER antagonist) Levels of ER β : - no effect of BPA after 30 min incubation - Similar absence of effect with 17 β -E2 and co-treatment BPA+17 β -E2.	BPA may rapidly affect dendritic morphology through ER- mediated pathways involving activation (phosphorylation) of NMDA receptor GluN2B (primarily expressed in immature synapses of the hippocampus during postnatal development).
Hasegawa <i>et al.</i> , 2013	Cultured hippocampal slices from adult (12-week old) male Wistar rats 10 or 100 nM BPA perfused 30 min before measurements	Modulation of synaptic transmission through long-term depression (LTD) with custom multi-electrode probes in various hippocampus areas: In the CA1 area: - dose -response \nearrow in LTD from 10 nM - effect suppressed by 4-OH-tamoxifen (ERR γ antagonist) - no effect of co-administration of ICI 182,780 (ER antagonist) In the dentate gyrus - \searrow LTD at 10 nM, no effect at 100 nM	BPA may affect synaptic transmission in adult through ERR γ - but not ER-dependent pathways

Table 23: In vivo studies reporting effects of BPA exposure on brain tissues

Reference	Study design	Route of exposure Dose	Investigation of brain tissues and potential indications of endocrine MoA
Developmental exposure			
Xu <i>et al.</i> , 2012	ICR mice Exposure for GD 7 to 20 or PND 1 to 14	Oral (gavage) 40 µg/kg/d or 400 µg/kg/d	Males and ovariectomised female - PND 56 Hippocampus - Down-regulation of expression of AMPA receptor subunit GluR1 in females and males after gestational or lactational exposure - Down-regulation of expression of NMDA receptor subunit GluN1 only in males exposed during lactation (40 µg/kg/d) Amygdala - Down-regulation of expression of AMPA receptor subunit GluR1 in females and males after gestational or lactational exposure - Up-regulation of expression of NMDA receptor subunit GluN1 only in females after gestational exposure (400 µg/kg/d).
Kundakovic <i>et al.</i> , 2013	BALB/c mice (males and females) Exposure: GD0-GD19	Oral 2, 20, 200 µg/kg/d	Male and female pups at PND28 Prefrontal cortex: Changes in Esr1 (ER α gene), Esr2 (ER β , sex-specific), Esr γ (sex-specific) Hippocampus: Changes in Esr2 (sex-specific) and Esr γ
Kumamoto & Oshio, 2013	ICR mice Exposures twice at GD6-GD15	Oral 0.0.2 and 50 mg/kg BPA	Female pups at PND2, PND4, PNW3 and PNW7 Cerebrum (cerebral cortex and subcortical structures (hippocampus, basal ganglia, and olfactory bulb) - Xist downregulated at PN21, 28 in BPA-50 and at PN28 in BPA-0,02. Tsix upregulated. - Changes in the expression of X-linked genes. - *Increased AR levels at PN2 in BPA-50, and lower at PN28 in BPA-50 and 0,02 - *Increased ER α levels at PN4 and 28 in BPA-50 and 0,02, and ER β at PN28 (BPA-50- General: E2 levels lower at PN21 in BPA-0.02 and BPA-50 Anogenital distance shortened at PN4-21-28 in BPA-50; at PN21 in BPA-0.02
Xu <i>et al.</i> , 2013a	ICR mice Exposure from GD 7 to PND 21	Oral (gavage) 0.04, 0.4 or 4 mg/kg bw/d	Hippocampus (only male pups analysed) – PND 14, 21, 56 - \searrow numeric synaptic density at PND 14, 21 and 56 at the dose of 4 mg/kg and at PND56 at 0.4 mg/kg. No effect at 0.4 mg/kg - Alteration of the structural modification of synaptic interface of pyramidal cells with the enlarged synaptic cleft, the shortened active zone, and the thinned postsynaptic density (PSD) on PND 14, 21, and 56 and the increased curvature of synaptic interface on PND 14 and 21 - \searrow expression of synapsin at 4 mg/kg at PND14, and all doses at PND21 and 56 (no dose-response) - \searrow expression of PSD-95 at 0.4 and 4 mg/kg at PND14, 0.4 mg/kg at PND21 and all doses at PND56 (no dose-response)

			<ul style="list-style-type: none"> - ↓ expression of GluR1 at 0.04 and 4 mg/kg at PND14, 21 and 56 - ↓ expression of GluN1 at 0.4 and 4 mg/kg at PND21 and all doses at PND56
Xu <i>et al.</i> , 2010a	SD Rat Exposure: GD7-PND21	Oral intragastric 0.05, 0.5, 5, 50, 200 mg/kg/d	<p>Hippocampus (only male pups analysed) – PND 4, 7, 21 and 56</p> <ul style="list-style-type: none"> - low doses [0.05-50] strongly decrease the expression of GluN1, GluN2A and GluN2B subunits of NMDA receptors, but at the higher dose (200 mg) the expression of GluN1 only was strongly inhibited - dose-dependent decrease in the expression of estrogen receptors ERβ. - dose-dependent increase of aromatase cytochrome P450 (P450arom).
Castro <i>et al.</i> , 2015	Wistar rat - Females Exposure: GD12-PND0 and PN1-PN21	SC 10 µg/kg/d BPA	<p>Female pups at PND21 Brain prefrontal cortex</p> <ul style="list-style-type: none"> - Important decrease of the expression of the gene and the protein of 5α-R2 and 5α-R3
Arambula <i>et al.</i> , 2016	SD rats GD 6-22	Oral (gavage) 2.5, 25, 250, 2500, or 25 000 µg/kg/d	<p>Gene expression characterised by transcriptome sequencing and RT-qPCR in pups sacrificed at PND1</p> <p>Hippocampus Minimal effects on hippocampal transcriptome. With RT-qPCR, <i>Esr1</i> not affected by BPA or EE in either sex. In males, <i>Esr2</i> expression significantly increased in the BPA25 000 and EE0.05 groups, <i>Oxt</i> significantly decreased in the BPA 2500 and BPA 25 000 groups. In females, no effects on <i>Esr2</i>; <i>Oxt</i> higher in the BPA 25 group.</p> <p>Hypothalamus Numerous transcriptional changes but only in males. With RT-qPCR, in males, no effect on <i>Esr1</i>, no effects of BPA or EE on <i>Esr2</i>. Higher expression of <i>Oxt</i> in the BPA 25 and EE 0.05 groups In females, higher expression of <i>Esr1</i> in the BPA 2.5, BPA 250, and BPA 25 000 groups; of <i>Esr2</i> in the BPA 2.5, BPA 250, and BPA 25 000 groups, and of <i>Oxt</i> in the BPA 2.5, BPA 250, and EE 0.05 groups.</p>
Adult exposure			
Xu <i>et al.</i> , 2015a	Adult mice (10-week old) Exposure: 12 Weeks	Oral intragastric 0.04, 0.4, 4, 40 mg/kg/d	<p>Hippocampus</p> <ul style="list-style-type: none"> - Decrease in the expression of ERβ protein in males (inverse U-curve dose response) but no effect in females. - BPA up-regulated GABA(A)α2 receptor in females but down-regulated in males - Decrease in the expression of ERβ estrogen receptors <p>The effect on ERβ in males is associated with:</p> <ul style="list-style-type: none"> - An increase of anxiety - A decrease of brain level of testosterone in males. <p>No effect on brain and serum estradiol in females Note: [0.4-40mg] doses decrease testosterone in the brain</p>
Castro <i>et al.</i> , 2013	Wistar rat Exposure: Adulthood (4	SC 50 µg/kg/d BPA	<p>Brain prefrontal cortex</p> <ul style="list-style-type: none"> - Reduced mRNAs and protein levels of 5α-reductase 1 in females but not males

	days)		<ul style="list-style-type: none"> - Increased mRNAs and protein levels of aromatase in males and females - No effect on tryptophane hydroxylase Tph1. Increase in the expression of genes coding for Tph1 and Tph2 in males and females. Increase in the expression of the protein Tph2 in males and females
Leranth <i>et al.</i> , 2008	Adult female African green monkeys ovariectomised Exposure: 28 days	SC implants: vehicle, estradiol alone, BPA (50 µg/kg/d), or estradiol + BPA	<ul style="list-style-type: none"> - BPA alone had no effect - Estradiol induced a synaptogenic effect, in hippocampal regions (CA1, CA3 and DG) and prefrontal cortex, which is abolished by the continuous exposure to BPA

4.5 Metabolism and obesity

4.5.1 Overview of previous evaluation of BPA's effect on metabolism and obesity

Effects of BPA on metabolism and obesity have already been assessed in previous EU reports (ANSES, 2014; EFSA, 2015; ECHA, 2015). It is not the aim of this dossier to detail the studies that were considered in these previous reports and only the recent studies or studies that could be used to investigate the MoA will be further considered.

The BPA restriction dossier (ANSES, 2014) concluded for hazard assessment in animals that:

“Studies examining effects on enzyme activity, growth and metabolism suggest that rodents exposed in adulthood or during gestation undergo metabolic changes in various organs such as the liver, adipose tissue and pancreas. Moreover, a few authors have noted changes in the expression of protein-coding genes intervening in the cell signalling pathways involved in lipogenesis and carbohydrate metabolism. There is a trend showing in vivo effects on lipogenesis. In vitro mechanistic studies support these observations.

However, the effects on carbohydrate metabolism cannot be confirmed on account of insufficient repeatability.

Thus, in animals, BPA increases blood lipid levels, leads to excess body weight and enhances lipogenesis. The effects on lipogenesis (in vivo and in vitro data), after pre- or perinatal exposure or exposure in adulthood, are considered to be recognised. The effects on glucose metabolism after pre- or perinatal exposure to BPA are considered to be controversial.

Changes in lipid metabolism are effects that are taken into account for the risk assessment.”

These studies are summarised in Table 24.

Table 24: 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	<i>In F1 offspring</i> , 6-month males had ↓ glucose tolerance, ↑ insulin resistance, and ↑ plasma levels of insulin, leptin, triglycerides and glycerol, altered calcium signalling in islets of Langerhans ↓ BrdU incorporation into insulin-producing β cells, whereas their surface was unchanged. <i>In mothers</i> , ↑ 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., 2010b	CD-1 mice	Oral	0.25 µg/kg bw/day GD0 to PND21	<i>In F1 offspring</i> , ↑ 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	Sprague	Oral	70 µg/kg	At birth: BPA treatment during gestation did not

<i>al., 2009</i>	Dawley rats		bw/day GD6 - PND21	<p>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.</p> <p><u>Conclusion:</u> Perinatal exposure to BPA. ↗ Adipogenesis at weaning in ♀. In adult ♂, ↗ BW observed if high-fat diet.</p>
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	<p>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.</p> <p>↗ 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</p> <p>↗ 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</p>

The EFSA opinion on the risks to public health related to the presence of BPA (2015) concluded: "Of the reviewed human studies on metabolic effects only two were prospective while 22 were cross-sectional and thus not suitable on their own to study exposure-disease associations. Inconsistent with the results of cross-sectional studies one prospective study found that a higher BPA concentration in maternal urine during pregnancy was associated with a lower level of obesity in daughters. A causal link between BPA exposure and metabolic effects in humans cannot be established.

A number of studies in pre- and postnatally exposed rats and mice indicate that BPA exposure could have an effect on metabolic function as evidenced by effects on glucose or insulin regulation or lipogenesis, and body weight gain (short-term studies). Based on the results from other studies with a longer duration (e.g. 90 days) there is no convincing evidence that BPA is obesogenic after intrauterine exposure or in longer-term studies. Using a WoE approach, the CEF Panel assigned a likelihood level of "as likely as not" to metabolic effects of BPA. Since the likelihood level for this endpoint is less than "likely", this endpoint was not taken forward for assessing the toxicological reference point, but was taken into account in the evaluation of

uncertainty for hazard characterisation and risk characterization.” See sections 3.7 and 4.3 of the EFSA (2015) opinion for more details.

In its opinion of **June 2015 (ECHA, 2015)**, RAC adopted the following conclusions in relation to the analysis of the effect of BPA on metabolism and obesity:

“The Dossier Submitter derived a LOAEL of 0.26 mg/kg bw/day based on increased body weight and increased cholesterolemia in female mice in Miyawaki et al. (2007). The EFSA (2015) opinion concluded: see above

Conclusion

RAC in principle agrees with EFSA’s conclusion on metabolism and obesity. Although RAC is of the opinion that the studies described are not sufficiently convincing for quantifying the dose-response, RAC considers it prudent to take the metabolic effects into account in hazard and risk assessment (by accounting for them in the setting of Assessment Factors) and in health impact assessment.”

Considering that the metabolic effects of BPA were taken into account in hazard and risk assessment at EU level under the restriction regulatory processes, it was decided not to further present and discuss the whole database in the main part of the present dossier.

Considering these previous assessments, metabolic effects of BPA could be evidenced by effects on glycaemia and its insulin regulation on the one hand or on lipogenesis and body weight gain on the other hand.

The pancreas is involved in the regulation of glucose homeostasis *via* two major hormones: insulin and glucagon, which are produced and released by the β - and the α -cells of the pancreatic islets, respectively (Quesada *et al.*, 2008; Rorsman and Braun, 2013). The pancreatic endocrine system 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 (see **Figure 17**).

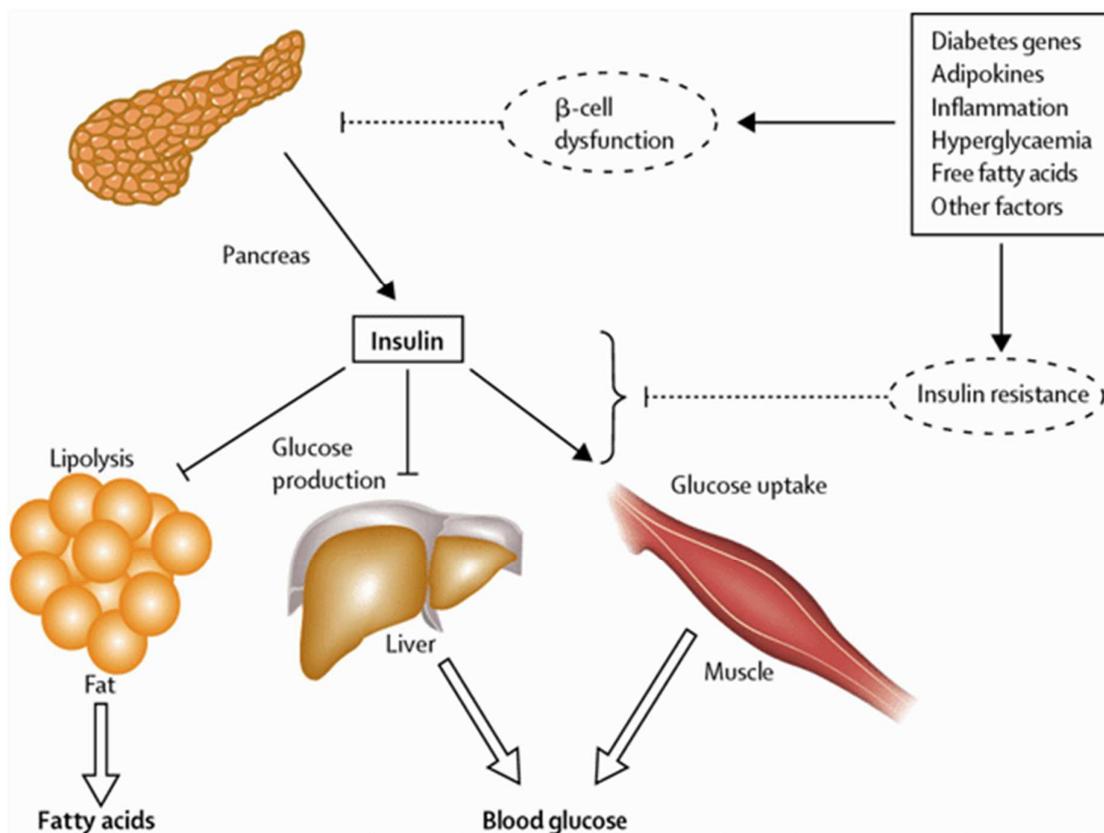


Figure 17: Pathophysiology of hyperglycemia and increased circulating fatty acids in type 2 diabetes from Stumvoll *et al.*, 2005.

Adiponectin and leptin are protein hormones secreted from adipose tissue. They modulate metabolic processes and regulate energy balance. Leptin (secreted by adipocytes in proportion to fat mass) acts as a satiety hormone, whereas adiponectin (its expression by adipocytes is reduced in proportion to fat mass) is involved in glucose regulation and fatty acid oxidation in the liver, improves pancreatic β cell function, enhances peripheral insulin sensitivity, suppresses hepatic glucose production and reduces inflammation (Chakraborti, 2015). Both hormones play a major role in glucose and lipid metabolism, insulin resistance and obesity.

When insulin action decreases (e.g. with obesity), glycaemia remains at values exceeding physiological range between meals, resulting in an increase in insulin secretion by the pancreas to lower glycaemia. Normal glucose tolerance occurs as long as β -cells counteract hyperglycaemia through enhanced insulin secretion. First signs of insulin resistance arise in the liver, muscle and adipose tissues. In that case, hepatic production of glucose namely gluconeogenesis is no longer controlled appropriately by insulin, resulting in higher levels of glucose in the blood. Concomitantly, the glucose uptake in muscle, which is dependent on the insulin-regulated recruitment of the glucose transporter GLUT4 to the membrane, will be less effective. In the adipose tissue, lipolysis, usually inhibited by insulin, will be enhanced leading to elevated levels of free fatty acids in circulation (lipotoxicity).

A decrease in insulin secretion and/or in 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). These processes are auto-amplified

since augmentation in free fatty acids in plasma and hyperglycemia increase insulin resistance and decrease insulin production in response to glucose.

In addition, adiponectin that is inversely secreted by adipocytes with enhanced fat mass will be less efficiently synthesised. Importantly adiponectin highly sensibilises metabolic tissues to insulin action. It reduces inflammation in the adipose tissue, suppresses glucose production and induces fatty acid oxidation in the liver and improves pancreatic beta cell function. Therefore, gradually, the body's ineffective use of insulin evolves as a tryptic of hyperglycemia, hyperinsulinemia and hypertriglyceridemia, in a vicious circle where hyperglycemia aggravates hyperinsulinemia and hyperinsulinemia aggravates hyperglycemia and hypertriglyceridemia. Eventually, type 2 diabetes develops with impaired β -cell function leading to persistent and progressive deterioration of glucose tolerance.

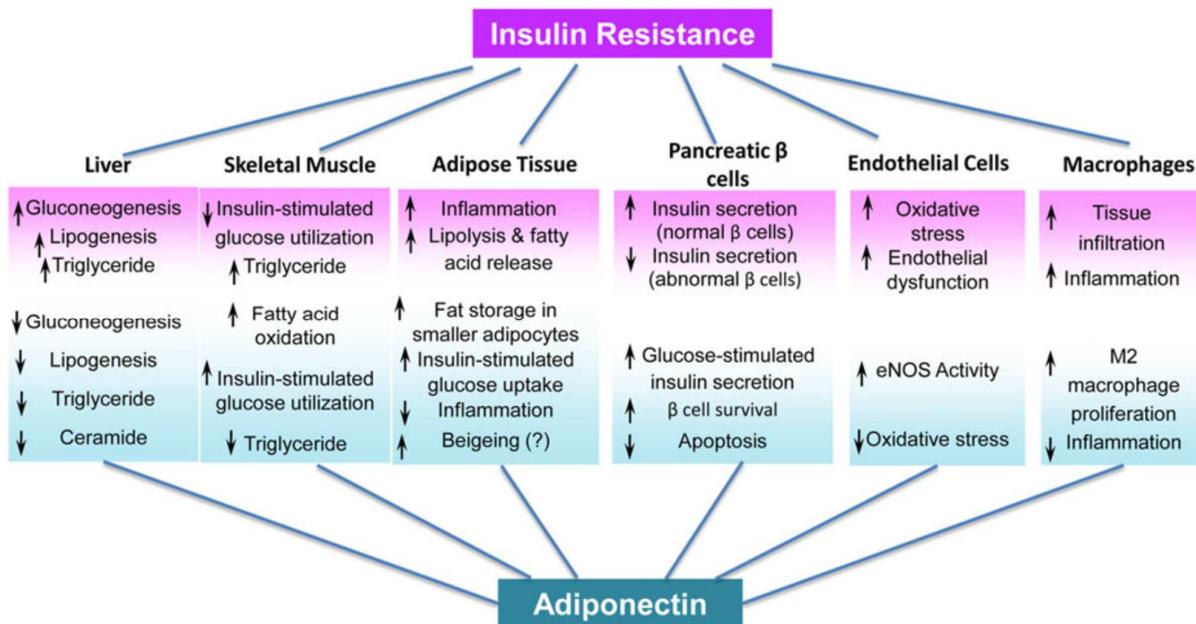
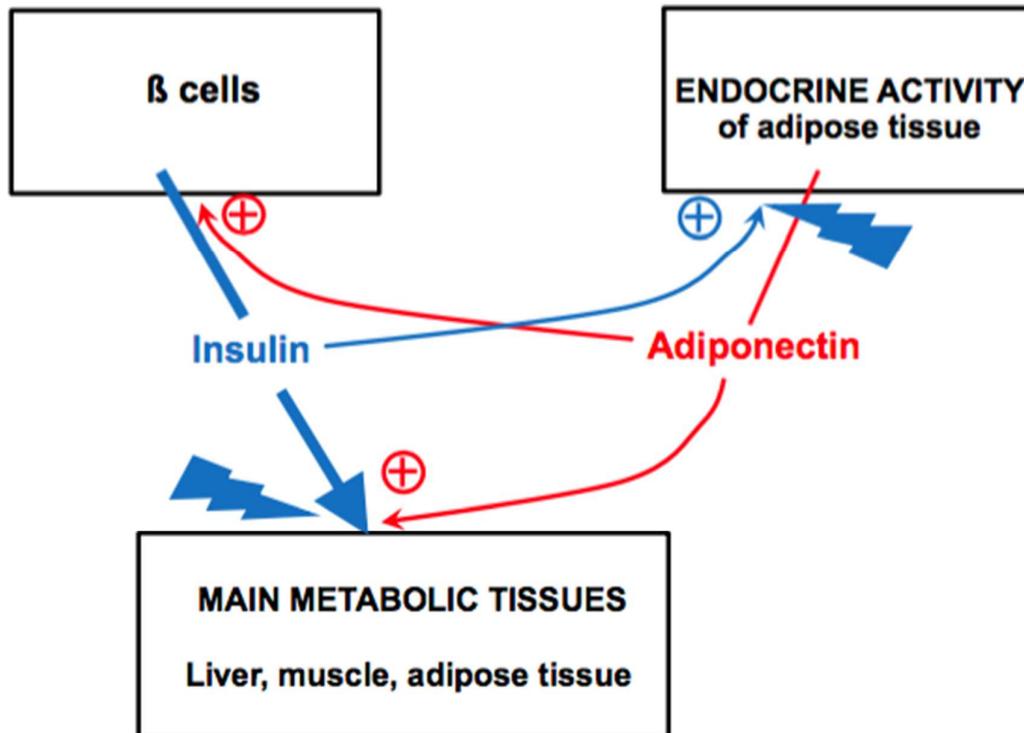


Figure 18: Summary of tissue-specific functions of adiponectin (Ruan and Dong, 2016).

Adiponectin and its downstream effector molecules are central for insulin sensitivity and homeostasis. Thus, dysregulation of adiponectin signaling will induce insulin resistance as follows. On the one hand, Adiponectin (shown in blue) acting *via* the adiponectin receptors (either I or II) expressed in liver, skeletal muscle, adipose tissue, pancreatic β -cells, endothelial cells or macrophages reduces metabolic effects favoring insulin resistance (e.g., inflammation in adipose tissue or in macrophages; lipogenesis and gluconeogenesis in liver) or enhances metabolic effects favoring insulin sensitivity (e.g., β cell survival in pancreas, insulin-stimulated glucose utilisation in the skeletal muscle or uptake in the adipose tissue). On the other hand, insulin resistance (shown in purple) is favored in liver by enhanced gluconeogenesis or by enhanced inflammation in the adipose tissue to name a few of the effects (quoted from Ruan and Dong, 2016).



A self-amplifying loop of an initial increase in insulin resistance in type II diabetic process.

Insulin acts on the endocrine activity of the adipose tissue to stimulate adiponectin secretion. Adiponectin increases the efficiency of insulin on its target tissues (i.e. main metabolic tissues) and stimulates β cells function. Thus, an initial decrease in insulin efficiency (i.e. an increase in resistance to insulin) () will provoke a reduction in the adiponectin secretion which will induce a supplemental increase in insulin resistance and a decrease of the ability of β cells to secrete insulin in response to glucose. These last two changes will result in a further development of the type II diabetes.

Figure 19: Interaction between insulin and adiponectin.

In addition to the *stricto sensu* metabolic hormones (e.g., insulin, ghrelin, leptin, adiponectin), a large amount of hormones contribute to regulate energy homeostasis, among which are the sexual hormones, but also the glucocorticoid hormones or the thyroid hormones. Specifically, estradiol is involved 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. Moreover, estradiol exhibits other several important effects in the pancreas, including promoting insulin secretion and synthesis, enhancing β -cell survival, increasing islet oxygenation during transplantation and preventing lipotoxicity (Mauvais-Jarvis *et al.*, 2013). Estradiol-17 β exerts a major role as evidenced with the phenotype of obesity and insulin resistance in both males and female mice deficient for the ER α (estrogen receptor alpha).

Lastly, it should be noticed that fetal pancreatic development occurs both in fetal and neonatal stages prenatally and post-natally in rodents whereas in humans, the major development is completed prenatally.

Overall, alterations in insulin secretion by β -pancreatic 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 considered by the experts as hallmarks of endocrine disruption mechanisms. This is especially true if there is a combination of effects, each leading to insulin resistance within the

different insulin-sensitive tissues. Therefore, this dossier is based on a new evaluation of these specific aspects examining both *in vivo* and *in vitro* experimental studies.

In the following sections, available evidence of both types of effects (β -cell dysfunction and insulin resistance), showing that BPA may have adverse effects and that these effects are related to an ED MoA in a causal way, will be considered.

Although the summary of the effects of BPA on metabolism and obesity presented above is mainly based on the literature collected for the restriction dossier, recent studies investigating "BPA and adipocytes" or "BPA and 3T3-L1 cells" or "BPA and obesity" or "BPA and steatosis" or "BPA and liver" or "BPA and pancreas" have been collected in Pubmed until May 2016.

4.5.2 Effects of BPA on glycaemia and insulin synthesis

4.5.2.1 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. However, some papers present the effect of BPA both on the pancreas and on the insulin-sensitive tissues. In order to avoid redundancy, they will be presented in the present section (and summarised in Table 25):

Alonso-Magdalena *et al.* (2010) studied the effects of BPA on glucose metabolism in female mice, during gestation, and their male F1 offspring. BPA was administered sub-cutaneously to the mothers, from GD9 to GD16, at doses of 0, 10 and 100 $\mu\text{g}/\text{kg}$ bw/day. In the F1 offspring, 6-month old males had reduced glucose tolerance, increased insulin resistance, and higher plasma levels of insulin, leptin, triglycerides and glycerol. Moreover, the islets of Langerhans presented altered calcium signaling. The authors noted that bromodeoxyuridine (BrdU, a synthetic nucleoside commonly used in the detection of proliferating cells in living tissues) incorporation into insulin-producing β cells was reduced. Therefore, taking into account isolation and culturing methods, cultured cells have different phenotypes than *in situ* cells. Such an approach is relevant when undertaking an instant analysis of the cellular state after rapid fixation and treatment of the tissues. However, it is not appropriate when examining differences in cell functioning between controls and individuals exposed to a stress agent.

Ryan *et al.* (2010b) tested the hypothesis that prenatal and lactational exposure to BPA, at a dose consistent with environmental exposure (0.25 μg BPA/kg bw/day), results in increased susceptibility to high-fat diet-induced obesity and glucose intolerance in CD-1 mice. F1 individuals were exposed to BPA (1 $\mu\text{g}/\text{kg}$ *via* the mothers' feed, equivalent to around 0.25 $\mu\text{g}/\text{kg}$ bw/day) from the embryonic stage GD0 to weaning (PND21). In the weaned F1 individuals, increased body weight was observed in males and females at 3 weeks and increased body length was observed in males at 4 weeks, these biometric differences disappearing in adulthood. No significant effects on glucose tolerance were observed. The authors concluded that the increased body length and weight were due to a faster rate of growth in the exposed mice rather than a state of obesity.

New experimental *in vivo* studies indicate that BPA exerts effects on the endocrine function of the pancreas (secretion of insulin). The potential impact of BPA on lipogenesis is confirmed by recent experimental (*in vivo* and *in vitro*) studies. These new data reinforce the observations previously reported by ANSES. Therefore, the evaluation of the potential endocrine disrupting effects of BPA was conducted in order to determine to which extent BPA could interfere in the balanced interplay between insulin secretion and insulin action that controls glycaemia.

β -cell mass is critical for proper functioning of the endocrine pancreas as well as insulin biosynthesis and secretion. Therefore, BPA as an estrogeno-mimetic and, depending on the exposure periods, is likely to interfere with pancreas development and function.

Searching for new publications from 2013 to November, 2016 with the search terms BPA and PANCREAS generated 13 original articles, 3 *in vitro/ex-vivo* studies and 10 *in vivo* studies mostly on the mouse model. These papers are presented successively and summarised in Table 26.

In vivo data and early life exposure

Liu et al. (2013) treated pregnant C57bl6 mice with either vehicle or BPA (100 µg/kg bw/day) on days 1–6 of pregnancy (P1–P6, preimplantation days); from day 6 of pregnancy (GD6) until postnatal day (PND) 0 (GD6–PND0, fetal exposure); from lactation until weaning (PND0–PND21, neonatal exposure); and from day 6 of gestation until weaning (GD6–PND21, fetal and neonatal exposure) *via* daily subcutaneous injection. Glucose homeostasis was impaired in males more than in females as assessed *via* handling glucose tolerance metabolic tests. After exposure to BPA, the β -cell mass increased while insulin secretion was either reduced or remained invariable. It suggested that β -cells were less functional in BPA-exposed mice. The alterations of insulin secretion rather than β -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, knowing that fetal pancreatic development occurs both in fetal and neonatal stages prenatally and post-natally in rodents.

García-Arevalo et al. (2014) treated pregnant OF-1 mice with a subcutaneous injection of 10 µg/kg bw/day of BPA or a vehicle from day 9 to 16 of pregnancy. At weaning, males were either fed a normal chow diet (CD) or a high fat diet (HFD) resulting in 4 groups of 8 mice/group. Body weight was recorded weekly and male mice were sacrificed by 17 or 28 weeks. BPA-treated males had enhanced body weight whether fed a control or a 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. The authors concluded that male offspring from BPA-treated mothers presented a form of diabetes which typically develops in later life and is associated with obesity.

García-Arévalo et al. (2016) demonstrated that exposure of pregnant OF-1 mice to either vehicle (control) or BPA (10 and 100 µg/kg·d, BPA10 and BPA100) between GD9 and GD16 (subcutaneous injection) to cover 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 post-natal day 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 (10µg/kg bw/d) between GD9 and GD16 also resulted in enhanced β -cell mass in the male offspring (PND30) as compared with controls, although it resulted from decreased apoptosis and not from changes in cell proliferation. Thus, it is 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 impaired glucose tolerance during adulthood.

Whitehead et al. (2016) fed pregnant C57BL/6 mice with a BPA diet (25 mg BPA/kg bw/day diet roughly corresponding to 5 mg/kg bw/day) from embryonic day 7.5 (E7.5) to E18.5. At E18.5, fetal pancreas were collected and analysed for morphological changes in the endocrine pancreas such as islet size, number and β and α cell distribution. It was observed that BPA altered the differentiation program of pancreatic cells resulting in enhancement of the glucagon expressing cells and thus in a decrease of the insulin β - pancreatic cells. There was also a change in the localisation of the α -cells, normally located in the periphery, as they were spread throughout the entire islet after BPA treatment. These data indicate that BPA may alter the differentiation program of the pancreas.

In vivo data on adult animals

Alonso-Magdalena et al. (2015) studied in OF-1 mice the metabolic status of mothers treated

with BPA (10 and 100 µg/kg bw/d) from day 9 to day 16 of gestation *via* subcutaneous route. Several months after delivery, it was shown 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. Mechanisms of action include 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 for the mother.

In the study from **Moon *et al.* (2015)**, 4 to 6-week-old C57BL/6 male mice on a high-fat diet (HFD) were treated with 50 µg/kg bw/day of BPA orally by gavage for 12 weeks. Although, body weight, percentage of white adipose tissue, and percentage of body fat did not differ between the treated and the control group of mice, long-term oral exposure to BPA along with an HFD for 12 weeks induced glucose intolerance in growing male mice. The origin of glucose intolerance did not result from any detrimental changes in the islet area or morphology or the insulin content of β cells. However, the authors observed decreased phosphorylation of AKT and GSK3 β in skeletal muscle indicative of insulin resistance which might be one mechanism by which BPA induces glucose intolerance. The effects are mostly subtle with the exception of the effects describing impairment of insulin signaling in the skeletal muscle.

Moghaddam *et al.* (2015) investigated the effects in adult male mice (strain not given) of BPA (0.5 and 2 mg/kg bw/day) dissolved in olive oil and injected intraperitoneally (n=6/group) for 4 weeks. BPA was found to enhance body weight with no differences between the 2 BPA-treated groups. BPA was also found to enhance glycaemia and plasma levels of triglycerides, LDL-C and cholesterol while HDL-C levels were significantly reduced. Importantly, in regard to the topic of this section, the authors evaluated the impact of BPA on the pancreas. Specifically, they studied oxidative stress. BPA injection increased malondialdehyde level and reduced the levels of glutathione (GSH) and the activities of SOD and CAT in the pancreas of the exposed compared to the control group. All these effects were significant and stronger in mice treated with the 2 mg/kg bw/day dose than in mice treated with the lower BPA dose. Taken together, it is suggested that BPA-induced hyperglycemia and hyperlipidemia may be associated with oxidative stress.

Jayashree *et al.* (2013) reported the effects of a single dose of BPA (20 or 200 mg/kg bw) diluted in corn oil and administered by gavage to male adult Wistar rats (n=6/group). Animals were sacrificed 30 days post dosing. The authors demonstrated enhanced serum insulin with the BPA dose but no change in fasting blood glucose level, indicative of an adaptive response of the pancreas to maintain glycaemia level. Glucose oxidation and glycogen content were found to be decreased in the liver of both high and low dose treated rats. In addition, there was impaired insulin response in the liver with decreased Akt phosphorylation, all indicative of hepatic insulin resistance. The authors also reported decreased testosterone levels which may also contribute to insulin resistance. However, unfortunately, there is no indication of the body weight of the animals and the doses used are high.

4.5.2.2 Mechanism of Action: secretion of insulin, insulin biosynthesis and secretion, and β -cell survival

Estrogens signaling occurs *via* at least the α - and β -estrogen receptors and *via* GPR30 or GPER1, a membranous form of estrogen receptor, resulting in distinct effects depending on the receptor activated. Activation of ER α enhances glucose-stimulated insulin biosynthesis, promotes β -survival from apoptotic stimuli and prevents lipotoxicity. Activation of ER β 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 ER β ^{-/-} mice, it was shown that ER β 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**).

Primary cultures of pancreatic islets recovered from C57BL/6 male mice were prepared by pancreas bile duct perfusion and collagenase P digestion (**Carchia *et al.*, 2015**). The authors

describe mitochondrial dysfunction and alteration of cell viability in pancreatic islets exposed to low dose of BPA (1×10^{-9} M) through mechanisms involving oxidative stress and enhanced cell apoptosis. The *in vitro* results were confirmed *in vivo* in diabetic mice transplanted with pancreatic islets previously treated with BPA. 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. Overall, it can be concluded that BPA exposure leads to disruption of insulin synthesis through enhanced oxidative stress and cell apoptosis.

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 (ROS) and cell apoptosis. Effects were dose-dependent from 5 to 50 μ M with first effects seen at 10 μ M. This study is of interest because it is the only paper on BPA and IAPP which is a hormone secreted by the pancreas. However, BPA doses used are quite high and there is little information on the number of experiments performed.

Song et al. (2012) isolated islets from pancreas of male Sprague Dawley rats and islet morphology and β -cell function in the isolated rat islets was assessed after exposure to different estrogenic compounds including BPA, E2 and DES. Selected concentrations were from 0.1 to 250 μ g/L. It was demonstrated 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. Glucose-stimulated insulin secretion (GSIS) and expression levels of genes involved in β -cell function were analysed by qPCR. All the data converge 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.

Two other chemicals have been tested within this experimental protocol. A total of 5 experiments were performed. It is not completely clear how the authors have taken into account the decrease of cell viability in the measurement of insulin secretion and the mRNA expression. Despite the fact that E2 while highly cytotoxic at the highest dose is also dose-dependently enhancing insulin secretion; at this highest dose, BPA is decreasing insulin secretion. These studies described above are reported in Table 26.

Epigenetic data

Mao et al. (2015) investigated epigenetic changes following BPA exposure (via oral administration) of Sprague Dawley rats. BPA exposure during early life can result 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.

4.5.2.3 Summary of the plausible link between adverse effects and endocrine MoA: Effects of BPA on insulin synthesis

Different studies, mostly from Nadal's group, have pointed to that BPA could impair glucose homeostasis through targeting both α (Alonso-Magdalena *et al.*, 2005) and β pancreatic cells (Garcia-Arevalo 2014, 2016; Alonso-Magdalena *et al.*, 2006; 2008; 2015; Soriano, 2012). Mechanisms of action were found to involve an estrogen receptor dependent mechanism, the β ,

the α or the membranous form depending on the species (mouse, rat, human) leading to changes in either cell proliferation and/or apoptosis and altering the β -cell mass.

Involvement of estrogen receptor-dependent mechanisms was highlighted using positive controls, estradiol-17 β or DES.

Two studies identified detrimental effects of BPA on insulin secretion and sensitivity with intolerance to glucose but the authors reported no changes in β -cell mass (Liu *et al.*, 2013; Moon *et al.*, 2015).

Other groups demonstrated that BPA exposure could provoke mitochondrial dysfunction decreasing ATP production and insulin release and generating oxidative stress leading to apoptosis of the pancreatic cells (Carchia *et al.* 2015; Song *et al.*, 2012; Makaji *et al.*, 2011). However, these authors did not examine whether E2 or DES had identical effects to those reported with BPA.

A total of 13 publications have been considered. Most of them are *in vivo* studies with early life exposure. Altogether, it is demonstrated that BPA exposure during early life when pancreas differentiation occurs with α - and β -cells differentiating into glucagon and insulin secreting cells, respectively, impairs pancreas development. Later in adult life, consequences are firstly hyperinsulinemia until the pancreas is exhausted and diabetes occurs. One paper did not identify adverse effects in the pancreas but reduced insulin signaling in muscles as the event initiating glucose intolerance in the BPA-exposed animals (Moon *et al.*, 2015). Oxidative stress leading to β -cell apoptosis also happens in case of exposure during adult life as well as impaired insulin-signaling transduction with reduced phosphorylation of AKT, for example. A paper from Soriano *et al.* (2012) recapitulates several findings from Nadal's laboratory indicating that BPA may act through the distinct estrogen receptors including the nuclear α - and β -receptors as well as the membranous form of estrogen receptor. In as much as these receptors signal distinct but overlapping mechanisms all converging to protect β -cell survival and β -cell mass as well as insulin biosynthesis and secretion, the harmfulness of BPA for pancreas development is likely to be due to an endocrine disrupting MoA.

Overall, it is suggested that the pancreas is targeted by BPA exposure and that mechanisms could 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 pancreas morphology and function during fetal life resulting in alterations of insulin synthesis and/or release.

4.5.3 Effects of BPA on insulin resistance

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

The available *in vivo* experimental studies retrieved since 2013 are detailed below and reported in a tabular format in Table 25.

Angle *et al.* (2013) treated pregnant CD-1 mice with BPA at doses ranging from 5 to 50,000 $\mu\text{g}/\text{kg}$ bw/day, from GD9 to GD18 that resulted in average unconjugated BPA between 2 and 200pg/ml in fetal serum (AUC_{0-24h}). BPA was fed to pregnant females once daily using a micropipetter and the volume was adjusted to 30 μl in corn oil. There were significant effects in adult male offspring: an age-related change in food intake, an increase in body weight and liver weight, abdominal adipocyte mass, number and volume, and in serum leptin and insulin, but a decrease in serum adiponectin and in glucose tolerance. For most of these outcomes inverted U-shape dose-response curves were reported by the authors but a more refined statistical analysis did not confirm these observations. This study is convincing because of the multiple doses with multiple outcomes surveyed including a positive control (DES) with 9-14 pregnant

mice and 13-17 male offspring per group. A 0.1 µg/kg/day dose of DES resulted in some but not all low-dose BPA outcomes (e.g. food consumption, renal and gonadal fat pad weight, more adipocytes and impaired glucose tolerance). However, the non-monotonic dose-response relationships suggested by the authors need to be confirmed.

Delclos et al. (2014) summarised the results of the US FDA/NCTR 2013 study. This US FDA/NCTR 2013 study was a large-scale animal study on continuous exposure to BPA from GD6 to postnatal day 90, when a number of Sprague Dawley rats were euthanised and tissues were harvested. It complied with the criteria of Good Laboratory Practice (GLP) and was conducted in accordance with the specific guidelines of the NTP. The study included a wide range of tested doses (2.5 – 8 – 25 – 80 – 260 – 840 – 2,700 – 100,000 – 300,000 µg/kg bw/day) administered by gavage using a stomach tube (mothers or pups after 5 days) or orally for newborns from the first day after birth. This descriptive study relied primarily on the observation of overall morphological-histological and biochemical criteria. Two positive control groups for the characterisation of estrogenic effects were included in the study: treatment with 0.5 and 50 µg ethinyl oestradiol (EE2) per kg bw/day. Two negative control groups were also included (vehicle and without treatment). It is noted that groups administered doses of BPA up to 80 µg/kg/d had the same serum levels of unconjugated BPA as the vehicle negative controls, which the FDA scientists attributed to inadvertent contamination of the negative controls (Churchwell et al., 2014). Contaminated negative controls would make it impossible to find effects in this low-dose range. Although the US FDA/NCTR 2013 study did not specifically deal with metabolism and obesity as such, the following parameters were studied: weight changes of the exposed animals, levels of thyroid hormones (on postnatal days 15 and 90), cholesterol, serum triglycerides, glucose, insulin and leptin. The US FDA/NCTR 2013 study concludes that BPA has no effects on the assessed parameters for doses ranging from 80 µg/kg bw/day to 2,700 µg/kg bw/day, with the exception of an increase in AST (aspartate aminotransferase) in females on postnatal day 90 for the dose of 2,700 µg/kg bw/day; this effect was not found at higher doses (100,000 and 300,000 µg/kg bw/day). F1 females and males had body weights that differed from vehicle controls across the study period only at the highest BPA dose tested (300,000 µg/kg bw/d), with a treatment-induced depression of body weight in both genders. The effect on adipose tissue (reduction of mean weight) was confined to the highest dose tested in both males and females F1.

Regarding clinical chemistry measured at post-natal day 90, in females, the 100,000 µg/kg bw/d dose significantly depressed cholesterol and triglycerides by 16 and 30%, respectively, but there was no effect on these endpoints at 300,000 µg/kg bw/d. Leptin was significantly decreased (56%) in the highest BPA dose group. In males, 100,000 µg/kg bw/d and 300,000 µg/kg bw/d reduced cholesterol by 16 and 21%.

Van Esterik et al. (2014) exposed C57BL/6 mice during gestation and lactation to 8 doses of BPA ranging from 0 to 3 mg/kg/day (4 mice per dosage group). Since BPA was introduced in the standard diet, these values are based on a body weight of 25g and a diet intake of 4.5 g per day which may be a problem because during gestation and lactation there is a high range of bw changes and food intake. Male and female offspring were surveyed for 20 weeks without further exposure to BPA. For every dose group on average 8 mice per sex (range 4–10, evenly recruited from available litters) were included for follow-up through juvenile and adult stages. Overall the authors describe sex-dependent effects with bw 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, these data are difficult to reconcile with the stable plasma levels of insulin and glucose; and food consumption could not be reliably surveyed because of spillages. In addition, there was an over-representation of small litters with the highest doses of BPA introducing a bias.

In females, given that no bw changes were detected up to the age of 17 weeks, the authors decided to challenge all female mice with a high-fat diet for 6 weeks. In contrast to males, the authors observed decreased bw 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 (BAT)).

Overall, the authors conclude that “*Although these results suggest that BPA can program for an altered metabolic phenotype, the sexual dimorphism of effects and diversity of outcomes among studies similar in design as the present study 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.*”

In the study of Moon *et al.* (2015) already cited in the previous section (see 4.5.2.1), the authors observed decreased phosphorylation of AKT and GSK3beta in skeletal muscle indicative of insulin resistance which might be one mechanism by which BPA induces glucose intolerance.

The aim of the following study from **Veiga-Lopez *et al.* (2016)** was to assess the effects of prenatal exposure to BPA on postnatal metabolic outcomes, including insulin resistance, adipose tissue distribution, adipocyte morphometry, and expression of inflammatory markers in adipose tissue, as well as to assess whether postnatal overfeeding would exacerbate these effects. Female sheep (groups of 6-9) were daily injected subcutaneously with BPA (0.05, 0.5, 5 mg/kg/day from day 30 to day 90 of gestation, term 147d). 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 high fat diet starting at the age of 14 weeks ending by 19 months of age.

The authors observed glucose intolerance and insulin resistance in the high-fat fed animals. BPA did not impact these parameters. The population of adipocytes was also analysed; normally there is a bimodal repartition with a population of small adipocytes and a population of large adipocytes full of lipids. Interestingly, there was a shift in response to BPA towards more hypertrophic adipocytes, also evidenced in response to the high-fat diet. Notably, there was no further aggravation in sheep exposed to BPA and fed the high-fat diet. This indicates that both challenges (BPA and High-fat diet) lead to similar defects.

Yang *et al.* (2016) prepared primary cultures of adipocyte progenitors from the stromal vascular fraction (SVF) recovered from the white adipose tissue (WAT) of C57BL/6 male mice. They found that addition of 50 µM BPA but not of lower doses on confluent cells resulted in an increase in the expression of C/EBPα, PPARγ, FABP4 through a mechanism involving GR (use of RU486 as a glucocorticoid antagonist). This study is presented together with *in vivo* data showing enhanced body weight and fat mass by oral intake of BPA for 30 days in both sexes when fed a chow diet but not a high fat diet (n=9-12/group). BPA doses ranged from 5 to 5000 µg/kg/day. In addition, mice exhibited increased circulating inflammatory factors and leptin plasma levels; and there was local inflammation in the WAT. Glucose tolerance was not changed and insulin sensitivity was not studied.

Biasiotto *et al.* (2016) treated a group of pregnant C57BL/6 mice with BPA daily by gavage at the doses of 0.5, 5, 5, 500 µg/kg/day (consistent with the 0.85 µg/L of BPA found in municipal water) and gavages continued on F1 males until the age of 140 days with an interruption from birth to weaning. Groups ranged from 16 to 19 mice. Mice were fed a standard diet. The number of pregnant females is not given but it can be estimated to be around 5-6 per dose level. Fat mass and body weight enhanced starting from day 90 in male mice dosed with BPA 5 µg/kg/day probably linked to the enhancement in the epididymal weight. Several genes including PPARγ, ATGL, HSL and LPL were analysed in epididymal fat and liver and they were found to be significantly enhanced in BPA-treated samples over control even though differences were subtle.

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 25 µg/kg/day, as assessed *via* handling Insulin Tolerance Test. Glucose tolerant 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.

Human studies

In the study from **Yang et al. (2016)** reported above, the authors have studied the association of BPA levels (subdivided in quartiles) with leptin and TNFα plasma levels within a cohort of 228 subjects. Leptin levels reflect adiposity because the hormone is mostly produced by adipocytes. TNFα is a marker of inflammation. The authors observed that urinary BPA concentrations were associated with leptin and TNFα plasma levels in lean female subjects but not in lean male subjects. No association was found in overweight/obese subjects of both sexes between leptin and TNFα plasma levels and the urinary BPA quartiles. The authors concluded that BPA may interact with body mass index (BMI) and/or diet composition to affect adiposity or inflammation.

Menale et al. (2016) prepared adipocytes from subcutaneous explants recovered from children undergoing orchidopexy surgery. Adipocytes were treated with BPA (1, 10, 100 nM) and adiponectin and resistin were measured by RT-qPCR as an index of insulin sensitivity. The authors demonstrated a significant down-regulation of adiponectin at the 2 highest doses of BPA. In addition, resistin could only be quantified in BPA-treated cells. These findings are indicative of a reduced sensitivity to insulin. Clinical and biochemical features of 141 obese children were collected. Serum resistin and adiponectin were evaluated. Insulin resistance and urinary BPA levels were assessed. The authors found a direct association between urinary BPA levels and HOMA ($r = 0.23$; $p: 0.0069$). The association remained significant when adjusted for BMI, sex, age (135 children, 80 boys, mean age of 10.5 ± 2.3 years). The authors also observed a strong inverse association between BPA and adiponectin ($r = -0.48$; $p < 0.0001$). In their conclusion, the authors suggested the involvement of BPA in the development of insulin resistance in childhood obesity highlighting that urinary BPA levels are directly associated with insulin resistance regardless of BMI.

4.5.3.2 Mechanism of action

Literature search analysis of the *in vitro* experiments was subdivided considering, on the one hand, studies using the 3T3-L1 cell line, which is a mouse cell line very commonly used and human mesenchymal cells or cultured cells from human adipose explants on the other hand. These studies are described below and reported in Table 26.

In vitro studies using murine 3T3-L1 cells

Three different experimental protocols were developed by **Biemann et al. (2012)** to cover the phases of undifferentiated growth, induction of differentiation and terminal differentiation of adipocytes which are occurring *in vivo*. The authors demonstrated that BPA (10 µM) had an inhibitory effect during the undifferentiated growth and no effect at the 2 other phases. Three independent experiments were performed. The authors conclude that "*These findings indicate BPA as an EDC, which reduces the determination of multipotent stem cells to the adipogenic lineage. (...) Regarding potential mechanisms of action estrogenic signaling triggered by BPA seems to be a plausible mechanism to reduce the commitment of Mesenchymal Stromal Cells (MSC) into adipocytes (...) in accordance with the general conclusion from in vivo studies that*

obesity is associated with reduced estrogen signaling and that estrogens may act antiadipogenic". The conclusions drawn by the authors are in accordance with the findings described in the study.

Pereira-Fernandes et al. (2013) developed a screening system for obesogenic compounds and the obesogenic properties of BPA were evaluated using the 3T3-L1 model (**Pereira Fernandes et al., 2014**).

First study: 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 PPAR γ transactivation and antagonist studies considering that PPAR γ signaling is a major regulator of differentiation. The culture system concerned the differentiation step and not the proliferation step. Cells were confluent in all the plates and BPA (or other compounds to be tested) was either added alone or in the presence of insulin to evaluate interactions. Outcomes included a lipid accumulation fluorescent test and the development of a PPAR γ CALUX cell line. Positive controls included the reference compounds rosiglitazone (ROSI) and TBT which are PPAR γ agonists and T0070907, a potent PPAR γ antagonist. Exposure to 12.5, 25, and 50 μ M of BPA resulted in enhanced lipid accumulation by less than 1.5 fold-change. There was a 2 fold-change at the highest doses when BPA was combined with insulin. Addition of ROSI 100 nM resulted in a 5 fold-change and 3.5 fold-change if combined to insulin. Finally, a very weak, although significant, increase in PPAR γ activity (<1.2 fold-change *versus* >15 with ROSI 1 μ M) was detected in cells treated with the highest dose of BPA. The authors conclude that there was weak obesogenic activity for BPA in their model.

Second study: The authors used the 3T3-L1 model to analyse and compare the transcriptomic profile of cells exposed to various compounds including ROSI, TBT and BPA. The authors stated that: "*Based on the transcription data, BPA was the most distinct compound of this group, indicating that this compound might act through a different mechanism of action. Indeed BPA is the sole compound of this cluster that only weakly activated the PPAR receptor. Together all these studies show that the obesogenic mechanism of action of BPA remains enigmatic, but seem to be different from the frequently observed PPAR mediated obesogenicity. Including other obesogenic compounds with low PPAR activation capacity in future microarray experiments could further confirm the distinction of the BPA gene expression profile compared with PPAR agonists*"

In conclusion, the authors considered BPA as weakly obesogenic with a mechanism of action distinct from PPAR γ -mediated.

Valentino et al. (2013) investigated the impact of nM doses of BPA (1 and 10 nM) on differentiated 3T3-L1 cells. While markers of differentiation (Glut4, PPAR γ) did not change with BPA exposure, the authors described reduced glucose utilisation and insulin signaling (measured by phosphorylation levels of IR, AKT/PKB and phosphor ERK), and leptin mRNA levels, all converging to BPA impairing insulin action.

Atlas et al. (2014) investigated the impact of BPA on differentiated 3T3-L1 cells 2 days post-confluence in the presence of insulin 100nM. No dexamethasone (DEX) was included in the differentiation medium. BPA was added at 0.1 nM, 1 nM and 10 nM. DEX 1 nM and 250 nM were used as a positive control. Within these conditions, addition of BPA resulted in a dose-dependent increase of lipid accumulation, FABP4, adiponectin. There was no induction of C/EBP α or PPAR γ in contrast to DEX (also shown using reporter genes coupled to luciferase). However, BPA could potentiate the transcriptional complex containing GR and C/EBP at the promoter of FABP4. Enhancing FABP4 is clearly indicative for an adipogenic mechanism of action but it is not PPAR γ -mediated.

Héliès-Toussaint et al. (2014) investigated the impact of 1fM, 1 pM, 1 nM and 1 microM (100 μ M was found cytotoxic) on confluent and differentiated 3T3-L1 cells. Findings include enhanced lipid accumulation (as shown with DES and ROSI), increased lipolysis (not seen with DES), no effect on glucose uptake (seen with ROSI not with DES) and no effect on Leptin (DES has a

negative effect and ROSI a positive effect); a very light effect on Srebp1C, PPAR γ and FABP4 mRNA levels and enhancement of ERR α and ERR γ mRNA levels (not shown for DES or ROSI). The authors conclude that BPA could activate adipocyte differentiation through binding to ERR α or ERR γ . Based on the data shown in the paper, it is not possible to determine if BPA modulated insulin sensitivity as no effect was described on glucose uptake.

Ariemma et al. (2016) investigated the effects of adding BPA (1nM) from plating throughout the phase of proliferation and differentiation of the 3T3-L1 cells. They demonstrated enhanced FABP4 mRNA and protein; PPAR γ and C/EBP α were also enhanced, so were leptin and IL6. Glucose utilisation and insulin signaling were reduced. All data converge to show adipocyte metabolic dysfunction and inflammation and decreased insulin sensitivity in 3T3-L1 cells.

Biasiotto et al. (2016) investigated the response of 3T3-L1 cells to BPA and found that it promoted adipocyte differentiation at the concentration of 50 and 80 μ M. BPA effect in 3T3-L1 cells was associated with the specific activation of the ER α in undifferentiated cells and the ER β in differentiated cells. BPA also activated the PPAR γ upregulating a minimal 3xPPRE luciferase reporter and the PPAR γ -target promoter of the Fabp4 (alias aP2) gene in adipose cells, while it was not effective in preadipocytes (undifferentiated cells). The pure estrogen receptor agonist DES displayed an opposite action to that of BPA inhibiting PPAR γ activity in adipocytes, preventing cell differentiation, activating ER α in preadipocytes and inhibiting ER α and ER β regulation in adipocytes. Three independent in vitro experiments were performed. The in vitro protocol is not classical with a mix of the proliferative and differentiation phases. Initial plating of cells is very low with 10³ cells per 12-well culture plate (instead of 50,000 cells).

Ahmed et al. (2016) treated 3T3-L1 cells with BPA 25 μ M. It resulted in enhanced lipid accumulation and increased mRNA and protein expression of key adipogenic markers (P<0.05) including lipoprotein lipase, adipocyte protein 2, PPAR γ , perilipin, and adipsin. Furthermore, using transcriptional assays, BPA was found to modestly activate PPAR γ using a PPRE (PPAR γ response element)-dependent luciferase construct by 1.5-fold (P<0.05). Co-treatment of cells with the selective PPAR γ antagonist GW9662 inhibits BPA-, ROSI- but not DEX-dependent adipogenic differentiation, indicative that BPA requires PPAR γ and not GR to induce adipogenesis.

The paper of **Dai et al. (2016)** was not read (not available). The abstract indicates that the authors treated 3T3-L1 adipocytes for 0, 2, 6, 12 and 24 h with BPA at 80 μ M in serum-deprived medium. The sensitivity of adipocytes to insulin was measured by the ability of insulin to phosphorylate the insulin receptor substrate 1 (IRS-1) and the AKT protein. Western blotting analysis indicated constant levels of AKT and IRS1 proteins but decreased levels of the phosphorylated forms in the BPA-treated cells.

In vitro studies using murine primary cells culture

Yang et al. (2016) have prepared primary cultures of adipocyte progenitors from the stromal vascular fraction (SVF) recovered from the white adipose tissue (WAT) of C57bl6 male mice. They found that treatment of confluent cells with BPA (50 μ M) but not of doses of BPA lower than 50 μ M resulted in a stimulation of C/EBP α , PPAR γ , FABP4 through a mechanism involving GR (use of RU486). This study is presented together with *in vivo* data (see 4.5.3.1).

In vitro studies using human cells (either cell lines or cultured from explants) published from 2013 to the end of May, 2016:

In the study from Chamorro-Garcia et al. (2012), murine and human multipotent mesenchymal stromal stem cells (MSCs) and murine 3T3-L1 cells were used to study the adipogenic capacity of BPA (1, 10, 100, 1000, 10000 nM) assessed by lipid accumulation measurement (oil red O staining and FABP4 mRNA and protein levels). BPA enhanced significantly lipid accumulation and FABP4 at the 3 highest doses in 3T3-L1 cells. In contrast, no effect was observed with the 2 MSCs populations used.

Wang et al. (2013) investigated the impact of 3 doses of BPA 10 nM, 1 µM, 80 µM in adipocytes recovered from omental biopsies of children (boys and girls neither overweight nor obese) with an age range of 3 to 13 years for girls (10 for boys). The authors demonstrated enhanced 11-bHSD1 mRNA and activity in adipocytes treated with BPA 10nM and 80 µM, and enhanced PPARγ and LPL mRNA levels. The enzyme 11-bHSD1 converts the inactive cortisone into the active hormone cortisol (corticosterone in rodents) in adipose tissues and promotes adipogenesis. To determine mechanisms of action of BPA, they used human cell lines isolated from human visceral fat tissue. The authors 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.

Valentino et al. (2013) investigated the impact of nM doses of BPA (1 and 10 nM) on human adipocytes prepared from a biopsy of subcutaneous WAT. The authors also used 3T3-L1 cells (see above). 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 BPA impairing insulin action.

In **Boucher et al. (2014)**, primary human pre-adipocytes were differentiated in the presence of 50 µM BPA or 1 µM DEX for 48 hours and gene expression microarray analysis was developed to determine potential mechanisms of BPA. Transcriptomic profiling shows enrichment in genes involved in adipogenesis and in other specific genes depending on treatment. Specifically BPA treatment resulted in enrichment of genes associated with the SREBF1 but also mTOR and Thyroid receptor/RXR signaling.

Ohlstein et al. (2014) demonstrated very strong effects of low doses of BPA (100 nM and 1 microM) on adipogenic differentiation of human adipose stromal cells with a 6-fold increase of PPARγ, 65-fold increase of Era and 20-fold increase of ERβ, effects that were inhibited if pre-treating cells with 100 nM ICI, indicating mechanisms largely under estrogenic regulation. However, it seems not plausible to get such extremely large changes as observed in this study.

Menale et al. (2015) prepared adipocytes from subcutaneous explants recovered from children undergoing orchidopexy surgery (total of 8 explants). Adipocytes were either treated with E2 (1nM) or with BPA (1, 10, 100 nM) and gene profiling performed. The analysis of deregulated genes in response to E2 allowed the identification of a small group of genes that are expressed in an opposite manner from that of adipocytes treated with BPA. 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. In addition, using a human pancreatic cell line, the authors found that BPA could decrease the expression of PCSK1, a gene involved in insulin production. These results indicate that BPA can exhibit opposite effects of those induced by E2.

Menale et al. (2016) have prepared adipocytes from subcutaneous explants recovered from children undergoing orchidopexy surgery. Adipocytes were treated with BPA (1, 10, 100 nM) and adiponectin and resistin were measured by RT-qPCR as an index of insulin sensitivity. The authors demonstrated a significant down-regulation of adiponectin at the 2 highest doses of BPA. In addition, resistin could only be quantified in BPA-treated cells; all these observations indicate a reduced sensitivity to insulin.

4.5.3.3 Summary of the plausible link between adverse effects and endocrine MoA: Effects of BPA on insulin sensitivity

Only studies showing reduced levels of adiponectin, which is a marker of insulin sensitivity have been considered here, as well as studies describing reduced insulin sensitivity coupled or not

with reduced glucose tolerance, as they demonstrate reduced capacity to respond to hyperglycemia.

Angle *et al.* (2013) demonstrated metabolic disruption through endocrine disruption in male mice exposed *in utero* to BPA with alteration in serum leptin, insulin and adiponectin and in glucose tolerance. They have also shown that some of the effects are mimicked by DES which is indicative of estrogen dependency.

Veiga-Lopez *et al.* (2016) demonstrated in *in utero* BPA -exposed female sheep, intolerance to glucose coupled with insulin sensitivity in post-pubertal, but not pre-pubertal F1 sheep illustrating an endocrine MoA for BPA.

Rubin *et al.* (2017) demonstrated in perinatal and peripubertal BPA-exposed female mice, impaired glucose/insulin homeostasis consistent with hyperinsulinemia and the development of insulin resistance.

Taken together, using distinct animal models, these 3 recent studies established that BPA acts on the efficiency of insulin on its target cells, and thus *via* an ED MoA.

However, two other studies from Van Esterik *et al.* (2014) and Delclos *et al.* (2014) did not provide evidence for strong effects indicative of BPA as an endocrine disrupter. 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 and vehicle used (gavage *versus* drinking water), 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). Furthermore, the animals were subject to fasting from the day before the study for Miyawaki *et al.* (2007), whereas this indication is not given in Delclos *et al.* (2014).

Thus, it can be concluded that there is some evidence that BPA elicits endocrine mechanisms of action to disrupt glucose homeostasis.

Mechanisms of action of BPA have mostly been examined using the 3T3-L1 cells: there are several papers indicating an alteration of endocrine activity with reduced insulin sensitivity upon exposure to BPA. 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 β (Biasiotto *et al.*, 2016), and altered the ability of insulin to phosphorylate downstream effectors strongly indicating that BPA was acting as an EDC (Dai *et al.*, 2016).

In other studies, BPA has been shown to decrease insulin sensitivity as a consequence of enhanced inflammation (Ariemma *et al.*, 2016). It is also debated whether BPA activates PPAR γ which is a master transcription factor of adipogenesis. For example, it has been shown that BPA could weakly activate the PPAR γ receptor (Pereira-Fernandes *et al.*, 2013; 2014); others demonstrated that BPA required PPAR γ to induce adipogenesis (Ahmed *et al.*, 2016) or initiated adipocyte differentiation through binding to ERR α or ERR ν (Héliès-Toussaint *et al.*, 2014); but with no indication of insulin sensitivity.

It has been demonstrated that metabolic actions of estrogen receptor beta (ER β) are mediated by a negative cross-talk with PPAR γ resulting in improved insulin sensitivity in male mice knockout for ER β , although obese because of enhanced activation of PPAR γ (Foryst-Ludwig *et al.*, 2008).

Human cells (either cell lines or cultured explants) have also largely been used to explore the effects of BPA. Down-regulation of adiponectin release, a marker of insulin sensitivity, was demonstrated 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 of normal weight children (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) or to a decrease in the expression of PCSK1, a gene involved in insulin production (Menale *et al.*, 2015).

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

4.5.4 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 effects in humans cannot be established. Most of the studies were indeed cross-sectional and thus not suitable on their own to establish a causal inference and only few were prospective studies.

In the EFSA risk assessment, five studies examining urinary BPA and **diabetes outcomes (Ning *et al.*, 2011; Shankar *et al.*, 2012; Silver *et al.*, 2011; Lakind *et al.*, 2012; Kim and Park, 2013)** were reported (EFSA, 2015). All were cross sectional by design and relied on spot urine sampling for BPA exposure assessment. The study by **Wang *et al.* (2012)** found that in addition to being associated with increased prevalence of obesity, higher urinary BPA was also associated with increased prevalence of insulin resistance in 3390 Chinese adults aged 40 years or older. Ning *et al.* (2011) studied 3423 Chinese adults and defined type-2 diabetes from fasting- and 2-h glucose tolerance test (GTT) and serum insulin levels. Increased risk of type-2 diabetes was seen for participants in the second and fourth BPA quartiles, but not in the third. A study in 1210 nationally representative Korean adults aged 40-69 years found no association between urinary BPA and self-reported type-2 diabetes (Kim and Park, 2013).

Two cross-sectional studies used NHANES data (Shankar *et al.*, 2011; Silver *et al.*, 2011). Shankar *et al.* (2011) examined 3967 adults in pooled data from 2003 to 2008 and examined type-2 diabetes diagnosed by fasting glucose levels and glycosylated haemoglobin according to the latest American Diabetes Associations guidelines. The risk of type-2 diabetes increased with increasing quartiles of BPA in a dose-dependent manner.

Silver *et al.* (2011) examined 4389 adults and also used pooled data from 2003 to 2008, and defined diabetes 2 as glycosylated haemoglobin $\geq 6.5\%$ or if participants used diabetic medication. A weak association between BPA and type-2 diabetes mellitus was seen in 2003-08 pooled data. Breaking down by year, the association was only significant in 2003/04, not 2005/06 or 2007/08. Results were similar when glycosylated haemoglobin was used as a continuous outcome. It is unclear whether the studies by Silver *et al.* (2011) and Shankar *et al.* (2011) report the same association or are independent studies. Both studies used a population in which the association was already described before by Lang *et al.* (2008) and Melzer *et al.* (2010).

Lakind et al. (2012) conducted a re-analysis of the associations between BPA exposure and chronic disease outcomes, including diabetes, using four available NHANES data sets, including the same data used in the studies above. 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 review of the literature published in 2013, 2014 and 2015 was performed to check if the conclusions, which were reported in these reports, are still valid. Among all publications retrieved, 4 were reviews and therefore excluded (Liu *et al.*, 2015; Ranciere *et al.*, 2015; Song *et al.*, 2015; Chrysant, 2015).

The other studies were assessed based on the title and summary and criteria were used to classify studies that could be used in a weight of evidence approach to assess a causal link between BPA exposure and effects on metabolism.

These criteria are:

- Type of study: cross-sectional studies are not designed to assess a causal link and therefore were not further considered. Only prospective studies may be used to discuss a causal link.
- Validity of BPA exposure estimation: most of the studies relied on spot urine BPA measures which are not appropriate to assess properly exposure, as explained above. The study of Agay-Shay *et al.* (2015) is the only one with 2 measures. Therefore this criterion was not used to exclude studies. This consideration should be taken into account when analyzing the retrieved studies.
- Assessment of the outcomes: some studies consider biological parameters (leptin, adiponectin), other considered diseases (hypertension, type 2-diabetes) or anthropometric characteristics such as BMI and body weight. Some of these parameters may be considered as adverse outcomes whereas others are only indicative of health status without any direct link with a disease.
- Size of the samples and representativity of the population compared to the general population.

The following studies that are based on a cross sectional design were not further assessed. However they may be used to elaborate some hypothesis.

The study of Leclerc *et al.* (2014) was excluded due to the small sample size and lack of representativity of the population included in the 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), were excluded because they were strictly transversal studies. The case-control study from Ahmadkhaniha *et al.* (2014) was also excluded due to the small sample size and limited information on the control group and their health status.

Four prospective cohort studies should be further considered.

In the study from Shapiro *et al.* (2015), no statistically significant associations were observed

between BPA with impaired glucose tolerance (IGT) or gestational diabetes mellitus (GDM).

In the study from Hu *et al.* (2015), the authors showed that serum BPA could predict the progression of chronic kidney disease (CKD) in patients with type 2 diabetes mellitus (T2DM).

In the nested case-control studies within the cohorts of the Nurses' Health Study (NHS) and of the Nurses' Health Study II (NHSII), Sun *et al.* (2014) reported some diverging results concerning the association between BPA exposure and type 2 diabetes. In the NHSII, BPA levels were not associated with incident T2DM in multivariate-adjusted analysis until BMI was adjusted: odds ratio (ORa) comparing extreme BPA quartiles increased from 1.40 (95% CI: 0.91, 2.15) to 2.08 (95% CI: 1.17, 3.69; $p(\text{trend}) = 0.02$) with such an adjustment. In contrast, BPA concentrations were not associated with T2DM in the NHS (ORa = 0.81; 95% CI: 0.48, 1.38; $p(\text{trend}) = 0.45$).

Concerning anthropometric characteristics, the study from Agay-Shayet *et al.*, (2015) in which measurements of BPA in two maternal pregnancy urine samples were performed did not show any association with body weight in the children until 7 years old. Song *et al.* (2014) reported a slight but significant increase in body weight. The study population was from the controls in a prospective case-control study of T2DM in the NHS and NHSII. A total of 977 participants provided first-morning-void urine samples in 1996-2002. Urinary concentrations of BPA were measured using liquid chromatography-mass spectrometry. Body weights were self-reported at baseline and updated biennially thereafter for 10 years. On average, the women gained 2.09 kg (95% confidence interval (CI), -2.27 to 6.80 kg) during the 10-year follow-up. In multivariate analysis with adjustment of lifestyle and dietary factors, in comparison with women in the lowest quartile of BPA concentration, those in the highest quartile had 0.23 kg per year (95% CI, 0.07-0.38 kg per year) greater weight gain during the 10-year follow-up ($P\text{-trend}=0.02$).

Finally, using 188 mother-child pairs from the CHAMACOS prospective study (Volberg *et al.*, 2013), BPA was measured in urinary spot samples during early and late pregnancy and in 9-year-old children. BPA concentrations during late pregnancy were associated with increased plasma lectin in boys and BPA concentrations during early pregnancy were associated with plasma adiponectin in girls. By contrast, no associations were found between BPA concentrations in 9-year old children and adiponectin or leptin at the same age.

In conclusion, in view of the limitations of using urinary BPA concentrations as a surrogate of exposure, the problems of interrelated dietary exposures, mostly cross-sectional designs and inconsistency of the results between cross-sectional and prospective studies, the conclusions that can be drawn concerning the relationship of BPA exposure and the reported findings are limited.

Notwithstanding this, there are indications from cross-sectional studies that higher BPA may be associated with increased body mass in children, and indication from a prospective study (Volberg *et al.*, 2013) that prenatal BPA exposure may be associated with reduced body mass and lower plasma adiponectin levels in girls and with higher plasma leptin levels in boys. There are no indications of note for other hormonal or metabolic endpoints. A systematic literature review of the epidemiological literature on the relation of BPA with obesity and markers of glucose metabolism and diabetes concluded that assertions about a causal link between BPA and obesity or diabetes are unsubstantiated (Lakind *et al.*, 2014).

4.5.5 General conclusion on metabolism and obesity

In conclusion, based on animal studies (rodents and non-rodents) after prenatal and/or perinatal or adult exposure, there is now evidence that BPA may increase the incidence of type-2 diabetes *via* an ED MoA. In particular, BPA has been shown to alter insulin secretion and/or release by β -pancreatic cells, or insulin signalisation (signaling mechanisms) within insulin-sensitive organs (i.e., liver, muscle, adipose tissues). This resulted in variations in the expression levels of hepatic

or adipose tissue markers which are indicative of a state of insulin resistance. These effects are considered by the experts as hallmarks of endocrine disruption mechanisms, especially if there is a combination of effects each leading to insulin resistance within the different insulin-sensitive tissues. In addition, while most studies were performed on males, a few studies have also examined the impact of BPA either on both sexes or on females. However, more studies should be undertaken before one can conclude on a sex-specificity or not of the metabolic impact of BPA.

Recent experimental *in vivo* and *in vitro* studies indicate that these effects may involve ER α , ER β or GPR30 pathways. 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.

Overall, it is suggested that the pancreas is targeted by BPA exposure and that mechanisms could differ depending on whether exposure occurs during the 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 indicate that BPA can elicit histopathological modifications during the fetal life, with consequences on insulin synthesis rate and/or release.

Moreover, most of the *in vitro* studies showing adverse effects of BPA on adipocyte differentiation and function point to alteration of endocrine mechanisms (e.g., adiponectin release, insulin signaling cascade effectors). It is not clear whether BPA activates PPAR γ and/or other nuclear receptors. Cross-talk between nuclear receptors may explain these uncertainties.

Even if available epidemiological studies are 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.

4.5.6 Summary tables of studies

Table 25: *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 of the expert
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 mg/kg	The authors conclude that BPA impairs hepatic glucose oxidation and glycogen content through defective signal transduction	There is no indication of the BW of 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 <u>*gluc tolerance in F1 females</u> - PND0-PND21: no effect of BPA - GD6-PND21: no effect of BPA - GD6-PND0: gluc intolerance at 3 and 6 months (m) - GD1-GD6: impaired gluc tolerance at 6 m but not at 3m - no difference in gluc tolerance at 8m in all groups <u>* gluc tolerance in F1 males</u> - 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 sensitivity (IpITT) <u>* in females:</u> -GD1-GD6: no effect - PND0-PND21: no effect - GD6-PND21: no effect - GD6-PND0: ↓ insulin sensitivity at 3m and 6m <u>* in males:</u> - GD1-GD6: no effect - GD6-PND21: ↓ insulin sensitivity at 3m - PND0-PND21: idem - GD6-PND0: idem and until 8m	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,	Glucose homeostasis was impaired in males more than in females as assessed through handling glucose tolerance metabolic tests. After exposure to BPA, the β-cell mass ↑ while insulin secretion was either ↓ or remained invariable. It suggested that beta cells were less functional in BPA-exposed mice. The alterations of insulin		

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					<p><u>* insulin release in F1 females:</u> - 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</p> <p><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</p> <p>Normal at 8 m in all groups</p>	<p>Insulin secretion after glucose stimulation <i>in vitro</i> ↗ in GD1-GD6 females ↓ in GD6-PND0 females and males and in PND0-PND21 and GD6-PND21 males</p> <p>No effects at 8m in all groups</p> <p>Islet morphologic analysis <u>β-cells mass:</u> - GD1-GD6: no effect in males and females -GD6-PND21: ↗ 'β-cells mass in males and females - GD6-PND0: :↗ β-cells mass in males only - PND0-PND21: ↗ β-cells mass in males only</p> <p><u>β-cells turnover:</u> - GD6-PND0 ↓ in females - PND0-PND21 ↓ in males - GD6-PND21: idem</p>	<p>neogenesis and apoptosis.</p>	<p>secretion and insulin sensitivity, rather than b-cell mass, were consistent with the development of glucose intolerance.</p> <p>Data also indicated that the fetal development stage may be a critical window of susceptibility to BPA exposure.</p>
<p>Van Esterik et al., 2014</p>	<p>C57bl6 mice</p>	<p>In diet (fed a BPA-containing CD)</p>	<p>3, 10, 30, 100, 300, 1000, 3000 µg/kg/d During gestation and lactation</p>	<p>(4 females /group); groups were made of 8 mice per sex (4-10)</p>	<p><u>in males:</u> increased body size (not fat mass) with altered energy balance (glucagon increase) (but overrepresentation of small litters)</p>	<p><u>in females:</u> no effect, so the authors decided to give a High Fat diet (HFD) 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</p>	<p>the authors concluded "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. »</p>	<p>conclusions consistent with the data</p>

<p>Garcia-Arévalo et al., 2014</p>	<p>Pregnant OF1-mice</p>	<p>sc</p>	<p>Vehicle or BPA 10 µg/kg/d From E9 to E16</p>	<p>n= 8 mice/group 4 groups: males exposed to CD and CD-BPA or to HFD and HFD-BPA during 13 or 24 weeks</p>	<p>Effects of BPA on weight, food intake, gonadal and retroperitoneal fat pad weight and plasma NEFA</p> <p>At birth: - ↓ bw in BPA rats - slight ↑ bw in BPA mice compared to CD</p> <p>17 weeks: HFD-BPA ↓ perigonadal and retroperitoneal fat pad weight compared to HFD</p> <p>28 weeks: CD-BPA ↑ perigonadal fat pad weight compared to control</p> <p>No effect on food consumption in ND groups but ↑ in HFD-BPA compared to HFD</p> <p>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</p> <p>no effect in ipITT</p>	<p>Effect of BPA on glucose stimulated secretion (GSIS) and insulin content</p> <p>Disruption of glucose stimulated insulin release, particularly in HFD-BPA group</p> <p>At 17 weeks: ↑ islet insulin content in BPA and HFD –BPA mice compared to control but not at 28 weeks</p> <p>Effects of BPA on gene expression in white adipose tissue, liver and skeletal muscle</p> <p>- ↓ mRNA expression of genes involved in fatty acid metabolism in white adipose tissue, comparable to HFD; - upregulation of <i>Pparγ</i> and <i>Prkaa1</i> genes in the liver; - ↓ expression of <i>Cd36</i></p>	<p>Impaired tolerance to glucose in BPA treated mice compared to control. The phenotype of the BPA group resembles that of HFD mice.</p> <p>Male offspring from BPA-treated mothers presented a form of diabetes which typically develops in later life and is associated with obesity</p>	<p>The experiment is well conducted. Only males but not females were studied. Mechanisms of action are not described nor did the authors use a positive control or inhibitor to explore the possible involvement of estrogeno-mimetic actions of BPA.</p>
<p>Moon et al., 2015</p>	<p>Growing male C57 BL/6 mice</p>	<p>Oral during 12 week High Fat Diet (HFD)</p>	<p>BPA 50 µg/kg/d</p>	<p>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</p>	<p>BW, % of WAT and % of body fat did not differ between BPA and control group.</p> <p>↑ glucose intolerance in HFD BPA mice (IpGTT)</p>	<p>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</p> <p>No changes in islet area or morphology or insulin content of β-cells.</p>	<p>Long-term oral exposure to BPA along with HFD for 12 weeks induced glucose intolerance and insulin resistance in growing male mice.</p>	<p>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.</p>

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<p>Alonso-Magdarena et al., 2015</p>	<p>OF-1 mice</p>	<p>sc</p>	<p>Pregnant mice: Vehicle (control<0) or BPA, 10 (BPA10) and 100 µg/kg/d (BPA100) From E9 to E16</p> <p>Nonpregnant mice:</p>	<p>n= 10-30/ group depending on the tests and ages</p>	<p>BPA 10 and 100 in BPA pregnant mice: PND 3 months: no # in glucose homeostasis (no effect on ipGTT) – no effect on ipITT PND 4 months: effects on glycemia homeostasis, slight effect on ipITT in BPA 100 PND 5 months: ↗ effects on glycemia homeostasis; effect on ipITT in BPA 100 PND 6 months: strong effects on glycemia homeostasis; 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 ←²¹↓ glucose –stimulated insulin secretion ↓ β-cells mass in BPA 10 and BPA 100 ←↓ proliferation ; ↓ expression of <i>Ccnd2</i> (in BPA100) but no effect on <i>Cdk4</i> ↗ expression of <i>p16</i> , no # in <i>p53</i> gene expression, ↓ expression of cyclin D2 and CDK-4 proteins in BPA10 mice ↗ expression of p16 and p53 proteins</p>	<p>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</p> <p>No change in glucose-stimulated insulin secretion</p>	<p>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</p>	<p>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</p>
<p>Moghaddam et al., 2015</p>	<p>Adult Male mice</p>	<p>ip.</p>	<p>Control and BPA 0.5 and 2 mg/kg 4 weeks</p>	<p>n= 6/group</p>	<p>↗ BW with BPA ↗ blood glucose with BPA dose dependently ↗ triglycerides, total cholesterol, LDL-C and ↓ HDL-C with BPA dose dependently</p>	<p>Oxidative stress parameters: – <u>serum</u>: ↗ malondialdehyde ↓ GSH with BPA dose dependently – <u>pancreas</u>: ↗ malondialdehyde ↓ GSH, TAS and SOD/CAT activities with BPA dose dependently No effect in proteins content</p>	<p>These results suggest that BPA exposure might induce hyperglycemia and its complications in adult male mice by induction of oxidative stress</p>	<p>Strain of mice not specified; composition of food not given; some authors not referenced.</p>

²¹ Where the symbol "←" is presented, this is intended to be read as "due to".

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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 containin	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

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		g BPA so 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				
Biasio 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 ED 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 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: <ul style="list-style-type: none"> Effects of BPA on body weight (m+f) <p>No difference in body weight by exposure level prior to 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</p> <ul style="list-style-type: none"> Effects on glucose homeostasis (f only) 	In the perinatal + peripubertal exposed group: <ul style="list-style-type: none"> Effects of BPA on body weight (m+f) <p>No difference in body weight by exposure level prior to 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</p> <ul style="list-style-type: none"> 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 were influenced by the precise window of BPA exposure. The addition of peripubertal BPA exposure following the initial perinatal exposure exacerbated adverse effects in the females but appeared to reduce differences in 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 liver, insulin/glucose homeostasis, were only measured in females. In addition,		

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				<p>No effects reported on glucose or insulin levels following 6h fast</p> <p>No effects following Insulin Tolerance Test (ITT) or Glucose Tolerance Test (GTT)</p> <ul style="list-style-type: none"> • Serum measurements (f only) <p>Dose dependant increase of serum leptin in BPA tretated females</p> <p>No significant effect on triglycerides</p>	<p>only in the females 2.5 and 25 µg BPA) (f only)</p> <p>Increased glucose levels at 34 weeks after 6h fasting (2.5 µg BPA but not 25)</p> <p>Increased insulin levels at 28 and 34 weeks after 6h fasting (2.5 µg BPA)</p> <p>Significant decrease of insulin sensitivity in the BPA exposed P+P mice (2.5 and 25 µg)</p> <ul style="list-style-type: none"> • Serum measurements (f only) <p>Dose dependant increase of serum leptin in BPA tretated females</p> <p>No significant effect on plasma triglyceride levels (but a tendency)</p> <p>Significant increase in hepatic triglyceride levels at the two highest doses of BPA (25 and 250 µg/kg/day)</p>	<p>weight and body 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.</p>	<p>insulin sensitivity and glucose tolerance tests were only performed in females.</p>
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Table 26: Summary of the available recent *in vitro* studies on bisphenol A on insulin

Ref.	Model	BPA doses	Outcome s: TG accumulation	Glucose uptake	GLUT 4	FABP4 (aP2)	Adiponectin / leptin	Possible MoA (MOA)				Insulin sensitivity (IS)	Comments of the authors	Expert's comments
								ER	Pparg	GR	PI3K			
Biemann et al., 2012	mesenchymal stem cells	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	0.1 nM, 1 nM, 10 nM	enhanced in the absence of DEX (presence of Ins)			dose-dep ↗			Not directly				BPA can interact with the transcriptional machinery at the promoter of FABP4	BPA is stimulating FABP4 but the weak activation of Pparg by BPA cannot explain the FABP4 increase
Hélie-Toussaint et al., 2014	3T3-L1 cells	1 fM, 1 pM, 1 nM, 1 µM	↗ with 1fM, 1pM, 1nM	no effect	not ↗ but Glut1 is increased	1pM ↗ srebp1c, pparg, aP2	no effect on leptin	ERRα (not ERRγ) ↗ 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 et al., 2016	3T3-L1 cells	1 nM added during	↗ TG accumulation	↘	not ↗	↗	no change in adiponQ;		↗	GR not regarded; ↗ of	↘ (WB Phospho)	↘ of IS	Glucose utilisation and insulin signaling were	BPA is reducing insulin sensitivity. However,

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		all phases					increase of leptin		C/EBP α				reduced. All data converge to show adipocyte metabolic dysfunction and inflammation; and decreased insulin sensitivity in 3T3-L1 cells.	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 μ M	\nearrow at 50 and 80 μ M			\nearrow in MDI		\nearrow ER α in BM, Erb in MDI (ICI 182,780)	\nearrow in MDI (TO)				specific activation of ER α in undifferentiated cells and ER β in differentiated cells. BPA also activated PPAR γ ; 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).
Dai et al., 2016	3T3-L1 cells	80 μ M	\searrow Socs3 expression (socs3 contributes to IR and leptin R)							\searrow (WB Phospho)	\searrow of IS	\searrow of IS	\searrow of IS	\searrow of IS as can be concluded from the reading of the abstract (only the abstract is available)
Ahmed, and Atlas, 2016	3T3-L1 cells	0.01 to 25 μ M	\nearrow lipid accumulation (2.5>25 μ M)			\nearrow		\nearrow ; further \nearrow with ROSI					BPA requires PPAR γ to induce adipogenesis	the use of GW9662 to demonstrate the involvement of PPAR γ in BPA induced effects is convincing (RT-qPCR and Western-blot analysis)

4.6 Summary and discussion of human health hazard assessment

This dossier has summarised the available evidence showing that BPA can affect a number of physiological functions and systems of a mammal organism through ED pathways. More specifically, BPA alters the reproductive function, mammary gland development, cognitive functions and metabolism through an ED MoA.

Most importantly, although the steps of the respective mechanisms of action are specific for each effect, the disruption of the estrogenic pathways is a common MoA consistently involved in each of the four effects. The primary target of BPA is however still not known with certainty. BPA binds to estrogen receptors (ER) but with a weak affinity. In addition, BPA binds also to other types of ER such as GPER or ERR- γ with a higher affinity and then these receptors may also be involved in BPA-mediated effects, particularly at low doses. The complexity of the toxic response to BPA also suggests multiple MoA that may interact. However, some evidence detailed in each section of this report for each effect specifically enables to establish that the estrogenic pathway is central and common in the MoA. Other findings also point toward alternative elements involved in BPA-mediated effects, e.g. epigenetic modifications. It is not known whether these modifications may be estrogen-dependent. It is also noted that some evidence points toward a disruption by BPA of the Hypothalamo-Pituitary-Thyroid axis. Thyroid hormones are known to regulate sex steroid synthesis and action in both the brain and gonads as well as in fat metabolism. The crosstalk between the sex steroid and the thyroid endocrine axes was recently reviewed by Duarte-Guterman *et al* (2014). However, the potential role of additional mechanistic pathways does not contradict the importance of the estrogenic pathways in the BPA-mediated effects and the ED nature of the MoA.

Estrogens are known to be central in the regulation of the sexual function and system but are also known to interact with many other physiological functions and developmental processes including neurobehaviour or metabolism. The pattern of the effects observed with BPA is therefore consistent with an ED MoA through estrogenic pathways.

There is recent emerging evidence that BPA may have immunotoxic effects (Ménard *et al.*, 2014a and 2014b). The variability of the effects makes the interpretation and the transposition of these effects to humans uncertain. It is however noted that the role of estrogens has been often reported in immunocompetence and in the development of innate and adaptive immune response (Fish *et al.*, 2008).

A recent review also reports evidence suggesting an effect of BPA on the cardiovascular system that may involve estrogen receptor rapid signalling (Gao *et al.*, 2014).

It has also been shown that the induction of androgen receptors in fetal mice by estradiol or BPA is permanent, leading to dramatically increased prostatic androgen receptors (vom Saal *et al.*, 1997). This increase may result in a marked increase in the sensitivity of the adult prostate to hormonal stimulation, which is associated with prostate enlargement and pre-cancerous cellular abnormalities (metaplasia) (Ogura *et al.* 2007).

These effects were however not further investigated because the level of evidence is considered insufficient at this point but it cannot be excluded that the range of effects related to the ED-properties of BPA may be wider than those described in this dossier.

Effects on environment are not addressed in this report. However, it is of importance to note that estrogen-related modes of action are reported as one of the predominant MoA potentially involved in the reproductive disturbance reported in several taxonomic groups. In particular, some data suggest an estrogen-agonist MoA in fishes (severe effect on reproduction affecting population (Yokota *et al.*, 2000) and vitellogenin induction (Kashiwada *et al.*, 2002)), in amphibians (skewed sex ratio towards females (Levy *et al.*, 2004) and vitellogenin induction (Oehlmann *et al.*, 2009)) and in molluscs (stimulatory effect of BPA on egg production antagonised by anti-estrogen (Oehlmann *et al.*, 2006))

and possibly an estrogen-like action in echinoderms (abnormal larvae development (Roepke *et al.* 2005)).

In conclusion, experimental evidence shows that BPA affects the reproductive function, mammary gland development, cognitive functions and metabolism and these alterations are mediated through disruption of estrogens and estrogenic pathways. These effects are considered predictive of serious health outcomes. Although they may be exerted through direct exposure (alteration of estrous cycles and of memory/learning performance), they are also observed for all four endpoints after exposure during development with consequences later in the life of offspring.

5. Environmental hazard assessment

Not assessed for this dossier addressing the identification of the substance as SVHC as an endocrine disruptor for human health in accordance with Article 57 (f) of REACH.

6. Conclusions on the SVHC Properties

6.1 CMR assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) of REACH.

It is however noted that BPA is included in the Candidate List as a SVHC under Article 57 (c) with ECHA decision ED/01/2017 of 4 January 2017.

Indeed, Bisphenol A is covered by index number 604-030-00-0 of Regulation (EC) No 1272/2008 in Annex VI, part 3, Table 3.1 (the list of harmonised classification and labelling of hazardous substances) and it is classified in the hazard class Reproductive toxicity category 1B (H360F 'May damage fertility') since its 9th Adaptation to Technical Progress (ATP) (Commission Regulation (EU) 2016/1179).

6.2 PBT and vPvB assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) of REACH.

6.3 Assessment under Article 57(f)

6.3.1 Summary of the data on the hazardous properties

This dossier has summarised the available evidence that BPA can affect a number of physiological functions and systems of mammalian organisms through an endocrine disruptive pathway. In particular, BPA can alter reproductive function, mammary gland development, cognitive function and metabolism through ED MoA.

- **Alteration of estrous cycles**

In humans, one epidemiological study investigated the link between BPA and the characteristics of menstrual cycles. An association with shorter luteal phases was observed. However, no definitive conclusion in humans can be drawn on the basis of a single study (Jukic *et al.*, 2015). In animals, although there was some disparities between studies, reliable results were repeatedly reported in several experimental studies in rats and mice showing an adverse effect of BPA on the estrous cycle, including irregular and

prolonged cycles and an alteration of estrous cycles dynamic by BPA after different periods of exposure:

- after exposure of adult females (Tyl *et al.*, 2008; Laws *et al.*, 2000; Lee *et al.*, 2013) and
- after exposure during the developmental phase of the reproductive system, i.e. *in utero* (Honma *et al.*, 2002; Nikaido *et al.*, 2004; Wang *et al.*, 2014a), perinatal (Rubin *et al.*, 2001; Mendoza *et al.*, 2011; Patisaul *et al.*, 2014; Delclos *et al.*, 2014), postnatal (Nah *et al.*, 2011; Adewale *et al.*, 2009; Fernandez *et al.*, 2009) or prepubertal exposure (Zaid *et al.*, 2014)

This effect was recognised by RAC in its opinion in support of classification of BPA as Repr 1B – H360F as summarised in the RAC opinion on restriction of BPA (ECHA, 2015): “RAC’s opinion (RAC, 2014) was based on adverse effects, such as disturbances in the estrous cycle, at a dose of 600 mg/kg bw/day (Tyl *et al.*, 2008) and at a dose of 100 mg/kg bw/day (Delclos *et al.*, 2014).”

Proper cyclicity is considered essential to reach successful ovulation. An alteration of cyclicity may therefore directly induce at least subfertility through disturbed (delayed or absent) ovulation. As synthesised by Kortenkamp *et al.*, (2012) an association between menstrual cycle characteristics (e.g. length of the cycle, duration of menstrual bleeding), sub-fecundity and spontaneous abortion has been observed in humans and menstrual characteristics have been associated with chronic diseases, including breast and ovarian cancer, uterine fibroids, diabetes and cardiovascular disease. Alteration of cyclicity is therefore considered as an effect clearly fulfilling the criteria of adversity.

In relation to alteration of cycle following adult exposure, a negative effect of BPA on ovarian estrogen production is clearly demonstrated in rodents *in vivo* (Lee *et al.*, 2013) in accordance with the effects observed on rodent and human ovarian cells *in vitro* (Kwintkiewicz *et al.*, 2010; Mansur *et al.*, 2016). Whereas the effect of BPA on theca-interstitial cells depends on the test model and the protocol, BPA constantly reduces the conversion of androgens into estrogens in granulosa cells. This reduction is, at least in part, a consequence of a decreased transcription of CYP450arom either *via* a direct effect in the granulosa cells or *via* changes in intrafollicular signaling factors that regulate follicular growth and endocrine activity. In both primates and non-primate mammals, follicle selection, growth, and maturation, as well as ovulation, oocyte quality, and subsequent *corpus luteum* function, all depend on subtle sequential actions of gonadotropins and intra-ovarian regulators. Furthermore, the ovary and the hypothalamo-pituitary system are in permanent endocrine dialogue with each other. Consequently, any disturbance in the cycle implies some disturbances in the endo/para/autocrine activities of the ovary and/or the hypothalamo-pituitary system. Given the regulatory scheme of the estrous cycle, an alteration in the preovulatory follicle steroidogenic activity - as observed with BPA - is very likely to be associated with a disruption of the estrous cycle as shown in the Lee *et al.* study (2013). These results demonstrate a clear endocrine MoA, namely the alteration of the ovarian steroidogenic activity, underlying estrous cycle disruption in adult rodents.

Although most of the reported evidence relies on rodent studies, there are *in vitro* data showing the same negative effect of BPA on the estrogen production in the human follicle cells. Furthermore, an indication of a negative association between the ability of the follicle to produce estrogens and exposure to BPA was observed in women (Mok-Lin *et al.*, 2010; Ehrlich *et al.*, 2012). Lastly, the role of estrogens in the maintenance of the cycle is similar in rodents and humans. Thus, we conclude that it is likely that BPA may alter the ovarian cycle in humans through the disruption of the endocrine activity of the ovarian follicle.

Alteration of cycle further to developmental exposure to BPA through endocrine or neuroendocrine mechanisms is less clearly demonstrated than after adult exposure.

Nevertheless, many studies show that the basic (neuro) endocrine mechanisms underlying the finely tuned regulation of the gonadotropic function underlying the estrous cycle can be altered in response to exposure to BPA, after developmental exposure. BPA has been shown to affect the hypothalamic expression of kisspeptin, a key neuropeptide in the regulation of the hypothalamic-pituitary-gonad (HPG) axis that is essential to later achieve the release of hormones at the appropriate time and concentrations during the cycle. In particular, studies by Monje *et al.*, (2010) and Fernandez *et al.*, (2009) provide a link between neuroendocrine changes and alteration of the cycles through concomitant observation of an alteration of hormones of the HPG axis and a cycle disturbance. The affected targets are similar to a large extent to targets affected by either estrogen agonist or estrogenic positive controls. Overall, animal and *in vitro* data support the hypothesis of an endocrine-relative MoA of BPA to induce perturbation of estrous cyclicity after developmental exposure.

In humans, the role of kisspeptin in the neuroendocrine control of the HPG axis has been demonstrated. In addition, the recent study by Kurian *et al.* (2015) on mid to late pubertal ovarian intact female rhesus monkeys suggests that persistent exposures to BPA could impair the female reproductive function by directly influencing the hypothalamic neuroendocrine function as evidenced by an alteration of kisspeptin release and GnRH pulsatility. Therefore, it can be considered that BPA-induced alterations of the hypothalamic kisspeptin/GnRH system are also relevant in humans and may impact estrous cyclicity long time after exposure in an irreversible manner.

- **Alteration of mammary gland development leading to an increased susceptibility to carcinogens through morphological modifications**

Very few epidemiological studies on risk of breast cancer after exposure to BPA early in life are available. Based on those studies, which were mainly case-control or cross-sectional studies, a link between exposure to BPA and human breast cancer could not be determined (Aschengrau *et al.*, 1998, Yang *et al.*, 2009, Sprague *et al.*, 2013 and Trabert *et al.*, 2014). It should be noted that the existing studies evaluate exposure to BPA on the date of the study whereas the effects looked at are long-term effects appearing several decades after exposure. Therefore, a more biologically relevant study design would be a longitudinal epidemiological study measuring BPA *in utero* as breast cancer most likely takes years to develop.

The mammary gland develops in three distinctive life stages: fetal, peri-pubertal, and pregnancy (Fenton, 2006) with hormonal implication such as mammary epithelial estradiol signaling, progesterone (PR) and prolactin (PL) receptor involvement depending on the developmental period. Moreover, mammary epithelium responds differently to hormonal stimulus depending on its developmental stage.

In terms of hormonal regulation, there seems to be substantial similarities across species. In most mammals, the ovaries first secrete estrogens in response to increased secretion of gonadotropins, and sexual maturity coincides with the establishment of cyclic peaks of ovarian progesterone secretion. Proliferative activity is observed in the human breast epithelium (Masters *et al.*, 1977; Longacre and Bartow, 1986) concomitantly with the peak of progesterone during the luteal phase. Thus rodents and human mammary epithelia may indeed be similarly regulated, at least with regards to hormonal control of cell proliferation.

Although the anatomy of the human breast is more complex than the rodent mammary gland, some human breast structures (Terminal Ductal Lobular Unit or TDLU) are structurally similar to rodent structures (Terminal End Bud or TEBs) during the same life stages. These structures are undifferentiated and highly proliferative, and as such they are sensitive to the effects of carcinogens and other chemicals.

There is evidence from rodents and non-human primate studies that prenatal and post-

natal exposure to BPA causes endocrine modifications in the mammary tissue, ultimately increasing its susceptibility to chemical carcinogens, as previously reported (ANSES, 2013b and review by Soto *et al.*, 2013).

Based on BPA data analysis, there is evidence from rodent and non-human primate studies that prenatal and post-natal exposure to BPA causes, depending on the period of exposure, modifications in the mammary tissue such as an increased number of TEBs relative to the ductal area, a fewer apoptotic TEB cells, an increased lateral branching and ductal hyperplasia, an increased cell proliferation and a decreased apoptosis in the glandular epithelium, a ductal (and occasionally lobuloalveolar) and an intraductal hyperplasia. This ultimately increases its susceptibility to chemical carcinogens, as previously been reported (ANSES, 2013b and review from Soto *et al.*, 2013) and the number of DCIS in rats treated with BPA (Durando *et al.*, 2007, Jenkins *et al.*, 2009 and 2012, Betancourt *et al.*, 2010). Delclos *et al.* (2014) observed ductal hyperplasia accompanied with a very limited number of mammary adenocarcinoma. Overall, based on all available *in vivo* studies, the structural similarity between humans and rodents, and some common hormonal controls of cell proliferation, suggested there is substantial evidence from rodent studies that early-life exposures to BPA may lead to increased susceptibility to breast cancer in humans.

In vitro experiments conducted on normal-like (MCF-10F or MCF-12A) breast cells show an increased cell proliferation due to exposure to BPA and the engagement of ER α and its co-activator (Sengupta *et al.*, 2013). In breast cancer cells and cancer-associated fibroblasts (CAF) that lack the classical ER, it was shown that G protein-coupled estrogen receptor (GPER) is required for growth effects and migration (Pupo *et al.*, 2012). *In vitro* 3D models for breast glandular structure development, using non-transformed breast epithelial MCF-10F and MCF-12A cells, indicated that BPA may alter the ductular and alveolar patterns in the collagen matrix, with a similar pattern as E2 (Fernandez and Russo, 2010). When BPA or 17 β -estradiol treatment was combined with ER and GPER inhibitors (ICI 182 780 and G15, respectively), the effects (deformed acini) were reversed suggesting a role of ER and GPER in the estrogenic disruption of acinar formation operated by BPA. As GPER is required for growth effects and migration in cancer (SKBR3) cells and in CAFs and since proliferative effects induced by BPA were cancelled when GPER expression was silenced by shGPER, it can be concluded that BPA induces stimulatory effects as a GPER agonist in these breast cancer cells and CAFs.

Some studies using transcriptional analyses on the stromal and epithelial compartments isolated from the fetal mammary gland (mouse) demonstrate that exposure to BPA in dams alters the mesenchymal and epithelial transcriptomes related to proteins involved in apoptosis (increased expression of the anti-apoptotic gene, Birc2, Abl1), myoepithelial differentiation, changes in the composition of ECM and in adipogenesis (Wadia *et al.*, 2013).

A series of studies from the same scientific group, Mandal's team (Bhan *et al.*, 2013, 2014a and 2014b) described how BPA can alter the epigenetic programming of the promoter of HOTAIR and EZH2. The expression of these genes has been shown to be EE2 dependent. In addition, these genes have been described as being involved when cell proliferation increases, during increased invasiveness seen in some breast tumors and to contribute to breast cancer progression.

Other studies support the possibility that BPA, through interaction with the nuclear ERs (Murray *et al.*, 2007; Wadia *et al.*, 2013) or GPER and indirectly with PR (Munoz de Toro *et al.*, 2005; Jenkins *et al.*, 2009; Ayyanan *et al.*, 2011) modulates estrogenic and progestin agonist activities. Furthermore, exposure to BPA during fetal life provokes an increased expression of both RankL (a critical connection between progesterone and epithelial cell proliferation) and Wnt4 (involved in progesterone-induced side branching in early adult life) at adult stage (see Jenkins *et al.*, 2009 and Ayyanan *et al.*, 2011,

respectively).

In conclusion, available data support the plausibility that BPA, through interaction with the nuclear ERs, or GPER and indirectly with PR, modulates estrogenic and progestin agonist activities. Emerging epigenetic studies have reported changes related to estrogen-dependent genes (such as EZH2 and HOTAIR), as well as HOX genes (involved in embryogenesis and post-natal development) associated with the BPA induced abnormal development and cancer increased susceptibility of mammary gland.

- **Alteration of learning and memory performances**

Epidemiological evidence on the potential role of exposure to BPA early in life on learning and memory performances is still insufficient to conclude. However, a substantial majority of rodent studies (total of 26/35) reported impaired spatial and non-spatial memory following exposure to BPA, regardless of the period of exposure. Nonetheless, the overall evidence has recently substantially increased and altogether strongly points toward the conclusion that BPA alters memory in rodents. In addition, as the 74% of studies reporting impaired cognitive behaviours were also performed under various experimental conditions (various doses, routes and periods of exposure or tested species), this means that this impairment is a robust BPA-induced effect. Another interesting finding, which can be drawn from this analysis, is the sex-dependent effect observed in several studies. Furthermore, although not systematically assessed in all these studies, the neural mechanisms associated with the behavioural alterations consist of a reduction in the level of expression of NMDAR subunits, kinases, enzymes involved in neurotransmitter regulation, and synaptic proteins as well as decreased spine density or neurogenesis. Such molecular, cellular and structural changes are fully relevant and could underlie the impaired learning and memory performances observed in the same animals.

Finally, although there is a limited number of studies conducted in non-human primates, these studies have shown that BPA during the prenatal stage of development has detrimental effects on the midbrain dopaminergic system and on spine synapses in the hippocampus, while it has no effect when applied at a juvenile stage (Elsworth *et al.*, 2013). In addition, adult BPA-exposed monkeys displayed a significant cognitive impairment (Elsworth *et al.*, 2015). It is also interesting to note that down-regulation of estradiol-induced increase in spine synapses in the hippocampus and prefrontal cortex in adult ovariectomised monkeys exposed to BPA was also reported in another study of the same laboratory (Leranth *et al.*, 2008).

Overall, BPA has been demonstrated to alter memory and learning after developmental, pubertal or adult exposure, based on multiple converging experimental studies reporting this functional effect as well as molecular and cellular changes in the brain in line with the functional changes observed pointing to a coherent adverse effect.

The possibility that BPA alters the cellular and molecular pathways involved in learning and memory processes through disruption of estrogen-dependent pathways was first suggested in the study of Xu *et al.* (2010a and 2010b) reporting decreased expression of ER β . In the study of Xu *et al.* (2014b), the link between BPA-induced effects on learning and memory processes and estrogenic pathway disruption was clearly established from the demonstration that the ER antagonist ICI 182,780 reversed both BPA-induced effects on ER α (modulation and regulation) and memory. Three other studies performed in adults indicated that BPA is also able to interfere with estradiol-induced effects on behaviour and spine density in rodents (Xu *et al.*, 2015b; Inagaki *et al.*, 2012) and on synaptogenesis in nonhuman primates (Leranth *et al.*, 2008). Additional evidence was provided by *in vitro* studies showing that BPA-induced effects on NMDAR signalling and synaptic proteins were reversed by the ER antagonist. In one *in vitro* study, BPA-induced disruption extended to other non-classical estrogen receptors (ERR γ).

The modulatory effects of estrogens on cognitive processes and behaviour in adults are now well established. Although they were studied more extensively in females (Galea *et al.*, 2013; Pawluski *et al.*, 2009), the importance of the estrogenic pathway in the regulation of cognitive behaviour and synaptic plasticity has also been reported in male rodents (Picot *et al.*, 2016). Detailed effects of adult estrogens on learning and memory and the mechanisms underlying these effects in both males and females are described in recent reviews (Frick *et al.*, 2015; Hamson *et al.*, 2016). Sex differences in hormonal impregnation during the critical periods of development and their influence on cognitive performance have been described by Roof and Havens (1992). This may explain the sex differences observed in the expression of cognitive behaviours and their alteration by BPA.

Altogether, these data support the plausibility that alteration of learning and memory by BPA is mediated by disturbance of the estrogenic pathways. Further details presented in the dossier also show that other steroid pathways might be involved.

Cognitive function in humans involves signaling pathways, which seem similar to those described above in rodents. The involvement of NMDAR signaling pathway in memory processes in healthy and diseased brain has been largely reviewed (e.g. in Gilmour *et al.*, 2012; Campos *et al.*, 2016; Arnsten *et al.*, 2017). Estrogens were shown to modulate hippocampus-dependent learning in women and non-human primates (Hampson, 1990; Lacreuse, 2006 and review of Hamson *et al.*, 2016). Testosterone also modulates cognitive functions in men. Given these similarities in the modulatory effects of sex steroids on cognitive functions between rodents and humans, it is likely that the MoA observed in rodents occurs in humans and affects similarly these processes in humans as in rodents. In support of this hypothesis, the *in vivo* study conducted on adult female non human primates showed that a subcutaneous exposure to BPA (50 µg/kg/d) counteracts the synaptogenic effect of estradiol in the hippocampus and prefrontal cortex (Leranth *et al.*, 2008).

Based on i) the significant amount of *in vivo* and *in vitro* animal data showing impairment of learning and memory following exposure to BPA and the potential alteration of cellular and molecular mechanisms underlying these processes through disturbance of the estrogenic pathway, ii) the similar types of signaling pathways underlying human cognition and iii) the numerous data showing sex steroid regulation of these behaviors, exposure to BPA could also alter human cognitive abilities through disturbance of estrogenic pathways.

- **Effects on metabolism**

Based on animal studies after prenatal and/or perinatal or adult exposure, there is now evidence that BPA may increase the incidence of type-2 diabetes *via* an ED MoA. In particular, BPA has been shown to alter insulin synthesis and/or release by β -pancreatic cells, or insulin signalisation (signaling mechanisms) within insulin-sensitive organs (i.e., liver, muscle, adipose tissues). This resulted in variations in the expression of hepatic or adipose tissue markers which are indicative of a state of insulin resistance. These effects are considered by experts to be hallmarks of hormonal adverse effects, especially if there is a combination of effects, each leading to insulin resistance within the different insulin-sensitive tissues. In addition, while most studies were performed on males, a few studies have also examined the impact of BPA on either both sexes or only females. However, more studies should be undertaken before concluding on a sex-specificity or not of the metabolic impact of BPA on insulin synthesis/release and signalisation.

Recent experimental *in vivo* and *in vitro* studies indicate that these effects may involve ER α , ER β or GPR30 pathways. Other hormones such as leptin or adiponectin, 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.

Overall, it is suggested that the endocrine pancreas is targeted by BPA and that mechanisms could differ depending on whether exposure occurs during the fetal life or in adulthood. Fetal differentiation of the pancreas appears highly sensitive to exposure to BPA 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. In addition, BPA can elicit histopathological modifications during the fetal life with consequences on insulin synthesis rate and/or release.

Moreover, most of the *in vitro* studies showing adverse effects of BPA on adipocyte differentiation and function indicate alteration of endocrine mechanisms (e.g., adiponectin release, insulin signaling cascade effectors). However, uncertainties remain as whether BPA activates PPAR γ and/or other nuclear receptors (possible cross-talk between nuclear receptors).

Even if available, epidemiological studies are inconclusive. These effects are considered relevant for humans because similarities exist in homeostatic regulation of insulin production and sensitivity between rodents (mostly investigated in the experimental studies) and humans and because of *in vitro* experimental data using human cells or tissues.

Overall conclusion on ED-mediated effects of BPA

This dossier displays extensive evidence showing that BPA can affect several physiological functions and systems of mammal organisms through ED pathways. As explained earlier, BPA alters the reproductive function, mammary gland development, cognitive functions and metabolism through an ED MoA.

Most importantly, although the steps of the respective mechanisms of action are specific for each effect, the disruption of the estrogenic pathways is a common MoA consistently involved in each of the four effects. The primary target of BPA is however still not known with certainty. BPA binds to estrogen receptors (ER) but with a weak affinity. In addition, BPA binds also to other types of ER such as GPER or ERR- γ with a higher affinity and then, these receptors may also be involved in BPA-mediated effects, particularly at low doses.

The complexity of the toxic response to BPA also suggests multiple MoA that may interact. However, some evidence detailed in each section of this report for each effect, specifically enables to establish that the estrogenic pathway is central and common in the MoA. Other findings also point toward alternative elements involved in BPA-mediated effects, e.g. epigenetic modifications. It is not known whether these modifications may be estrogen-dependent. It is also noted that some evidence points toward a disruption by BPA of the Hypothalamo-Pituitary-Thyroid axis. Thyroid hormones are known to regulate sex steroid synthesis and action in both the brain and gonads as well as in fat metabolism. The crosstalk between the sex steroid and the thyroid endocrine axes was recently reviewed by Duarte-Guterman *et al* (2014). However, the potential role of additional mechanistic pathways does not contradict the importance of the estrogenic pathways in the BPA-mediated effects and the ED nature of the MoA.

Estrogens are known to be central in the regulation of the sexual function and system but are also known to interact with many other physiological functions and developmental processes including neurobehaviour or metabolism. The pattern of the effects observed with BPA is therefore consistent with an ED MoA through estrogenic pathways.

There is recent emerging evidence that BPA may have immunotoxic effects (Ménard *et al.*, 2014a and 2014b). The variability of the effects makes the interpretation and the transposition of these effects to humans uncertain. It is however noted that the role of

estrogens has been often reported in immunocompetence and in the development of innate and adaptive immune response (Fish *et al.*, 2008).

A recent review also reports evidence suggesting an effect of BPA on the cardiovascular system that may involve estrogen receptor rapid signalling (Gao *et al.*, 2014).

It has also been shown that the induction of androgen receptors in fetal mice by estradiol or BPA is permanent, leading to dramatically increased prostatic androgen receptors (vom Saal *et al.*, 1997). This increase may result in a marked increase in the sensitivity of the adult prostate to hormonal stimulation, which is associated with prostate enlargement and pre-cancerous cellular abnormalities (metaplasia) (Ogura *et al.* 2007).

These effects were however not further investigated because the level of evidence is considered insufficient at this point but it cannot be excluded that the range of effects related to the ED-properties of BPA may be wider than those described in this dossier.

Effects on environment are not addressed in this report. However, it is of importance to note that estrogen-related modes of action are reported as one of the predominant MoA potentially involved in the reproductive disturbance reported in several taxonomic groups. In particular, some data suggest an estrogen-agonist MoA in fish (severe effect on reproduction affecting population (Yokota *et al.*, 2000) and vitellogenin induction (Kashiwada *et al.*, 2002)), in amphibians (skewed sex ratio towards females (Levy *et al.*, 2004) and vitellogenin induction (Oehlmann *et al.*, 2009)) and in molluscs (stimulatory effect of BPA on egg production antagonised by anti-estrogen (Oehlmann *et al.*, 2006)) and possibly an estrogen-like action in echinoderms (abnormal larvae development (Roepke *et al.* 2005)).

In conclusion, experimental evidence shows that BPA affects the reproductive function, mammary gland development, cognitive functions and metabolism and these alterations are mediated through disruption of estrogens and estrogenic pathways. These effects are considered predictive of serious health outcomes. Although they may be exerted through direct exposure (alteration of estrous cycles and of memory/learning performance), they are also observed for all four endpoints after exposure during development with consequences later in the life of the offspring.

6.3.2 Equivalent level of concern assessment

6.3.2.1 Human health

There is currently no guidance on how to assess that an adverse health effect represents an equivalent level of concern (ELoC) to a CMR substance, thereby fulfilling criteria for SVHC identification according to article 57(f) of REACH for human health. A discussion paper of ECHA is however available (ECHA, 2012) with a specific focus on sensitizers. However, the criteria identified in this document to evaluate the ELoC are considered fully relevant for the present case. These factors are listed below and are used in the present analysis:

- Health effects:
 - o Type of possible health effects
 - o Irreversibility of health effects
 - o Delay of health effects
- Other factors:
 - o Quality of life affected
 - o Societal concern
 - o Is derivation of 'safe concentration' possible?

Type of effects

The type of effects of BPA is comparable to CMR substances that have the potential to induce serious and permanent organ dysfunction. This section focuses on whether the ED-

related effects of BPA are serious effects. Persistency of the effect is addressed in the following section related to irreversibility.

In relation to its ED-mediated MoA, BPA exerts a range of experimental effects described in this document that are predictive of serious adverse health outcomes:

- BPA can affect the proper regulation of the reproductive function and can be involved in alteration of fertility, as reflected by its harmonised classification Repr 1B. On this basis, it unequivocally fulfills the criteria of seriousness, as well as *per se* of ELoC compared to CMR.
- BPA has no harmonised classification for carcinogenicity. However, pre- and/or post-natal exposure to BPA induced structural changes including proliferative effects on mammary tissue. These types of changes have been associated with increased vulnerability of the mammary gland to develop breast cancer at a later stage in life. The carcinogenic effect of BPA has been described: "BPA may be reasonably anticipated to be a human carcinogen in the breast and prostate due to its tumor promoting properties" (Seachrist *et al.*, 2016) and based on experimental data, RAC acknowledged that " BPA has been shown to have a proliferative effect on mammary tissue". BPA may therefore lead to a serious effect.
- BPA is also implicated in alteration of learning and memory as well as of brain histological structures which is also considered to be a serious effect. As summarised in Annex 2.2 of the recent JRC report (JRC, 2015) discussing ELoC for neurotoxicants (and immunotoxicants), all these changes are recognised as adverse changes by groups of experts on neurotoxicity within the WHO Environmental Health Criteria Programme and EFSA. They are also considered as serious effects eligible for a STOT RE classification according to CLP
- The effects of BPA on metabolism are associated with serious chronic pathologies such as type-2 diabetes after prenatal and/or perinatal or adult exposure in animals (rodents and non-rodents). The two major pathophysiologies of concern for diabetes mellitus (type 2) are a decreased insulin secretion and an increased insulin resistance. We have shown in this report that BPA exposure may act on both pathways. Diabetes and obesity are associated with serious co-morbidities and reduced life span. Moreover, BPA exposure during pregnancy induces an alteration of glucose homeostasis in dams, which is characterised by the development of severe glucose intolerance and aggravated insulin resistance, hyperinsulinemia and hyperleptinemia. This is a situation similar to that of gestational diabetes mellitus (GDM). GDM is associated with increased risks of birth complications (complications related to excessive birth weight, preterm birth and respiratory distress syndrome, hypoglycemia) and of type 2 diabetes later in life. The effects of BPA on metabolism are therefore associated with a serious health outcomes. In addition, there are no criteria on how to consider these effects are available for classification & labelling. Despite the seriousness of the effect, a CLH dossier for this endpoint is not foreseen.

Altogether, the ED MoA of BPA is considered to raise concern in relation to serious health effects.

Irreversibility of the effects and delay of health effects

Some of the ED-related effects of BPA have been identified after concomitant exposure. However, these ED-related effects are also all characteristically observed after developmental exposure to BPA, with consequences that are observed later in life (estrous cycle, mammary gland, neurotoxicity, insulin synthesis and resistance), without a direct exposure. As the effects appear long time after the exposure, they are indeed considered

as **permanent and irreversible**.

It is also noted that some studies although limited in number have reported transgenerational effects of BPA on cognitive function.

This delay between exposure and manifestation of the effects creates difficulties in detecting the link between exposure and effect. It raised a difficulty in the protocol design, more specifically in human epidemiological studies. An absence of observed effects does not mean necessarily an absence of effect. In this case, risk management measures and prevention of exposure may not be taken in time and individuals might be exposed for a long time before action is taken. It is especially applicable to developmental effects for which exposure occurs during the developmental period and they are therefore very difficult to characterise *a posteriori*. This relates to the concept of developmental origins of health and disease (DOHaD). Moreover, the latency and irreversibility of the effects may also pose some ethical concern due to the fact that they may also affect future generations that might not be protected. Due to the difficulty posed by latency both in terms of detection of the effect and responsibilities for later generations, it is considered as an additional concern.

Quality of life affected

Altogether, the adverse effects of BPA such as demonstrated in this document may concur to a **reduced quality of life**.

- The effects of BPA on the reproductive function encompass a wide range of manifestations that can significantly impact the quality of life. Puberty is a difficult period of life associated with significant psychological distress and a need for social identification. Modification of the onset of puberty can increase these difficulties. The disruption of ovarian cycles may occur triggering various outcomes in women: extension or shortening of the menstrual cycle, erratic period cycles, irregularity of menstruation flows, etc. These outcomes may have various and more or less serious impacts on everyday life such as abnormal bleeding (menstruation flow), disruption of fertility (due e.g. to fewer ovarian cycles and thus a lower probability of getting pregnant in the case of elongation of cycles), disruption of sexuality, discomfort and inconvenience, and generally a lower quality of life. This kind of disruption may occur from puberty to menopause. The magnitude of adverse impacts depends on the severity of the effects likely to appear, from a slight extension of ovarian cycles to complete amenorrhoea. Ultimately, potential infertility is associated with a major source of worries and psychological distress for the individuals affected. It may involve medical assistance that involves constraining and time demanding protocols, most often over a certain period of time to reach success, if any.
- Pre- and/or post-natal exposure to BPA induced structural changes including proliferative effects on mammary tissue. These types of changes have been associated with increased vulnerability of the mammary gland to develop breast cancer at a later stage in life. Therefore, the health impacts associated with the effect of BPA on mammary glands may correspond to increased occurrence of breast cancers. Breast cancer is an uncontrolled growth of breast cells leading to a malignant tumor. Initially, breast cancer may not cause any symptoms. A lump in the breast may be too small to feel or to cause any unusual changes (swelling of all or part of the breast, breast pain, nipple turning inward, etc.). Breast cancer can be treated by surgery, medical therapies (radiotherapy, chemotherapy) and/or medication such as anti-estrogen, cytotoxic, and endocrine drugs. Medicines can also be prescribed to treat patients, in order to mitigate potentially severe side effects of medical therapies. Besides treatments, breast cancer might cause many other adverse consequences for patients such as absenteeism, social isolation,

psychological depression, anxiety, and more generally a lower quality of life; the worst adverse effect being death. Subsequent to the breast cancer itself, additional physical and psychological suffering may be imposed by breast reconstructive surgery, if any.

- A decrease of cognitive functions can have an impact on daily life by impairing the ability of an individual to effectively and autonomously cope with daily tasks and difficulties. It may occur through many various forms such as disorientation, weak memory, disrupted learning capacities, etc. In children and adolescents it may also affect school performance and may contribute to situations of school failure that may impact childrens' self-esteem as well as compromise future educational and occupational achievement. It may also create a significant source of worries in parents. Emerging evidence of anxiety is also associated with possible impact on well-being and social abilities of these individuals. At a population level, this effect could lead to a global displacement of the Gaussian IQ distribution impacting the average IQ of the population.
- BPA exposure may enhance diabetes risk due to an increase in insulin resistance. Diabetes is a metabolic disease accompanied by important co-morbidities and reduced life span. In addition, it generally requires daily glycemic control as well as careful adherence to specific dietary habits that may in particular affect also social life. The quality of life may also be altered by symptoms of low or very high blood glucose and fears about potential or real complications.

Societal concern

The adverse effects of BPA such as demonstrated in this document all raise indisputable **societal concern**.

- Regarding reproduction, there is a general societal concern associated with possible conception difficulties. Infertility rates have remained stable (Kortenkamp *et al.*, 2012) in the last decades ranging from 3.5 to 16.7% in developed countries (Boivin *et al.*, 2007) but the demand for assisted reproductive technologies (ART) treatment in Europe – as expressed in treatment cycles performed in European countries – has increased by 59% in the five years from 1997 to 2002 (HEAL 2014). Although increasing age at the time of conception may be a preponderant factor to explain conception difficulties, a potential role of EDCs is generally considered as plausible as well (Marques-Pinto *et al.*, 2013). In addition, there is an increasing concern related to disorders associated with the female reproductive system such as endometriosis and PCOS which might additionally affect fertility. Additionally to ethical and demographical concerns associated with conception difficulties, those adverse effects affecting fertility might be costly for the society as a whole. Indeed, recent literature provides evaluations of such costs although there is no specific estimate of the burden that may be potentially related to BPA. For example, the HEAL study (HEAL 2014) concluded that EDC-related male and female infertility direct cost associated with ART is between 48 and 155 million € per year in the EU28 as quoted by Rijk *et al.* (2016)²². Likewise, Norden *et al.* 2014 study estimated the direct (healthcare) and indirect (productivity losses) cost of EDCs-related male infertility cases associated with fertility treatments such as ICSI treatments to be about 72.3 million € for the EU28 (Norden, 2014).

²² Proportion of 2 to 5% of the total annual ART cycle cost in EU28 (€2.4 - 3.1 billion) attributed to EDC exposure

- The risk factors for breast cancer are numerous and there is no specific estimate of the breast cancer burden related to BPA. Within the EU, breast cancer is the most common form of cancer in women, accounting for 28% (WHO website²³). Breast cancer is the cancer type causing most cancer related deaths in women (17.2% of the total) (WHO website²⁴, 2010). Breast cancer can occur from puberty to end of life. The age-standardised incidence of breast cancer is reported to be around 90 per 100,000 in EU women (89.7 per 100,000 women in Western Europe by WHO²⁵ and 94.2 per 100,000 EU women by IARC (Ferlay *et al.*, 2013)). The life time risk²⁶ for women of getting breast cancer in EU28 is around 1 in 8 women, or approximately 13% (e.g. 12.5% in France, 12.90% in the UK²⁷, 14.3% in the Netherlands (Paap *et al.*, 2008)). In terms of societal burden, recent literature shows that breast cancers also represent significant costs. The HEAL study (HEAL 2014) concluded that EDC-related female breast cancers direct (healthcare) costs are between 128 and 320 million € per year in the EU28. Including also indirect (productivity losses and informal care) costs, the total societal burden accounts for between 320 and 800 million € per year in the EU28. How large a part of this burden can be attributed to BPA itself has however not been calculated.
- In its state-of-the-art report Kortenkamp *et al.* (2012) reports that although population-based statistics are not generally available for neurodevelopmental outcomes, surveys indicate that, for example in the US, several hundred thousand children have disabling childhood mental health conditions including mental retardation, learning disabilities, autism and attention deficit hyperactivity disorder (ADHD). Learning difficulties may affect up to 10% of school children. Endocrine disruption is unlikely to be the only cause of such trends but may importantly contribute. In its assessment of health costs associated with ED chemicals, Rijk *et al.* (2016) stated that neurodevelopmental and behavioural diseases and disorders include several pervasive disorders that persist for a lifetime, thereby leading to prolonged costs. They concluded that these disorders comprise the largest contributors to the total EDC-associated socio-economic cost estimates. Especially the contribution of IQ loss dominates the cost, accounting for between 32 and 184 billion € per year for the EU28 (indirect cost). HEAL 2014 also provided an evaluation of neurological disorders affecting child brain development and behavior due to EDCs but only focusing on autism and ADHD. The study reported a direct cost of between 4.5 and 11.3 billion € per year for the EU28, autism being the major contributor. Bellanger *et al.* (2015) estimated a cost of €80-400 million for EDC-related autism spectrum disorders. Which part of this burden can be attributed to BPA itself has however not been calculated and no BPA-specific data are available to our knowledge. The emergence of an elevated prevalence of neurobehavioural disorders in particular in children (that may be partly linked to EDCs) as well as their important costs for the healthcare systems in particular, and for society at large when also considering possible indirect costs of future decreased productivity, is therefore a major societal concern.
- The number of people with diabetes has doubled since 1980, rising from 33 million in 1980 to 64 million in 2014 in Europe (WHO 2016). The European prevalence of diabetes among adults over 18 years of age has risen from 5.3% in 1980 to 7.3% in 2014 and the associated mortality has also increased. Likewise, according to

²³ <http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-common-cancers/breast-cancer>, accessed on 3 February 2017

²⁴ <http://www.euro.who.int/en/media-centre/sections/press-releases/2010/02/up-to-40-of-cancer-cases-could-be-prevented>, accessed on 3 February 2017

²⁵ <http://www.who.int/cancer/detection/breastcancer/en/index1.html>

²⁶ The lifetime risk of cancer is an estimation of the risk that a newborn child has of being diagnosed with cancer at some point during its life.

²⁷ <http://www.cancerresearchuk.org/cancer-info/cancerstats/incidence/risk/statistics-on-the-risk-of-developing-cancer>

WHO (2016), the (age-standardized) mortality rate due to high blood glucose for adults over 20 years old (both sexes) is estimated to be 55.7 per 100,000 in Europe and around 10% of all deaths can be attributed to high blood glucose in 2012 (both sexes). Due to these increasing prevalences, there is a growing societal concern about diabetes and related-diseases. For example, HEAL 2014 estimated the direct and indirect cost of diabetes due to EDCs between 6 and 15 billion € per year for the EU28. How large a part of this cost can be attributed to BPA itself has however not been calculated.

Is derivation of a 'safe concentration' possible?

- With regard to alteration of the reproductive function and of estrous cyclicity in particular, when considering oral studies, it is noted that effects are detected in some studies at relatively high doses (100, 300 and 600 mg/kg in Laws *et al.*, 2000, Delclos *et al.*, 2014 and Tyl *et al.*, 2008, respectively) and at relatively low doses in other studies (0.0005, 0.001, 1.2 and 1.2 mg/kg in Wang *et al.*, 2014, Lee *et al.*, 2013, Rubin *et al.*, 2001 and Mendoza-Rodriguez *et al.*, 2011, respectively). The uncertainty in the dose response was acknowledged by RAC in its restriction opinion (ECHA, 2015) that concluded that "*effects on the reproductive system have been observed at and below the range where kidney effects occur, RAC considers it prudent to take them into account in hazard and risk assessment and in health impact assessment. RAC however acknowledges that the available information does not allow a quantification of the dose-response relationship.*"
- Regarding the mammary tissue proliferative effect, RAC agrees "*that BPA has been shown to have a proliferative effect on mammary tissue at doses below the doses causing general toxicity (such as kidney weight changes). The effects on mammary gland development should be taken into account in hazard and risk assessment and in health impact assessment. In line with EFSA (2015), no individual study is however considered robust enough by RAC to serve as critical study for the identification of a starting point for DNEL derivation. Therefore the effects will be accounted for in the setting of Assessment Factors.*" When considering oral studies, it is noted that most of the oral studies identified a rather low NOAEL/LOAEL couple of 25/250 µg/kg/d (see Moral *et al.*, 2008; Jenkins *et al.*, 2009; Betancourt *et al.*, 2010). Whereas in Delclos *et al.*, 2014, doses from which BPA causes hyperplastic ductal lesions were difficult to estimate.
- A similar conclusion as for reproductive toxicity was reached by RAC regarding neurobehavioural effects. In addition, a number of recent studies have confirmed since then, that an alteration of cognitive performance is observed in the vast majority of the studies at doses below 9 mg/kg (point of departure used for DNEL derivation - BMDL10 for kidney effects in mice) and the form of the dose-response relationship is still under discussion. Several studies consistently identify effects from/at 40-50 µg/kg/d. In some studies, a single dose of exposure was used which does not allow to assess the dose-response relationship. The study by Inagaki *et al.* (2012) well illustrates that the time of exposure may be a more critical parameter than the dose of exposure. An effect on object placement memory was only observed in adult females during proestrus and not during the other phases of the cycle. This result was consistent with other results in the study showing that the effect is dependent on the concomitant presence of estrogens. Therefore, other parameters than the dose could impact the 'safe concentration'.
- With regard to metabolism, in the restriction dossier (ANSES, 2014), the effects of BPA on metabolism were considered as critical effects to be used for the risk assessment. The study from Miyawaki *et al.*, 2007 was selected as the key study for this type of effect and a LOAEL of 260 µg/kg bw/day was derived based on the

results reported by the authors. This LOAEL was below the NOAEL used by EFSA and RAC to derive a safe level with BPA. The recent studies that have been published and assessed for this dossier (and presented above see Table 25) also reported effects of BPA at doses equivalent or below the LOAEL from Miyawaki *et al.* (2007). The level of sensitivity will also depend on the window of exposure and the susceptibility of the exposed population. Based on the available data, it will be difficult to define a safe level with a sufficient level of confidence.

- In addition, it is noted that our knowledge of the ED-related effects of BPA may not be complete. In particular, there is some emerging evidence of an effect of BPA on the immune function that has been recently investigated and also raises concern on possible effects at doses lower than starting point for DNEL derivation (5 µg/kg and possibly 0.5 µg/kg in Menard *et al.*, 2014a and 2014b).

Altogether, the database shows important uncertainties in establishing a quantitative dose-response, as well as safe levels, with some studies identifying effects at doses below the point of departure used for DNEL derivation.

The synthesis of these elements for each effect as well as for ED-mediated effects of BPA in general are presented in Table 27 below.

Overall assessment

Overall, based on the WoE presented, BPA is identified as an SVHC according to article 57(f) for probable serious effects on human health, due to its endocrine disrupting properties, which are of ELoC.

It is noted that the MSC has agreed in December 2016 to identify BPA as an SVHC according to article 57 (c) in relation to its recent harmonised classification Repr 1B – H360F and BPA is included in the Candidate List as an SVHC under Article 57 (c) by decision of ECHA ED/01/2017 of the 4 January 2017.

The reproductive toxicity of BPA is also one of the effects linked to the ED properties of BPA and it is considered that the additional identification of the ED MoA for this effect, as well as for the three other effects, through SVHC 57(f) criteria is fully justified as this additional identification has specific regulatory consequences:

- So far, agreeing on a SVHC-57(f) property for a substance is the only way to identify it as an ED. Indeed, there is no other regulation allowing an identification/classification of a substance as an ED.
- In the prioritisation process for inclusion into Annex XIV, a score is calculated. The different inherent properties have different weight attributed and 57f (ED) is given a medium weight, higher than the low score allocated to 57 a, b or c. In the scoring system a higher score is given to a combination of inherent properties only when PBT or vPvB properties are involved. For all the other properties, and in the present case, identifying BPA as an ED in addition to e.g. reproductive toxicity will not result in summing the different scores or increasing the existing score, the score for ED alone will apply (ECHA, 2014b). Technically, there is therefore no double counting in relation to prioritisation. Additional identification as an ED is therefore important for the prioritisation process without any double counting effect.
- If a new identified SVHC property (57(f) endocrine disruption) is to be included in the entries of Annex XIV, a concentration limit of 0.1% would apply to the use of the substances in mixtures, with regard to authorisation obligations, whereas currently a higher concentration limit of ≥0.3% applies for substances identified under Article 57 (c).

- In accordance with Article 60(3), applicants may have to apply for authorisation under the socio-economic route, unless a threshold can be determined for which the risk for humans and the environment can be demonstrated to be adequately controlled for any exposure scenario. So far, the discussion on the capability of scientists to determine a threshold for ED effects is still open. Specificities of EDs have been raised to justify that even if a threshold might exist at an individual level, it would be very difficult if not impossible to determine it at a population level. Indeed, critical windows of susceptibility and uncertainties on dose-response characterisation are specificities rendering classical risk assessment complicated for EDs. These specificities are particularly relevant for BPA, as described above. More generally, with respect to risk assessment for authorisation applications, European Commission recognised in a report published in December 2016, that the possibility of demonstrating a threshold for EDCs is expected to be difficult given the current state of scientific knowledge, but cannot be excluded. As a consequence, European Commission confirmed that it will be up to the applicants to demonstrate a threshold for the uses of the substance they applied for (to be scrutinised then by RAC) and in case they cannot, they shall apply under the socio-economic route (European Commission, 2016).

To enable an appropriate assessment of potential future applications under the socio-economic route, it is critical that the scope of the health effects associated with the ED MoA is appropriately defined and is as comprehensive as possible.

In addition, it makes much sense to present and analyse all the possible well-established effects linked to an ED MoA together as they present strong common features that highly support their overall plausibility.

Finally, it is noted that there is no legal provision in the REACH regulation that prevents the identification of a substance as an SVHC according to several criteria, whatever the possible link between them may be.

Table 27: Summary of factors to be considered in ELoC assessment for the different ED-related effects identified

	Alteration of reproductive function	Disruption of mammary gland development	Effect on brain and behaviour	Effect on metabolism and obesity	Overall conclusion for BPA ED-related effects
Possible serious health effects?*	<ul style="list-style-type: none"> ▪ Serious dysfunction as illustrated by its harmonised classification Repr 1B for fertility 	<ul style="list-style-type: none"> ▪ Associated with increased vulnerability of the mammary gland to develop breast cancer 	<ul style="list-style-type: none"> ▪ Serious dysfunction having consequences on the cognitive performance 	<ul style="list-style-type: none"> ▪ Associated with serious chronic pathologies such as diabetes and obesity. Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation. 	<p>YES</p> <ul style="list-style-type: none"> ▪ Pattern of ED-related effects associated with serious dysfunction or pathologies clearly related to serious illness

<p>Delay of health effects?</p>	<ul style="list-style-type: none"> ▪ Effects on the reproductive function can be induced after only developmental exposure and do not systematically involve a direct exposure. In this case, they appear later in time when the reproductive system becomes functional. 	<ul style="list-style-type: none"> ▪ Developmental and/or post-natal exposures can induce effects on the mammary gland which do not systematically involve a direct exposure. In this case, they appear later in time when breast (or mammary) tissues further develop. 	<ul style="list-style-type: none"> ▪ The effects of developmental exposure impacts cognitive function later in life. 	<p>Developmental exposure has been shown to have consequences in relation to ED-related metabolism effects later in life</p>	<p>YES</p> <ul style="list-style-type: none"> ▪ Developmental exposure has been shown to have consequences in relation to ED-related effects later in life. Future generations not protected.
<p>Irreversibility of health effects?</p>	<ul style="list-style-type: none"> ▪ Developmental exposure will lead to late effects when exposure has stopped showing that these modifications are irreversible. 	<ul style="list-style-type: none"> ▪ Developmental and/or post-natal exposures may lead to late effects even when exposure has stopped suggesting that these modifications are irreversible. 	<ul style="list-style-type: none"> ▪ Impairment of cognitive function can be induced after developmental exposure. These modifications are irreversible as shown by the fact that they are visible when exposure has stopped. 	<ul style="list-style-type: none"> ▪ BPA-related effects may be induced after developmental exposure without direct exposure 	<p>YES</p> <ul style="list-style-type: none"> ▪ All ED-related effects may be induced after developmental exposure without direct exposure.
<p>Quality of life impaired?</p>	<ul style="list-style-type: none"> ▪ Mental/psychological impact, modification of normal physiology. 	<ul style="list-style-type: none"> ▪ Impaired quality of life associated with breast cancer. ▪ Possible impact on well-being, social isolation, psychological depression, anxiety. 	<ul style="list-style-type: none"> ▪ Impact on ability to cope with daily tasks and difficulties, alteration of school performance, school failure affecting self-esteem and later achievement, parental worries; possible impact on well-being and social abilities. 	<ul style="list-style-type: none"> ▪ Diabetes is associated with serious co-morbidities and reduced life span. Impact of monitoring and control of diabetes on daily quality of life. 	<p>YES</p> <ul style="list-style-type: none"> ▪ Impaired quality of life associated with several ED-related effects of BPA

<p>Societal concern?</p>	<ul style="list-style-type: none"> ▪ Widespread concern about fertility issues ▪ Cost implications for society in terms of healthcare 	<ul style="list-style-type: none"> ▪ Concern about breast cancer. ▪ Cost implications for society in terms of healthcare. 	<ul style="list-style-type: none"> ▪ Increasing concern about neurobehavioral disorders in particular in childhood ▪ High socio-economic impact of neurobehavioral effects 	<ul style="list-style-type: none"> ▪ Diabetes is a metabolic disease that represents a growing public health concern worldwide. ▪ High socio-economic impact of diabetes 	<p>YES</p> <ul style="list-style-type: none"> ▪ Multiple ED-related effects associated with major societal/ethical health concerns and socio-economic burden for the society as a whole
<p>Is derivation of a 'safe concentration' possible?</p>	<ul style="list-style-type: none"> ▪ Difficulties in defining the 'safe concentration' based on inconsistent information across studies on dose response including effects at doses below starting point for DNEL derivation in some studies ▪ Critical windows of exposure may be more important than doses ▪ Derivation of safe concentration associated with large uncertainties 	<ul style="list-style-type: none"> ▪ Effects observed below starting point for DNEL derivation in a few studies ▪ Critical windows of exposure may be more important than doses ▪ Derivation of safe concentration associated with uncertainties 	<ul style="list-style-type: none"> ▪ Effects observed below starting point for DNEL derivation in some studies ▪ Critical windows of exposure may be more important than doses ▪ Derivation of safe concentration associated with large uncertainties and many co-factors to be still determined. 	<ul style="list-style-type: none"> ▪ Effects observed below starting point for DNEL derivation in some studies ▪ Critical windows of exposure may be more important than doses ▪ Derivation of safe concentration associated with large uncertainties and many co-factors to be still determined. 	<p>MOST PROBABLY NO</p> <ul style="list-style-type: none"> ▪ Difficulty in the quantification of dose-response acknowledged by RAC ▪ Possible immunotoxic effects not well investigated (low dose potential) ▪ Critical windows of exposure may be more important than doses ▪ Derivation of safe concentration associated with large uncertainties

* This factor is intended to discuss the severity of the effects and not their probability

6.3.2.2 Environment

Not relevant for this dossier

6.3.3 Conclusion on the hazard properties and equivalent level of concern assessment

Bisphenol A is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with *endocrine disrupting properties* for which there is scientific evidence of probable serious effects to human health which gives rise to an equivalent level of concern to those of other

substances listed in points (a) to (e) of Article 57 REACH.

Bisphenol A has a harmonised classification for the hazard class Reproductive toxicity category 1B (H360F 'May damage fertility') based on effects on reproductive function. BPA has been identified recently as SVHC according to Article 57(c) of REACH and included in the Candidate List by decision of ECHA ED/01/2017 of 4 January 2017.

BPA has been shown to affect the reproductive function, mammary gland development, cognitive function and metabolism through pathways that commonly involve disruption of estrogenic regulation. The effects on female reproductive function include the induction, after both developmental and adult exposures, of cystic ovaries, changes in the uterus morphology, alteration of fertility parameters as well as estrous cycle disturbance. The estrous cycle is a perfectly synchronised and sequenced event that relies on a permanent endocrine dialogue between the ovary and the hypothalamo-pituitary system. Those pathways differentiate during fetal life and are largely influenced by numerous factors and in particular the steroid environment of the foetus. BPA at the adult stage alters the endocrine steroidogenic function of the ovary and more specifically the production of estrogens by the follicle, leading to disturbance in the estrous cycle. At the neuroendocrine level, BPA can also act during the perinatal/postnatal organisation or adult activation of the hypothalamus-pituitary system through changes in kisspeptin, gonadotrophin-releasing hormone (GnRH) expression, activity or liberation and sex steroid receptor expression that impact estrous cyclicity.

The effects on the mammary gland, depending on the period of exposure, include: modifications in the mammary tissue such as an increased number of terminal end buds (TEBs) relative to the ductal area, fewer apoptotic TEB cells, increased lateral branching and ductal hyperplasia, increased cell proliferation and decreased apoptosis in the glandular epithelium, ductal (and occasionally lobuloalveolar) and intraductal hyperplasia - ultimately increasing its susceptibility to chemical carcinogens. These effects were observed in rodent or in non-human primate following prenatal and/or post-natal exposure to BPA. Available data also support the plausibility that BPA, through interaction with the nuclear estrogen receptors (ERs), or G protein-coupled estrogen receptor (GPER) and indirectly with the progesterone receptor (PR), modulates estrogen and progestin agonist activities. Emerging epigenetic studies have suggested changes related to estrogen-dependent genes (such as EZH2 and HOTAIR), as well as HOX genes (involved in embryogenesis and post-natal development) which could be associated with BPA induced abnormal development and increased cancer susceptibility of the mammary gland.

BPA has been reported to alter memory and learning after developmental, pubertal or adult exposure, based on multiple converging experimental studies reporting this functional effect as well as molecular and cellular changes in the brain (reduced expression of NMDAR, altered synaptogenesis). These effects involve disturbance of estrogenic pathways as evidenced by the reversal of the functional, cellular and molecular effects of BPA by an ER antagonist and interference of BPA with estradiol-induced effects on behavior and spine density/neurogenesis.

The effects of BPA on metabolism in rodent and non-rodent after prenatal and/or perinatal or adult exposures include alteration of insulin secretion and/or release by β -pancreatic cell, or of insulin signalisation (signaling mechanisms) within insulin-sensitive organs (i.e., liver, muscle, adipose tissues) leading to variations in the expression levels of hepatic or adipose tissue markers which are indicative of a state of insulin resistance. It is therefore considered that BPA may increase the incidence of type-2 diabetes. Additionally, *in vivo* and *in vitro* experimental studies indicate that these effects may involve ER α , ER β or GPR30 pathways. 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. Most of the *in vitro* studies showing adverse effects of BPA on adipocyte differentiation and function point to alteration of endocrine mechanisms (e.g., adiponectin release, insulin signaling cascade effectors). Overall, it is suggested that the pancreas is targeted by BPA, that the mechanisms could differ depending on the period of exposure (fetal life or adulthood) and that an ED MoA is involved. Lastly, mainly based on similarities in homeostatic regulation of insulin production and sensitivity between animals and humans, these effects are considered relevant for humans.

The steps of the respective mechanisms of action are specific for each effect. The complexity of the toxic response to BPA suggests multiple MoA that may interact but **most importantly, the available evidence shows that disruption of the estrogenic pathway is central and consistently involved in each of the four effects.**

In conclusion, on the basis of evidence available in relation to alteration of reproductive function, mammary gland development, cognitive function and metabolism, BPA can be considered an endocrine disruptor for human health.

It is not excluded that BPA may also alter other physiological functions, e.g. the immune function, through a similar ED MoA but the level of evidence is considered insufficient at the moment for this effect to be presented.

The range of experimental effects induced by BPA in relation to its ED MoA is considered predictive of serious health outcomes. All these ED-related effects are characteristically (but not only) observed after developmental exposure to BPA, with consequences that are observed later in life. As they appear a long time after the exposure, they are indeed considered permanent and irreversible. In addition, the effects of BPA are associated with conditions that may lead to a reduced quality of life. In particular breast cancers, neurobehavioural disorders and diabetes are observed with high prevalence and increasing trends during the last decades in Europe and raise indisputable societal concern, also in relation to their potential economic burden on the health systems. Finally, for each of the four effects, the database shows important uncertainties in establishing a quantitative dose-response as well as safe levels, with some studies identifying effects at doses below the point of departure used by RAC for DNEL derivation and on-going discussions on the shape of the dose-response relationship and the parameters impacting the dose-response (period of exposure and concomitant presence of estrogen in particular).

Overall, based on the WoE presented, BPA is identified as an SVHC according to article 57(f) for probable serious effects on human health, due to its endocrine disrupting properties, which are of ELoC.

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