Nordic Council of Ministers

New EU criteria for endocrine disrupters

Consequences for the food chain

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Preface

There are growing concerns that environmental contaminants; for example substances migrating from food packagings, food additives, or natural substances in foods (*e.g.* phytoestrogens) may have adverse effects on endocrine functions in humans. Such adverse effects could be mediated by mimicking the body's own hormones or interfere with hormone production, secretion, degradation or transport of hormones in the body. These substances are known as endocrine disruptors (EDs). It is suspected that EDs can cause decreased fertility, hormone-related cancers, behavioral changes, adverse effects on the immune systems, and metabolic disorders.

Setting scientific criteria for their identification is highly complicated and will have important consequences for a wide range of stakeholders. The process of defining these criteria has moved forward slowly since there is a lack of consensus between scientists and regulators.

However, work is now under way in the EU to develop common criteria at EU level for the hazard identification of endocrine disruptors/hormone-disrupting substances and to scrutinize the possibilities to establish safe exposure levels for these.

Therefore a two-day workshop was held in November 29–30 in Uppsala within the Nordic co-operation (NKMT; Nordic Working Group for Diet, Food and Toxicology) with invited representatives from Nordic food authorities (risk assessors and risk managers), trade, industry, trade associations, consumer organizations and with national and international experts to assist in giving the Nordic food authorities a common knowledge base and thus the possibility of a uniform handling of endocrine disruptors in foods. This knowledge will also be used to assess the impact of new EU legislation in this area and possibly affect this new legislation.

The organizing project group consisted of the following persons:

- Denmark: Mette Holm and Maja Kirkegaard, Danish Veterinary and Food Administration, Anne Marie Vinggaard, Technical University of Denmark
- Finland: Johanna Suomi and Kimmo Suominen, Finnish Food Safety Authority Evira
- Iceland: Grimur Eggert Olafsson, Iceland Food and Veterinary Authority
- Norway: Julie Tesdal Håland and Rune Jemtland, Norwegian Food Safety Authority
- Sweden: Emma Ankarberg and Kettil Svensson (project manager), National Food Agency, Sweden

Summary

Endocrine disruptors (EDs) are substances that adversely affect the endocrine system's normal functions by mimicking the body's own hormones or interfere with hormone production, secretion, degradation or transport of hormones in the body. It is suspected that EDs can cause decreased fertility, hormone-related cancers, behavioural changes, effects on the immune systems, and metabolic disorders.

The effects of EDs are thought to depend on both the level and timing of exposure. EDs are suspected of being capable of acting even at very low doses and sensitive windows of exposure appear to be during critical periods of development (for instance, foetal development and puberty).

Defining scientific criteria for their identification is highly complex and has important consequences for a wide range of stakeholders. Work on the issue has been conducted at EU and international level for a long time. The European Commission's delay in adopting scientific criteria has provoked strong reactions from various stakeholders. One reason is the lack of consensus between scientists and regulators about these criteria.

However, work is now under way in the EU to develop common criteria at EU level for the identification of endocrine disruptors/hormone-disrupting substances and the possibilities to establish safe exposure levels for these. Chemicals that are suspected endocrine disruptors can be found in foods, including drinking water, for several reasons. They may contaminate food because some of them are contaminants in the environment (*e.g.* dioxins, PCBs, cadmium, perfluorinated alkyl acids), migrate from packaging materials (*e.g.* phthalates, bisphenol A), used as a food additives, veterinary drugs or pesticides, or occur as a natural component of some common food plants and fungi (soy phytoestrogens, mycotoxins *etc.).*

The upcoming EU criteria identifying EDs could ban EDs in foods from a health point of view as for CMR (carcinogenic, mutagenic or toxic to reproduction) substances and this may have implications for food production, trade and authorities.

A Nordic workshop comprising several actors within the food chain discussed possible consequences, both positive and negative, of such a scenario and how this could be handled. Phasing-out of EDs can give some players a positive image, for others it would imply significant costs. From the production chain perspective the challenge is in particular the limited or non-existent availability of alternative solutions. However, within the food sector, harmonized EU legislation in this area, based on scientific risk assessment, seems to be preferred instead of a horizontal framework regulation or national specific legislation as it treats all the actors in an equal manner.

Abbreviations

ACTH	Adrenocorticotropic hormone
ADI	Acceptable Daily Intake
ADME	Absorption, distribution, metabolism and excretion
ARfD	Acute reference dose
AR	Androgen receptor
BBP	Benzyl butyl phthalate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BPA	Bisphenol A
BPR	Biocidal products (EU)
CAGs	Common assessment groups of pesticides
CHD	Coronary heart disease
CMR	Carcinogenic, mutagenic or toxic to reproduction
COPHES	Consortium to Perform Human Biomonitoring on a European Scale
CVD	Cardiovascular disease
DBP	Dibutyl phthalate
DEHP	Diethylhexyl phthalate
DEMOCOPHES	Demonstration of a study to COPHES
DHT	Dihydrotestosterone
DINP	Diisononyl phthalate
DNEL	Derived no effect level
E20	17β-oestradiol
EAS	Endocrine active substance
EATS	Estrogen, Androgen, Thyroid and Steroidogenic pathways
ECHA	European Chemicals Agency
ECPA	European Crop Protection Association
EDCs	Endocrine disrupting chemicals
EDs	Endocrine disruptors
EDC	Endocrine disrupting chemicals
EEA	European Economic Area
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ERα	Estrogen receptor alpha protein
ERβ	Estrogen receptor beta protein
FCM	Food contact materials
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid

LOAEL	Lowest Observed Adverse Effect Level
MoA	Mode of Action
MoE	Margin of Exposure
MRL	Maximum residue limit
NGOs	Non-governmental organisations
NKMT	Nordic Working Group for Diet, Food and Toxicology
NOAEL	No Observed Adverse Effect Level
PBK	Physiologically-based kinetic (modelling/method)
PBTs	Persistent, bioaccumulative and toxic substances
PCBs	Polychlorinated biphenyls
PFC	Perfluorinated compounds
PGR	Progesterone receptor
PPAR	Peroxisome-proliferator activating receptor protein (α, β/δ, γ)
PPPR	Plant Protection Products Regulations (EC)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SCOPAFF	Standing Committee on Plants, Animals, Food and Feed (EC)
TDI	Tolerable Daily Intake
T2D	Type 2 diabetes
T_3	Triiodothyronine
T_4	Tetraiodothyronine, Thyroxine
vPvBs	Very persistent and very bioaccumulative substances
WHO/IPCS	World Health Organisation/International Programme on Chemical Safety

Introduction

Within the Nordic co-operation (NKMT; Nordic Working Group for Diet, Food and Toxicology) a project *Consequences for the food chain of new EU criteria for endocrine disrupting chemicals* was applied for in 2015 and was funded the same year by the Nordic Council of Ministers.

There is a growing concern that environmental contaminants, substances migrating from food packagings, food additives or natural substances in foods (*e.g.* phytoestrogens) may adversely affect the normal functions of the endocrine systems. Such EDs could act by binding to hormone targets, or by interfering with hormone production, degradation or transport of hormones. With work underway in the EU to develop common criteria for the identification of endocrine disrupting properties under the plant protection products and biocidal products regulations it seemed important to discuss potential consequences for the food chain and how to deal with it.

Therefore, a two-day workshop was held in November 29–30 2016 in Uppsala with invited representatives from Nordic food authorities (risk assessors and risk managers), trade, industry, trade associations, consumer organizations and with national and international experts to assist in giving the Nordic food authorities a common knowledge base and thus the possibility of a uniform handling of endocrine disruptors in foods. This knowledge will also be used to assess the impact of new EU legislation in this area and possibly affect this new legislation.

The first day included lectures that mainly focused on legislation, occurrence and exposure of EDs/potential EDs. The second day dealt with risk assessment and health effects of EDs/potential EDs and different stakeholders' (food producers, trade and NGOs *etc*.) view on the consequences of these new criteria.

Part of the workshop was devoted then to discuss what consequences, both positive and negative, how these EU criteria would impact the primary actors in the areas of plant protection products and biocides but also in the future other regulated food areas. Nordic examples of risk management of EDs/potential EDs were given both from government agencies, industry and trade as a start of the discussions. A follow-up to this workshop is this report.

In addition annexed to this report is an overview of current knowledge on how the endocrine system is believed to be affected and their putative mechanisms-of-action.

Program – NKMT Workshop 2016

November 29

Table 1: November 29

09.30	Registration and coffee/tea
10.00–10.15	Welcome, background, purpose and introduction – Kettil Svensson, National Food Agency, SE
10.15–10.20	Presentations of participants
10.20–11.00	Endocrinology – Olle Söder, Karolinska Institute, SE
11.00–11.30	EDC – 2020, From Science to Policy – Mattias Öberg, Swetox, SE
11.30–12.00	Potential EDCs in foods – Julie Boberg, Danish Technical University, DK
12.00–12.30	The REACH regulation: significance on risk assessment of endocrine disruptors in food – Tero Hirvonen, Finnish Food Safety Authority (EVIRA), FIN
12.30–13.30	*Lunch*
13.30–14.00	EFSA's Opinion on endocrine active substances – Tony Hardy, EFSA
14.00–14.30	Update on the EU Commission process on establishing criteria for identification of EDs – Pia Juul Nielsen, Danish Environmental Protection Agency, DK
14.30–14.50	*Coffee and tea break*
14.50–15.30	First Nordic example: A risk assessment dealing with a potential endocrine substance in foods? – Common assessment groups – experiences from a cumulative pesticide risk assessment – Juha Laakso, Finnish Safety and Chemicals Agency (Tukes), FIN – Dietary heavy metal exposure of Finnish children: risk assessment and risk management decisions – Johanna Suomi, Finnish Food Safety Authority (Evira), FIN
15.30–16.00	An alternative approach for prioritizing chemicals for *in vivo* testing for endocrine disruption– Anne Marie Vinggaard, Danish Technical University, DK (speech was cancelled)
16.00–17.20	Workshop 1 – Discussions in small groups with presentations in plenum. Questions related to the presentations.
19.00	*Dinner*

November 30

Table 2: November 30

08.30–08.50	Second Nordic example (DK): A risk management dealing with a potential endocrine substance in food and food contact materials – Mette Holm, Födevarestyrelsen, DK
08.50–09.40	Health effects of EDCs – epidemiological – Anna Maria Andersson, Center for Endocrine Disruptors, DK
9.40–10.00	The European Human Biomonitoring Initiative (EHBMI) – Anna Maria Andersson, Center for Endocrine Disruptors, DK
10.00–10.30	*Coffee and tea break*
10.30–10.50	Third Nordic example: Exposure to possible EDs in a Norwegian population – assessment of total exposure, examples – Cathrine Thomsen, Folkehelseinstittutet, NO
10.50–11.10	FCM manufacturer's view – Natural Greaseproof – an alternative to the use of fluorochemicals in packaging paper – Henrik Kjellgren, Nordic Paper, SE
11.10–11.30	Pesticide producer's view – Euros Jones, European Crop Protection Association (ECPA)
11.30–11.50	Trade organization's view – Malene Teller Blume, COOP, DK
11.50–12.10	Consumers view? – Claus Jørgensen, Forbrugerrådet,TÆNK Kemi, DK (speech was cancelled)
12.30–13.30	*Lunch*
13.30–13.50	Fourth Nordic example (SE): Exposure to some potential EDs in Sweden – Kettil Svensson, National Food Agency, SE
13.50–15.30	Workshop 2 (including coffee/tea). Discussions in small groups with presentations in plenum
15.30	Conclusion – Mette Holm, Födevarestyrelsen, DK

Speaker's abstracts

Olle Söder

Olle Söder, MD, PhD, is Professor and Head of the Department of Women's and Children's Health, Karolinska Institutet, and Senior Clinical Consultant of Paediatric Endocrinology at Astrid Lindgren Children's Hospital, Stockholm, Sweden. He has had multiple national and international assignments in the field of paediatric endocrinology and was the President of the Swedish Paediatric Society, 2012–2014. He has 200 hits on Web of Science on research covering reproductive biology, late adverse effects of paediatric cancer treatment, and health effects and mechanisms of actions of endocrine disrupting chemicals.

Endocrinology

Multicellular organisms need regulatory systems to integrate and fine-tune cooperation between cells, tissues and organs into functional units at the systemic level. This control is important in the entire life-span from the early prenatal stage to senescence. For such purposes the nervous system is responsible for immediate signalling via nerve fibres and synapses while the endocrine system works through soluble long and medium distance acting messengers (hormones) transported in the blood stream to mediate less rapid responses. Important functions under endocrine control are regulation of energy supply and consumption, reproduction, growth and development, and control of internal environment and homeostasis. This includes control of glucose metabolism, sexual differentiation and puberty, longitudinal growth, stress response, water and electrolyte balance, and blood pressure. Chemically, hormones constitute a mixed bag of biological substances including proteins, peptides, steroids and low molecular weight factors such as catecholamines and modified amino acids. Hormones act via well-defined receptor systems encompassing two different principles; the plasma membrane bound and the intracellular nuclear receptors. Endocrine disorders are typically of two major kinds; hypo- or hyperfunction; which may be due to defective ligands (hormones), receptors or post receptor signalling. The endocrine system is frequently subject to autoimmune perturbations resulting in disease. Many but not all endocrine axes are controlled by feedback circuits. Typical examples are the feedback loops executed by hypothalamic releasing factors acting on the pituitary gland to produce tropic hormones that stimulate peripheral endocrine glands such as the thyroid, adrenals and gonads. The hormones secreted by these glands act on target tissues, but are also sensed at the central levels to control their circulating concentrations by negative feedback. The set points for this control are believed to be programmed in foetal or early postnatal life. Endocrinology is probably

the clinical discipline in which the underlying physiology is most tightly linked to pathophysiology and disease. A good knowledge of the basic principles of endocrinology is required for a thorough understanding of endocrine disorders. Disruption of endocrine functions may lead to a multitude of disorders with immediate onset but also to late-onset consequences due to inadequate early programming of endocrine set-points for metabolic control and feedback circuits. The aim of this lecture is to give an overview of the endocrine system to set the scene for the forthcoming presentations and discussions on endocrine disruption.

Mattias Öberg

Mattias Öberg, PhD, ERT is Associate professor at Karolinska Institutet with a long experience across research and education within the field of toxicology and environmental medicine. He is senior research fellow with focus on risk assessment at Swetox, the Swedish Toxicology Sciences Research Center – a unique collaboration between eleven Swedish universities.

EDC – 2020, From Science to Policy

Some commentators argue that we have become addicted to safety: that the risk regarding chemicals has been hyped up – a bit of muck purges the stomach. Others look with concern as allergic disorders continue to rise among children, more and more young people have difficulties with concentration and learning, and as hormone-related cancers become increasingly common.

In my opinion, there is much to suggest that we may have underestimated the risks of chemicals in general, and EDCs in specific. Firstly: We have only studied a tiny number of chemicals. The proportion of environmental toxicants that are regularly measured in the population is just a few dozen of the 145,000 different substances that are registered in the EU. Secondly: We have done research on individual stages of the life cycle. Knowledge of the body's ability to form and develop new cells well into adulthood shows that we need to take a long view over entire lifespans. New results show that foetal exposure affects disease incidence in much later life. Thirdly: We have looked at one disease at a time. The immune systems and endocrine systems affect every cell in our bodies, and experiments have shown these systems to be susceptible to the effects of chemicals. Now we need to generate a new understanding of how the underlying mechanisms are interrelated and create effects that can vary from one individual to another.

Significant changes are already taking place in the view of how chemical substances can damage our health and how we should be working with chemical safety. We researchers have begun to speak in terms of "the new toxicology", which seeks the answers to how different substances affect cell function rather than at what amount of exposure individual diseases can be observed. The new toxicology presupposes that we are different as people, and that throughout the life cycle, we are exposed to a cocktail

of natural and synthetic substances. But to create a chemically safe society in the future, the new toxicology also has to be out there among the innovative chemical and biotechnological companies. The regulatory criteria for EDC have an important role to align science and policy. However, the European Commission has until today not been able to specify the scientific criteria for the identification of EDCs applicable to plant protection products and biocidal products, criteria that may have an impact on other pieces of EU legislation dealing with chemicals. We need therefore to consider the interface between science and policy as an increasingly important area for translational toxicological research, and strive to develop and use tools for an efficient translation of findings in toxicological sciences into risk assessment and regulatory practice.

Julie Boberg

Julie Boberg, Senior Scientist at the Technical University of Denmark, has a MSc in Human Biology from University of Copenhagen, and she finished her PhD in toxicology in 2007. She is an expert in reproductive toxicology, chemical mixture studies and histopathology in rodent studies. Since 2002, she has worked with research on endocrine disrupting chemicals in food and human environment, and she is also an advisor on endocrine disrupting chemicals and chemical mixture effects for the Danish Environmental Protection Agency and the Danish Food and Veterinary Administration. She is involved with several projects collecting exposure and toxicity information for endocrine disruptors and other groups of chemicals for cumulative risk assessment.

Potential endocrine disrupting chemicals in foods

Humans are exposed to endocrine disruptors from various sources, and our foods are among the important exposure sources. Endocrine disrupting chemicals may enter the food chain as contaminants from environment or food contact materials, or may be deliberately added as *e.g.* pesticides or food additives. Also natural components of our food may have endocrine disrupting potential, as seen for natural oestrogens in plants (phytoestrogens).

In this presentation, an overview of food sources of endocrine disruptors will be presented, and an introduction to current sources of exposure data (including a new Nordic report on phytoestrogens)[1] will be given. This will be put into context of past and ongoing projects on cumulative risk assessment of EDs from foods and other exposure sources.

[1] Phytoestrogen levels in food (Authors Linus Carlsson Forslund and Hans Christer Andersson).

Tero Hirvonen

Tero Hirvonen is Senior Researcher at the Risk Assessment Research Unit, Finnish Food Safety Authority Evira. He has an MSc in human nutrition and general toxicology and a PhD in epidemiology. He is currently working in the following projects as a principal researcher: risk assessment of polycyclic aromatic hydrocarbon compounds and heterocyclic amines, risk profile of plant food supplements.

REACH regulation: significance on risk assessment of endocrine disruptors in food

REACH regulation concerns *R*egistration, *E*valuation, *A*uthorization and *R*estriction of industrial chemicals. For all substances produced or imported in quantities of 1 ton or more per year, manufacturers and importers must submit a registration dossier to European Chemicals Agency (ECHA). ECHA can check the compliance of any registration dossier with the requirements of REACH, and examine and endorse the testing proposals provided by the industry (= dossier evaluation). The Member State competent authorities carry out substance evaluation and may require registrants to perform tests which are not necessarily required in REACH regulation. Authorisation will be required for each use of substances of very high concern: CMRs, PBTs (persistent, bioaccumulative and toxic substances), vPvBs (very persistent and very bioaccumulative substances) *etc.* identified as causing serious effects on humans or the environment. Authorisation will be granted for those uses if the manufacturer or importer is able to demonstrate that risks can be adequately controlled.

REACH also has a link to food: human dietary exposure is a part of exposure assessment of man via environment. REACH does not concern the use of chemicals directly to food. It should be noted that manufacturers and importers of chemicals are responsible for the safety of chemicals. Therefore, these actors should also take care that chemicals ending up in food is not a health risk.

EDs are tackled in REACH article 57: substances that should not be placed to market. These include substances meeting the criteria for classification in the hazard class reproductive toxicity category 1A or 1B, adverse effects on sexual function and fertility or on development (art. 57 c) and substances having endocrine disrupting properties (57 f). Identification of substances with endocrine disrupting properties is mainly based on information in the registration dossier and information obtained in substance evaluation. The majority of the information in dossiers is only published in ECHA website, especially for hazard information.

Tony Hardy

Tony Hardy is currently Chair of EFSA's Scientific Committee. Professor Tony Hardy is a biologist and environmental chemist with degrees at Oxford and Aberdeen Universities in the UK. He subsequently worked in the UK's Ministry of Agriculture, Fisheries and Food, which later became the Department for Environment, Food and Rural Affairs, at its Central Science Laboratory for 33 years before retiring. He has worked on the impact of agricultural pesticides on wildlife, the wider environmental impact of chemicals, the risk assessment and management of agricultural pests and diseases and food safety issues. Professor Hardy has been involved in national and international risk assessment, ecotoxicology and food safety for over 30 years. Prior to his appointment as Chair of EFSA's Scientific Committee, Professor Hardy chaired the EFSA Panel on Plant Protection Products and their Residues (dealing with pesticides) for 9 years since EFSA's establishment in 2003.

EFSA's Scientific Opinion on the hazard assessment of endocrine disruptors: Scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment.

Upon request of the European Commission, the Scientific Committee of the European Food Safety Authority reviewed existing information related to the testing and assessment of endocrine active substances (EASs) and endocrine disruptors (EDs). This work was conducted by a working group of experts in endocrinology, risk assessment and toxicology, together with observers from other EU agencies, namely EMA, ECHA and EEA. To distinguish between EDs and other groups of substances with different modes of action, it was concluded that an ED is defined by three criteria: the presence of i) an adverse effect in an intact organism or a (sub) population; ii) an endocrine activity; and iii) a plausible causal relationship between the two. As scientific criteria for adversity have not been generally defined, specific criteria for endocrine disrupting effects could not be identified. Hence, expert judgement is required to assess on a case-by-case basis the (eco) toxicological relevance of changes at the molecular to individual and/or (sub) population level following exposure to an EATS. The Scientific Committee concluded that a reasonably complete suite of standardised assays for testing the effects of EATSs is (or will soon be) available for the oestrogenic, androgenic, thyroid and steroidogenic modalities in mammals and fish, with fewer tests for birds and amphibians. Shortcomings in current tests and for other endocrine modalities and species were reviewed. Critical effect, severity, (ir) reversibility and potency aspects are part of the hazard characterisation of EDs. To inform on risk and level of concern for the purpose of risk management decisions, risk assessment (taking into account hazard and exposure data/predictions) makes best use of available information. Levels of concern are not determined exclusively by risk assessment but also by protection goals set by the risk management.

Pia Juul Nielsen

Pia Juul Nielsen is Chief Adviser at the Danish Environmental Protection Agency (EPA), Chemicals Division, with focus on endocrine disruptors and combination effects. She holds a Master in Pharmacy and has been working as consultant in toxicology and at the Danish EPA with regulatory toxicology and hazard and risk assessment of chemicals for more than 25 years. She has been responsible for the activities under the Danish strategy on endocrine disruptors for many years and has been the initiator of several activities in relation to endocrine disruptors and combination effects both nationally and internationally and further, coordinated the establishment of a Danish Centre on EDs. During the last years she has particularly been involved in work with the establishment of criteria for EDs and scientific discussions on low dose effects, non-monotonic dose responses and the existence of thresholds for ED effects. She worked in 2014–15 as a seconded national expert at the European Commission, DG Environment, on the EDs file.

Update on the EU Commission process on establishing criteria for identification of endocrine disruptors

According to legislation on Plant Protection Products (PPPR) (EC 1107/2009) and Biocidal Products (BPR) (EU 528/2012) the European Commission is obliged to establish scientific criteria for the determination of endocrine disrupting properties by 13 December 2013. Legislations on cosmetics and medical devices make reference to the criteria, whereas substances with endocrine disrupting properties are identified as Substances of Very High Concern under REACH by a case-by-case assessment. Furthermore, the 7th Environmental Action Programme commits the EU to develop harmonised hazard-based criteria for the identification of endocrine disruptors (ED) that can be implemented across legislation and to ensure the minimisation of exposure to endocrine disruptors. After several years of intensive work led by DG Environment with involvement of an expert group, member states and stakeholders, draft criteria were ready in June 2013 where after the work was set on hold. In particular, inclusion of potency in the criteria for ED identification has been disputed as this will result in fewer substances meeting the cut-off criteria in the legislation. In June 2014 the Commission launched a roadmap for defining criteria under the BPR and PPPR that included an impact assessment of 4 options for criteria and 3 options for regulatory approaches. In December 2015 the General Court of the European Union very clearly concluded in the court case where Sweden has sewed the Commission for not meeting the legal deadline for setting ED criteria in BPR, that the Commission should immediately comply with the law and ED criteria should be established based on science, only. In June 2016 the Commission launched a communication on endocrine disruptors accompanied by draft legal acts on plant protection products and biocidal products setting out criteria for determination of endocrine disrupting properties. These draft criteria have been intensely discussed among member states, NGO's and industry and at the Environment Council meeting 17 October 2016, it was questioned whether the

Commission has exceeded their legal mandate by changing the legal text and thereby lowering the protection level. Especially, the very high level of evidence required to identify endocrine disruptors, the lack of consistency with current legislation and the derogations in the PPPR to be based on negligible risk instead of negligible exposure have been criticized. The Commission has circulated revised draft legal acts 4 November addressing some of these issues and these will be subject for discussion and potentially voting at committee meetings on 18 November 2016.

Follow-up comment

After a process with several technical meetings and revised criteria proposals during winter and spring 2017, Member States representatives voted in favour of the European Commission's revised proposal on scientific criteria to identify endocrine disruptors under the PPPR at a SCOPAFF (Standing Committee on Plants, Animals, Food and Feed) Pesticides legislation meeting on the 4 July 2017. The text agreed will be sent to the Council and the European Parliament. They will have three months to examine it before final adoption by the Commission. Correspondingly, on the 4 September 2017 the European Commission adopted the scientific criteria for identification of endocrine disrupting properties under the BPR. The Council and the European Parliament have two months to raise objections to the legislation before its passage into law.[2]

Juha Laakso

Juha Laakso is Senior Adviser at the Finnish Safety and Chemicals Agency. He has a PhD in Pharmacology (University of Helsinki, Faculty of Medicine) and he is a European Registered Toxicologist, ERT. His PhD research was comprised of experimental studies on molecular mechanisms of salt-induced hypertension and metabolic syndrome. He has participated as an expert on cardiovascular toxicity and as one of the authors of EFSA Scientific Opinion on Erucic acid in feed and food, which was published in 2016 in EFSA Journal. In addition Juha Laakso was the main author of a study on cumulative risk assessment of pesticides, published in 2010 by Finnish Food Safety Authority.

Common assessment groups – experiences from a cumulative pesticide risk assessment

Pesticides are agents, which have been selected on basis of their biological adverse effects towards the pests being limited and thus inherently may give rise to risks also for human consumer. These substances are regulated under their own legislation including pre- and post-authorisation procedures. While the former deals with the

[2] Biocides: 4 September 2017, the Commission (2017/2100) decided on scientific criteria for the determination of endocrine-disrupting properties in accordance with Regulation (EU) No 528/2012 of the European Parliament and Council. It will apply from 7 June 2018. Pesticides: the 19th April 2018, the Commission (2018/605) amended Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties. It will apply from 20 October 2018.

acceptability of the substances in terms of their toxicity and exposure, the latter deals with acceptability of uses, MRLs and subsequent monitoring the commodities being sold. Up to 75% of the active substances, which were on the market in 1993 in EU, have been withdrawn. As a consequence, more active components are often used in a single formulation and found in the commodities.

The safety assessments are based on evaluation of substances on one-by-one basis. Although not yet in regulatory use, cumulative risk assessments have been developed, which are based on common assessment groups of pesticides (CAGs). A general endocrine mode of action is not as such feasible for CAG assignments, because of the plethora of underlying complex and overlapping systems, which even include counteracting components. Mostly CAGs are based on phenomenological effects. Few specific modes of action have been proposed, *e.g.* antiandrogenic activity to define a CAG.

In our work different CAGs were arbitrarily based on chemical similarity. We took all monitoring data available in Finland covering years 2002–2008 and subjected the data to probabilistic exposure assessment. New dietary intake data were generated for adults in addition to 2 and 4 year old children on basis of available national surveys. Any data were not obtained for pregnant women. While legislation pays specific attention to protect pregnant women, the issue is practically ignored in the regulatory EU risk assessments. For a singleton pregnancy, mean total energy cost corresponds to approximately 77,000 kcal, *i.e.* to a mean 30% increase in energy requirements of the mother.

Dietary exposure to residues of active substances is characterized by a low chronic exposure level, on which higher acute exposures occasionally take place. Cumulative acute carbamate and organophosphate exposure showed the acute reference dose (aRfD) in children was exceeded with a probability higher than 0.1% (*i.e.* more than in one case out of thousand). While the last two years of the time period showed a gradual decrease of the probability, acceptable levels were still not reached for three-year old children. Most of the exposure was derived from commodities imported from outside of EU.

Cancer risk could not be studied using this left-censored data as the MRL (*i.e.* limit of quantification, LOQ) levels already indicated risk. Epidemiological literature comparing different profession groups shows that farmers generally have the lowest overall and specific risks for cancer in the Nordic countries. One epidemiological study linked the annual amounts of pesticides sold to changes in gender ratios of Finnish general population. However the authors stated that the change had already started in 19th century and thus cannot be a consequence of environmental chemicals. There are many underlying environmental factors derived from nutritional, chemical, physical (radiation), life-style and biological (infections) environment of humans, the role of which have not been fully worked out. In Finland low intake of iodine raises concerns for thyrotoxic substances. Careful risk communication is essential, in order not to undermine the already successful preventive messages concerning *e.g.* cancer and other well-established risks of cigarette smoking.

Johanna Suomi

Johanna Suomi (PhD in Analytical Chemistry, title of docent in Pharmacognosy at University of Helsinki) is an expert in dietary chemical risk assessment. She works as Senior Researcher at Risk Assessment Unit of the Finnish Food Safety Authority Evira and she is also member of the EFSA Scientific Network on Chemical Occurrence Data. Recently, she was main author in a risk assessment research project on dietary heavy metal exposure of Finnish preschool children, which also briefly covered cumulative exposure to several heavy metals.

Dietary heavy metal exposure of Finnish children: risk assessment and risk management decisions

Heavy metals cadmium, lead, arsenic, and mercury are elements and thus found widely in nature, but pollution may increase their levels in industrialized areas. The levels of these contaminants in many foods are controlled by (EU) Regulation No 1881/2006. Exposure to heavy metals can occur through many pathways, but the main exposure for Finnish children was through food.

The toxicological reference values set for these heavy metals are based on their ability to damage the developing central nervous system or the kidneys, or on increased cancer prevalence (inorganic arsenic). In addition, these four heavy metals have been named in literature as metalloestrogens and they may also have other endocrine mimicking activity, either through replacement of zinc in zinc fingers of an oestrogen receptor, or through actually binding to a glucocorticoid receptor. The endocrine activity of heavy metals has not yet been widely studied, and no dose-response values for the effects have been reported yet.

The dietary heavy metal exposure of Finnish children of 1, 3 and 6 years was determined through probabilistic exposure assessment using individual consumption data and concentration data mostly consisting of control sample results. The exposure levels were found to be slightly lower than previously estimated by EFSA, but the tolerable weekly intake value for cadmium was exceeded by a large portion of all age groups, and the lowest benchmark dose levels for lead and arsenic were also exceeded by a part of each age group. The heavy metal exposure of some of the children is therefore at a level that cannot be guaranteed to be safe.

Different risk management practices are in place in Finland to decrease the heavy metal exposure. Tightened maximum levels in Commission Regulation (EC) No. 1881/2006 are of use in helping to decrease the exposure at EU level. National recommendations on fish use and on avoidance of rice drinks as only drink also help. On the basis of the new risk assessment, no new limitations were put in place. Instead, the risk communication concentrated on the importance of a diverse and varied diet in moderation, as it will help to avoid high levels of contaminants and the adequate intake of nutrients also gives some protection. New information was also published on the Evira web pages for the consumers' use.

Anne Marie Vinggaard

Ann-Marie Vinggaard, is Professor at the National Food Institute, Technical University of Denmark, where she is heading the Molecular Toxicology group. She has more than 20 years of experience within toxicology with a focus on endocrine activity of chemicals, cocktail effects of chemicals and mechanisms of toxicant action.

An alternative approach for prioritizing chemicals for in vivo testing of male reproductive health effects

Today, humans are exposed to a great number of chemicals, and every man, woman and child is carrying with them a chemical footprint of their environment. Research increasingly suggest that this chemical burden can impact on human health, especially for people that are most exposed. The number of chemicals currently in use is estimated to be 40–80,000. Among these, we only have sufficient information to carry out full risk assessments for about 1000 chemicals. This means that there is a huge gap in our knowledge pertaining to potential adverse effects of chemicals on biological systems, including humans.

The current paradigm for chemical risk assessment is based primarily on animal studies, which are valuable but also resource-demanding. Thus, there is a pressing need to develop alternative methods for risk assessing chemicals. To tackle the sizeable societal challenge of safeguarding humans against all environmental chemicals, an integrative approach using technologies from chemistry, biology and bioinformatics is needed. One such approach is the use of a panel of *in vitro* assays utilizing primarily human cells for toxicity prediction, coupled with physiologically-based kinetic (PBK) modelling approaches to derive an alternative to the traditional "Acceptable" or "Tolerable Daily Intake" used for risk assessment of chemicals.

Here, we will showcase a promising integrated approach based on *in vitro* profiling, combined with PBK modelling for predicting adverse effects on male reproductive development. Predictions were made for nine pesticides, of which three were selected for *in vivo* testing and subsequently verified to affect male reproductive development *in vivo*. These results hold great promise for improved protection of humans and a more sustainable development in the chemical field.

Mette Holm

Mette Holm, is a biologist and scientific adviser in the Danish Veterinary and Food Administration. She has for more than 10 years worked with chemical food safety in relation to e.g. migration from food contact materials and pesticide residues in food.

A risk management dealing with a potential endocrine substance in food and food contact materials

Endocrine disrupting chemicals (EDCs) – or suspected endocrine disrupting substances, can be found in food. One of the sources of EDCs in food is food contact materials *i.e.*, packaging material or equipment in contact with food. In Denmark the Veterinary and Food Administration have focus on EDCs in food contact materials (FCM). There are several steps to take in order to risk manage EDCs in FCM. Some of which have been taken are to reduce the intake of bisphenol A and fluorinated substances from FCM of paper and board.

Anna-Maria Andersson

Anna-Maria Andersson is a biologist (cand.scient.) with a Ph.D. in cellular and molecular biology. She is senior researcher and laboratory leader at Department of Growth and Reproduction, Rigshospitalet, University of Copenhagen, Denmark as well as part of the research and management team of the International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), same place.

In addition, she is the leader of the Danish Centre for EDs, which is a knowledge generating centre financed by the Danish government (Kemikaliehandlingsplanen 2014–2017) and refers to the Danish Environmental Protection Agency.

She has more than 20 years expertise in human male reproductive development and physiology; how male reproductive hormones function, how they are regulated, and how they are affected by environmental factors.

Human health effects of EDCs – clues from epidemiology

The research area of endocrine disruption was from the start spurred by observations of increasing trends in the incidence of hormone related diseases and conditions in wildlife and human populations; especially adverse trends related to reproductive health. Although trends in some reproductive problems seen in human populations have slowed down in some countries, they do not seem to have reversed (for review see Skakkebaek *et al.*. 2016)

The fact remains that endocrine disruption is occurring in human populations; perhaps best documented by the increase in incidence rates of cancer in some endocrine organs, including breast-, testicular-, pancreas- and thyroid cancer. In

particular, trends in the incidence of testicular cancer can be viewed as a "whistle-blower" for adverse male reproductive health trends. Environmental factors are undoubtedly involved in these adverse trends. Based on the endocrinology of the observed disease trends and on the results of mechanistic and "proof-of-concept" laboratory studies, endocrine disrupting chemicals (EDCs) are among the prime suspects. Human biomonitoring studies have documented widespread human exposures to a large range of known and suspected EDCs. The realistic general human exposure scenario in most developed countries is therefore characterised by mixed and simultaneous exposure to multiple EDCs; albeit human exposure levels for individual compounds are generally at much lower levels than those observed to cause adverse effects in experimental animal studies.

Increasing incidences of cancer in hormone sensitive organs is presumably just the tip of the iceberg of environmental endocrine disruption effects in the populations. Environmental endocrine disruption is also likely to impact on fertility, fecundity, metabolic diseases, immunology, neuroendocrinology, and general health and comes with great personal and societal cost.

Cathrine Thomsen

Cathrine Thomsen (Dr. Scient.) is Director of the Department of Environmental Exposure and Epidemiology at the Norwegian Institute of Public Health. She has more than 18 years of experience in environmental medicine and analytical chemistry, with key expertise in the field of human exposure to environmental pollutants, particularly using human biomonitoring approaches, and the linkage and use of such data in epidemiology. She is presently involved in the EU projects A-TEAM (Marie Curie ITN), HELIX (7FP), Euromix (H2020) and HBM4EU (H2020).

Exposure to possible EDCs in a Norwegian population – assessment of total exposure, examples

A study group of 48 households with mother-child pairs was established by the Norwegian Institute of Public Health during spring 2012. The overall aim of the cohort was to serve as basis for two research projects assessing human exposure pathways to phthalates, BPA and flame retardants by comparing estimates of exposure from the indoor environment and food with integrated biomarkers of exposure measured in blood or urine. The recruitment was done by sending invitations to households through nine different primary schools. A total of 700 different samples were collected comprising urine, hair, saliva, blood, indoor air and dust. Information on dietary habits, the indoor environments, and different factors related to lifestyle including use of cosmetics were recorded through interviews and questionnaires. Methods for chemical analysis of the biomarkers were established and validated. An overview of the total exposures assessed so far will be given.

Henrik Kjellgren

Henrik Kjellgren is Product Manager at Nordic Paper AB. He has 18 years with the company at different positions such as Area Sales Director, Manager Technical Support & Product Development, Development Engineer, Process Engineer, Laboratory Engineer. Education: PhD (Influence of paper properties and polymer coatings on barrier properties of greaseproof paper) 2007 at Karlstad University.

Natural Greaseproof – an alternative to the use of fluorochemicals in packaging paper

There are already existing natural alternatives to fluorochemical treated papers on the market. The differences between a Natural Greaseproof paper (Nordic Paper AB) and a paper treated with fluorochemicals will be explained in the presentation.

Grease-barrier properties in paper are required in several end-use areas, in particular paper wrapping for sandwiches or other greasy and fatty foods. In these applications, as well as for baking paper and paper for baking cups, Natural Greaseproof paper is available on the market and does not have any fluorochemicals added. When a Natural Greaseproof paper is produced, the wood fibres are treated mechanically in a refining process. This process causes the fibres to bind very tightly together, resulting in a paper surface with a very dense layer of pure cellulose. In contrast, a fluorochemically-treated paper is a more open structured paper, and the fluorochemicals provide the chemical repellence of the grease.

Hence, there are two alternative options for manufacturing a grease-proof packaging paper: a natural method using no additions, where a mechanical treatment changes the physical properties of the cellulose fibers, or a chemical method that uses addition of hazardous fluorochemicals. Natural Greaseproof paper is produced without any addition of fluorochemicals but traces of fluorine can bee detected due to natural occurrence of fluorines in the raw material. These values are however much lower than those detected in fluorochemically-treated papers.Therefore, it is of greatest importance that official limits for fluorochemical doesn't exclude the use of natural alternatives. Natural alternatives for baking paper, baking cup, and wrapping papers for fatty foodstuffs should be promoted.

Euros Jones

Euros Jones has worked for the European Crop Protection Association (ECPA) since May 2001, and has held the post of Director, Regulatory Affairs since January 2006. Euros is Welsh and holds a bachelor's degree in Agricultural Economics from the University College of Wales, Aberystwyth. He has worked in Brussels since 1994, having previously worked as Deputy Director of the Brussels office of the UK National Farmers' Unions, and as Secretary General of the European Council of Young farmers

(CEJA). In his current post, Euros' responsibilities include supporting ECPA's advocacy on regulatory issues, with a particular focus on the implementation of the new Regulation on the Placing of Plant Protection Products on the Market Regulation 1107/2009.

Pesticide producer's view

EU Regulation 1107/2009 introduced new hazard based authorization criteria for plant protection product active substances. Annex 2 of the Regulation include criteria that active substance shall only be approved if it is not considered to have endocrine disrupting properties that may cause adverse effect in humans (point 3.6.5.) or non-target organisms (3.8.2). While interim criteria are included in Regulation 1107/2009, the European Commission is currently finalising draft measures concerning specific scientific criteria for the determination of endocrine disrupting properties.

The crop protection sector has concerns with the Commission's current proposal, as many substances which present little or no concern to human health or the environment will be identified as endocrine disruptors. The criteria for decision making need to provide tools to identify those substances which have a real potential to cause harm, from those that do not. To do this, we believe that the criteria should incorporate key elements of hazard characterisation – the Commission's own Impact Assessment concludes that this would have less impact on agricultural competitiveness and trade while offering the same high level of protection for human health and the environment.

The application of the proposed criteria will have a substantial impact on agriculture and trade. Recent evaluations have highlighted that EU production would be impacted with farmers being more dependent on a smaller number of crop protection tools – which raises concerns about managing pest resistance. Information is also available on the potential negative impact on food and feed imports into the EU if Maximum Residue Level (MRL) trading standards are removed.

Malene Teller Blume

Malene Teller Blume, is Nonfood Quality and CSR Manager At Coop Danmark A/S. She has worked in Coop DK for almost 15 years and is responsible for Coop's Quality and Safety program for nonfood consumer products. She is responsible for Coops Chemical Strategy and therefore closely involved with chemicals in consumer goods, and responsible for Coop's approach and requirements in this important area. Appointed member of Boards: Dansk Miljømærkenævn (Environmental labels, Nordic Swan and European Flower), Kosmetikrådet (administered by Danish Environmental Agency) and ChemSec's Business Group (International NGO, owner of Sin-List, other co-member are multinationals brands and retailers like Apple, Adidas, Ikea, Dell, Sony, Skanska, Boots). Education: 1995, Profession Bachelor in Nutrition and Environmental project Management, Suhrs Seminarium.

Trade organization's view

Coop has a long and proud tradition of concern about harmful chemicals, and for many years it has set up requirements which go beyond the legislation. Our responsibility is therefore not only to ensure that Coop meets legal requirements, but also to secure that Coop takes the necessary responsibility and covers known risks for chemicals in consumer products. This applies to both environmental and health issues.

Coop has especially focused on using the precautionary principle. For example, endocrine disruptors in chemicals have a very high priority. She is also responsible for Coop's program for social compliance (CSR).

I will tell about how Coop has worked to identify and phase out potential EDCs in food packaging. When it comes to EDC, Coop Danmark have used the precautionary principle and banned them in private label products. We recognize the long term risk, *e.g.* from the concern from the cocktail effect.

We do not think that the legislation covers the risk for the consumers and environment, especially not when it comes to fluorinated compounds. So we have banned all PFC's in food packaging in 2014. There are other and useful alternatives. But Coop did struggle with one product, Microwave Popcorn. And our suppliers could not inform, when a substitution was possible, and then we decided to remove all microwave popcorn in May 2015.

We have also banned BPA in cans and lids, and are still working on to remove them from all private label brands.

We really encourage that the criteria and regulation for the EDC will be settled and clear as soon as possible. We realize that many suppliers have difficulties to control this, when it is only Coop demanding PFC free packaging. If it was a legal requirement, it would be much easier to handle. We strongly recommend that when it comes to PFC´s we need to regulate them with a group approach. Our consumers are with us in our actions and I will show some good business cases for safe substitutions.

Claus Jørgensen

Claus Jørgensen, is Senior Project Manager at the Danish Consumer Council THINK Chemicals. Claus Jørgensen has been working on the issue of 'unwanted' chemicals in consumer goods for almost 20 years, the last 12 years at the Danish Consumer Council.

In 2014 he started the project Danish Consumer Council THINK Chemicals, which is a government funded project. Based at the Danish Consumer Council, we are a team of six people, who help consumers avoid problematic chemicals in their everyday life. We test consumer products in order to provide consumers with information on how to live a toxic free life.

We are the consumers' chemical watchdog. In our tests we examine consumer products such as earphones, diapers, cosmetics, chewing gum, food, food packaging, cleansing tissues and much more.

Children and pregnant women are particularly vulnerable to problematic chemicals. Consequently we put in an extra effort in testing consumer products aimed

at these groups - so far we have tested for example pushchairs, cleansing tissues and loom bands.

Danish Consumer Council THINK Chemicals creates working relationships with other NGO's in Denmark as well as in other countries of the European Union. That is to supplement the efforts of the authorities with a strong and common NGO voice.

Danish Consumer Council THINK Chemicals is independent of authorities, business communities and political parties. Our activities are continuously coordinated with the Danish Environmental Protection Agency – mainly as regards tests and examinations of health and environmentally damaging substances in consumer products.

Chemical surprises – what might also be in the food?

In 2016 the EU parliament has published an own initiative report on the regulation of FCM. The report was drafted by rapporteur Christel Schaldemose (DK) and had focus on the lack of regulation of FCMs.

At the DCC THINK Chemicals we map the unwanted chemicals in FCMs. In 2016 we performed a number of tests on FCMs, and I will present our findings. We have tested among others these products: canned tomatoes, canned mackerel, jars of jams, jars of pesto, pizza boxes, cake wrappings and canned coconut milk.

The results show that substances which the EU parliament, but also authorities around Europe, are calling unwanted, are present in the products that ordinary people buy and eat every day.

Per Ola Darnerud/Kettil Svensson

Per Ola Darnerud, Assistant Professor in Toxicology, has a long experience in food toxicology issues at the National Food Agency (NFA), Sweden. His main present focus is exposure analysis, *i.e.* using different methods to estimate intake on individual, or population level, for compounds present in food.

Kettil Svensson, is a Risk Assessor and Toxicologist at the NFA, Sweden. His focus has been on food contact materials for many years both within Nordic cooperation, Council of Europe and EFSA. Other areas include chemicals in drinking water and radioactive contaminants in foods.

Exposure assessment of potential endocrine disrupting chemicals in foods

Exposure assessment is a crucial part of the risk assessment procedure, forming the bridge between hazard and risk. Thus, exposure assessment activities play an important role in risk benefit assessments made at the NFA, Sweden. In the traditional assessment, the exposure is subsequently compared to reference points for adverse health effects, beneath which the exposure should be without risk. The assessment of carcinogenic compounds is somewhat different, but routines for assessing these

compounds have also been developed. However, risk assessment methods for EDCs are still much debated, including the issue of how exposure data should be dealt with.

At present, exposure studies at NFA are performed by the use of both external and internal methods. External methods use production and purchase statistics or consumer questionnaires to obtain data on estimated consumption on population or individual level. Internal methods for assessing exposure mean that the actual body burden of the actual compound, or its metabolite(s), is measured by analysing levels in *e.g.* blood, urine or breast milk. For both these methods, the risk assessment is made by comparing the exposure values with reference points for adverse health effects.

Focusing on exposure assessment of EDCs, there are several challenges, including the low doses and various exposure pathways that must be covered, possible contamination of food samples, the potentially fast degradation, and how to deal with "natural" EDCs, *e.g.* phytoestrogens. By studying some examples of potential EDCs, *i.e.* BHA (a food additive), several pesticides and phytoestrogens, the margin of exposure (MOE) seems to be sufficiently high, at least when comparing to generally acknowledged reference points. However, there are much new data questioning the use of only "established" end points in toxicity testing, and the dose-effect relationship is also under debate, as new low dose effects are reported and the dose-effect curve might have new, non-linear shapes. Thus, even if traditional exposure methods in many cases find EDCs in food at relatively safe levels, development of methods for risk assessment of EDCs are needed.

Workshop: Summary of questions and answers – group discussions

In the group discussions representatives from Nordic food authorities, Nordic chemical, environmental and public health agencies, EFSA, Nordic research institutes, trade organisations and producers (food and packagings) participated. However, considering the small representation of various areas within the food chain, the summarised answers below reflect the ideas and views of those present and should not be interpreted as consensus views of the food sector.

Day 1 Risk assessment

Question:

Food can contain endocrine disruptors that are intentionally added (*e.g.* food additives), of natural origin (*e.g.* phytoestrogens in soy products and mycotoxins), or contaminants (*e.g.* dioxins). Which food-derived endocrine disruptors are the most problematic from a health point of view?

Answer:

Contaminants that are already present (and emerging ones) in the environment and cannot be regulated, especially persistent ones. On the contrary food additives have been thoroughly investigated and natural compound have always been present.

Question:

Should intentionally added chemicals be assessed differently from unintentionally occurring substances? Or should intentionally added chemicals be assessed differently from naturally occurring substances?

Answer:

They should all be assessed in the same way, but risk management may be different. Since it is not possible to perform a full risk assessment of all EDs, *in vitro* profiling could be used for prioritizing chemicals for *in vivo* testing.

Question:

Should all endocrine disrupting effects be assessed in the same way, regardless of the severity and type of effects on human health?

Answer:
They should all be assessed in the same way, but risk management may be different. Since it is not possible to perform a full risk assessment of all EDs, *in vitro* profiling could be used for prioritizing chemicals for *in vivo* testing.

Question:

How important is the exposure to EDs in food? Are other routes of exposure more important? In some cases the ED effects occur at doses far higher than the dose that provides the critical effects for the setting of TDI/ADI.

Answer:
It depends on what compounds we are talking about. Food is probably the most important source of EDs in general followed by cosmetics, dust and substances in contact with the skin. But for some individuals, skin/lung uptake (indoor/outdoor) or use of medicines may be equally or more important. One problem is that knowledge about exposure routes is often insufficient. ED effects even at high doses are still relevant to determine to consider the possibility for combination effects. Furthermore, many substances are not tested in ED relevant tests or with test methods that include endpoints relevant for detection of endocrine disruption.

Question:

Is the concept of TDI/ADI appropriate for regulation of endocrine disruptors considering that they may have effects at low-doses or no threshold dose? Is it necessary to use other approaches in risk assessment such as specific uncertainty factors or margin of exposure (MoE, as for genotoxic carcinogens) or something else?

Answer:
TDI/ADI is not always appropriate – multiple pathways may be affected at different doses – may appear as a non-monotonic affect. Mode(s) of action are critical for setting any safe dose. Margin of Exposure (MoE) might be used if no ADI/TDI is available. An alternative approach is to use specific or extra uncertainty factors. Risk assessment of mixtures is a further problem.

Question:

What are the present sources of information on EDs (*e.g.* risk prioritization based on *e.g. in silico* methods like SAR, research)? What information about EDs is the most urgently required?

Answer:

In silico methods may be used to identify endpoints/critical organs and some mode of action (MoA). It is important to know whether the chemical will affect the unborn foetus. This can be done *in vivo* or by applying *in vitro*/PBPK modelling approaches in the future. In addition, indications from epidemiological studies may be used. Also information on possible "cocktail effects". Further, it is important with adequate and transparent information of chemical used downstream from the chemical producer (or raw material producer) of the supply chain, all the way to the end-user, the consumer. Another priority is to set the criteria for EDs. Sources of information on EDs may be found at ECHA, EFSA, US EPA, databases (*e.g.* VEGA in Italy and one in DK (QSAR)).

Question:

Presently, epidemiological studies implicate the "usual suspects" (*i.e.* phthalates, bisphenols, fluorinated compounds, PCBs, parabens, pesticides) as causes to endocrine disruption. How can we in the future identify and stratify EDs that confer large impact on human health?

Answer:

Epidemiological studies on food intake/specific subpopulations (Nordic collaboration, large enough studies) are important. Follow subpopulations with high exposure (*e.g.* occupational). Should be a *non-silico* approach in order to get the full picture. Projects like HBM4EU and other biomonitoring studies/projects. More cooperation between big bodies, like WHO, FAO, EFSA. Criteria for suspected ED's is also needed, to set up a category approach as for CMR substances. This would stimulate research and identification of ED's.

Day 2 Risk management

Question:

EDs in food can be derived from different sources: Should broad risk management be directed more to EDs in regulated areas (*e.g.* plastic food packaging materials, food additives, pesticides) than to unregulated EDs (*e.g.* non-plastic food packaging materials, some environmental compounds, natural compounds) and why?

Answer:

It is easier within regulated areas, since risk assessments are available. Unregulated areas are more important but more difficult. Environmental contaminants should be stopped at the source, then you do not have to take care of the problems downstream and do the risk management for *e.g.* food.

Question:

Should the control measures be risk-based?

Answer:
Yes, if possible.

Question:

What are suitable measures for assessing the effects of control and risk mitigation management to lower the risk of exposure? For example by legislation (banning/limiting the use of chemicals) or information (via food consumption recommendations and risk communication).

Answer:
Both ways, however, best if legislation is strict enough to make all food safe. Sometimes you have to combine maximum limits with recommendations/advice to vulnerable groups (example raisins for small children in DK; ochratoxin A risk). A dialogue with industry to lower levels of pesticides or other contaminants in food may be another option. It is easier to choose risk management options that are easy to perform and gives large health effects, rather than the ones that require a big effort with less benefit for health.

Question:

What are the positive and negative consequences of ED restriction in food for different stakeholders (food industry, retail, authorities and consumers)? For example a positive for the food and retail industry could be supporting a company profile as sustainable and responsible. A negative consequence might be that there is a lack of alternatives for *e.g.* pest control.

Answers:
At the end of the day, we should focus on the consumers. Do you know what the consumers want? Are they informed enough? Most are worried about chemicals/pesticides! We need to help consumers prioritize! What is really healthy for you! How do you reach the consumer with information? Social media instead of webpages! If some chemicals that are important for farmers or food producers are not approved due to identification as EDs the consequences may be that food prices rise and more food is wasted. Some industry request rules to follow, such as for FCM paper and board. Some companies can benefit from a weak or unclear legislation and thus phasing out EDs or mark their products with "green labels". A strict harmonized legislation could drive innovation. In summary: Positive consequences: image of companies, safer food. Negative consequences: scared consumers, high costs for testing and developing safer chemicals.

Questions:

What are the consequences for industry in applying ED definition according to Option 1 or 2–4 (see below for clarification)?

Table 3: European Commission roadmap defining criteria for identifying endocrine disruptors

Option	
1	No criteria are specified; the PPPR and BPR interim criteria continue to apply
2	WHO–IPCS definition to identify EDCs (hazard-based)
3	WHO–IPCS definition to identify EDCs plus introduction of additional categories based on the different strengths of evidence (namely "suspected endocrine disruptors" and "endocrine-active substances")
4	WHO–IPCS definition to identify EDCs plus inclusion of potency as element of hazard

Source: DG SANTE, June 2015.

Answers:

- Option 1: Gives false positives and false negatives.
- Option 2: More "true" EDs will be identified.
- Option 3: Same as for option 2 and in addition, this enables authorities to request for additional data. Includes also "Suspected EDs" and increased burden for industry (more data).
- Option 4: As for option 2, however, fewer substances will be identified as EDs. Even more burden for industry (more data).

Question:

What are the plus and minus of applying the substitution principle – replacement?

Answers:

There is a "risk" for substitution to worse chemicals? Food legislation/FCM is not detailed enough (based on migration) for applying the substitution principle. There is a need to force industry to move away from "bad" chemicals.

Option 1 is not a good definition for EDs, since it can give false positive and false negative. It could impact different substances differently. The problem is that the legislation is there, and now you try to fit the criteria with the legislation (e.g. from negligible exposure to negligible risk).

Question:

How can the regulatory framework take into account the cocktail effect *i.e.* that the general population are concurrently exposed to multiple substances with potential endocrine disrupting effects that may enhance or counteract each other?

Answers:
Risk assessment must take into account cocktail effects. There is a need of priority tools to identify the most contributing compound in "the mixture". An option may be to add an extra uncertainty factor?

Question:

How can Nordic Managers impact EU legislation concerning EDs, in particular, most efficiently?

Answers:
The Nordic synergy effect! Support each other's actions! Presenting new scientific knowledge!

Question:

When the criteria for EDs are established, how should they be implemented in other areas than pesticides/ biocides?

Answers:
They should be written in the guidelines for risk assessment. It is important with more scientific knowledge and there should be exchange between different areas.

Question:

Are the costs for replacing/substituting some of the EDCs proportional to the benefits for health and the environment? Could the limited resources be used in a way that increases health more?

Answers:
There is no simple answer. There are possibilities for development/innovation. There is a need to support those who develop alternative methods/promoting alternatives. There should be time lines for phasing out – give time to find alternatives.

Additional Questions and Answers to companies within the food sector

A questionnaire was sent out to some trade organisations within the food sector in the Nordic countries on behalf of the Nordic Food Authorities. Three companies replied and you will find their answers below.

Question:

Are you aware of any potential endocrine disrupting chemicals in your products and the sources of them (*e.g.* natural compounds: phytoestrogens; pollutants: perfluorinated – compounds; food contact materials: phthalates; residues of pesticides or biocides). Are you aware on impacts and risks of EDCs in food?

Answers:

- *Company 1)* We are aware of potential PCB and dioxin residues in some foods, originating from environmental pollution. Their harmful effects are known to us. Regular studies are conducted for these chemicals (sampling by authorities and according to in-site control system).
- *Company 2)* Naturally occurring phytoestrogens are fairly common in foodstuffs, but their possible physiological role is not well understood. Many of them are considered beneficial rather than detrimental, but there is also disagreement (*e.g*. soy-based infant formulas). They cover a wide variety of structurally different compounds such as isoflavones in soy, lignans in grains, and stilbenes in grapes. Unwanted exogenous EDCs such as pesticides, biocides and other EDCs may find their way to food if production chain contains weak links (*e.g.* dioxin contamination in animal feeds in Belgium some years ago). Food contact materials are a known potential source of EDCs (*e.g.* plasticizers).
- *Company 3)* We are aware of potential EDCs in our products. Known EDCs are included in all risk assessments on all raw materials and food contact materials (FCMs). Our priority is on certain foods and food contact materials. Our raw material specifications give instructions on pesticide residues, and the materials are controlled by analyses in accordance with an annual schedule. Records demonstrate that we keep pesticides under control.

Question:

What are the positive and negative consequences of ED restriction in food for different stakeholders (food industry, retail, authorities and consumers)? For example a positive for the food and retail industry could be supporting a company profile as sustainable and responsible (an example is the phase out of potential EDs in consumer products). A negative consequence might be that there is a lack of alternatives for *e.g.* pest control.

Answers:

- *Company 1)* Food produced/grown in cleaner environment may get an advantage in the market together with a better price.
- *Company 2)* Reduction of the excessive EDC burden is good for all the stakeholders, but contradictory information about their possible health impact, good/bad/neutral (*e.g.* bisphenol A), makes it very difficult to determine what kind of restriction would make sense. From the production chain perspective the challenge is in particular the limited or non-existent availability of alternative solutions.
- *Company 3)* Our company – producing and distributing food to consumers and catering – base their existence on trust. We will not, and cannot, put consumers at health risk or contribute to environmental damage. We are dedicated to follow regulatory limits and, where such do not exist, the latest scientific recommendations. However, we need to be confident that the alternative(s) to the EDCs in question have been thoroughly tested and that there has been a science based process and conclusion for the change. A process which should not be repeated is the regulatory process of removing BPA, where national provisions were implemented before scientific research and industrial development had concluded on safe and available alternatives. Additionally, we must be confident that laboratories are accredited for the specific analyses. The analytical methodology must be validated and give true results.

Question:

Are the costs for replacing/substituting some of the EDCs proportional to the benefits for health and the environment? Could the limited resources be used in a way that increases health more?

Answers:

- *Company 1)* There are multiple challenges ahead to ensure healthy food, and a priority list, where the microbiological and food fraud risks come on top, will be supported. However, in our opinion, EDCs represent one of the most serious threats to humans and wildlife in our time. EDCs that can be safely replaced, should therefore be replaced, and efforts to avoid and restrict their entrance to nature should be established. The signs of a nature balancing on the edge, where

hormonal processes are disrupted and where species disappear in an increasing speed, should be a warning sign for all stakeholders. The good stories, like banning of/restriction on DDT, dioxin and PCB, and the following recovering of wildlife in some threatened areas, should be an inspiration to us all. Resources to research and communication on EDCs should be given high priority. This work needs involvement from a wide range of stakeholders, as mentioned initially.

- *Company 2)* Benefits can be evaluated at public health level but industry see only the possible additional costs; benefits/costs need to be determined at national level. The need for replacement needs to be scientifically proven and the availability of substitutes has to be confirmed before making changes.

Question:

What are the plus and minus of applying the substitution principle – replacement of chemicals (pesticides, biocides, food-contact materials and food additives)?

Answers:

- *Company 1)* If an ingredient or a substance is proven to contain a health-threatening effect, and a company – knowing this – continues to use it, it will destroy their trust and reputation. To oversee the scientific evidences for health-threatening substances will cause dramatic consequences for a food operator. The cost of replacing a substance is minimal compared to the loss of reputation, trust and business they then will face. However, the alternative(s) must be based on science. They must be tested, reliable and proven safe. In case of strong opinions based on false assertions, we may need (and will expect) communication support from Food Safety Authorities.
- *Company 2)* If the need for replacement is scientifically sound and applies to all the actors it is positive for all the stakeholders. If technically viable alternatives do not exist it will be a burden to the value chain. If replacement is not economically viable it will hurt export industry unless the same restrictions apply globally.

Question:

In your sector, what are the best suitable measures to lower the risk of exposure: by legislation (banning/limiting the use of chemicals), by information (via food consumption recommendations and risk communication), or by substituting to safe chemicals? Have you discussed or used any risk mitigation measures (*e.g.* as described above) for EDCs in your company?

Answers:

- *Company 1)* Lowering the environmental pollution by legislation. Informing people about the risks associated with different foods (guidelines for usage, *i.e.* frequencies of consumption).
- *Company 2)* The best suitable measures will be different on different product categories. Legislation is only the final step. If legislation does not allow a substance in a material, or describes a defined limit to follow, we will follow that. However, keeping ourselves up-dated on scientific literature and participation in sector federations/ association projects is just as important, giving us understanding of the scientific development on health-related issues, enabling us to establish measures before the regulations are in place. A good example is the international and intercompany acrylamide projects. Other examples are the cod liver oil purification process, selecting the cod size and age for cod liver used for cod liver oil and cod liver products, and reformulating products to reduce the content of specific EDCs that cannot be avoided. Information and risk communication from authorities to manufacturers when specific issues are in an early stage of research or monitoring is very important. Authorities and manufacturers will then be able to work out common projects and common communication tools for mutual benefit. Finally, our most important key to controlling EDs lies in the supply chain. We have developed a Supplier Approval System and a Material Approval System, including supplier self-assessments, a supplier audit programme performed by trained auditors, material specifications and data sheets, DoCs for FCMs, verification measures (documentation control and analyses) etc. If a supplier fails to comply, he will be removed as a supplier, and if a material fails, it will be replaced.
- *Company 3)* Legislation, based on scientific risk assessment, is preferred as it treats all the actors in an equal manner. Information is a good addition but world is already full of misinformation and it is difficult for citizens to judge which information is based on solid basis and valid sources, and which is not. Our organization has made its homework concerning this topic and risk mitigation plans are in place.

Conclusion

Endocrine disruptors are substances that interfere with the functioning of hormones, with potentially harmful effects on health. A wide range of chemicals are suspected of being responsible for endocrine-disrupting activity. Defining scientific criteria for their identification is highly complex and has important consequences for a wide range of stakeholders. There is a lack of consensus among both scientists and regulators about these criteria. Work on the issue has been conducted at EU and international level for a long time. The European Commission's delay in adopting scientific criteria has provoked strong reactions from various stakeholders. However, after a process with several technical meetings and revised criteria proposals during winter and spring 2017, Member States representatives voted in favour of the European Commission's revised proposal on scientific criteria to identify endocrine disruptors under PPPR at a SCOPAFF Pesticides legislation meeting on 4 July 2017. On 4 September 2017 it was followed by a decision of the EU Commission on a new EU Regulation (2017/2100) for scientific criteria for the determination of endocrine-disrupting properties of a biocide, taken into force from June 7 2018 in all member states.

If EDs are identified by the new EU criteria, legislation will put restrictions or bans of such substances used in foods or as contaminants in a similar same way as for CMRs (carcinogenic, mutagenic or toxic to reproduction) and this may have implications for food production, trade and authorities. Some views from the Uppsala workshop follows here: Phase-out of EDs can give some actors a positive image as being responsible and working for sustainability. Not acting on knowledge about EDs in your products may destroy trust and reputation. The cost of replacing a substance is minimal compared to the loss of reputation, trust and business they will face. However, use of alternative(s) must be based on scientific assessments. They must be tested, reliable and proven safe. EDs that can be safely replaced should therefore be so. Efforts to avoid and restrict ED entrance into the environment should be established. Benefits can be evaluated at public health level but industry sees only the possible additional costs; benefits/costs needs to be determined at national level.

However, within the food sector, legislation in this area based on scientific risk assessment, seems to be preferred as it treats all the actors in an equal manner.

Information and risk communication from authorities to manufacturers when specific issues are in an early stage of research or monitoring is of utmost importance.

Participants

Table 4: Participants

Surname	First name	Representing Organization
Andersson	Anna-Maria	Center for Endocrine Disruptors, (CED), Denmark
Aspenström Fagerlund	Bitte	National Food Agency, Sweden
Boberg	Julie	Technical University of Denmark (DTU), Denmark
Busk	Leif	National Food Agency, Sweden
Dammand	Henrik	Fødevarestyrelsen, Denmark
Dirven	Hubert	Norwegian Institute of Public Health, Norway
Ekroth	Susanne	National Food Agency, Sweden
Fotland	Øystein	Norwegian Environment Agency, Norway
Gabrielsson	Britt	National Food Agency, Sweden
Gylllenhammar	Irina	National Food Agency, Sweden
Haarklou Mathisen	Gro	Norwegian Scientific Committee for food, Norway
Halldin Ankarberg	Emma	National Food Agency, Sweden
Hardy	Tony	EFSA, Italy
Hirvonen	Tero	Finnish Food Safety Authority (EVIRA), Finland
Holm	Mette	Fødevarestyrelsen, Denmark
Jestoi	Marika	EVIRA, Finland
Jones	Euros	European Crop Protection Association, (ECPA)
Juul Nielsen	Pia	Danish Environmental Protection Agency (EPA), Denmark
(Jörgensen	Claus	Forbrugerådet, TAENK, Denmark)[1]
Kirkegaard	Maja	Fødevarestyrelsen, Denmark
Kjellgren	Henrik	Nordic Paper, Sweden
Laakso	Juha	Finnish Safety and Chemicals Agency (TUKES), Finland
Leeves	Sara Ann	Norwegian Food Safety Authority, Norway
Lignell	Sanna	National Food Agency, Sweden
Njålsson	Runa	Chemicals Agency, Sweden
Olafsson	Grimur	Iceland Food and Veterinary Authority, Iceland
Otterstedt	Jens	Nordic Paper, Sweden
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Tesdal Håland	Julie	Norwegian Food Safety Authority, Norway
Thomsen	Cathrine	Norwegian Institute of Public Health, Norway
Totlandsdal	Annike Irene	Norwegian Food Safety Authority, Norway
Viljakainen	Sanna	EVIRA, Finland
Widenfalk	Anneli	National Food Agency, Sweden
(Vinggaard	Anne-Marie	Technical University of Denmark (DTU), Denmark)[1]
Zuberovic Muratovic	Aida	National Food Agency, Sweden

Note: [1] = no participation.

Sammanfattning (sv)

Hormonstörande ämnen eller endokrinstörande (ED) är ämnen som negativt påverkar våra hormonsystem antingen genom att efterlikna kroppens egna hormoner eller störa hormonproduktion, utsöndring, nedbrytning eller transport av hormoner i kroppen. Det misstänks att hormonstörande ämnen kan orsaka en minskad fertilitet, en ökad risk för vissa cancerformer, beteendeförändringar, effekter på immunsystemet samt ämnesomsättningsrubbningar.

Det antas att effekter av hormonstörande ämnen beror både på dos och när exponeringen skett. Dessutom, antas det att under vissa diskreta, kritiska perioder i tidig utveckling (t ex fosterutveckling och pubertet) kan även låga doser öka risken för att utveckla sjukdom senare i livet. Följaktligen är det en utmaning att utforma vetenskapliga kriterier för identifiering av ämnen om de är hormonstörande eller inte.

Hur kriterierna för ett hormonstörande ämne utformas kommer att få betydande konsekvenser för ett flertal intressenter. EU kommissionens kraftiga försening för fastställande av vetenskapliga kriterier för identifiering av hormonstörande ämnen har kritiserats från flera håll. Dessutom finns en klar skillnad mellan forskares och tillsynsmyndigheters synsätt. EU kommissionen fastslog nyligen vetenskapliga kriterier för hormonstörande ämnen som används som biocider och växtskyddsmedel.

Kemikalier med misstänkt hormonstörande aktivitet kan förekomma i livsmedel eller dricksvatten som miljöföroreningar (PCB, kadmium, perfluorerade alkylsyror eller dioxin); via migration från förpackningsmaterial (bisfenol A, ftalater), eller som tillsatser till mat (konserveringsmedel), veterinärläkemedel eller växtskyddsmedel eller förekomma som komponent i vanliga växter eller i svamp (t ex fytoöstrogener i soja eller mögelgifter). De nya kriterierna för identifiering av EDs kan komma att förbjuda dessa på samma sätt som för CMR ämnen (carcinogena, mutagena eller reproduktionstoxiska) och detta kan få långtgående konsekvenser för olika aktörer med fokus på livsmedel, exempelvis produktion, handel och reglerande myndigheter.

I en nordisk workshop med representanter från olika delar av livsmedelskedjan, diskuterades både positiva och negativa följder av en utfasning av hormonstörande ämnen. Ett förbud av hormonstörande ämnen/kemikalier kan ge vissa aktörer en mer positiv image, men ökad kostnad för andra. Inom livsmedelsområdet är det framförallt inom produktionen som de stora utmaningarna kommer då alternativen till t ex dagens växtskyddsmedel är begränsade eller saknas. Deltagarna var dock överens om att en harmoniserad EU lagstiftning som grundar sig på vetenskaplig riskvärdering, är att föredra framför en horisontell ramlagstiftning eller nationell specifik lagstiftning eftersom den behandlar alla aktörer lika.

Annex 1:
EU-Legislation

- Plant Protection Products Regulation (EC) 1107/2009 : https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32009R1107&from=EN
- Biocidal Products Regulation (EU) 528/2012: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32012R0528&from=EN

Annex 2: Endocrine disruptors in food and their mechanisms of action

Author Britt Gabrielsson, National Food Agency, SE

Abbreviations

ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone, see AVP
ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism and excretion
AGA	Adequate for gestational age
AR	Androgen receptor
AVP	Arginine vasopressin, a.k.a. ADH
BBP	Benzyl butyl phthalate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BPA	Bisphenol A
bw	Body weight
CamKII	Ca^{2+}/Calmodulin-dependent protein kinase II
CDRS	Children's Depression Rating Scale-Revised
CHD	Coronary heart disease
COPHES	Consortium to Perform Human Biomonitoring on a European Scale
CRH1	Corticotropin-releasing hormone
CVD	Cardiovascular disease
CYP19A1	Cytochrome P450 19A1, aromatase
DBP	Dibutyl phthalate
DEHP	Diethyl hexyl phthalate
DEMOCOPHES	Demonstration of a study to COPHES
DHEA/DHEAS	Dehydroepiandrosterone/Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DINP	Di-isononyl phthalate
DNEL	Derived no effect level
E2	17β-oestradiol
ECHA	European Chemicals Agency
ED	Endocrine disruptor

EDC	Endocrine disrupting chemical
EFSA	European Food Safety Authority
EPM	Elevated platform maze
ERα	Oestrogen receptor alpha protein
ERβ	Oestrogen receptor beta protein
ESR1/ESR2	Oestrogen receptor alpha and oestrogen receptor beta gene
ESRRG	Oestrogen-related receptor-gamma protein (ERRγ)
FP7	EU 7th Framework Programme
FSH	Follicle-stimulating hormone
FXR	Farnesoid X receptor
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
H295R	Human adrenocortical carcinoma cell line
hCG	Human chorionic gonadotropin
HeLa	Immortalized epithelial cell line from human cervical cancer tissue
HGNC	HUGO Gene Nomenclature Committee
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
HSD11B1	11-beta hydroxysteroid dehydrogenase 1; both 11-beta-dehydrogenase (cortisol to cortisone) and 11-oxoreductase (cortisone to cortisol) activities
HSD11B2	11-beta hydroxysteroid dehydrogenase 2; 11-beta-dehydrogenase activity (cortisol to cortisone)
IPCS	International Programme on Chemical Safety
IVF	*In vitro* fertilization
KISS1R	Kisspeptin 1 receptor a.k.a. GPR54
LGA	Large for gestational age
LH	Luteinizing hormone
LOAEL	Lowest Observed Adverse Effect Level
LXR	Liver X receptor
MCF-7	Immortalized epithelial cell line from human breast cancer tissue
NHANES	National Health and Nutrition Examination Survey
NOAEL	No Observed Adverse Effect Level
PAI-1	Plasminogen activator inhibitor type 1
PGR	Progesterone receptor
POMC	Pro-opiomelanocortin
PPAR	Peroxisome-proliferator activating receptor protein (α, β/δ, γ)
PTK	Protein tyrosine kinase
PXR	Pregnane X receptor
RCMAS	Revised Children's Manifest Anxiety Scale
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction

RXR	Retinoid X receptor protein (α, β, γ)
SCF	Scientific Committee of Foods (EU)
SCN	Suprachiasmatic nucleus
SGA	Small for gestational age
SVHC	Substances of very high concern
T2D	Type 2 diabetes
T_3	Triiodothyronine
T_4	Tetraiodothyronine, Thyroxine
TDI	Tolerable daily intake
THBQ	Tert-butylhydroquinone, a metabolite to BHA
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone (Thyrotropin)
u-BPA	urinary BPA
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein
WHO	World Health Organization

Abstract

Background: The endocrine system is composed of about one hundred hormones that maintain the internal balance by coordinating organ functions and respond to environmental cues. It has therefore a high degree of adaptability and is particularly sensitive to environmental stimuli during foetal and early postnatal life. This is because the regulation of hormone action on target cells and interaction between hormonal pathways are largely being shaped during early development. This ability to prepare for the postnatal environment was previously an advantage, to survive and reproduce. This adaptability is now cause for concern since it can predispose for adult disease. We are presently exposed to novel substances, endocrine disruptors (EDs), which could adversely affect human health. The challenge is to identify ED targets and the short- and long-term consequences of the ED interference.

Aim: To present an overview of mechanism and mode of action of selected endocrine disruptors at doses below the established no or lowest observed adverse effects levels (NOAEL/LOAEL); effects of early and exposure on disorder/disease development (fertility, cardiovascular disease, diabetes, cancers, obesity, behaviour).

Methods: The selected EDs were BPA; phthalates (DEHP, BBP, DBP, DINP, DIDP); antioxidants (BHA, BHT); parabens (Me-, Et-, Pro-); dithiocarbamate pesticides (mancozeb, maneb, metiram, propineb, thiram, ziram) and phytoestrogen (genistein). PubMed searches were performed and manually curated to exclude studies that did not fill the criteria (non-endocrine effects, non-oral administration, non-mammalian species; ED-mixtures; hypothesis/review/opinion papers; only doses above NOAEL).

Results: BPA, genistein and DEHP are the most studied of the selected EDs, with established mechanisms of action as oestrogen agonists (BPA and genistein) or as androgen antagonist (DEHP). New research show that many of the selected EDs can

bind and modulate the signal cascade of several other nuclear receptors such as the glucocorticoid receptor, the thyroid hormone receptors, the PPAR receptors and hepatic nuclear receptors involved in detoxification. Further these EDs are not perfect match to binding sites of the receptors and are therefore likely to act as partial receptor agonists/antagonists resulting in different responses from that of the natural ligands. EDs has also been shown to modulate enzyme activities; to block or open different ion channels in heart and pancreas, interfere with mitochondrial energy production and to induce DNA methylation. Studies in animals suggest that ED exposure below the current NOAELs during early development can have long-term effects in the adult animal with changes in gonadal organ/cell development, responses to stress or predisposal to metabolic disorders and cardiovascular disease in the adult animals. However, some studies did not identify any long-term effects after early ED exposure. Human studies usually report detection of several EDs (BPA, phthalates and parabens) in urine, plasma/serum and in cord blood. Detection in cord blood or placenta shows that the ED is transferred from the mother's circulation to the foetus. The effects of phthalates on gonadal development in humans have varied but the studies largely agree on a negative effect on male genitalia development. Cohort studies have found that subjects with high urinary BPA or phthalate concentrations are more likely to have insulin resistance/diabetes and high blood pressure. Soy protein intake, which contains genistein, had cardioprotective effects in postmenopausal women but a slight increase in CVD mortality and morbidity risk in men. High intake of soy phytoestrogens is protective in Asian, but not Western women, on the incidence of breast cancer. The reason why is not clear but may involve epigenetic modifications.

Discussion: Cell studies are important in identifying the mechanism of action of a specific EDs. The weaknesses of this approach are the use of immortalized cells, which often are abnormal, or the use of transfected cells where other important cofactors may not be expressed and that can affect the results. Animal studies are important for studying the mode of action of EDs; to identify the primary target organs, sensitive windows of exposure and of course the effects after oral intake of EDs. The drawback is the differences in study design; time, dose, species, sex, control of background contamination, choice of study end-points, inadequate description of study design, methods etc. However, that the experiments are repeated in independent laboratories is vital to gain new knowledge and not only indications of effects. Human studies are of course important since it is effects on human health that in this context is the focus. The results obtained from human studies are also limited by study design (selection of subjects: age, gender, ethnic origin, socioeconomic factors etc.); prospective/retrospective; diet and choice of study end-points. In addition, human exposure studies are hampered by the use of spot urine samples which are likely to reflect ED exposure from the last meal and/or exposure via other routes than diet. There have been reports of confounding factors that affect the interpretation or the results. For example, urinary phthalate levels were positively correlated with a western style diet pattern as well as multiple risk lifestyle factors (smoking, low physical activity) which in turn may predispose to obesity and metabolic dysfunction. Further, severe stress during pregnancy may mask antiandrogenic effects of phthalates on male

genitalia development. Even in animals studies it was shown that physical exercise could block behavioural changes in rats induced by early DEHP exposure.

Conclusion: We have to realize that the endocrine system by its nature is plastic and highly adaptable to the changing environment. This makes the endocrine systems vulnerable to novel environmental stimuli, particularly during early life when the pathways are formed and control connections between them are being set. The number of research reports in this field will surely increase but this new flood of information may also increase the confusion. There are obvious differences in point of view between scientists in academia and in legislative authorities. There is a need for the legislative procedures to with certainty predict ED effects on human health outcome. There is also a need for scientists in academia to produce and publish original research to raise research funding for continued survival in academia. Perhaps it is time for a top-down strategy; to identify (adult) disease areas of interest from epidemiological data before any mechanistic approach; to provide funding for conformational studies and to facilitate publication of negative data to avoid publication bias.

1. Introduction

From an epidemiological point of view, there is concern about the global increase in non-communicable diseases[3] that has been difficult to explain in terms of changes in lifestyle habits alone [1]. With new knowledge on developmental origins of disease and epigenetics [2], the area on how endocrine disruptors (EDs) affect state of health now engages scientists from several research fields: endocrinology, neuroscience, immunology, toxicology, epidemiology and epigenetics [3,4]. The decision of 4 September 2017 by the EU Commission on a new EU Regulation (2017/2100) for scientific criteria for the determination of endocrine-disrupting properties of a biocide will be taken into force from 7 June, 2018 in all member states. Briefly, the criteria are shifted from a risk- to hazard-based identification of potential EDs used as biocides and are limited to human health. This means that only the inherent properties of the substance will be taken into account for the decision-making and not exposure to the compound. However, the evaluation of such inherent properties is still based on established toxicological principles. The new criteria enable the regulation of EDs that are pluripotent, acting on several endocrine systems, at low levels that may have adverse effects in foetal development that predisposes to adult non-communicable disease.

The purpose was to review studies from 2010 and onward, to present an overview of ED effects on disease/disorder development. This includes mechanisms-of-action of the selected EDs, affected endocrine systems, developmental long-term effects and association with disease end-points. In

[3] Certain cancers, type-2 diabetes, cardiovascular diseases and fertility disorders.

particular, endocrine effects below the current no or lowest observed adverse effect (NOAEL or LOAEL, respectively) of selected EDs were prioritized. Critical review of the included references was carried out as standard procedure but criteria for systematic evaluation of the clinical studies were not used.

1.1 What is an endocrine disruptor?

An endocrine disruptor (ED) was defined by the World Health Organization's (WHO) International Programme on Chemical Safety (IPCS) in 2002[4] as follows: "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".

This definition is by far the most commonly used definition of ED and has been adopted by the EU Commission in June 2016.[5] In December 2016, EFSA and ECHA published an outline of Draft Guidance Document to implement the scientific criteria concerning the hazard-based identification of EDs in the context of Regulations (EC) No 1107/2009 and (EU) No 528/2012[6] and on 4 July this year Member States representatives (pesticide legislation meeting) voted in favour of European Commission's proposal on these criteria. EFSA and ECHA state that a multitude of endocrine modes of action of EDs are possible, but due to time limitation only the EATS pathways (an acronym for Estrogen, Androgen, Thyroid and Steroidogenesis) was covered in the draft guidance document. That disruption of endocrine systems must be shown in intact organisms is essential due to the fact that hormones are released from endocrine glands into the circulation and act on distant target organs. The definition is however vague for the following reasons:

- "Exogenous substance or mixture" – food is a mix of exogenous substances and, to our body, foreign (xenobiotic) compounds. Several of these xenobiotic compounds have endocrine activity either in their intact form, their metabolites or as precursors to bioactive compounds. An example is vitamin D_3, present in fish and liver, where the D_3 metabolite calcitriol acts on the nuclear receptors for Vitamin D (VDR) in mammalian cells. VDR is a ligand-activated transcription factor that belongs to the steroid nuclear receptor superfamily. Similarly, the long-chain polyunsaturated fatty acids (LC-PUFA), EPA and arachidonic fatty acids (fish, meat), are precursors to eicosanoid synthesis and ligands to the PPAR nuclear receptors.

[4] Joint Programme of the World Health Organisation (WHO), the United Nations Environment Programme and the International Labour Organization: Global assessment of the state-of-the-art-science of endocrine disruptors.
[5] http://europa.eu/rapid/press-release_IP-16-2152_en.htm
[6] https://www.efsa.europa.eu/sites/default/files/documents/161220_ed_guidance_outline.pdf: Note that on 17 November 2017 , the Commission-delegated regulation 2017/2100 the new scientific criteria for determination of endocrine-disprupting properties (persuant to EU Regulation 528/2012) was published in the Official Journal of the European Union.

- "Adverse health effects" – an adverse effect was defined by the WHO/ International Programme on Chemical Safety (IPCS) in 2009 as: "Change in the morphology, physiology, growth, development, or life span of an organism, or (sub)population that results in an impairment of functional capacity to compensate for additional stress, or an increase in susceptibility to other influences". The time perspectives are important here: are we for practical reasons mainly focussing on the acute effects or also changes that confer an increased risk for disease later in life? According to the development origin of health and disease, also known as the Barker hypothesis (see section 2.4), adverse events during early development affects susceptibility for the development of several non-communicable diseases decades later in adulthood [5,6], *i.e.* cancers, cardiovascular diseases (CVD), type 2 diabetes (T2D) and chronic respiratory diseases.

The American Endocrine Society used a shorter operational working definition of an ED in their Scientific Statement from 2015 [7], reflecting that interference of hormone action is a disorder: "An exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action". This might be too generous as a general definition but it is suitable for the purpose of this report since the scope of this overview is to identify putative endocrine effects below the already established critical toxicological effects.

1.2 Endocrine disruptors covered in this report

The following EDs are discussed in Chapter 3: Bisphenol A; BPA (Section 3.1) and five phthalates; DEHP – diethylhexyl phthalate, BBP – benzyl butyl phthalate, DBP – dibutyl phthalate, DINP – diisononyl phthalate, DIDP – diisodecylphthalate (Section 3.2). These EDs can be detected in food packaging materials. Other EDs that are considered are BHA – butylated hydroxyanisole, BHT – butylated hydroxytoluene (Section 3.3) and parabens (methyl-/ethyl-/propyl-p-OH-benzoates; Section 3.4). These EDs are added to food as preservatives. As a comparison, the endocrine effects of dithiocarbamate pesticides (mancozeb, maneb, metiram, propineb, thiram, ziram; Section 3.5) and a naturally occurring phytoestrogen, genistein, present at high levels in soybeans is also included (Section 3.6).

2. Background: an introduction to endocrinology

The word hormone was first used in 1905 by Starling with the definition "the chemical messengers which speeding from cell to cell along the blood stream, may coordinate the activities and growth of different parts of the body" [8].

It is estimated that the human endocrine system is constituted by over 50 different signalling pathways involving more than 100 hormones. There are three main classes of hormones; the protein and peptide hormones (*e.g.* the pituitary hormones), hormones that are modified amino acids (*e.g.* thyroid hormones) and steroid hormones (*e.g.* oestrogen, testosterone, cortisol). The functions of hormones can broadly be separated into the following categories:

- Reproduction and sexual differentiation
- Development and growth
- Homeostasis – maintenance of the internal environment
- Regulation of metabolism and nutrient supply.

Each hormone may be involved in one or several of these functions; each function can be controlled by one or several hormones. Examples of hormones in the different categories are:

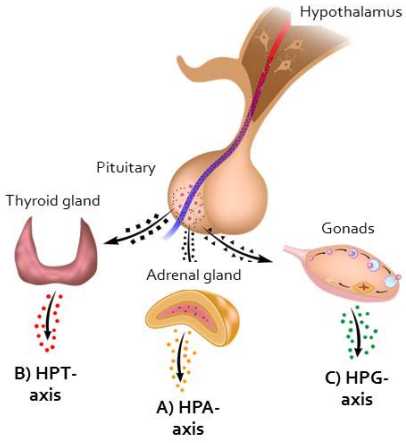
- The gonadotropins; luteinizing hormone (LH), follicle-stimulating hormone (FSH), and the sex steroid hormones (oestrogen, progesterone and androgens)
- Insulin, growth hormone (GH), thyroid hormones, cortisol and sex steroids
- Aldosterone, arginine-vasopressin (AVP/ADH), atrial natriuretic peptide (ANP), renin-angiotensin, thyroid hormones, insulin
- Insulin, glucagon, thyroid hormones, GH, cortisol, leptin, adiponectin, gut hormones.

The endocrine and the neuronal systems interphase the organism to its environment. They function as interpreters between the environment and the organism and initiate responses in the organism. Environmental cues can be light/darkness, hunger/satiety, danger, and the body acts by the release of specific hormones to mobilize appropriate responses in specific organs. Hormones in endocrine systems are also communicators between organs to coordinate anticipated physiological and behavioural activities during the 24 hour day. For this reason, several hormones typically exhibit an endogenous circadian secretion (24 hour cycles, see further section 2.5) that sometimes is overlaid by a more frequent pulsatile pattern triggered by hunger/food, physical activity. Due to these plastic or adaptable features of the endocrine system, it is perhaps not surprising that endocrine disruption early in life could have long-lasting effects on physiological functions. The concept "homeostasis" is central in both physiology and endocrinology and can be explained as a dynamic equilibrium where the

biological system strives for balance in a continuously changing environment. For example, if the concentration of a hormone is elevated in circulation, the effect can be counterbalanced by downregulation of receptor density or sensitivity in the target tissue cells. In this way the outcome is preserved.

This chapter gives an overview of the neuroendocrine systems that has been implicated in the mechanisms-of-actions of EDs, namely the hypothalamic-pituitary (HP)-adrenal (HPA)-, HP-thyroid (HPT) – and HP-gonadal (HPG)-axes (Sections 2.1 – 2.3). The organs and main hormones that make up each axis are shown in Figure 1. Sections 2.4, 2.5 and 2.6 introduce foetal/perinatal programming, epigenetics, circadian rhythm and nuclear receptors. Section 2.7 deals with other endocrine systems that are dysregulated in non-communicable diseases (obesity, CVD, cancer, T2D), which are gaining increasing interest in the field of ED effects on health.

Figure 1: Overview of the three neuroendocrine axes: HPA, HPT, HPG



Note: A) HPA; the hypothalamus-pituitary-adrenal axis which is triggered by stress causing release of CRH and AVP from the hypothalamus and acts on the pituitary to secrete ACTH. Plasma ACTH acts on the adrenal glands causing secretion of cortisol and androgens. A feedback loop exists where the elevated blood cortisol levels to inhibit further release of CRH from the hypothalamus and ACTH from the pituitary. B) HPT; the hypothalamus-pituitary-thyroid axis is involved in growth, development, energy regulation and metabolism. TRH from the hypothalamus causes TSH release from the pituitary that acts on the thyroid gland causing release of the thyroid hormones (T_4 and T_3). A feedback loop from the thyroid to the pituitary regulates serum levels of thyroid hormone by increasing pituitary TSH when serum levels of T_4 and T_3 are low, and reducing TSH when serum T_4 and T_3 levels are high. T_3, the active hormone is derived from T_4 and this conversion occurs mainly in target tissue. Therefore the activity of the HPT-axis is not necessarily reflected in serum thyroid hormone levels. C) HPG; the hypothalamus-pituitary-gonadal axis is involved in breed and feed. GnRH is released from the hypothalamus causing secretion of LH and FSH into the circulation. LH and FSH acts on the gonads to produce and release sex steroid hormones. There is a feedback loop of the sex steroids to the hypothalamus and the pituitary to decrease secretion of GnRH, and LH and FSH, respectively.

2.1 The hypothalamus-pituitary-adrenal (HPA)-axis[7]

2.1.1 Components of the HPA-axis

The HPA-axis is composed of the paraventricular nucleus of the hypothalamus where arginine-vasopressin (AVP; a.k.a. antidiuretic hormone, ADH) and corticotropin-releasing hormone (CRH) are synthesized. AVP is transported via neurones to the posterior pituitary, and CRH is secreted into the hypophyseal portal blood system. CRH and AVP synergistically stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the general circulation. ACTH in the circulation acts on the adrenal gland and stimulates synthesis and release of glucocorticoids and androgen from adrenocortical cells. The adrenal glands are situated on top of the kidneys and consist of an outer cortex and an inner medulla. The cortex is divided into three layers: the *zona glomerulosa* (aldosterone production and secretion, important for blood pressure regulation); *zona reticularis* (non-gonadal production and secretion of androgens) and *zona fasciculate* (production of cortisol/corticosterone: basal and ACTH-induced cortisol/corticosterone secretion). Adrenalin and noradrenalin is synthesized in the medulla under the regulation of the sympathetic nervous system. Secreted glucocorticoids suppress further CRH and ACTH production in the hypothalamus and pituitary, respectively, by a feedback inhibition loop.

2.1.2 Function of the HPA-axis

The main function of the HPA-axis is the fight or flight response. The increased blood levels of cortisol caused by an activated HPA-axis results in raised blood glucose levels by dual actions: a reduction in target tissue insulin sensitivity, and an increase in gluconeogenesis in the liver. Cortisol also suppresses immune processes, which are high in energy demand, and thereby further increasing availability of glucose. This system is activated by stressors that can be either physiological (temperature, injury, fasting, exercise) or psychological. Psychological stressors are further divided into absolute (*e.g.* earth quakes, war, terror actions) or relative (subjective) stressors.

2.1.3 The HPA-axis in pregnancy and foetal development

During normal pregnancy, maternal serum cortisol levels in the third trimester increase about three-fold compared to levels in non-pregnant subjects. This is due to higher corticosteroid-binding globulin levels and that the placenta secretes large quantities of CRH during the second and third trimester. Placental CRH stimulates the maternal pituitary gland, which increases ACTH in circulation and consequently elevates serum cortisol levels. Despite the increasing circulating levels of cortisol, the circadian secretion of cortisol is maintained throughout pregnancy with high levels in the morning and low in the evening (see Section 2.5). In late pregnancy, hypothalamic CRH is downregulated and the responsiveness of the HPA-axis to both physiological and

[7] For review see Spiga, F., Walker, J.J., Gupta, R., Terry, J.R., Lightman, S.L. (2015) 60 YEARS OF NEUROENDOCRINOLOGY: Glucocorticoid dynamics: insights from mathematical, experimental and clinical studies. J Endocrinol 226: T55-66. doi: 10.1530/JOE-15-0132.

psychological stress is dampened. In addition, the foetus is normally protected from maternal cortisol levels by the activity of the placental enzyme 11-beta-hydroxysteroid dehydrogenase, II isoform (HSD11B2), which deactivates cortisol to the less potent cortisone. However, excess maternal cortisol can cross to the foetus, and the placental-blood barrier can be compromised by infections, inflammation and maternal anxiety (see further Section 2.4).

2.1.4 Dysfunction of the HPA-axis: causes and effects

Chronic stress results in an activated HPA-axis that affects the immune system, metabolism, growth and reproduction. A chronic activation of the HPA-axis is associated with learning disabilities, mood and behavioural disorders [9]. The hippocampus and the paraventricular nucleus in the hypothalamus are the central targets for glucocorticoid action. The hippocampus is crucial for establishing new memories and a chronic high cortisol level is likely to negatively affect hippocampal plasticity [10]. In adults, high serum glucocorticoid levels as in Cushing's disease or syndrome is associated with central obesity, muscle atrophy, T2D, high blood pressure, memory defects and mood swings. At the other end of the spectra, adrenal insufficiency or Addison's disease is associated with extreme fatigue, low blood pressure, weight loss and depression.

2.1.5 Clinical chemistry reference values for cortisol and ACTH

As mentioned above, both ACTH and cortisol (corticosterone in rodents) have a circadian secretory pattern, with highest levels in the early morning and lowest in the late evening in healthy subjects (see further Section 2.5). There is however a large inter-individual variation and the reference range used in Swedish Clinical Chemistry for serum cortisol is 170 to 500 nmoles/L in the morning, and 70 to 280 nmoles/L in the evening (see Appendix).

2.1.6 Glucocorticoid mechanism of action

Glucocorticoids act via the glucocorticoid nuclear receptors in target cells and, to a lesser extent, mineralocorticoid nuclear receptors, which are ligand-activated transcription factors. For general mechanism of action see Section 2.6. The glucocorticoid receptor (GR; NR1C3) pre-mRNA is alternatively spliced and produces two isoforms, alpha and beta. The alpha isoform confers transcriptional activity of glucocorticoids and the beta isoform inhibits glucocorticoid action by binding to DNA without activating transcription.

2.2 The hypothalamus-pituitary-thyroid (HPT)-axis[8]

2.2.1 Components of the HPT-axis

The HPT-axis is composed of the paraventricular nucleus of the hypothalamus where thyrotropin-releasing hormone (TRH) is synthesized and secreted into the hypophyseal portal blood system. TRH acts on specific cells of the anterior pituitary to stimulate the release of thyroid stimulating hormone (TSH) into the general circulation. TSH regulates the endocrine function of the thyroid gland, located at the front of the base of the neck, by stimulating release of the thyroid hormones precursor T_4 and the active hormone T_3. T_4 is synthesized in the thyroid gland from tyrosine and iodine. Following this, dietary intake of iodine is important for thyroid gland and thyroid hormone production. T_4 is activated by deiodinase function 2 (DIO_2) to form the active hormone T_3 [11]. Only about 20% of this activation of T_4 to T_3 takes place in the thyroid gland itself, the main conversion occurs in target tissues. T_3 and/or T_4 are actively concentrated in target tissue cells with up to 10-fold concentration to the concentration in serum. DIO_2 is present in the endoplasmic reticulum in many cell types with highest expression in the CNS, the pituitary, the brown adipose tissue and the placenta. In contrast, deiodinase 3 (DIO_3) which is present in cell plasma membranes inactivates T_3 and to a lesser extent prevents T_4 from being activated [11]. DIO_3 is expressed at high levels in the CNS and the placenta. The third deiodinase, DIO_1, is mainly expressed in the liver and the kidney and to a lesser extent in the thyroid. It has a dual function; to produce a small amount of circulating T_3 (contributing about 25% of circulating T_3) and to preserve iodide by removing iodine from inactive metabolites of T_3 and T_4 in the liver and kidney. In conclusion, there are several control systems in the regulation of the activity of thyroid hormones, which mainly occur in the target tissues. The levels of thyroid hormone in the circulation are regulated by a feedback loop to the pituitary where low serum thyroid hormone levels cause increased TSH release from the pituitary by increasing TRH receptor expression in pituitary thyrotrophs. Conversely, high serum thyroid hormone levels suppress the expression of TRH receptor in the thyrotrophs resulting in reduced secretion of TSH into the circulation.

2.2.2 Function of the HPT-axis

The HPT-axis is essential for several physiological and organ functions; foetal/perinatal brain development, regulation of food intake and thermogenesis, energy expenditure and regulation of cardiovascular, bone and liver function. Cold exposure increases hypothalamic TRH release whereas negative energy balance (fasting, anorexia, infections and critical illness) inhibits the HPT-axis at the hypothalamic level.

[8] For review see Joseph-Bravo, P., Jaimes-Hoy, L., Uribe, R.M., Charli, J.L. (2015) 60 YEARS OF NEUROENDOCRINOLOGY: TRH, the first hypophysiotropic releasing hormone isolated: control of the pituitary-thyroid axis. J Endocrinol. 2015 Aug;226(2):T85-T100. doi: 10.1530/JOE-15-0124.

2.2.3 The HPT-axis in pregnancy and foetal development

Thyroid hormone receptors are expressed in the foetus already in the first trimester of pregnancy, before the foetal thyroid becomes functional. It is therefore assumed that the foetus is largely dependent on the maternal TH supply. During pregnancy, maternal plasma levels of T_4 and T_3 are increased caused by increased secretion of T_4 binding globulin. This increase occurs early in pregnancy, and by week 16–20 the plasma level of T_4 is doubled. During normal pregnancy and sufficient iodine intake, the required increase in T_4 secretion by the thyroid is met without difficulty. The HPT-axis in the neonate responds similarly as adults to various conditions; TSH release is suppressed by neonatal hyperthyroidism or prenatal exposure to glucocorticoids, and elevated by cold exposure.

2.2.4 Dysfunction of the HPT-axis: causes and effects

Early disturbances of the HPT-axis can lead to cretinism, caused by iodine deficiency, which is the most common cause for preventable mental handicap. Postnatal screening for hypothyroidism is therefore important to prevent permanent brain damage. An underactive thyroid gland can lead to hypothyroidism, which is more common in women over 60 years. Symptoms may include sensitivity to cold, fatigue, joint and muscle pains, muscle weakness and/or weight gain. Hyperthyroidism, an overactive thyroid gland, is associated with sudden weight loss, increased/irregular heart-beat, heat intolerance, fatigue, enlargement of the thyroid gland and/or anxiety/nervousness. Excess of glucocorticoids such as in Cushing's syndrome reduces serum levels of thyroid hormones and TSH. Oestrogen, testosterone and obesity are associated with increased serum TSH levels. The increase of TSH in obesity may be associated with leptin insensitivity since leptin/leptin receptor deficiencies are associated with hypothalamic hypothyroidism, characterized by low free T_4 and high TSH levels in serum [12].

2.2.5 Clinical chemistry reference values for thyroid status

Thyroid status is usually evaluated by measuring serum levels of TSH, free T_3, reverse T_3 (inactive isomer of T_3), free T_4, thyroperoxidase and thyroglobulin antibodies. Circulating TSH levels vary during the 24-hour day with a peak after onset of sleep and lowest in the late afternoon [13]. It has also been suggested that serum TSH and free T_3 levels vary with season with lower values in spring and summer [14]. The variation in serum markers for thyroid function measured in repeated measures from the same subject is however relatively small compared to the inter-individual variability. A Belgian study of male siblings with normal thyroid function (euthyroid; n=941, 25–45 years) showed that serum levels of free T_4, total T_4, reverse T_3 and thyroglobulin had the highest genetic or familial component of 80–90% [15]. Serum levels of free and total T_3 had a moderate genetic influence of 60% and TSH had the lowest, 49%. Lifestyle parameters such as smoking, education level and body composition had significant influence on thyroid hormone status, whereas iodine intake had only minor effect in this cohort. Free T_3 was the variable that appeared to be most influenced by environmental factors.

The reference ranges for free and total T_3, T_4 and TSH used in Swedish Clinical Chemistry are shown in Appendix, indicate only minor effects of sex and age on serum thyroid hormone levels.

2.2.6 Thyroid hormone mechanism of action

Free T_3 acts via thyroid hormone nuclear receptors which are ligand-activated transcription factors (see Section 2.6). The thyroid hormone receptors are coded by two different genes; *THRA* and *THRB*, each giving rise to different receptor protein isoforms through alternative splicing of the pre-mRNA. Thyroid hormone receptor-α and thyroid hormone receptor-β form monomers, homodimers, or heterodimers with retinoid X receptors (RXR) and regulate gene expression by binding to thyroid response elements in gene promoters. Usually the thyroid hormone receptor without ligand is bound to DNA and inhibits transcription, and is activated when the ligand binds to the receptor. However, thyroid hormone receptors are also detected in the cytosol and translocate to the nucleus after ligand binding.

2.3 The hypothalamus-pituitary-gonadal (HPG)-axis[9]

2.3.1 Components of the HPG-axis

The HPG-axis is composed of gonadotropin-releasing hormone (GnRH) which is secreted in a pulsatile manner from the hypothalamus. GnRH acts on the anterior pituitary where it regulates the synthesis and release of gonadotropins, LH and FSH. LH and FSH acts via their cell surface receptors on the gonads to initiate production of sex steroid hormones. The LH/choriogonadotropin-receptor is expressed in ovarian cells and in the Leydig cells in the testes, inducing synthesis and release of 17β-oestradiol (E2) and testosterone, respectively. The gonadal steroid hormones in turn regulate hypothalamic GnRH synthesis and release through a negative feedback system between the gonads and the brain.

2.3.2 Function of the HPG-axis

The HPG-axis controls gonadal maturation and reproduction in humans and other animals. GnRH is the principal regulator of reproduction that integrates cues from sex hormone steroids, stress/glucocorticoids, nutritional/metabolic status and prolactin to control secretion of gonadotropins and subsequently gonadal function. FSH is important in the early stages of follicular maturation in females and in spermatogenesis in males. Progesterone dominates during the luteal phase and plays an important role in the attachment of the blastocyst to the uterine wall and maintenance of early pregnancy. Components of the HPG-axis are also involved in early imprinting of sex differences in the brain. In women, oestrogen modulates the immune system, bone health and affects body fat distribution. In men, testosterone is positively associated to increased muscle mass and indirectly also via oestrogen to bone health.

[9] For review see Plant RM (2015) 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-pituitary–gonadal axis. . J Endocrinol. 2015 Aug;226(2): T41-T54. doi: 10.1530/JOE-15-0113.

2.3.3 The HPG-axis in pregnancy and foetal development

GnRH can be detected in the foetal hypothalamus by 10 weeks of age. FSH and LH are produced in the foetus at 10–13 weeks of age when the hypophyseal portal blood system has developed and these gonadotropins reach the highest levels in foetal circulation during the middle of pregnancy. The gonadotropins decline towards birth due to negative feedback at both the hypothalamic and pituitary level caused by increased secretion of oestrogen and progesterone from the placenta. In puberty, pituitary secretion of LH and FSH is resumed in a sexual dimorphic manner by activation of GnRH neurons by kisspeptin. Although very rare, loss-of-function mutations in the kisspeptin gene or the kisspeptin GPR54 receptor (*KISS1R*) gene have been described and are associated with hypogonadotropic hypogonadism, whereas gain-of-function mutations in these genes are associated with precocious puberty [16]. In males, gonadotropin and presumably GnRH are released in a pulsatile pattern every two hours. In premenopausal adult females, the GnRH pulse pattern varies depending on the time of the menstrual cycle with more frequent pulses and lower amplitude during the follicular phase, which stimulates LH secretion. The slower GnRH pulse frequency in the late luteal phase favours FSH secretion. This phase is also associated with high progesterone and lower oestrogen levels that may contribute to the preferential release of FSH. The increased secretion of FSH is important for recruitment of follicles in the next cycle. With age, the follicular number will decrease with subsequent drop in serum oestrogen levels and GnRH pulses decrease in frequency.

2.3.4 Effects of dysfunction of the HPG-axis in adults

There is an obvious and necessary crosstalk between breeding and nutritional status with leptin as one likely candidate. Serum leptin levels correlate with amount of body fat and are higher in women suggesting an oestrogen regulation. Normal pubertal development does not occur in adults with leptin or leptin receptor deficiency, two very rare disorders [12]. In analogy, women with hypothalamic amenorrhea, *i.e.* cessation of ovulation due to negative energy balance, can restore their menstrual cycles with leptin treatment. Conversely, hypogonadism and low fertility in obese subjects may be associated with leptin insensitivity [17]. Indeed, leptin receptors are found on kisspeptin neurons suggesting a direct link between body energy stores and reproduction [18].

2.3.5 Clinical chemistry reference values for gonadal hormones

The reference ranges for the gonadotropins and the sex steroid hormones are strongly dependent on sex, age and, for women, oestrus cycle phase and postmenopausal state (Appendix). Excess of glucocorticoids, such as in Cushing's disease or syndrome, results in hypogonadism. This is due to multiple actions by glucocorticoids on the hypothalamus, the pituitary and on the gonads resulting in reduced testosterone in men and follicular development in women. However, suppressed glucocorticoid levels such as in Addison's disease also results in testicular dysfunction.

2.3.6 Steroid hormone mechanism of action

The sex steroids regulate gene transcription by binding to respective nuclear receptors: the oestrogen receptors ERα and ERβ (coded by the *ESR1* and *ESR2* genes, respectively), the androgen receptor (AR) and the progesterone receptor (PGR) (see also Section 2.6). These nuclear receptors are present in the cytoplasm of target cells and upon binding by a ligand, dissociate from a protein complex and translocate to the nucleus. The ligand-activated receptors bind to specific response elements in gene promoters, usually as homodimers, and induce gene transcription. There are differences in tissue expression patterns of the two oestrogen receptors. ERα is highly expressed in the hypothalamus, smooth muscle and female reproductive organs. ERβ is expressed in the testes, the ovary and adrenal gland. There are differences in ligand affinity between the two receptors, although oestrogen binds equally well to both receptors, genistein has higher affinity to the ERβ. There are two isoforms of the PGR; A and B, due to alternative splicing of the pre-mRNA. The B-isoform binds to the PGR response elements in gene promoters and activates transcription. In contrast, the A isoform represses progesterone-induced transcription by binding to the response elements but lacks binding sites to other proteins necessary to form a functional transcription complex. The androgen receptor is activated by androgens, primarily testosterone but also dihydrotestosterone (DHT) and androstenedione. In analogy with the oestrogen and progesterone receptors, the ligand-activated AR complex translocates to the nucleus, homodimerizes and activates gene transcription by binding to specific response elements in gene promoters. Oestrogen, progesterone and androgen can also bind to cell membrane-bound receptors, which trigger activation of classical intracellular pathways.

2.4 Foetal exposure: programming and epigenetics[10]

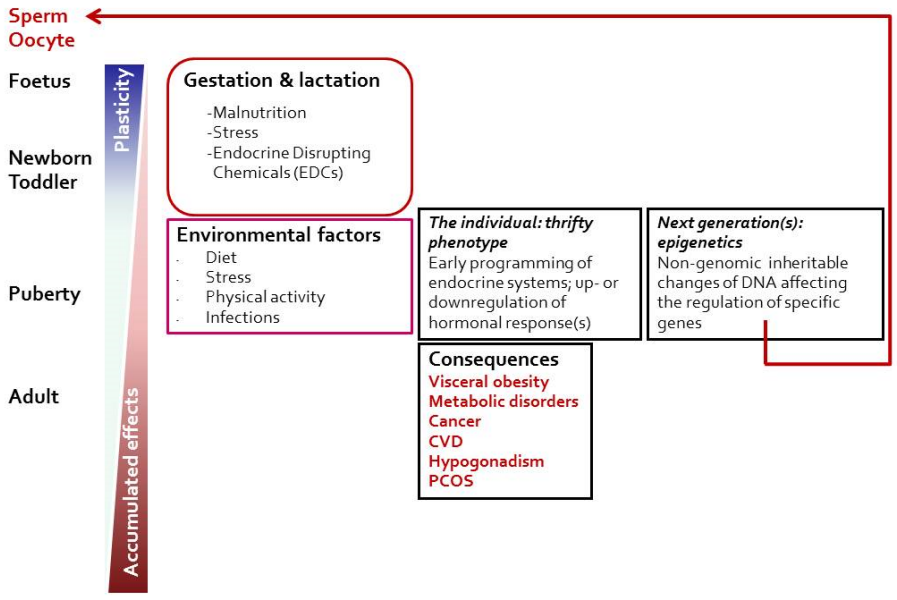
2.4.1 Developmental origins of health and disease

The rationale for the developmental origins of health and disease hypothesis is that environmental cues during development (foetal or early postnatal) cause permanent changes of tissue structure and function. Such changes are considered to be adaptive responses to increase the chances of survival and reproduction, and thereby securing future generations. However, these adaptions may also increase the susceptibility to develop chronic diseases later in life (Figure 2). One of the first to highlight the associations between foetal development and adult disease was the British epidemiologist David Barker. He showed that low birth weight was correlated to increased risk for developing ischaemic heart disease in adult age. He did so by tracing morbidity and mortality data from over 5,500 men born between 1911 to 1930 in England and Wales [6,19]. His conclusions from these and other studies were that the foetus adapts to the nutrient supplied by the mother via the placenta. Further,

[10] For review see Wadhwa, P.D., Buss, C., Entringer, S., Swanson, J.M. (2009) Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Semin Reprod Med. 2009 Sep;27(5):358-68. doi: 10.1055/s-0029-1237424.

environmental factors that disturb the foetal growth have an effect on future risk for chronic disease. Later studies point to the existence of both critical (absolute) and sensitive time frames or windows during development during which external influences can affect tissue maturation and hence function. These critical or sensitive windows differ depending on organs but also depending on the type of environmental cue or challenge. For example, the brain has a high plasticity throughout life, but CNS regulation of peripheral functions, *e.g.* hunger/satiety, are formed during early foetal/postnatal development. The brain is also more sensitive to environmental factors before the blood-brain-barrier is stabilized. There are several study protocols of perinatal programming in animal experiments, involving exposure to excess of glucocorticoids [20], or exposure to specific nutrients or contaminants via the maternal diet [21]. Long-term effects are then studied in the adult offspring.

Figure 2: Endocrine adaption to environmental factors during development



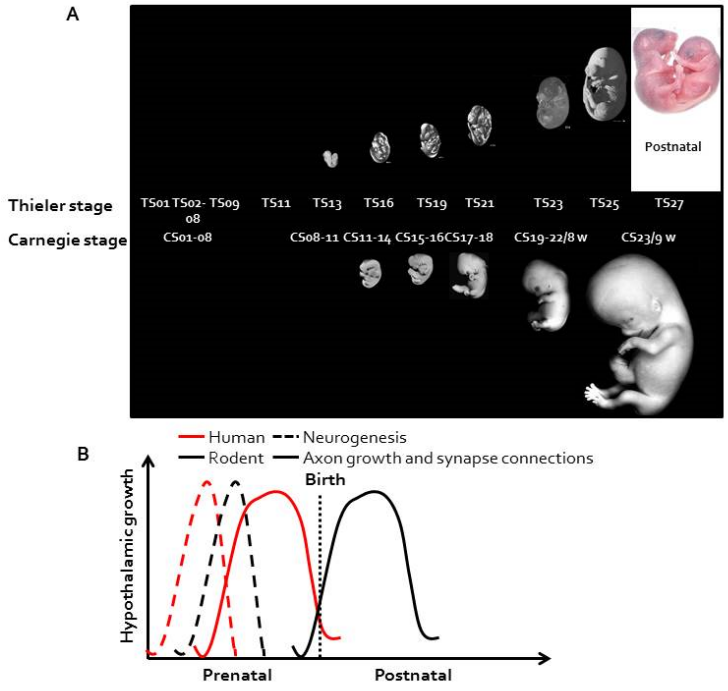
Note: The hypothesis of developmental origins of health and disease states that environmental factors (diet, stress, infection, EDs) during early development (pregnancy, lactation and early childhood) can cause long-term effects in adulthood. Consequently the same genotype can give rise to different phenotypes dependent on modifications of DNA in the early environment. Later research also point at epigenetic changes of DNA in the germ cells (haploid sex cells) can cause changes in disease risk over several generations.

2.4.2 Epigenetic modulation of DNA

Figure 2 also points to the possibility that environmental challenges *in utero* can cause effects over several generations through epigenetic modifications of the DNA. Environmental challenges can be the maternal diet, specific macro-/micronutrients, maternal stress, maternal hormone status or xenobiotic compounds such as EDs. This means that the same genotype can through epigenetic modulation of the DNA give rise

to different phenotypes. The most commonly studied epigenetic mechanism is the methylation of DNA, which is controlled by specific DNA methyl transferases. Hypermethylation of a gene promoter suppresses transcription of the gene and hence lowers expression of the protein. Modification of histones by specific enzymes (histone acetyltransferases, deacetylases, methyltransferases, and demethylases) affects the chromatin structure, and consequently the activation of specific genes. Epigenetic changes of microRNAs affect the expression of specific proteins. Exposure of a compound must occur during development to cause effects over several generations and these effects must be observed in the third (F_3) generation. This is because when a pregnant Fo female is exposed to epigenetic stimuli, the germ line cells in the foetus (F_1 generation) are directly exposed to the stimuli. These altered F_1 germ line cells produces the F_2 generation and therefore the F_2 generation is also directly exposed to the stimuli. Therefore the F_3 generation is the first generation on the maternal line that is not directly exposed to the stimuli. In the paternal lineage, the F_2 generation is the first generation not being directly exposed to the stimuli.

Figure 3: Comparison of foetal developmental stages in human and rodents



Note: Comparison of embryonal developmental stages in humans and in rodents. A) The development of a mouse pup at birth corresponds in late human first trimester [22]. Hence a substantial part of organ growth occurs after birth in mice and rats. B) Shows a graph comparing pre- and postnatal development of nerve cell proliferation (dotted lines) and nerve cell connectivity (solid lines; axon growth, synapse-dendrite connections) in human (red) and rodent hypothalamus (black). Reproduced from [23] by permission from authors.

Source: Xue, L., Cai, J.Y., Ma, J., Huang, Z. & MX Guo et al.. (2013) Global expression profiling reveals genetic programs underlying the developmental divergence between mouse and human embryogenesis. BMC Genomics, 14: 568.

2.4.3 Differences in embryonal/foetal development between species

It is important to take into account differences in foetal and postnatal development between species when using data from animal studies. Figure 3 compares pre- and postnatal development in mice and rats, and compares to humans.

Mice and rats are born prematurely in comparison to humans. The first ten postnatal days in the mouse or rat corresponds roughly to the third trimester in humans. This must be kept in mind when applying data from rodent studies using pre-, post- or perinatal protocols. Figure 3B shows a comparison between human and mouse development and maturation of the hypothalamus. The hypothalamus is of great interest in developmental programming since it links the endocrine and the nervous systems, and controls food intake and energy expenditure. Changes in hypothalamic development will have downstream effects on the pituitary and subsequently the HPA-, HPG- and HPT-axes. For example, there is a leptin surge in rodent pups at postnatal day 8–11, which is important for the maturation of the postnatal brain and peripheral organs (pancreas, kidney, thymus, ovaries) in rats and pigs [24,25].

2.4.4 Perinatal development of components of the HPA-axis

The foetal adrenal gland initiates *de novo* glucocorticoid synthesis during the second half of gestation, at around gestational week 28 in humans and embryonal day (E) 14.5 in mice. Foetal glucocorticoid and maternal production increases markedly over a narrow time window shortly before birth, which cause a surge in foetal glucocorticoid levels in late gestation. This prenatal surge is crucial for the maturation of the foetal lung before birth. The foetal heart also undergoes extensive growth, remodelling and maturation in late gestation to support the rapid growth in late gestation and to prepare for life after birth [26]. In late gestation, glucocorticoids accelerate the maturation of energy metabolism in the foetal heart preparing for a fuel switch from carbohydrates to fatty acids, a more efficient source of ATP and necessary for the increased energy demand of the heart after birth. Animal studies show that exposure to excessive glucocorticoid levels in utero, by bypassing or overwhelming placental HSD11B2, causes short-term effects such as foetal growth restriction and long-term effects that include increased risk of CVD in the adult offspring. Maternal nutritional restriction also increases foetal glucocorticoid levels by prematurely activating the foetal HPA axis, which confers a greater risk of CVD later in life.

2.4.5 Perinatal development of components of the HPT-axis

The major points of foetal development of the HPT-axis have already been described in Section 2.2. However, since the HPT-axis is involved in energy homeostasis and thermogenesis it is likely that links exists between other endocrine systems such as leptin that are developed during the postnatal period.

2.4.6 Perinatal development of components of the HPG-axis

The reproductive endocrine system is sexually dimorphic from production of oocytes vs. sperm, steroid hormone synthesis to sexual behaviour. These processes are controlled by the limbic system in the brain, often named the "reptile brain" since all base instincts are regulated by the limbic system. The hypothalamus, hippocampus and the amygdala are parts of the limbic system imprinted in a sex-specific manner by sex steroids during critical periods of development in the foetal or early postnatal development. In rodents, the early androgen-dependent developmental phase of gonads is called the male programming window, which occurs at E13–E17 in rodents, and at about gestational week 8–15 in humans. In primates this foetal testosterone surge is also important for the sexual differentiation of the hypothalamus. The anogenital distance is a sex-specific indicator of androgen action in foetal life, and is larger in males than females. In humans, normal androgen action during gestational week 8–15 is critical for normal development of the genitalia; penile malformations arise at this time window, whereas the development of sperm production capacity in humans occurs in a much broader time window that continues to puberty. A second testosterone peak occurs in the male neonate around birth or shortly after and is important for the sexual differentiation of the hypothalamus in rodents. The function of this second testosterone peak around birth in primates is less clear. In female foetuses, primordial follicles originate from a pool of primordial germ cells that are localized to the developing gonad early in gestation. The primordial germ cells enter meiosis at E13.5 in mice, and gestational week 11–12 in humans becoming primordial follicles, each containing an oocyte. Before birth these cells arrested and do not become activated until puberty.

2.5 Circadian rhythms[11]

Virtually all multicellular organisms have pre-programmed endogenous physiological and endocrine rhythms that anticipate the demands of the 24-hour day; the activity-feeding and the resting-repairing periods, and this is named circadian rhythm (Figure 4A). The master clock in animals resides in the brain and there is a molecular clock in each cell. To coordinate the time of day there are two major circadian timekeeper hormones: melatonin and cortisol [27]. Disturbances in circadian rhythm are associated with metabolic dysfunction and have been observed in shift workers.

Night: Melatonin is released in the evening (from about 9 pm) from the pineal gland, which is situated at the centre of the brain but outside the blood-brain barrier. This secretion is regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. Body fuel usage is switched during the resting period towards lipid metabolism to spare glucose for the brain and thereby avoiding insulin-induced hypoglycaemia. In humans, this switch is associated with release of GH from the pituitary, but also other pituitary

[11] For review see Jonathan, D., Johnston, J.D., Skene, D.J. (2015) 60 YEARS OF NEUROENDOCRINOLOGY: Regulation of mammalian neuroendocrine physiology and rhythms by melatonin. J Endocrinol. 2015 Aug;226(2): T187-T198. doi: 10.1530/JOE-15-0119.

hormones like prolactin and TSH, at the onset of slow wave sleep at about midnight. In agreement, glucose tolerance tests show reduced glucose tolerance and increased insulin secretion rate during night-time compared to daytime in lean glucose-tolerant subjects [28]. In the early morning, about 4 to 5 am, healthy subjects show dips in both body core temperature and in blood pressure. Blunting of this dip in blood pressure is an independent predictor for future cardiovascular events.

Day: Cortisol, a glucocorticoid hormone synthesized from cholesterol, is secreted from the adrenal gland in the morning (≈6 am) as a wake-up signal to prepare the body for alertness and activity. During the day, the body prepares for activity and re-fuelling. The vagal nerve and a number of gut hormones are involved in hunger/satiety signalling to the brain. One of the hunger hormones is ghrelin, which is secreted from the stomach as a response to an empty stomach. The secretion of ghrelin ceases when the stomach is distended. Ghrelin activates receptors in neuropeptide Y (NPY) neurons of the arcuate nucleus of the hypothalamus. In the fasting period, glucagon is released from the pancreas as response to lower glucose levels and stimulates hepatic gluconeogenesis. After food intake, insulin is released from the pancreas to stimulate glucose uptake into tissues and inhibit lipolysis. Insulin can also signal satiety to the brain through activation of specific neurons of the arcuate nucleus, a part of the hypothalamus that is at the border of the blood-brain barrier.

Figure 4: Cartoon showing endogenous circadian rhythm of hormone levels and physiological parameters

Note: The circadian clock depicted above represents a number of endogenous physiological processes that are self-sustained and prepares the organism for the anticipated variation in the 24 hour activity-rest cycle. Two hormones are vital in maintenance of this rhythm: melatonin in the evening and cortisol in the morning. The text in green shows circadian effects on hormone levels, for example growth hormone and melatonin. The text in blue shows circadian effects on physiological parameters such as blood pressure, which drops in the morning, or insulin sensitivity which is higher in the morning than in the afternoon.

2.6 Nuclear receptors[12]

The nuclear receptors compose a superfamily of transcription factors whose ligands mediate the physiological state of the body. It is therefore not surprising that several of these nuclear receptors are circadian regulators or exhibit circadian regulation. Endogenous ligands are for example steroid hormones, thyroid hormones and cholesterol metabolites, whereas other nuclear receptors act as metabolic sensors by responding to exogenous ligands; 9-cis- and all trans-retinoids (retinoic acid receptors; RARs and RXRs), polyunsaturated fatty acids (PPARs) or vitamin D3 (VDR) present in the diet. However, several nuclear receptors are still orphan, where the ligand has not been identified yet.

2.6.1 Structural domains in nuclear receptor proteins

The first nuclear receptors were cloned from human in the late 1980's; the glucocorticoid (GR), progesterone (PGR), oestrogen (ERα), vitamin D (VDR), thyroid (TRα), mineralcorticoid (MR) and retinoic acid (RXR, RAR) receptors. The nuclear receptors have a structural homology; the N-terminal is highly variable and can contain one or two activation function domains, *i.e.* binding sites for corepressors/coactivators (see below). The middle region is the DNA-binding domain (DBD), which is the most conserved region between the receptors and contains Zn-fingers which can bind DNA. The ligand-binding domain (LBD) is located at the C-terminal. Between the DBD and the LBD there is a hinge-region that contains the nuclear translocation signal.

2.6.2 Activation of nuclear receptors

The classical mechanism of action by nuclear receptors can be described as a three-step process: repression, de-repression and transcriptional activation. The unbound receptor (the aponuclear receptor) is found in the cytoplasm (EGR, PGR, ERs, AR, PPARs) or in the nucleus (TRs, RARs, RXRs) bound to a corepressor complex in the repressed state. In this form the ligand-binding domain is highly flexible but upon ligand binding, the conformation becomes more stable, causing the corepressor proteins to dissociate from the ligand-receptor complex, which is the de-repression step. The ligand-bound receptor usually forms dimers and cytoplasmic ligand-receptor dimers translocate to the nucleus where it binds to specific response elements on DNA. The receptor can form homodimers (the classical sex steroid receptors) or heterodimers with a different nuclear receptor usually with RXR (TRs and PPARs). Additional proteins, coactivators or corepressors, are recruited to the receptor-ligand complex on the DNA to regulate gene transcription. Presently there are some 200 identified nuclear proteins that can bind to nuclear receptor-ligand complexes, and these proteins are expressed differently depending on cell and tissue type. The consequence is that the same ligand can elicit different responses in different cells depending on the expression of other proteins in the nucleus.

[12] For review see Evans, R.M., Mangelsdorf, D.J. (2014) Nuclear Receptors, RXR, and the Big Bang. Cell. 2014 Mar 27;157(1):255-66. doi: 10.1016/j.cell.2014.03.012.

2.6.3 Non-genomic actions of steroid hormones

Several of the nuclear receptors (AR, ERs, GR, MR, PPARs, PXR and RXRs) have non-genomic actions as evidenced by their expression in platelets which lack nucleus [29]. The GRs, ERs, the TRs and a truncated isoform of the PR have all been detected in mitochondria in different cell lines and hormone-response elements (HREs) are present in the mitochondrial genome [30]. This suggest that glucocorticoids, oestrogens and thyroid hormones can affect the production of energy and reactive oxygen species (ROS) in the cells by regulating expression of mitochondrial- and/or nuclear-encoded genes for proteins involved in oxidative phosphorylation [31]. ERα has also been detected in endothelial cells from cerebral arteries where it was co-localized with caveolin-1 in the cell plasma membrane [32]. E2 stimulation increased NO production via the PI3-Akt signalling pathway and thereby inducing vasorelaxation.

There are also membrane-bound receptors for several of the steroid hormones [33]. These membrane-bound receptors mediate a much faster response to steroid hormones by activating phosphorylation cascades in the cells compared with the nuclear receptors that act via regulation of gene expression. For example, the G-protein-coupled oestrogen receptor 1 (GPER1; mER or GPR30) is an endoplasmic reticulum receptor involved in calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate, both important intracellular second messengers [34]. Non-genomic effects of thyroid hormones have also been reported [35]. These effects by thyroid hormones initiates by binding to a cell surface receptor, integrin αvβ3, or a truncated isoform of TRα. Binding to these receptors initiates an intracellular signalling cascade via MAPK or ERK and Akt, or activation of integrin αvβ3 induces TRβ1 translocation from the cytosol to the nucleus.

2.6.4 Nuclear receptor partial agonism/antagonism

The action of EDs has so far been focused on steroid hormone receptors and therefore other receptors that bind oxysterols or bile acids may be of relevance. Examples of sterol-binding nuclear receptors are the liver X receptors (LXR) α and β, the farnesoid X receptor (FXR), the pregnane receptor (PXR) and the constitutive androstane receptor (CAR). Further, studies of graded or partial agonist/antagonist functions that have been carried out by the pharmaceutical industry are of high interest since most EDs bind with lower affinity compared with the endogenous ligand [36]. For example, it has been shown that full PPARγ agonists provided robust stabilization of the nuclear receptor conformation, and that weak partial PPARγ-agonists provided less stabilization. Similar results have been observed in ligand-induced conformational changes of the ER-α. Why is this interesting? It suggests that a less stable ligand-receptor complex may recruit and bind coactivators/repressors different from that of the physiological ligand-receptor complex. As a consequence, the transcriptional response will differ between the physiological ligand and xenobiotic ligands with graded agonist function. This mechanism is beyond the concept of agonists or antagonists since it suggest that entirely different set of genes may be activated. Similar levels of complexity may also apply to how EDs or phytoestrogens interfere with steroid hormone signalling.

2.7 Obesity and obesity-related disease

2.7.1 Obesity and adipose tissue dysfunction[13]

Obesity is increasing in virtually all countries in the world. As a consequence, obesity-related diseases are increasing in parallel; type 2 diabetes (T2D), certain cancers (breast, colorectal, liver, gallbladder, pancreatic), CVD (atherosclerosis, ischemic disease and stroke). These non-communicable diseases reduce the quality of life for the individual and increase the health care costs. The WHO estimates that overweight and obesity presently contribute to half of the incidence of diabetes and a quarter of ischemic heart disease. Further, the strongest associations of foetal origin of adult disease are linked to diseases associated to obesity; CVD, T2D and certain cancers.

The WHO originally classified adult obesity based on increased risk of death, irrespective of cause, resulting in a J-shaped curve where the BMI range with lowest mortality (18.5 ≤ BMI <25 kg/m2) was defined as normal range BMI [37]. This simplified relation between BMI and morbidity has since been criticized since the outcome depends on sex, ethnic origin and age. Several Asian populations show increased risk for T2D at much lower BMI-range than Caucasian or African populations, whereas BMI in the overweight range is protective in the elderly and is referred to as the obesity paradox.

The simplest explanation to obesity uses the first law of thermodynamics which states that energy cannot be destroyed or created. Hence, the energy consumed as food will either be used to produce energy needed for basal metabolism and physical exercise, or stored in the form of glycogen or fat. However, this may be somewhat simplistic view on metabolism. First, it is known that some individuals are more efficient in energy absorption in response to the same meal than others, and also that some individuals are more efficient in preserving energy during fasting. Second, the distribution of adipose tissue is important and intra-abdominal (visceral) obesity confers a much higher risk for development of metabolic dysfunction and CVD compared to subcutaneous obesity [38]. The physiological role of subcutaneous adipose tissue is to provide energy for future demands whereas visceral adipose tissue appears to be a short-term fat storage with some immune properties. Hence, visceral obesity can paradoxically be suggestive of an inability of subcutaneous adipose tissue to store fat. Consequently, excess energy is stored as fat in other internal organs, such as liver, pancreas and skeletal muscles, and negatively affects their function. In non-alcoholic fatty liver disease (NAFLD), the extent of fat in liver is strongly correlated with visceral obesity and cardiovascular disease, and increased lipid storage in skeletal muscle cells is a predictor for development of insulin resistance and T2D. In obese rodent models there is a clear relation between obesity and the HPA-axis. However, it has been

[13] Speakman, J.R., Levitsky, D.A., Allison, D.B. et al.. (2011) Set points, settling points and some alternative models: theoretical options to understand how genes and environments combine to regulate body adiposity. Dis Model Mech. 2011 Nov;4(6):733-45. doi: 10.1242/dmm.008698.

more difficult to identify stress-related mediators to the development of obesity in humans. Other factors that are likely to influence body composition are genetic predisposition and/or epigenetic programming to conserve energy. These factors, in combination of a sedentary life-style, are likely to predispose to the development of obesity and related disease.

2.7.2 Endocrine systems that are affected in obesity[14]

The most obvious endocrine system that is affected by obesity is the insulin signalling pathway. There are different opinions whether insulin resistance originates from the liver, the skeletal muscles or the adipose tissues. Irrespectively, there will be knock-on effects on other tissues resulting in reduced removal of glucose from the circulation (hyperglycaemia), increased hepatic output of glucose, increased VLDL levels (hyperglycaemia and hyperlipidaemia) and enlarged fat cells in adipose tissue (increased adipokine release). The elevated blood glucose levels will stimulate the pancreatic β-cells to produce and secrete more insulin, which can lead to hyperinsulinemia and eventually to T2D.

An activated HPA-axis have been proposed to be a component in developing obesity in humans since excessive glucocorticoids, caused by medical treatments or ACTH-secreting tumours, is associated with visceral adiposity, muscle wasting and insulin resistance [39]. The HPT-axis is also implicated since there is an association between non-alcoholic fatty liver disease/disease progression and degree of hypothyroidism [40]. This may be explained by that T_3 regulates several aspects of hepatic lipid metabolism via TRβ [41]. Further, a positive correlation between serum TSH and BMI was reported from the National Health and Nutrition Examination Survey (NHANES study) 2007–2008 [42]. The circadian pattern of glucose tolerance differs between lean and obese subjects. Plasma glucose levels in obese subjects are stable from morning to evening despite a steady decrease in insulin secretion rate, indicative of an improved glucose tolerance as the day progresses. In contrast, lean subjects show decline in glucose tolerance toward the end of the day. However, other endocrine systems are affected by obesity or may be part of the cause to obesity [39]. GH, which counteracts insulin action and favour lipid metabolism, is suppressed in obese subjects and adult GH-deficiency is associated with abdominal obesity [28].

Obesity is associated with increased circulating levels of the adipocyte-derived hormone leptin due to the increased fat mass. In contrast to the genetic obese rodent models (ob/ob) obese humans exhibit central/peripheral leptin resistance. The action of the other adipocyte hormone adiponectin is also suppressed in human obesity. This reduction in leptin and adiponectin action is likely to increase lipid storage in muscle and liver and thereby inducing insulin resistance in these tissues. So paradoxically, an inability to store excess energy in adipose tissue is a driver of obesity-related diseases.

[14] O'Rahilly, S. (2002) Insights into obesity and insulin resistance from the study of extreme human phenotypes. Eur J Endocrinol 147: 435-441. doi: 10.1530/eje.0.1470435.

3. Mechanisms of action and implicated disorders/diseases

The crucial questions are how to define endocrine disruption, is disruption different from endocrine interference (if so how) and how can we assess risk for EDs? The focus on risk assessment of EDs has been on EATS. The Organisation for Economic Co-operation and Development (OECD) presented guidelines for testing of chemicals with putative endocrine disrupting properties in 2012.[15] These tests are focussed on the HPG- and the HPT-axes, and are based on classical acute and long-term toxicity studies. Here, the ambition was to identify adverse effects on endocrine systems from an endocrine point of view. Due to time limitation and a number of excellent reviews on the subject, articles that were published from 2010 and on were the main focus. Studies that investigated endocrine effects below the NOAEL (or LOAEL) of respective substances were prioritized. This is due to the fact that the risks have already been identified through toxicological studies and therefore whether or not there are endocrine effects above these doses is immaterial. Studies of mode of action (*e.g.* effects on the HPA-axis) or mechanisms of action (cellular targets) were prioritized, as well as articles that investigated effects in humans. Further, articles that investigated ED effects on organ development, endocrine pathways, or effects of chronic exposure of ED on endocrine signalling were prioritized.

Sections 3.1 to 3.6 are structured as follows:

- Where the compound is detected *i.e.* in food contact materials, additives, pesticide remnants or natural compounds; the NOAEL if established;
- Criteria for the PubMed searches;
- Metabolism/metabolites after ingestion;
- The HPA-axis: adrenal weights and histology; effects on basal and stress-induced ACTH and corticosterone/cortisol levels, and behavioural responses to stress tests in animals. The HPA-axis, through glucocorticoid action, has long been recognized to be involved in memory and learning, mood and behaviour [9]. Studies that relate ED exposure to measures of anxiety, depression, learning and attention are also included here;
- The HPT-axis: effects on thyroid hormone receptor-induced gene transcription; effects of perinatal ED exposure in rodents on thyroid hormone status and thyroid gland development in the offspring; associations of ED exposure and thyroid hormone status in human studies. Effects on perinatal brain development, thermogenesis and/or cold adaption;

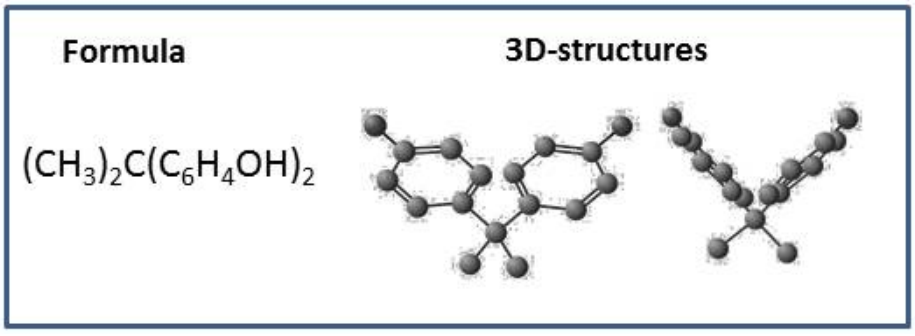
[15] OECD, Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption. Series on Testing and Assessment no 150, Paris 2012.

- The HPG-axis: expression of sex steroid receptors; binding to ERs and AR; effects on oestrogen and androgen signalling; developmental effects on the gonads; serum levels of E2, testosterone, SHBG, LH and FSH; sperm motility and count, infertility; pregnancy outcome;
- Programming studies: transport of ED from maternal to foetal circulation; ED content in foetal tissues; animal programming studies with ED exposure before conception, during gestation and/or during lactation; effects on HPA-, HPT-, HPG-axis in the offspring; effects on organ development:
- Other: effects on body composition, T2D, CVD;
- Summary.

3.1 Bisphenol A

BPA (4,4'-isopropylidenediphenol; CAS 80-05-7)

Figure 5: Bisphenol A



Note: Molecular formula and 3D-structures of the molecule (PubChem ID 6623).

Source: National Center for Biotechnology Information. PubChem Compound Database; CID=6623, https://pubchem.ncbi.nlm.nih.gov/compound/6623 (accessed 7 May 2018).

BPA has been used in food packaging materials since the 1960s and is used in epoxy resins as coatings on the inside of almost all food and beverage cans. It is also one of the monomers of polycarbonate plastics. Food is considered to be the primary source of BPA exposure in humans [43]. However, reported values of free BPA in blood/plasma/serum should be treated with caution since BPA is a common component in medical and laboratory disposables (tubing, tubes etc.). This may result in overestimation of blood-derived BPA content due to contamination from the blood sampling equipment [44]. In the last years it is apparent from temporal trend studies that BPA is being phased out and replaced by other bisphenols such as BPF and BPS [45]. Due to the fact that these compounds have not been on the market for as long as BPA, less is also documented about their effects. The Swedish Chemicals Agency recently identified 37 bisphenols with similar ED properties as BPA and that also pose a risk for consumer exposure [46].

EFSA: A NOAEL of 5 mg/kg bw/day was set in 2002 and confirmed in 2008 based on two multi-generation reproductive toxicity studies in rodents [47,48]. However, in 2015 EFSA reported a bench mark dose (BMDL10) of 8.96 mg/kg bw/day based on changes in the mean relative kidney weight in the two generation toxicity study in mice [48], resulting in a temporary new TDI for BPA set at 4 µg/kg bw/day [49]. EFSA is expected to publish a new report on BPA in 2018.

ECHA: BPA was added to the candidate list of substances of very high concern (SVHCs) as a substance meeting the criteria for classification as toxic for reproduction category 1 or 2 (REACH Article 57c) in 12 January 2017.[16]

The NOAEL for BPA based on rat studies corresponds in mice to 10 mg/kg bw/day taking into account their higher body surface to body weight ratio and in rabbits to 2.5 mg/kg bw/day due to their higher weight to body surface ratio [50].

So far four countries in the EU: Denmark (2010), Sweden (2013), Belgium (2013) and France (2013), have banned the use of BPA in food contact materials intended for 0–3 year old children [49].

3.1.1 Criteria for literature search

The PubMed search resulted in 1951 articles, filtered for humans or rat/mice (Table 1). Excluding duplicates and references written in other languages than English left 1744 items. This was followed by manual curation to remove articles which not filled the criteria; doses only above NOAEL; focus on non-mammalian species; other routes of administration; other EDs than BPA was the focus of the study or that BPA was a minor component in mixtures of several EDs. Reviews, opinion or hypothesis papers were excluded with the exception of systematic reviews of clinical data.

Table 5: PubMed search 8 December 2016 Bisphenol A and endocrine action published after 2009-12-31

Search	Query	Items found
#7	#5 AND #6	1,951
#6	#3 Filters: Publication date from 2010/01/01 to 2017/12/31; Humans	1,180
#5	#3 AND #4 Publication date from 2010/01/01 to 2017/12/31	771
#4	((rat OR rats)) OR (mouse OR mice)	2,935,513
#3	#1 AND #2	5,208
#2	Bisphenol A	10,429
#1	(((((((endocrine) OR endocrinol*) OR estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

[16] BPA: Proposal for the identification of a substance of very high concern on the basis of the criteria set out in REACH Article 57. https://echa.europa.eu/documents/10162/13640/ec_201-245-8_bpa_annex_xv_svhc_en.pdf/93bf4be3-9af6-d7ca-8b07-4e8fb42bad11

3.1.2 Metabolites/metabolism oral route

Ingested BPA is predominantly metabolized in the liver to glucuronide and sulphate conjugates being the major and minor metabolite, respectively [51]. In contrast, exposure of BPA via the skin or through inhalation gives rise to free BPA in the circulation. Rapid metabolism of BPA is observed for oral exposure (4–5 hours), while other routes of exposure that bypass liver metabolism increase the half-life of circulating BPA [52]. In monkeys and rats, the gastrointestinal absorption is greater than 85% with plasma peak values after 80 minutes of single dose ingestion.[17]

Studies in non-human primates suggest that the clearance of BPA is age-dependent. The serum peak value of BPA is higher in neonatal monkeys than older monkeys indicating a lower phase II metabolism [53]. This means that BPA has a higher half-life in the very young and consequently their tissues are exposed to BPA over a longer time than an adult would from an equivalent dose. Dietary components such as probiotics can significantly affect intestinal absorption and excretion of BPA [54]. Subjects with high consumption of some common over-the-counter drugs (*e.g.* naproxene, salicylic acid) have reduced ability to form BPA-glucuronide in the liver [55]. Further, detoxification of BPA has been suggested to be impaired in patients with fatty liver, diabetes and liver cirrhosis (non-alcoholic and alcoholic) due to a reduced ability to form BPA-sulphate conjugates [56]. From this follows that since the metabolism of BPA varies between subjects, the exposure to the same dose of BPA would also be expected to vary between subjects in terms of urinary BPA (u-BPA) levels and dose effects.

There are some differences in metabolism of BPA between humans and rodents. BPA acts as an agonist to human PXR but not to the mouse or rat PXR [57]. The PXR nuclear receptor is a transcription factor in the liver, small intestine and colon that induces enzymes involved in steroid metabolism and xenobiotic detoxification through the induction of CYP3A4, a phase I enzyme. There are also some controversies in extrapolating rodent BPA data to humans in ADME studies due to species differences in the transporter proteins that are involved [58].

Biomonitoring studies in humans show that over 90% of the subjects had detectable levels of u-BPA [59-61].

3.1.3 The HPA-axis

3.1.3.1 Animal studies; effects on plasma hormones and stress response in offspring to dams exposed to BPA during pregnancy and/or lactation.

Adrenal gland weight was increased in adolescent C57BL/6 mouse offspring (8 weeks old) to dams fed a BPA-containing diet (5 mg/kg bw/day; 0.5 x BPA NOAEL) the last two thirds of pregnancy [62]. Accordingly, plasma corticosterone levels were increased in offspring to the BPA-exposed dams. Plasma ACTH levels were not different from control, suggesting that BPA acted directly on the developing adrenal gland. In Wistar rats, adolescent female offspring (46 days of age) to dams fed BPA via cornflakes (40 µg/kg bw/day; 0.008 x BPA NOAEL) throughout pregnancy and lactation had higher

[17] ECHA/RAC/RES-O-0000001412-86-56/F

basal corticosterone levels and increased anxiety-like behaviour after mild stress tests compared with control female and BPA male offspring [63]. Using the same study design, it was shown that perinatal exposure to BPA was associated with changes in adrenal gland structure with reduced *zona reticularis* layer (androgen-producing cells) in the males and increased *zona fasciculata* layer (glucocorticoid-producing cells) in both sexes [64]. The female offspring to the BPA-exposed dams had higher basal levels of plasma corticosterone, blunted corticosterone response in stress tests and lower Gr expression in the hypothalamus compared to the control females. Further, the perinatal BPA exposure affected regulation of the *Fkbp5* gene in the hippocampus in the male BPA-exposed offspring [65]. The Fkbp5 protein is a negative regulator of glucocorticoid action by reducing hormone affinity to the receptor and also reducing nuclear translocation of the receptor. At basal conditions, hypermethylation of the *Fkbp5* gene in the hippocampus was associated with suppressed protein levels. However, following stress, Fkbp5 protein levels were increased to similar levels as the control males. The BPA effect on *Fkbp5* gene and protein regulation was further investigated *in vitro*, using differentiating murine hippocampal neurons of male origin (HT22 cells). As the *in vivo* study, BPA exposure to HT22 differentiating cells induced DNA hypermethylation of the *Fkbp5* gene, which resulted in decreased Fkbp5 protein levels three days after the exposure. BPA exposure potentiated the dexamethasone (a synthetic glucocorticoid) induced increase of Fkbp5 protein levels. These effects appeared to be mediated by antagonizing ERβ since ERβ knockout produced the same effects as that of BPA and blocked any further effects by BPA.

Pregnant CD-1 mouse dams were trained to voluntarily feed oil from a syringe. From gestational day 11 to lactation day 8, the mouse dams were given oral vehicle or BPA-containing oil (10 µg/kg bw/day; 0.001 x BPA NOAEL) [66,67]. The pups were cross-fostered from birth; pups exposed to BPA before birth suckled vehicle-treated dams, and pups to vehicle-treated dams suckled BPA-exposed dams. In addition, pups from vehicle-treated dams were cross-fostered to other vehicle-treated dams and served as controls. There were clear sex differences in the behavioural tests[18] in the juvenile (30 days) and adult (70 days) control offspring. However, after prenatal or postnatal BPA exposure resulted in that the sex differentiated behaviours were decreased or even reversed in both juvenile and adult offspring. The reversal of behavioural sex differences was more prominent in offspring that were exposed to BPA postnatally, indicating that the sensitive windows for changes behavioural responses were mainly postnatal in rodents (see also Figure 2 and 3).

In contrast, no effects on the HPA-axis or behaviour were reported in two studies of pregnant NCTR[19] Sprague-Dawley rats administered vehicle or BPA by gavage (2.5, 25 µg/kg bw/day; 0.0005, 0.005 x BPA NOAEL, and in the second study also 2,500 µg/kg bw/day; 0.5 x BPA NOAEL) from gestational day 6 to birth, after which the pups were given the same treatment until 21 days of age [68,69]. There were no differences in serum corticosterone levels or in circadian activity or play/aggressive

[18] Open field, novelty testing, elevated plus maze.
[19] National Center for Toxicological Research/FDA, U.S.

behaviour in offspring from the different treatment groups [68]. The second study, using the same experimental protocol, found no compelling evidence of BPA-related effects on anxiety or exploratory behaviour in the adult rats (age 77–125 days) [69]. However, in the juvenile rats (age 26–27 days), there were effects in the elevated platform maze in the 0.005 and the 0.5 x BPA NOAEL groups, suggesting heightened anxiety behaviour in the female offspring. There is no consensus in the perinatal animal studies since the NCTR studies are largely negative despite using exposures up to 60 x BPA NOAEL. The NCTR studies have been criticized for using gavage as the preferred method of administrating BPA to the pups, especially in studies using stress tests as endpoints since gavage is known to induce stress. However, naïve controls were also included and did not differ from vehicle controls.

3.1.3.2 Human studies; associations between maternal or child u-BPA levels and child behaviour.

The association between prenatal exposure to BPA (spot u-BPA at gestational week 16 and 26) and behavioural measures in the offspring at the age of three (n=239) was investigated in a prospective pregnancy cohort (Health Outcomes and Measures of the Environment Study, Cincinnati, U.S) [70]. They found that BPA exposure in late gestation was associated with anxious, depressive and hyperactive measures related to impaired behavioural regulation, and was more pronounced in girls than in boys. Another study found that prenatal BPA exposure (spot u-BPA at gestational week 27) was associated with an increase in adverse behaviours[20] in six to ten-year old boys (n=77), but not in girls (n=76), in a multi-centre pregnancy cohort (Study of Future Families, U.S.) [71]. Similar findings of behavioural effects after high prenatal BPA exposure (mean u-BPA spot samples at gestational week 14 and 26) were reported from a longitudinal birth cohort study from California (n=133 and 155, boys and girls, respectively) where the boys at seven years of age exhibited increased internalization problems, including anxiety and depression[21] [72]. The association between third trimester maternal spot u-BPA levels and anxiety/depression rating has also been investigated in ten to twelve year old children (n=348) from a New York City cohort [73]. There were positive associations between maternal third trimester spot u-BPA and the Revised Children's Manifest Anxiety Scale (RCMAS) total score (p=0.04) and the social concern subscale (p=0.025) in boys but not in girls[22] [73]. These studies are suggestive of an association between perinatal BPA exposure on mood and behavioural disturbances later in childhood. However, the studies are hampered by the differences in estimating prenatal BPA exposure and tests for valuating behavioural/mood.

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[20] Adverse behaviours included moderately increased internalizing, externalizing and withdrawn/depressed behaviours, somatic problems and oppositional/defiant disorder traits in the boys Covariates included in the model; age (months), creatinine, sex, mother's education (no college vs. college) and continuous family stress.

[21] Adjusted for mother's country of birth, maternal education, marital status, child's age, HOME score, household income, number of siblings, child's BPA at 5 years and maternal DAP metabolite levels during pregnancy.

[22] Adjusted for potential confounding variables; ethnicity, gestational age of the child, mother's intelligence (TONI-3), maternal education (+/-completion of high school prior to birth of this child), maternal demoralization (measured by the PERI-D scale), child age (in months) at testing, quality of the child's home environment at 3 years of age (measured by the HOME), prenatal exposure to environmental tobacco smoke (yes/no), and SG-adjusted maternal u-MnBP (3rd trimester).

Higher u-BPA levels at five years of age were associated with increased internalizing problems and increased attention deficit hyperactive disorder (ADHD) behaviours in both sexes, and in girls at age seven years also increased externalizing behaviour [72]. In a prospective population-based study from Granada, Spain, total BPA was measured in non-fasting morning (8–10 am) spot urine samples, and behavioural and emotional tests were performed in 269 of the original 668 boys at age nine to ten years [74]. They found that boys in the highest quartile of u-BPA ranked higher in somatic complaints and thought problems (excessive worry) compared with children in the lowest quartile.[23]

3.1.3.3 Human studies: associations between BPA exposure and cortisol levels

High u-BPA levels in the second trimester of pregnancy were associated with disturbed cortisol circadian rhythm with suppressed morning and day-time levels of saliva cortisol [75]. In adults, a Czech study of fertile and infertile men (n=191) found a positive correlation between plasma BPA levels[24] and the ratio of plasma cortisol/cortisone ratio [76]. This could suggest that BPA has an effect on the activity of the cortisol/cortisone converting enzymes, HSD11B1 and HSD11B2.

3.1.4 The HPT-axis

3.1.4.1 Cell studies: BPA mechanisms-of-action on thyroid hormone signalling

BPA at 10 µM concentration suppressed T_3 activation of thyroid hormone receptor-α in transiently transfected cells derived from a human embryonal kidney cell line [77]. The BPA effect was attributed to the recruitment of the N-CoR corepressor protein to the thyroid hormone receptor-α transcription complex. In another study, BPA at nanomolar concentrations suppressed thyroid hormone receptor-β-mediated transcription in transfected CV-1 cells (epithelial cell line derived from monkey kidneys) [78]. Although the inhibition of thyroid hormone receptor-β was a consequence of recruitment of N-CoR, BPA acted upstream of the receptor by blocking T_3/T_4 binding to an integrin cell plasma membrane receptor, necessary for activation of cytoplasmic THR. Primary rat cerebellar cultures +/- glia cells were exposed to 0.1 nM BPA, alone or in combination with E_2 (0.12 nM), T_3 (0.9 nM), or T_4 (65 nM) for 6 h (RT-qPCR) or 18 h (Western blot) [79]. BPA exposure alone suppressed thyroid hormone receptor-α mRNA levels, whereas BPA combined with E_2, T_3 or T_4 synergistically increased thyroid hormone receptor-α mRNA levels in the glia+ cells. In the glia-negative cultures, thyroid hormone receptor-α gene expression was increased with BPA in combination with E_2 or with T_3. Similar effects of BPA and combination treatments were found for thyroid hormone receptor-β gene expression. These data show that BPA can interfere with T_3/T_4 signalling both via regulation of thyroid hormone receptor-α and thyroid hormone receptor-β gene expression and inhibition of thyroid hormone receptor signalling in target cells.

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[23] Adjusted for child's age, IQ score, BMI, exposure to tobacco smoke in the home (yes/no), mother's IQ, mother's age at the time of assessment, education level (university/secondary school/up to primary), marital status (married/not married), maternal smoking during pregnancy (yes/no), breastfeeding (yes/no).

[24] The method included blanks in form of charcoal stripped plasma to control for putative contamination during sample handling and analyses.

3.1.4.2 Animal studies; effects on foetal growth and thyroid gland development

Groups of pregnant Wistar rats were administered BPA at two doses (0, 20 µg and 40 µg/kg bw/day; 0.004 and 0.008 of NOAEL, respectively) by gavage from gestational day 1 to the end of the experiment at gestational day 20 [80]. The BPA-treated dams were hypothyroid with suppressed serum levels of T_3 and T_4, and increased serum TSH compared to the vehicle treated control dams. These changes in thyroid hormone serum levels were also reflected in their foetuses at embryonal day 20. Foetuses from dams of both BPA groups exhibited histopathological changes of the thyroid gland with hyperplastic irregular follicles. This was associated with a dose-dependent reduction in foetal body weight. No studies investigating the effects of BPA on thermogenesis or cold adaption were identified.

3.1.4.3 Human studies; associations between u-BPA levels and thyroid hormone status in adults

There were no association in 167 men recruited through an infertility clinic (age 37±6 years; BMI 27±5 kg/m2) between serum levels of free T_3, total T_4 or TSH and spot u-BPA (single samples or in a subgroup, repeated samples) using linear regression analysis [81]. There are contradictive results from cross-sectional studies on the association between BPA and thyroid hormones in pregnant women. One study found positive association between free T_3 and free T_4 and spot u-BPA at the two measured time-points during pregnancy (gestational week 16–20 and week 24-28) [82]. In another cohort of pregnant women, u-BPA concentrations were negatively associated with total T_4 in samples taken around gestational week 26 [83]. A third study found no associations between thyroid hormone status at gestational week 16 or week 26 with u-BPA [84]. The relation between u-BPA and thyroid gland volume was investigated in 718 children in a cross-sectional study from the south-east coast of China [85]. Background BPA from disposables and equipment was controlled for and they reported that the levels of u-BPA were inversely correlated with thyroid hormone volume and risk for developing multiple nodules. A South Korean cross-sectional study in adults (M, n=2638; F, n=3365; adults age >19 years) reported a negative correlation between u-BPA and serum TSH levels, but not with serum levels of total T_4 or T_3[25] [86]. In contrast, a smaller case-control study of thyroid nodular disease in women from Cyprus and Romania (n=212; age 47 and 52 years, respectively) found a positive relation between u-BPA and serum TSH levels by multivariate analyses[26] [87]. The authors reported that plastic bottled water and personal care products usage were significant predictors of spot u-BPA levels. In summary, the correlation between u-BPA concentrations and serum markers for thyroid status show variable outcome in the different studies. This may reflect high variability in serum thyroid hormone levels between subjects.

[25] Adjusted for age, smoking status, monthly household income, ln-BMI, and urinary creatinine.
[26] Adjusted for urinary creatinine, age, BMI, study site and disease status.

3.1.5 The HPG-axis

3.1.5.1 Cell studies; BPA mechanisms-of-action on steroid hormone signalling

BPA had a weak oestrogenic action in HEK293 cells (unclear cell type derived from a human embryonal kidney) transfected with a reporter gene [88]. The BPA concentrations required to yield the same response as E2 on the reporter gene were 400 and 100 times higher, respectively. However, BPA exhibited high affinity to the ERα with an EC50 of 200–210 nM in the activation of a reporter gene in two human epithelial cancer cell lines (human breast cancer, MCF 7 and human cervical cancer, HeLa) [89]. BPA had oestrogen-mimicking effects in MCF-7 cells by inducing apoptosis in these cells and increasing the expression of known ERα-regulated genes [90]. The oestrogen activity of BPA has also been assessed in human cell lines of different cell types that were transfected with ERα or ERβ; Ishikawa (endometrial adenocarcinoma cell), HeLa (cervical epithelial cancer cells) and HepG2 (hepatocellular cancer cells) [91]. BPA (100 nM) induced ERα binding to oestrogen response elements in all three cells lines. However, activation of ERβ by BPA was only detected in the HeLa cells [91]. BPA also exhibited oestrogen-antagonizing effects in Ishikawa cells by suppressing E2-induced PGR expression [92]. Therefore, the oestrogen-mimicking effects by BPA could be mediated through block of PGR thus reducing progesterone-induced inhibition of oestrogen action. BPA activated GPER1 in human breast cancer cells and primary human breast tumour fibroblasts. Further, BPA binds to the human oestrogen-related receptor-gamma (hERRγ; ESRRG) with a high affinity Kd of 5.5 nM in transfected HeLa cells [93]. The ERRγ is a self-activated nuclear receptor, which is deactivated by inverse agonists such as 4-hydroxy-tamoxifen, a drug used in breast cancer therapy. Despite that BPA had no apparent effect on the high basal activity of ERRγ, BPA blocked the ER-antagonist 4-hydroxy-tamoxifen deactivation in a dose-dependent manner [93]. Exposure of 50 nM BPA to HeLa cells transfected with the mAR blocked DHT binding to the receptor by 40% in a non-competitive manner [94]. BPA also inhibited AR induction by 10 nM testosterone in two different reporter assays, with IC50-values in the nanomolar range [94]. Further, BPA suppressed release of testosterone and androstendione in a dose-dependent manner in a human adrenocortical carcinoma cell line [95].

3.1.5.2 Animal studies; effects on mammary gland and testes in offspring to dams exposed to BPA during pregnancy

Pregnant Sprague Dawley rats were given two doses of BPA (25µg or 250µg/kg bw/day; 0.005 and 0.05 x BPA NOAEL, respectively) or vehicle oil by gavage from gestational day 10 to delivery. At birth, the offspring was transferred to surrogate mothers to suckle [96]. Exposure of 0.05 x BPA NOAEL caused changes in cell composition and structure of the mammary gland in the female adult offspring, with higher number of undifferentiated structures. The lower dose of BPA also modified the gene expression profile of the gland as a function of age. Low doses of BPA (2.5 or 25 µg/kg bw/day; 0.0005 and 0.005 x BPA NOAEL, respectively) or vehicle oil was administered by gavage to pregnant Long Evans rats from gestational day 12 to the end of pregnancy [97]. BPA exposure did not affect litter size nor pup sex ratio. There was

no treatment effects on postnatal body weights or testis weight in the male offspring followed up to the age of 90 days. Serum levels of anti-mullerian hormone were increased in adult male offspring from the 0.005 x BPA NOAEL group, which was associated with increased protein expression of Cyp19a1 (aromatase) and Ar in the testis. *Ex vivo* studies showed an increased production of E2 in testes from offspring after the BPA exposure, whereas the production of testosterone did not differ from control. However, high-fat feeding for four weeks decreased serum testosterone levels in adult offspring exposed in utero to 0.005 x BPA [98].

3.1.5.3 Animal studies; effects on pregnancy outcome, reproductive toxicity, organ weights, serum hormone levels in offspring after perinatal BPA exposure

Pregnant NCTR Sprague Dawley rats were given daily gavage of low doses of BPA (2.5, 8, 25, 80, 260, 840, 2700 µg/kg bw/day; range 0.0005 to 0.54 x BPA NOAEL), high dose BPA (100; 300 mg/kg bw/day; 20 AND 60 x BPA NOAEL), ethinyl-E2 (0.5, 5.0 µg/kg bw/day) or vehicle control from gestational day 6 until delivery [99]. The pups were dosed daily by gavage from birth to day 90. Primary endpoints were reproductive toxicity (time of vaginal opening, testicular descent, sex organ weights, and day 69–90 oestrous cycle); secondary endpoints were organ weights, leptin, triglycerides, and insulin levels. Although the primary focus on the study was on the low dose groups, there were no consistent results at the lower BPA doses. In contrast, changes in hypothalamic gene expression was observed in one day old pups from NCTR Sprague Dawley dams administered BPA by gavage from gestational day 6 (0, 2.5, 25, 250, 2,500 or 25,000 µg/kg bw/day; 0, 0.0005, 0.005, 0.05, 0.5, 5 x BPA NOAEL, respectively). Even at the 0.005 x BPA dose, *Esr1* and *Esr2* mRNA levels were affected in the hypothalamus and oxytocin mRNA levels in the hypothalamus and hippocampus in sex-specific manner [100]. Oxytocin is important for brain development and plasticity in the neonate. However, the same group previously showed that gavage per se in new-born pups affected the gene expression of *Esr* in the neonate brain [101]. Female agouti mice, an obese mouse model used for epigenetic studies [102], were fed phytoestrogen-free diets supplemented with BPA (approx. 7 ng, 7 µg and 7 mg/kg bw/day; 0.0000007, 0.0007, 0.7 x BPA NOAEL, respectively) two weeks before mating, throughout pregnancy and lactation (3+3 weeks) [103]. Neither litter size nor pup sex ratio was affected by any of the BPA exposures. These studies show no effects on pregnancy outcome or toxicological endpoints, even at very high perinatal BPA exposure of 20 x BPA NOAEL.

3.1.5.4 Animal studies; effects on serum hormone levels after BPA exposure

Young adult male Sprague-Dawley rats were given placebo or BPA doses of 0.5 or 5 mg/kg bw/day (0.1 and 1 of NOAEL, respectively) by gavage for eight weeks [104]. Serum E2 levels were increased in animals given the low BPA dose, whereas serum testosterone and LH levels were decreased with the high BPA dose. There was no effect on serum FSH levels. Sperm motility was reduced and sperm abnormalities were increased by both BPA doses but there was no effect on sperm count.

3.1.5.5 Human studies; association of serum BPA levels and pregnancy outcome

A retrospective study from Stanford, U.S., reported that high serum levels of conjugated BPA in early pregnancy were positively associated with increased risk for miscarriage (n=115; 68 miscarriages) [105]. A Japanese prospective study found that the presence of antinuclear antibodies was associated with significantly higher serum BPA levels in patients with three or more miscarriages (n=45) [106]. However, high serum BPA levels alone did not predict early miscarriage in these patients.

3.1.5.6 Human studies; association of u-BPA levels and fertility measures in men

A prospective study on healthy young men in Denmark (n=303 and 298, hormone and semen analyses, respectively) showed that men in the highest quartile of u-BPA concentrations had higher serum levels of LH, testosterone and E2 compared to men in the lowest u-BPA quartile[27] [107]. Serum FSH, inhibin B, SHBG, and ratios between hormones were not significantly associated with BPA exposure. Sperm motility in semen was reduced in subjects in the highest u-BPA quartile compared to men in the first u-BPA quartile.[28] There was no association between u-BPA and with semen volume, sperm concentration, total sperm count or normal sperm morphologically. Similarly, a Czech study of fertile men and men with impaired fertility (n=89 and 59, 25, 18; healthy and slightly, moderately, severely infertile men; mean age 35–36 years) found that seminal BPA concentration was negatively correlated with sperm concentration, count and morphology, and plasma BPA was negatively correlated with sperm motility[29] [76]. Plasma BPA levels were higher in slightly or moderately infertile men compared with healthy men and severely infertile men. Furthermore, plasma BPA levels correlated positively with plasma levels of E2, oestrone, pregnenolone, 17-OH-pregnenolone and dehydroepiandrosterone (DHEA) and negatively with plasma DHT levels. In semen, seminal BPA correlated negatively with seminal levels of pregnenolone, 17-OH-pregnenolone and DHEA, and positively with seminal E2 and oestriol levels. These studies show a correlation between high BPA exposure and reduced sperm motility, in analogy to the findings in adult rats exposed to 0.1 or 1 x BPA NOAEL.

3.1.5.7 Human studies; association of serum BPA levels and nuclear receptor expression

Women from metropolitan areas had higher serum BPA levels than with women from urban or rural areas in Italy [108]. The same study reported that in metropolitan areas, infertile women had higher mRNA levels of several nuclear receptors (*ESR1*, *ESR2*, *AR*, *AHR* and *PXR*) in peripheral blood monocytes compared with fertile women [108]. Serum BPA levels in both fertile and infertile men were positively correlated with gene expression of *ESR1*, *ESR2*, *AR*, *AHR* and *PXR*, but not *PPARG* in peripheral blood monocytes [109]. These studies suggest a correlation between high serum BPA levels and increased gene expression of steroid hormone receptors in blood monocytes.

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[27] Adjusted for BMI, smoking, and time of day of blood sampling.

[28] Adjusted for smoking, varicocele, cryptorchidism, genital conditions, and time to motility analysis.

[29] Adjusted for age, BMI and abstinence time.

3.1.5.8 Human studies; association of u-BPA levels and serum hormone levels

A cross-sectional study in adult men (China; n=560; age 32.2±6.5 years, range 19 to 54 years) found positive association between serum LH and u-BPA but not with serum FSH or testosterone[30] [110]. This association was strengthen in the sub-group of current smokers with detectable u-BPA (n=225), who also had a positive association between serum FSH and u-BPA. In non-smokers with detectable u-BPA (n=163) the association between u-BPA and serum LH was lost. Further, the association between u-BPA and LH was found in lean but not overweight/obese subjects (BMI cut-off[31] 25 kg/m^2; n=283 and 111, respectively). However, in the overweight/obese subjects there was a negative association between u-BPA and serum testosterone levels. These findings suggest that the effects of BPA exposure in serum LH/FSH levels may be modified by other environmental factors.

3.1.6 Foetal exposure: programming and epigenetics

BPA has been detected in human foetal serum, in the placentae and amniotic fluid at full-term, confirming placental transfer of BPA to the foetus [111–113]. However, reported values of unconjugated or total BPA in body fluids should be treated with caution since BPA is present in some medical and laboratory plastic disposables (*e.g.* tubes, tubing). The methods used have been ELISA [111], LC-MS/MS using polyethylene lab ware disposables [112] and GC-MS using glass vials [113]. BPA has been detected in human milk collected in BPA-free polypropylene tubes [114]; BPA and chlorinated BPA has been detected in human colostrum collected in glass vials [115] and chlorinated BPA has been detected in human white adipose tissue [116].

3.1.6.1 Prenatal exposure: placental transfer of BPA

Cell viability was reduced by high BPA concentrations (≥ 100 µM) in BeWo cells (human placental epithelial cell line) and *ex vivo* cultures of human first trimester chorion[32] villi explants exposed to 1 nM BPA induced caspase-3 (CASP3) cleavage, a marker for apoptosis [117]. Whereas the high BPA dose suppressed β-hCG secretion in BeWo cells, indicative of loss of trophoblast function, the low BPA dose increased β-hCG secretion in the explants, suggesting an increase in the differentiation to syncytiotrophoblasts. BPA could be transported in both maternal-foetal and foetal-maternal direction over a BeWo monolayer, but with higher permeability in the foetal-maternal direction. The foetal to maternal clearance was investigated in toxicokinetic studies in sheep showing that about 70% of the BPA entering the foetal circulation (5 mg/kg) was rapidly removed by clearance of free BPA to the maternal circulation with a half-life of 20 min [118]. However, about a quarter of the

[30] Adjusted for age, BMI, nationality, alcohol intake and history of chemical exposure.

[31] WHO experts for Asian China Obesity Task Force define overweight as BMI 24 kg/m2 and obesity as BMI 28 kg/m2.

[32] The chorion is the double layer foetal membrane; the outer layer consists of trophoblasts and the inner of somatic mesoderm which is in contact with the amniotic fluid.

BPA was glucuronidated by the foetus and was slowly eliminated from foetal circulation (> 150 h). BPA-sulphate was also formed in the foetus and showed similar elimination profile as BPA-glucuronide. These studies confirm that BPA can be transported to the foetus and that BPA can be conjugated by the foetal liver and trapped in foetal circulation.

3.1.6.2 Prenatal exposure: placental and foetal tissue BPA content

BPA content in human placenta has been measured in a number of studies. Data from pregnancy cohort (n=200; Tennessee, U.S.) found average BPA placental concentrations of 103.4 ± 61.8 ng/g dry weight (range 4.4, 273.9 ng/g dry weight) [119]. Birth weight centiles were calculated taking into account parameters known to affect foetal growth, and the calculated birth weight centiles were negatively correlated with placental BPA concentrations [119]. Data from a prospective study (n=80; Michigan U.S.) found that a two-fold increase in maternal plasma unconjugated BPA levels at first trimester was associated with 183 g lower birth weight in female pregnancies only[33] [120]. Further, a two-fold increase in maternal unconjugated BPA levels at term was associated with 1.1 days longer gestation in the female pregnancies. These studies reported a negative correlation between placenta BPA content and birth weight, and also first trimester unconjugated BPA concentration and female baby birth weight.

Foetal liver [121], foetal kidney and placenta [122] were obtained from elective termination of pregnancy in the second trimester from 12 subjects and free and conjugated BPA content analyzed. The concentrations varied significantly across matched tissues for both free BPA (foetal liver: 0.54–50.5 ng/g; foetal kidney: 0.08 to 11.1 ng/g; placenta: below detection to 25.4 ng/g), and conjugated BPA (foetal liver: 0.45 to 15.9 ng/g; foetal kidney: below detection to 0.75 ng/g; placenta: below detection to 9.81 ng/g). Global DNA methylation and gene expression were analyzed in first trimester foetal livers (gestational days 70–120; n=18), divided into three BPA exposure groups: high (35.44 to 96.76 ng/g), low (3.50 to 5.79 ng/g) and non-detectable (<0.83 ng/g) [123]. Similarities between the low and high BPA exposure group versus the non-detectable suggest that BPA exposure in general results in hypomethylation of foetal liver DNA. The results are also indicative of dose-dependent effects since the largest difference in methylation was between the low and high BPA exposure groups.

3.1.6.3 Animal studies: effects of perinatal BPA exposure on pancreas and glucose homeostasis (for effects on the HPA- and the HPG-axes: see Sections 2.1.3 and 2.1.5, respectively)

Studies using Wistar rats reported that 50 µg BPA/kg bw/day (0.01 x BPA NOAEL) but not doses ≥ 250 µg BPA/kg bw/day (0.05 x BPA NOAEL) during pregnancy and lactation caused early β-cell dysfunction with swollen mitochondria and late onset of liver damage in the offspring [124,125]. *In vitro* studies in hepatocytes showed that BPA acted directly on mitochondria by affecting mitochondrial ultrastructure, inducing

[33] Blood samples were stored in glass vials, avoiding contact with plastic. Adjusted for race, education, marital status, occupation, smoking exposure, and maternal age.

mitochondrial permeability transition with swelling and causing release of proteins leading to apoptosis [125]. Using slightly lower maternal exposure of BPA, 40 µg/kg bw/day (0.008 x BPA NOAEL) during gestation and lactation, confirmed later development of fatty liver in the adult male rat offspring that was present from the age of 15 weeks [126]. In addition, mitochondria from the 3-week old BPA-exposed rat offspring showed early signs of dysfunction of fatty acid oxidation in the liver. These data suggest that low perinatal BPA exposure cause dysfunction of mitochondria in pancreatic insulin-producing cells and liver that predisposes to development of non-alcoholic fatty liver.

3.1.6.4 Animal studies: effects of perinatal BPA exposure on adipose tissue and fat storage

In obesity research, there is a difference between hyperplastic (many cells) and hypertrophic (large cells) adiposity since paradoxically the metabolic consequences to obesity are due to inability of adipose tissue to store excess energy. This is the case with hypertrophic adiposity where large fat cells cannot store more lipids and the recruitment of new fat cells is suboptimal. Male and female adult Wistar rat offspring of dams exposed to 50 µg BPA/kg bw/day (BPA-50; 0.01 x BPA NOAEL) during pregnancy and lactation had increased bw, elevated serum insulin levels and impaired glucose tolerance compared to controls [124]. On high fat diet (HFD), these effects were enhanced with earlier onset of obesity, dyslipidaemia and glucose intolerance. The 0.01 x BPA NOAEL resulted in enlarged adipocytes irrespective of post-weaning diet. The perinatal exposure of 0.01 x BPA NOAEL also affected developmental commitment of mesenchymal stem cells to the adipogenic pathway, reflected in hypertrophic (large cells) but not hyperplastic (large number of cells) adipocytes. This was in agreement with an *in vitro* study showing that BPA exposure to mesenchymal stem cells reduced the number of cells that could differentiate to adipocytes [127]. In CD-1 mice, gonadal adipocyte number and size, and renal adipocyte size were increased in the offspring to dams exposed to BPA (50 µg BPA/kg bw/day; 0.01 x BPA NOAEL) during mid- to late pregnancy, and was accompanied by increase in serum insulin levels and impaired glucose tolerance [128]. Young adult offspring to pregnant California mice fed BPA (5 mg/kg bw/day; 0.5 x BPA NOAEL) two weeks before conception and throughout pregnancy and lactation showed sexual dimorphic effects in feeding pattern, physical activity and energy source. Both female and male offspring from the BPA-fed dams exhibited a disturbed circadian rhythm in feeding with increased eating during the resting period (day) compared to control animals [129]. The female offspring had reduced voluntary physical activity and higher respiratory quotient, indicating a preferential use of carbohydrates as energy source rather than fat. There were no differences in serum levels of glucose, insulin, leptin or adiponectin or body composition. However, the reduced physical activity, and disturbed circadian rhythm confer an increased risk for developing of age-onset obesity and diabetes.

Male adult Wistar rats, fed either standard diet or HFD, were treated with either vehicle or BPA (50µg/kg bw/day; 0.01 x BPA NOAEL) mixed in the diet [130]. After 21 weeks of age, the males were mated with adult female rats. The pregnant rats were fed with standard diet during gestation and lactation. In the male rats treated with BPA,

blood glucose homeostasis was deteriorated (increased fasting glucose, reduced glucose tolerance and decreased insulin sensitivity) in both diet groups but HFD feeding worsened the condition. However, isolated paternal BPA exposure did not affect metabolic function in the offspring fed standard diet.

These animal studies show that developmental exposure to low doses of BPA resulted in increased adiposity with enlarged fat cells, impaired glucose handling and increased blood lipid levels. BPA appeared to affect the commitment and/or recruitment of adipocyte precursor cells causing reduction in adipocyte number and increase in adipocyte cell size, which associated with higher metabolic risk. Low BPA exposure in adult males also impaired glucose handling in the males but had no effects in their offspring.

3.1.7 Other

3.1.7.1 Obesity/type 2 diabetes

Human primary adipose-tissue-derived stromal/stem cells were pooled from three female lean donors and cultured in the presence of 100 pM to 10 µM BPA or 10 nM E2 [131]. BPA at 100 nM and 1 µM concentrations increased fat cell differentiation through an oestrogen-dependent pathway since an ER-specific antagonist blocked BPA action. Exposure of 1 µM BPA enhanced the rate of differentiation to mature adipocytes. Human primary subcutaneous preadipocytes, pooled from several lean donors were induced to differentiate and a PPAR-γ agonist was added together with 50 µM BPA or 1 µM dexamethasone in the early stage of differentiation [132]. Both BPA and dexamethasone induced changes in gene expression consistent with adipocyte differentiation. Other pathways (the SREBF1, thyroid hormone receptor-RXR and the mTOR pathways) were identified as potential mechanisms of action for BPA-induced adipogenesis [132]. *Ex vivo* cultures of human omental adipose tissue or isolated omental adipocytes obtained from children (n=17) were exposed to BPA at three concentrations; 10 nM, 1 µM, and 80 µM [133]. All doses of BPA increased *HSD11B1* gene expression and HSD11B1enzyme activity via the glucocorticoid receptor, since a specific glucocorticoid receptor antagonist blocked the effects. The adipose tissue HSD11B1 catalyzes the conversion of cortisone to the active hormone cortisol and promotes adipogenesis. Increased adipogenesis was also induced by 10 µM BPA in the mouse fibroblast cell line 3T3-L1 [134]. BPA at low concentration (1 nM) triggered insulin release from primary human and mouse pancreatic beta-cells through depolarization of the cells by blocking KATP channels [135]. This effect was dependent on the presence of ERβ since it was abolished in pancreatic cells from ERβ-knockout mice.

Male WHHL rabbits, given oral BPA (400 µg/kg bw/day; 0.16 x BPA NOAEL)[34] by gavage for 12 weeks, had increased subcutaneous and visceral adipose tissue mass that was characterized by enlarged fat cells [136]. Further, these animals also had mild hepatic steatosis with inflammatory cell infiltration. In contrast, there were no effects of oral BPA at 50 µg/kg bw/day (0.01 x BPA NOAEL) in adult male Wistar rats fed with

[34] NOAEL for rabbits was calculated to be 2.5 mg/kg bw/day according to Reagan-Shaw 50. Reagan-Shaw, S., Nihal, M., Ahmad, N. (2008) Dose translation from animal to human studies revisited. FASEB J, 22: 659-61.

either low-fat or HFD for 35 weeks on serum and liver lipid levels or hepatic lipid metabolism [137].

Plasma BPA concentrations in men were positively correlated with waist circumference, triglycerides, glucose homeostasis and inflammatory markers. Multivariate analysis identified waist circumference and plasma IL-6 remained as the main predictors of plasma BPA levels [138].

The *in vitro* studies show that BPA exposure enhanced adipogenesis in human stem cells via ERα-activation and in PPARγ-induced differentiation of human primary preadipocytes BPA enhanced adipogenesis via the thyroid hormone receptor, SREBF1, and mTOR pathways. Cultures of intra-abdominal human adipose tissue biopsies with BPA induced HSD11B1 activity that activates cortisone to cortisol, and promotes fat storage but inhibits insulin action.

3.1.7.2 Cardiovascular disease

In vitro studies showed that treatment with BPA and E2 caused arrhythmia in primary rat cardiomyocytes from female but not male Sprague-Dawley rats [139]. The arrhythmia was caused by dysfunction of the myocyte calcium handling and was dependent on an intact ERβ signalling. Exposure of BPA to primary cultures of rat cardiomyocytes from 8–10 week old female Sprague-Dawley rats in the nM to µM range resulted in both monotonic and non-monotonic dose responses for different intracellular calcium signalling endpoints [140]. BPA stimulated both Ca2+ release and reuptake in the sarcoplasmic reticulum in a monotonic manner; dose response had an EC50 of 0.81 and 0.15 nM, respectively. At higher concentrations BPA inhibited L-type Ca-channels in a monotonic dose response manner with an EC50 of 27.4 nM. The net effect of BPA stimulating sarcoplasmic reticulum Ca2+ release and uptake at low doses, and inhibiting L-type Ca-channels at high doses mimicked a non-monotonic dose response of BPA on Ca2+ fluxes in the cell. These effects by BPA were mediated by ERβ, since blocking this receptor abolished BPA effects on both sarcoplasmic reticulum and L-type Ca-channels. It has also been shown that 1 nM BPA activated both protein kinase A (PKA) and Ca2+/Calmodulin-dependent protein kinase II (CamKII) in female primary rat cardiomyocytes [141]. Phosphorylation of PKA contributed to BPA-induced Ca2+ leakage of the sarcoplasmic reticulum, but activation of both PKA and CamKII were necessary for BPA-induced arrhythmia.

Eight-week old CD-1 mice given oral BPA through drinking water (0.4 nM to 400 µM; 0.4 µM corresponds to approx. 12 µg/kg bw/day;[35] 0.0001 x BPA NOAEL) had increased systolic and diastolic blood pressure after 30 days [142]. The increase in blood pressure appeared to have a monotonic dose response, reaching a plateau between 4–40 µM BPA. BPA induced angiotensin-II that caused uncoupling of eNOS and subsequent endothelial dysfunction. BPA also induced CamKII-activation in the endothelium, and blocking either angiotensin-II or CamKII normalized the BPA-induced hypertension [142]. The effect of BPA on development of atherosclerosis was

[35] BG calculation: assuming 30 g mouse and drinking 4 ml water/day; Mw of BPA 228.29 g/mole gives 0.4 x 10-9 moles/ml x 4 ml/day x 229 g/mole divided with 0.03 kg.

investigated in male hPXR+/+ApoE-/- mice [143]. BPA was given via the diet (50 mg/kg diet, corresponding to approx. 7 mg/kg bw/day; 0.7 x BPA NOAEL) and yielded similar u-BPA levels in the mice as found in humans. Chronic exposure of dietary BPA for 12 weeks doubled atherosclerotic plaques in the aorta from humanized hPXR+/+ApoE-/- mice but not in their PXR-/-ApoE-/- littermates. WHHL rabbits given oral BPA (400 µg/kg bw/day; 0.16 x BPA NOAEL) for 12 weeks increased atherosclerotic lesions in the aortic arch by over 50% and increased gene expression for markers of endoplasmic reticulum stress, inflammation and lipid metabolism.

Higher serum BPA levels were detected in patients diagnosed with dilated cardiomyopathy [144]. A systematic review with meta-analysis of epidemiological data of u-BPA levels and cardiometabolic risk factors (33 studies, sample size from 239 to 4811) found that subjects with high u-BPA concentrations are significantly more likely to suffer from diabetes, general and abdominal obesity and hypertension than subjects with low u-BPA levels [145].

These studies show that BPA exposure caused arrhythmia in primary cardiomyocytes via an ERβ-dependent pathway. BPA induced abnormal intracellular Ca2+ concentrations by causing leakage from intracellular stores, and by inhibiting voltage-gated L-type Ca-channels in the cardiomyocytes. The BPA effects were also dependent on PKA and CamKII activation both affecting intracellular Ca2+ homeostasis. BPA exposure in adult atherosclerotic mouse and rabbit animal models enhanced the formation of atherosclerotic plaques in the aorta at doses below the current NOAEL (0.7 and 0.16 x BPA NOAEL, respectively). Meta-analysis of cohort data showed that subjects with high u-BPA concentrations are more likely to have diabetes, central obesity and high blood pressure.

3.1.8 Summary

Of the selected EDs in this overview, BPA had by far the largest number of references, with the exception of genistein. This appears mainly due to that BPA has been used as a model compound for the study of endocrine effects at doses lower than the current NOAEL of 5 mg/kg bw/day established by EUs Scientific Committee of Foods (SCF) in 2002. Previous studies have established that the main mode of action of BPA is acting as an oestrogen agonist. Therefore earlier studies have focussed on adverse effects by BPA on female reproductive health, in particular effects on ovarian function.

Metabolism: Ingested BPA is predominantly metabolized in the liver by phase II conjugation. The half-life of BPA after oral exposure is short, estimated to 4–5 hours, which makes spot u-BPA levels unreliable proxies as exposure measures.

Mechanism-of-action: BPA binds both ERα and ERβ in transfected cells but the activation of the receptors depended on which cells that were used in the studies, suggesting that other unidentified proteins modified BPA action. BPA also activated the nonnuclear oestrogen receptor mER/GPER1 suggesting interference with intracellular Ca2+ homeostasis, an important second messenger in neurones, cardiomyocytes and pancreatic β-cells. BPA interfered with thyroid hormone signalling *in vitro* by recruiting a corepressor protein to transcription complex and blocking the binding of T_4/T_3 to a cell surface receptor. Exposure of BPA to human stem cells or

primary preadipocytes induced adipogenic effects via both the ERα and the thyroid hormone receptor pathways. Exposure of BPA to isolated pancreatic β-cells caused insulin release in an ERβ-dependent manner.

HPA-axis: Exposure of BPA during pregnancy or lactation in rats at doses below NOAEL had effects on the HPA-axis. There were developmental changes of the adrenal gland and enhanced plasma glucocorticoid levels in the adolescent rat offspring exposed to 0.008 x BPA NOAEL from conception to weaning. The perinatal BPA exposure affected stress responses in rats with reduced glucocorticoid signalling in the hypothalamus and hippocampus. In mice, postnatal exposure to low levels of BPA (0.001 NOAEL) via maternal diet in mice caused sex-reversal changes in behaviour of the offspring. In contrast, two large studies in Sprague Dawley rat offspring to dams given BPA doses below NOAEL (0.0005, 0.05 or 0.5 x NOAEL) by gavage before, and to the pups after birth, showed no differences from control in serum glucocorticoid levels or behaviour.

HPG-axis: Adult male rats given low oral BPA dose (0.1 NOAEL) for 2 months had increased serum E2 levels. A higher oral BPA dose (1 x NOAEL) also suppressed serum testosterone and LH levels. Both doses decreased sperm motility but not count, and increased proportion of abnormal sperms. In humans, higher u-BPA concentrations were associated with higher serum LH, testosterone and E2 levels. Plasma BPA levels were positively correlated with plasma E2 levels and negatively with plasma dihydrotestosterone levels. Sperm motility was negatively correlated to both u-BPA and plasma BPA, whereas seminal BPA levels were correlated negatively with sperm concentration, count and morphology.

Foetal exposure: programming and epigenetics: The transfer of BPA to the foetal compartment is a prerequisite to consider BPA as factor in foetal origin of adult disease. BPA has been detected in human milk, placenta, amniotic fluid, foetal circulation and foetal tissues. Infants with low-birth weight had significantly higher placental concentration of BPA than normal birth weight infants, indicating a negative association between BPA exposure and foetal growth.

Other: BPA caused arrhythmia in primary myocytes from female, but not male rats. The net effect of BPA stimulating sarcoplasmic reticulum Ca^{2+} release and uptake at low doses, and inhibiting L-type Ca-channels at high doses mimicked a non-monotonic dose response of BPA on Ca2+ fluxes in the cell. The BPA induction of arrhythmia was ERβ-dependent.

In vivo studies in rats reported that low perinatal exposure of 0.01 x NOAEL BPA caused an early onset dysfunction of pancreatic β-cells characterized by swollen mitochondria that was associated with liver damage in the adult offspring. However, exposure to higher BPA (> 0.05 x BPA NOAEL) levels did not affect these parameters. Further, the adult offspring to rat dams exposed to a low BPA dose had increased body weights and impaired glucose handling, which further deteriorated by high fat feeding. Similar experiments were performed in pregnant mice where a low dose of BPA (0.005 x NOAEL) during the latter half of pregnancy resulted in increased adipose tissue mass, increased serum insulin levels and impaired glucose tolerance in the adult offspring.

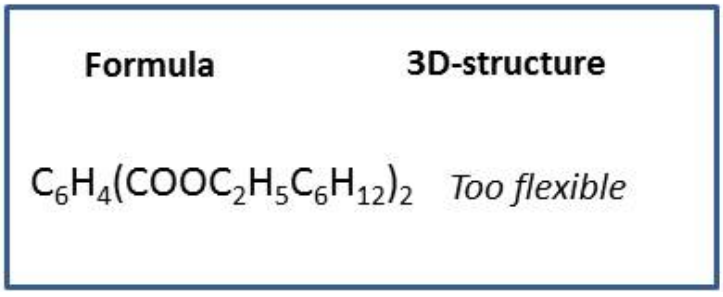
Meta-analysis of cohort data showed that subjects with high u-BPA concentrations are more likely to have diabetes, central obesity and high blood pressure. This could infer that the development of these non-communicable diseases, known to be strongly associated with socioeconomic parameters in several countries, could further be worsened by poor lifestyle choices, such as high consumption of soft drinks with high sugar content from plastic bottles containing BPA.

3.2 Phthalates

Phthalates and their esters are used as plasticizers, *i.e.* softeners to increase flexibility, and are found in a large range of plastic products. The phthalates are not covalently bound to the plastic, consequently the phthalates can migrate from the food packaging and leach into the food by diffusion. Phthalate-containing plastics are found in food containers and medical devices including tubing and bags for intravenous administration of drugs or parenteral nutrition. Phthalates are ubiquitous environmental contaminants due to their widespread use. Humans are exposed to phthalates via ingestion, dermal absorption and via the lungs by inhalation of dust. This overview focusses on the five phthalates authorized for use in plastics in contact with foodstuffs. Other phthalates are also used in the food industry and their metabolites have been detected in human tissues. Di-iso-nonylcyclohexane 1,2-dicarboxylate (DINCH), regarded as less toxic than DEHP, is a common replacement for DEHP and other phthalates. Some other new phthalates appearing in foods from packaging are di-(2-ethylhexyl)-terephthalate (DOTP) and di-(2-propylheptyl)-phthalate (DPHP).

DEHP (di-(2-ethylhexyl) phthalate; CAS no 117-81-7)

Figure 6: Molecular structure of DEHP



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=8343, https://pubchem.ncbi.nlm.nih.gov/compound/8343 (accessed 7 May 2018).

The primary source of DEHP exposure for the majority of people is ingestion via food. DEHP migrates into foods, particularly fatty foods, from DEHP-containing materials that are used to process and package food. Indoor air and dust are other common sources of exposure. DEHP is rapidly absorbed from the gastrointestinal tract following oral administration, but there is little evidence of accumulation. The metabolism of DEHP involves several pathways and yields a variety of metabolites. The major step in the metabolism of DEHP is hydrolysis by lipases to MEHP (mono (2-ethylhexyl)-

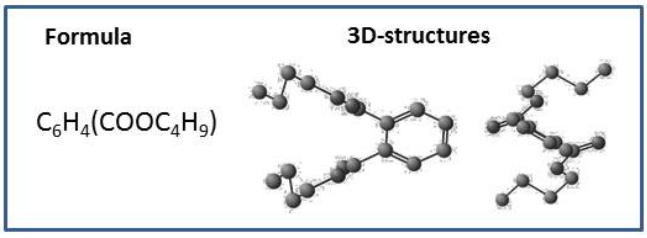
phthalate) and 2-ethylhexanol with 5-OH-MEHP and 5-oxo-MEHP identified as the main urinary metabolites [146].

EFSA: The critical toxic effects of DEHP relate to reproduction. A three-generation reproductive study in which DEHP was administered to rats in the diet gave a NOAEL of 5 mg/kg bw/day for testicular and developmental toxicity, and a TDI of 0.05 mg/kg bw/day was established by EFSA in 2005 and further supported by the Scientific Committee on Health and Environmental Risks (SHER).

ECHA: According to the harmonised classification and labelling (CLP00) approved by the EU, DEHP may damage fertility and may damage the unborn child. DEHP is a substance of very high concern (SVHC). DEHP requires authorization before it is used (Annex XIV of REACH) and some uses are restricted (Annex XVII of REACH). The oral DNEL is set to 36 µg/kg bw/day.

DBP (Dibutyl phthalate; CAS no 84-74-2)

Figure 7: Molecular structure of DBP



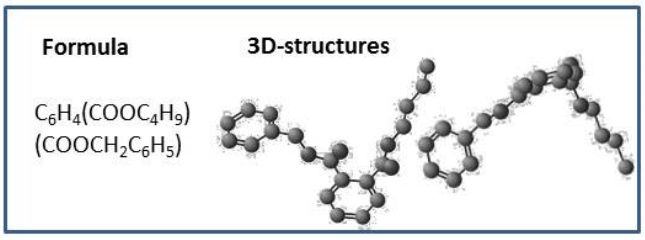
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=3026, https://pubchem.ncbi.nlm.nih.gov/compound/3026 (accessed 7 May 2018).

EFSA: a toxicity study of DBP showed effects on the male reproductive system with a NOAEL of 50 mg/kg bw/day. However, a study in rats showed that feeding DBP to mothers in late pregnancy and during lactation affected the development of both male and female offspring with a LOAEL of 20 mg/kg bw/day. TDI for DBP was therefore set to 0.01 mg/kg bw/day in 2005.

ECHA: DBP is classified as a substance toxic to reproduction (REACH Article 57c) and is included in Annex XIV of REACH, known as the Authorisation List. The DNEL for oral exposure is set to 7 µg/kg bw/day.

BBP (Benzyl butyl phthalate, CAS no 85-68-7)

Figure 8: Molecular structure of BBP



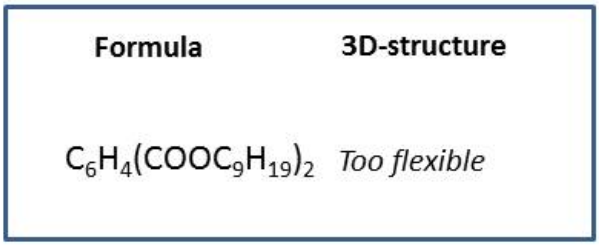
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=2347, https://pubchem.ncbi.nlm.nih.gov/compound/2347 (accessed 7 May 2018).

EFSA: a TDI of 0.5 mg/kg bw/day has been set that is derived from a NOAEL of 50 mg/kg bw/day on testicular toxicity found in a multi-generation study. The NOAEL was based on the reduction in anogenital distance in F1 and F2 offspring at 250 mg/kg bw/day.

ECHA: BBP is classified as a substance toxic to reproduction (REACH Article 57c) and is included in Annex XIV of REACH. The DNEL for oral exposure is set to 500 µg/kg bw/day.

DINP (Diisononyl phthalate; CAS no 68515-48-0[36] and CAS no 28553-12-0)[37]

Figure 9: Molecular structure of DINP



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=590836, https://pubchem.ncbi.nlm.nih.gov/compound/590836 (accessed 7 May 2018).

DINP and DIDP (the C-10 analogue) are widely used as plasticizers in polymer manufacturing. Due to their relatively low toxicity, DINP and DIDP have been seen as suitable replacements for more toxic phthalates (such as DEHP) in the manufacturing of consumer products. Although the human biological monitoring data on DINP and DIDP are currently limited, the available data suggest widespread human exposure to these compounds.

EFSA: The main toxicological effects for DINP are changes in the liver; there was an increased incidence in the type of hepatitis associated with increased liver enzyme levels; increases in absolute and relative liver and kidney weights in both sexes in a two-year chronic toxicity study in rats. Based on these data EFSA decided on a NOAEL of 15 mg/kg bw/day for non-peroxisomal proliferation-related chronic hepatic and renal effects and a TDI of 0.15 mg/kg bw/day.

ECHA: DINP2 (CAS no 68515-48-0) is restricted for use in toys which can be placed in the mouth (REACH Annex XVII). The DNEL for oral exposure is set to 4.4 mg/kg bw/day.

[36] DINP1 is a mixture of isomers C8-C10 chains, with a majority of C9-chains

[37] DINP2 is composed of C9-chains only.

3.2.1 Criteria for literature search

The PubMed search yielded an initial 1149 articles that are published after 2010-01-01. Further exclusion of articles written in other languages than English gave a final of 1092 references. This was followed by manual curation to remove articles which did not filled the criteria; doses only above NOAEL;[38] focus on non-mammalian species; other routes of administration; other EDs was the focus of the study or that the selected phthalates were a minor component in mixtures of several EDs. Reviews, opinions or hypothesis papers were also excluded with exception of systematic reviews.

Table 6: PubMed search 8 December 2016 phthalates and endocrine action published after 31 December 2009

Search	Query	Items found
#3	#1 AND #2 Filters: Publication date from 2010/01/01 to 2017/12/31	1,149
#2	((((DEHP OR di-(2-ethylhexyl) phthalate OR Bis(2-ethylhexyl) phthalate)) OR (DBP OR Di- butyl phthalate)))) OR (BBP OR Butyl benzyl phthalate)) OR (DINP OR Diisononyl phthalate) (((((((endocrine) OR endocrinol*) OR	16,195
#1	estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

Note: Note that there were substantially fewer studies in animals that investigated the effects of phthalates at doses below the current NOAELs compared to BPA exposure.

3.2.2 Metabolites/metabolism oral route

The main exposure of DEHP is via the food in adults, although in some subjects inhalation of dust can also be a source [147].The primary metabolite of DEHP is MEHP, which is rapidly metabolized to the secondary metabolites 5-OH-MEHP, 5-oxo-MEHP, 5-carboxy-MEPP and 2-carboxy-MMHP [148]. In a study of 20 volunteers (10 males, 10 females; age >18 years), the half-life of the DEHP metabolites was 4-8 hours, with 90% excretion during the first 24 hours [149].

DBP is rapidly absorbed and excreted after oral administration; over 90% of the oral doses given to rats or hamsters were excreted in urine within 24–48 hours. The major part of DBP is hydrolyzed to mono-n-butyl phthalate (MBP) and the corresponding alcohol before absorption by the small intestines, but hydrolysis can also occur in the liver and the kidneys. The metabolites that are detected in urine are MBP, MBP-glucuronide, various oxidation products of MBP and small amount of free phthalic acid.

BBP is mainly metabolized to monobenzyl phthalate (MBzP) in humans. There is no report on BBP half-life, but available data indicate a half-life of less than 24 hours.

Since DINP is a mixture with C8–C10 alkyl side chains, various isomers of OH-, oxo- and carboxy-metabolites are excreted in the urine. In the same study as DEHP of 20 volunteers, the half-life of the DINP metabolites is estimated to be in the range of 4 to 8 hours, and 90% of ingested dose was excreted in the first 24 hours [149]. The authors pointed out that due to the short half-lives of DEHP and DINP, exposure

[38] DINP2 is composed of C9-chains only.

measured in spot urine samples are likely to reflect what has been consumed in the last previous meal.

To summarize, diester phthalates are metabolized quickly primarily to their monoester form, and are excreted in the urine as monoesters, free or conjugated, and various oxo- and carboxy-metabolites usually within 24 hours after a single dose. The main exposure in humans is believed to be via food, but inhalation of dust is also an important source of exposure. Hence it is likely that humans are continuously exposed to phthalates.

3.2.3 The HPA-axis

3.2.3.1 Animal studies: effects of pre- and postnatal phthalate exposure on the adrenal gland

Sprague-Dawley dams received vehicle or DEHP (0.5, 1, 50, 100 or 300 mg DEHP/kg bw/day; 0.1, 0.2, 10, 20 or 60 x DEHP NOAEL) by gavage from gestational day 14 to birth [150]. The two highest doses of DEHP were associated with increase in cholesterol biosynthesis pathways and decrease in lipid metabolism pathways in the adrenal glands from adult offspring. The high DEHP doses also enhanced angiotensin II and potassium signalling pathways, suggesting an effect on blood pressure. Later they showed that young adult offspring prenatally exposed to the lower doses of DEHP (0.1 and 0.2 x DEHP NOAEL) had increased expression of Nr4a1 [151]. This transcription factor is important for function of the zona glomerulosa of the adrenal gland and implies that even a low dose of DEHP may affect the long-term regulation of blood pressure via aldosterone secretion. Even though basal serum aldosterone levels were not affected in the adult male offspring by these low doses, a PPARγ-antagonist lowered aldosterone levels in the DEHP-exposed but not in control male offspring. Another group reported that male offspring exposed perinatally to 0.2 x DEHP NOAEL had suppressed adrenal gland gene expression of Kcnk5 a potassium ion channel involved in aldosterone production.

Sprague Dawley dams were given DEHP (10 mg/kg bw/day; 2 x DEHP NOAEL) or vehicle control, n = 6) by gavage during lactation [152]. The control and DEHP-exposed female offspring were subdivided into two subgroups after weaning; one naïve group and one group trained to run on a treadmill 5 x á 30 min per week. At eight weeks of age, anxiety-like behaviour was evaluated by EPM. The naïve DEHP-exposed female offspring showed increased anxiety-like behaviour compared to their controls, whereas there was no effect of treatment in behaviour between the two exercised groups. Baseline and stress-induced plasma Acth levels were increased, whereas stress-induced plasma corticosterone was suppressed in the naïve DEHP-exposed offspring compared to their controls. There was no effect of DEHP exposure on any of the parameters in the exercised offspring. Physical exercise in adolescent female rats that were exposed to DEHP during lactation reduced their anxiety-like behaviour and normalized their regulation of the HPA-axis.

3.2.3.2 Effects of phthalate exposure on behaviour and CNS in animal experiments

Juvenile female ICR mice were administered DEHP by gavage (1, 10, 50 or 200 mg/kg bw/day; 0.1, 1, 5 or 20 x DEHP NOAEL, respectively) for 14 days [153]. Behavioural tests[39] were performed six weeks after treatment. There was no effect on the uteri weight or serum E2 levels in the DEHP-exposed mice. DEHP increased anxiety-like behaviour at all exposures, whereas increased social avoidance and reduced social interaction was only observed in animals at exposures ≥ 1 x DEHP NOAEL. All doses of DEHP reduced protein expression of ERβ and the dopamine D2 receptor in striatum, a subcortical brain area that is involved in movement and reward, suggesting that DEHP effects on the dopamine and central oestrogen signalling may be related to the behavioural changes.

Male adult NMRI mice received vehicle or DBP by gavage (6.25, 12.5, 25, 50, 100 or 200 mg/kg bw/day; 0.125, 0.25, 0.5, 1, 2 or 4 x DBP NOAEL) for 14 days [154]. Behavioural tests were performed on day 15 suggesting increased anxiety and impaired memory function in mice treated with DBP at doses ≥0.25 and 0.5 x DBP NOAEL, respectively. This was supported by brain histology showing that DBP exposure caused abnormal nuclei in granular cells in the dentate gyrus of the hippocampus, a brain area important for memory function. DINP administration to Kunming mice by gavage for two weeks (0, 0.2, 2, 20 or 200 mg/kg bw/day; 0.013, 0.13, 1.3 or 13 x DINP NOAEL) increased anxiety-like behaviour and negatively affected memory[40] in a dose-dependent manner [155]. Pathological changes in the hippocampus were observed even at the low doses of DINP and endoplasmic reticulum content in hippocampal cells was markedly reduced in mice exposed to the two highest DINP doses. Later the same group showed that DBP exposure (≥ 0.5 x DBP NOAEL) also resulted in an increase in anxiety-like behaviour in mice [156].

3.2.3.3 Human studies: association between phthalate exposure and adrenal gland hormones

In a prospective birth cohort of 202 mother-infant pairs in Japan (the Hokkaido study on environment and children's health) there was inverse correlation between maternal blood MEHP quartiles (week 23 to 35) and cord blood levels of cortisol, cortisone levels and glucocorticoid/adrenal androgen ratio [157]. However, the ratio between cortisol and cortisone exhibited a U-shaped curve where the first three maternal MEHP quartiles showed a negative trend and the highest MEHP quartile was associated with an increased cortisol/cortisone ratio. This may be a random effect or suggesting changes in the activity of HSD11B1/HSD11B2 during pregnancy.

[39] Open field, elevated plus maze, social play and social interaction tests.
[40] Open field test and Morris water maze, respectively.

3.2.4 The HPT-axis

3.2.4.1 In vitro studies on the effects of phthalate exposure on thyroid cells

An *in vitro* study showed that primary human thyroid cells metabolized DEHP and DBP to their respective monoesters [158]. The cells metabolized DBP faster than DEHP but the metabolism of both compounds was reduced at higher concentrations. MEHP suppressed thyroglobin and/or cAMP secretion. Gene expression of thyroid specific genes were unaffected by phthalate exposure.

3.2.4.2 Effects of phthalate exposure on thyroid hormone status during pregnancy and in the neonate

The correlation between phthalate exposure and thyroid hormone status in cord blood was investigated in a nested case–control cohort (n=439; Boston U.S.) [159]. Repeated measurements of urinary concentrations of nine phthalate metabolites (DEHP metabolites: MEHP, MEHHP, MEOHP and MECPP; DBP: MBP; BBP: MBzP; DINP: MiBP) during pregnancy were analyzed in samples collected at four visits (10, 18, 26 and 35 weeks of gestation). Maternal plasma samples were also collected up to four times and thyroid hormone status (TSH, total T_3, total T_4 and free T_4) was analyzed. There was an inverse correlation between several urinary phthalate metabolites, especially DEHP metabolites, and plasma TSH levels at visit 1 and 2 but not at the later visits. Plasma free T_4 levels were positively correlated with urinary phthalate metabolites at visit 1, 2 and 4 but not at visit 3.

In a prospective birth cohort from Puerto Rico (n=106), maternal spot urine and blood was collected at two time-points; at gestational week 18 and 26 [159]. The results showed an inverse correlation between total urine DEHP metabolites and free T_4 at late but not early second trimester.[41] One hundred and forty-eight maternal and infant pairs were recruited in southern Taiwan; spot urine and blood samples in the third trimester and cord blood samples at delivery were collected [160]. Metabolites of DEHP, DBP, BBP and DINP were analyzed in urine, and TSH, T_4, free T_4 and T_3 were analyzed in maternal blood and cord blood. Multivariate analysis showed negative correlation between cord blood TSH concentration and maternal urine MBzP levels.[42] There was no association between maternal serum MEHP levels (collected between gestational week 25 to 41) and infant serum T_4 or TSH levels nor on infant neurodevelopment at ages 6 and 18 months in the Hokkaido study from Japan (n=328 mother-infant pairs) [161]. The association between exposure markers of DEHP and T_4 levels was studied in new-born infants in a mother-child cohort in the Netherlands [162]. DEHP metabolites were measured in cord blood or breast milk, and information on T_4 levels in heel prick blood spots was obtained through the neonatal screening programme in the Netherlands. There were no correlation between cord blood or breast milk DEHP levels and infant serum T_4 levels in linear regression analyses.

[41] Linear regression models were adjusted for age at enrolment, BMI before pregnancy and urinary specific gravity.

[42] Including covariates age, gender, body mass index before the pregnancy (pre-BMI), weight gain during pregnancy, parity, education level, cigarette smoking, and alcohol drinking and other phthalate urine metabolites.

3.2.4.3 Effects of phthalate exposure on thyroid hormone status in adults

The correlation between DEHP,DBP,BBP and DINP metabolites in urine and thyroid hormone status was investigated in a cross-sectional study, the Nutrition and Health Survey in Taiwan, composed of 279 adults (mean age 53.4 years) and 79 children (mean age 12.6 years) [163]. The geometric mean levels of urinary MBzP and several DEHP metabolites in adults were significantly lower than those found in minors. They reported negative associations between urinary MEHHP or sum of DEHP metabolite levels and serum T4, and between urinary MEHP or MEOHP and serum free T4 in adults.

3.2.5 The HPG-axis

3.2.5.1 Phthalate effects on cells of the reproductive organs, gonadal hormone and sex steroid levels

MEHP exposure to rat ovarian granulosa cells acted as partial antagonist to both PPARα and PPARγ, causing suppressed Cyp19a1 (aromatase) mRNA levels and genes related to metabolism and differentiation [164]. DEHP treatment of primary human endometrial cells (epithelial cell layer of the uterus) caused decreased gene expression of antioxidant enzymes and an increased ROS production [165]. Both exposure of E2 and DEHP increased gene expression of ESR1 but in contrast to E2, DEHP decreased ESR2 and increased PGR mRNA levels.

In mouse spermatocytes, DEHP treatment induced apoptosis via the testicular nuclear receptor (Tr4)-Bcl2 pathway in a dose dependent manner [166]. Pregnant ICR mice were exposed to vehicle or DEHP (0.02, 0.2, 2, 20 or 200 mg/kg bw/day; 0.004, 0.04, 0.4, 4 or 40 x DEHP NOAEL) by gavage throughout pregnancy [167]. DEHP exposure affected the testes dose-dependently in the 21-day old male offspring with increased germ cell aggregation, and loss of germ and Sertoli cells. In agreement, in situ hybridization showed reduced gene expression of the Sertoli cell markers Sox9 and Fgf9 in testes from the DEHP-exposed male pups at doses from 0.4 x DEHP NOAEL and above. This reduced expression of Sox9 and Fgf9 was apparent already at E13.5. This could provide a novel explanation to how DEHP affects sperm production.

Linear regression analysis[43] data from the Hokkaido study (see also ref [157]) showed an inverse association between maternal blood MEHP levels (week 23 to 35) and cord blood levels of testosterone/E2 ratio, progesterone and inhibin B [168]. When the analysis was stratified by sex, the inverse associations between maternal MEHP levels and testosterone/E2 ratio, progesterone, inhibin B and insulin-like 3 (INSL3) were significant for male infants only. The authors proposed that since inhibin B and INSL3 are major secretory products of Sertoli and Leydig cell, respectively, the data indicate that prenatal DEHP exposure may have adverse effects on both Sertoli and Leydig cell development.

[43] Adjusted for maternal age, smoking during pregnancy, alcohol consumption during pregnancy, gestational age, blood sampling week, infant sex, and interaction of sex and MEHP.

3.2.5.2 Correlation between serum or urine phthalate levels and measures of fertility

The findings on how phthalate exposure affects female fertility studies, measured as time-to-pregnancy, vary. Serum levels of DEHP and DINP metabolites were analyzed in a subset of pregnant women (n=938; 24-33 weeks) and their partners (n=401) from the INUENDO study (cohorts from Greenland, Poland and Ukraine) [169]. Women and men from Greenland with high serum levels of DEHP metabolite had lower time-to-pregnancy compared to those with low levels. However, when only first-time pregnant women were investigated, the risk for infertility increased in women from Greenland with high serum levels of DINP metabolite. Results obtained from the Danish First Pregnancy Planner Study (n=229, repeated samples before/after ovulation cycle 1 and 5) found no association with urinary DEHP metabolite levels and decreased probability of pregnancy within the first six cycles of trying [170]. High levels of urinary DEHP metabolites have been associated with increased odds for Caesarean section as well as delayed delivery [171].

3.2.5.3 Human studies; association of phthalate exposure and pregnancy outcome

The effects of prenatal DEHP, BBP, DBP and DINP exposure on birth weight was investigated in a prospective multi-centre pregnancy cohort study, The Infant Development and the Environment Study (US) [172]. In preterm female infants, the log sum of DEHP metabolites in maternal first trimester urine was associated with an increase in birth weight (0.5 kg).[44] All individual DEHP metabolites were also significantly associated with birth weight in models adjusting for gestational age in the preterm female infants with the exception of MEHP. In male preterm infants, there was a negative correlation between first trimester maternal urine levels of MPB and birth weight (0.36 kg). However, the results should be interpreted cautiously due to heterogeneity (≤ 37 weeks) and small size of the preterm group (35 male and 33 female infants).

3.2.6 Foetal exposure: programming and epigenetics

3.2.6.1 Perinatal exposure

Maternal blood, cord blood and the infant's first stools after birth (meconium, used as a measure of foetal exposure) were collected from 201 new-born infants at the Shanghai Medical Center for Maternal and Child Health (China) and analyzed for levels of non-persistent organic pollutants including MEHP [173].There was a high correlation between MEHP levels in maternal and cord blood (r^2=0.65). The content in meconium was about twice that found in cord blood indicating that the developing foetus is continuously exposed to DEHP metabolites. MEHP and MBP were detected in amniotic fluid whereas other phthalate monoesters were only detected at trace levels [174]. Correlation between urine metabolite levels of DEHP, BBP and DBP collected in the early third trimester and placental expression of genes involved in trophoblast differentiation and steroidogenesis was investigated in a pregnancy cohort from New York City (n=54) [175]. The correlation between quintile content of urinary phthalates

[44] The multiple regression analyses were adjusted for race, smoking during pregnancy, study centre, parity, income.

and placental gene expression showed high variability. The authors reported a negative correlation of urine phthalate metabolite levels and placental gene expression of PPARG, aryl hydrocarbon receptor and HCG, involved in trophoblast differentiation. The results were not consistent for genes in the steroidogenesis pathway. The exposure of phthalates in the first trimester from 179 women[45] was correlated with lower placenta miR-185 expression, suggesting a mechanism of phthalates in foetal programming or epigenetics [176]. The function of miR-185 is not clear but it has been shown that hypoxia increases miR-185 transcripts in human and primary mouse lung epithelial cells where it was associated with increased apoptosis [177].

3.2.6.2 Prenatal studies

Sprague Dawley dams were treated with testosterone propionate, an AR antagonist (flutamide) or DEHP (10, 100, 500 mg/kg bw/day; 2, 20 and 100 x DEHP NOAEL) from gestational day 11 to birth [178]. At nine weeks of age, the male offspring to dams given 10 mg DEHP/kg bw/day had lower sperm count and reduced sperm motility and viability compared to controls. Sprague-Dawley dams were administered vehicle, genistein, DEHP or genistein + DEHP, (both at 10 mg/kg bw/day) by gavage from gestational day 14 to birth [179]. The doses were selected because foetal exposure of 10 mg/kg bw/day of genistein and DEHP alone did not affect plasma testosterone levels or germ cell numbers in adult offspring. The weight of the testes was increased in the offspring from the genistein + DEHP animals at 120 days of age and was associated with increased expression of inflammatory mast cell and macrophage markers. Increase in mast cell and macrophage markers are signs of inflammatory events in the testis and is often associated with testicular fibrosis. Using the same rat model and treatment groups, the group later found that DEHP was the main inducer of testicular changes in three day old pups and genistein counter-acted these effects [180].

3.2.6.3 Perinatal studies

Pregnant C57BL/6 mice were fed vehicle or DEHP-coated cocoa puffs at three doses (5, 40 or 400 µg/kg bw/day; 0.001, 0.008 or 0.08 x DEHP NOAEL, respectively) from gestational day 1 to lactation day 10 [181]. Serum levels of MEHP and 5-OH-MEHP were measured in dams and their foetuses three hours after ingestion of the cocoa puffs at gestational day 18. Mean serum levels of MEHP and 5-OH-MEHP were 160 and 1.6 ng/ml, respectively, in the 0.08 x DEHP NOAEL-exposed dams, and in the foetuses 96.7 and 1 ng/ml, respectively. In the 0.008 x DEHP NOAEL exposure group, two of three samples had detectable levels of MEHP in the dams, whereas dams from the control and low DEHP exposure groups had no detectable levels of MEHP. MEHP was not detected in foetal sera from these three groups. The anogenital distance/bw measured at day 1 was decreased in male pups to dams exposed to 0.001 x DEHP NOAEL, and in female pups from dams exposed to 0.08 x DEHP NOAEL. There were no effects on

[45] Enrolled from two large birth cohorts, the Harvard Epigenetic Birth Cohort (HEBC) and the Predictors of Preeclampsia Study (POPS) at the Brigham and Women's Hospital in Boston U.S.

DEHP-exposure on total interactive or independent behaviour[46] in juvenile offspring of either sex, but a moderate increase in anxiety-like behaviour was observed in both male and female offspring from dams exposed to 0.001 or 0.008 x DEHP.

C57BL dams were administered vehicle or MEHP (0.05, 0.25 or 0.5 mg/kg bw/day; no NOAEL for MEHP) by gavage from gestational day 12 to lactation day 7 [182]. Eight week old male offspring to dams given the lowest MEHP dose had increased adiposity with elevated levels of serum total cholesterol, serum triglyceride and blood glucose. These physiological changes were associated with induction of *PPARa* and *PPARg* mRNA levels in liver and adipose tissue, respectively. In contrast, there were no effects on these parameters in the female offspring in any MEHP group. Pregnant C3H/N mice were fed control or DEHP-containing diet (0, 0.05, 5 and 500 mg/kg bw/day; 0, 0.01, 1 and 100 x DEHP NOAEL, respectively) one week before conception and throughout pregnancy and lactation [183]. In the 100 x DEHP NOAEL, there were no pregnancies due to early spontaneous abortions. There were no differences in litter size or pup sex ratio in the remaining groups. At 84 days of age, body weight and fat mass were increased in male and female offspring from dams fed DEHP-diet compared with control. These data suggest that early exposure to DEHP predisposes to weight gain and obesity later in life.

Wistar dams were administered DEHP (1.25 mg and 6.25 mg/kg bw/day; 0.25 and 1.25 x DEHP NOAEL, respectively) by gavage from conception until weaning of the pups [184]. There was no difference in litter size or sex ratio of the pups between the groups. Birth weights of pups to dams of both DEHP-exposed groups were lower than control pups and remained so throughout the suckling period. Body weights remained lower in offspring from both DEHP-exposed groups throughout the study period. There was no difference in relative food intake between the groups. At weaning, the offspring from both DEHP-exposed groups had smaller fat cell size, and were more glucose tolerant and insulin sensitive compared to the controls. The response to oral glucose gradually deteriorated with age. Pancreas in the adult offspring to DEHP-exposed dams did not respond properly to the glucose challenge. A pre-diabetic state had developed in the female offspring, whereas the males maintained blood glucose levels but with higher insulin levels. Already at weaning, the pancreas from offspring to the DEHP-exposed dams had lower β-cell mass and swollen mitochondria. At week 27, the β-cells in the female offspring started to fail with decreased β-cell area and mass, and insulin content compared to controls. In the males, the area of the β-cells was increased but insulin content was similar to that of control.

3.2.6.4 Human studies: effects of prenatal exposure and genital development

Preterm birth and reduced anogenital distance are the two outcomes related to phthalate exposure that have been most studied and where effects have been observed after moderate exposure (for review see [185]). Reports on the effects of phthalates on gonadal development in humans have varied but the studies largely agree on a negative

[46] Interactive behaviours: side-by-side sitting, social grooming, sniffing, following, approaching, crawling and circling the partner. Independent behaviours: exploring the cage, self-grooming alone, sitting alone, and jumping.

effect on male genitalia development [174,186–188]. However, there was no effect on genital development by prenatal phthalate exposure in male babies to mothers that experienced some stressful life events during the pregnancy [189,190]. Only baby boys born by mothers without stressful life events during pregnancy exhibited a positive association between prenatal first trimester urine DEHP and anogenital distance at birth. These data suggest that major stressful events also have androgenic developmental effects in humans and mask putative ED effects.

3.2.6.5 Epigenetic effects of DEHP

Pregnant CD1 mice were treated with DEHP from gestational day 7 to 14. The offspring (F1) were then bred to produce the F2, F3 and F4 generations in maternal, paternal or maternal-paternal breeding lines, without any further DEHP treatment [191]. Fatal exposure to DEHP was found to disrupt testicular germ cell organization and male stem cell function in a transgenerational manner. First generation offspring (F1) to C57Bl/6J mouse dams fed vehicle or DEHP-coated cocoa puffs (5, 40 and 400 µg/kg bw/day; 0.001, 0.008 and 0.08 x DEHP NOAEL, respectively) from conception to postnatal day 10 [181] were paired with dose-matched non-siblings to create F2 offspring. The same breeding protocol was followed with the F2 mice to create F3 mice. There were transgenerational effects in female but not in male offspring. F3 female offspring from the F1 exposed to perinatal 0.08 x DEHP NOAEL had smaller anogenital index than control or the other DEHP exposure lineages. This was largely driven by body weight since F1 females were heavier than F3 females, and the controls and the two highest DEHP exposure groups were heavier than the low DEHP exposure group. F3 juvenile males from the F1 lineage exposed to perinatal 0.08 x DEHP NOAEL had increased interactive and decreased independent behaviour[47] compared with males of the F1 control and low DEHP-exposure lineages. Juvenile males derived from the highest DEHP-exposure group also exhibited increased anxiety-like behaviour.

Effects on behaviour of DEHP in the F3 generation were observed in C57Bl/6J mice. Females of the DEHP lineage had lower corticosterone response to restraint stress. These observations may be related to epigenetic modifications in expression of several pituitary hormones of the HPA axis [192].

3.2.7 Other

3.2.7.1 Obesity/Type 2 diabetes, in vitro studies

Studies from early 2000 have shown that phthalate monoesters can activate the PPARs (α, β, γ) in transactivation assays [193–195]. MEHP has been the focus of several studies with a preferential activation of both mouse and human PPARα and PPARγ. However, activation of receptor subtype, level of activation differed between the different studies which may depend on differences in the receptors depending on species, reporter gene constructs or the cell-type that was used in the studies. More recently, chromatin immunoprecipitation of mouse 3T3-L1 adipocytes, which express PPARγ, showed

[47] Interactive behaviours: side-by-side sitting, social grooming, sniffing, following, approaching, crawling and circling the partner. Independent behaviours: exploring the cage, self-grooming alone, sitting alone and jumping.

difference in the recruitment of co-regulators to PPARγ bound to DNA by rosiglitazone (a synthetic PPARγ-agonist) or MEHP [196]. MEHP also promoted adipogenesis, albeit to a lower extent than rosiglitazone. Importantly, MEHP induced a selective activation of different PPARγ target genes. DEHP exposure to murine pluripotent mesenchymal cells increased adipogenesis [127]. DEHP and DBP activated mouse and human hepatic PPARα as well as CAR. The activation of PPARα was stronger in mPPARα mice than in hPPARα mice, while the opposite was true of CAR [197]. MEHP increased the transcriptional activity of both *mPXR* and *hPXR* with 5- and 15-fold, respectively, in transfected HepG2 cells. The transcriptional activity of mPXR and hPXR were also activated up to 6-fold by MBzP [198]. Exposure of 10 nM BBP in HepG2 cells caused downregulation of Sirt1 and Sirt3, which are histone deacetylases acting as cell protecting factors in stress response, PPARγ corepressor and sensors of nutrient/energy levels in the cell [199].

3.2.7.2 Obesity/Type 2 diabetes, animal studies

Adult female C3H/N mice were fed control or DEHP-containing diet (0, 0.05, 5 and 500 mg/kg bw/day; 0, 0.01, 1 and 100 x DEHP NOAEL, respectively) for eight weeks [183]. Food intake was 20% higher in all DEHP-treated groups compared with controls. Body weights and visceral (gonadal and mesenteric) fat pads were increased in the DEHP-treated animals compared to control in a non-dose dependent manner. The increased fat mass was due to an increase in fat cell size associated with increased leptin and reduced adiponectin mRNA levels.

3.2.7.3 Obesity/Type 2 diabetes, human studies

The association between urinary phthalates[48] and insulin resistance (HOMA-IR),[49] was investigated in 766 fasting adolescents (12–19 years) in the 2003–2008 NHANES [200]. Insulin resistance was positively associated with urinary DEHP metabolite levels in continuous linear regression models.[50] All individual DEHP metabolites (MEHP, MEHHP, MECPP, and MEOHP) also correlated with insulin resistance but not BPA. Urine levels of DINP were associated with increased insulin resistance in a cross-sectional study of adolescents (12–19 years) [201].

BPA and nine phthalate metabolites were analyzed in urine samples 10, 18, 26 and 35 weeks gestation in 350 controls from the Lifecode prospective nested case-control study from Boston U.S. [202]. Women in the highest quartile of urinary DEHP metabolites at second trimester had reduced odds of impaired glucose tolerance compared with the lowest quartile. Similarly, women in the highest quartile of urinary MnBP levels across all time-points had reduced odds of excessive gestational weight gain compared to women in the lowest quartile.

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[48] Low (MBP, MEP) and high molecular weight (BBP) phthalates, and DEHP.

[49] Homeostatic model assessment- insulin resistance: f-glucose (mM) x f-insulin (mU/L)/22.5.

[50] Controlled for urinary creatinine, continuous age, BMI category, gender, PIR, parental education, serum cotinine, race/ethnicity and caloric intake.

Total phthalic acid was measured in first-morning urine samples after an overnight fast in men from the Australian cross-sectional cohort the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study, wave two (n=1527; mean age 59.6 years) [203]. They found that dietary intake was the main source to total phthalate exposure with carbonated soft drinks as a major contributor. The authors concluded that the positive association of total phthalate content in urine with an unhealthy (western) dietary pattern as well as multiple lifestyle risk factors (including smoking, obesity, insufficient physical activity) suggest that phthalate exposure co-associates with other lifestyle factors that increase the risk for chronic disease.

A study from Belgium included 123 adult obese subjects (38 m/85 f; mean age 41 years, range 18, 84) without known history of type 2 diabetes [204]. Anthropometric data, physical activity and OGTT were measured, and major urinary mono-ester phthalate metabolites (including DEHP, BBP, DBP metabolites) in 24-hour urine were analyzed. MBzP was positively associated with AUC glucose in the OGTT curve; 5-oxo-MEHP and MnBP levels were positively associated with AUC insulin in a linear regression model after multiple adjustments.[51] The authors concluded that exposure to phthalates was associated with increased insulin resistance, decreased insulin sensitivity and possibly impaired beta cell function in an overweight and obese population.

3.2.7.4 Cardiovascular diseases

Animal studies point to the possibility that DEHP exposure can be linked to secondary hypertension due to changes in the regulation of aldosterone from the adrenal gland (see Section 3.2.3.1).

The association between urinary phthalates, with focus on DEHP, DINP and DIDP, and blood pressure, triglycerides and lipoproteins was investigated in a cross-sectional subsample of US children (6 to 19 years of age) in NHANES 2009 2012 [205]. Data on urinary phthalate levels, fasting serum triglyceride levels, nonfasting serum lipid levels and blood pressure were available in 2838, 906, 2,555 and 2,447 subjects, respectively. Urine metabolite levels of DEHP and DINP were positively associated with systolic blood pressure but not with serum triglycerides or lipids levels.[52] Serum MEHP levels were negatively associated to serum LDL-cholesterol levels but not to the Framingham risk score for CVD[53] in the Prospective Investigation of the Vasculature in Uppsala Seniors (n=1016, age 70 years; Uppsala, Sweden) [206].

[51] Multiple-adjusted models include gender, age, physical activity, current smoking behaviour, current medication use and BMI as confounders.

[52] Standardized for age, sex and height; controlled for sex, caloric intake, television watching, poverty–income ratio, parental education, serum cotinine, urinary creatinine, BMI, race/ethnicity and age.

[53] Sex, BMI, serum cholesterol and triglycerides, hypertension, smoking and diabetes mellitus were used as confounders in the analysis.

3.2.8 Summary

It has previously been established that phthalates have anti-androgenic effects and focus has been on male reproductive function, including developmental effects on male reproductive organs and effects on sperm function and fertility.

Metabolism: Absorption and metabolism of phthalates in mammals is rapid and phthalate metabolites have half-lives of 12 to 48 hours. Therefore exposure estimates in humans from spot urine samples are likely to reflect what was consumed in the last meal, causing large variations in exposure estimates.

Mechanism-of-action: In general, the mechanism-of-action of phthalates is focussed on the PPARα and PPARγ pathways. However, DEHP and its metabolites have shown to activate several different pathways in cells of the reproductive organs. For example, MEHP acted as a partial antagonist to both PPARα and PPARγ in rat ovarian granulosa cells and DEHP acted as a partial ER-agonist in human endometrial cells. DEHP dose-dependently induced apoptosis via Bcl2 in mouse spermatocytes. In liver, both DEHP and DBP activated mouse and human hepatic PPARα as well as CAR, and their respective metabolites MEHP and MBzP activated mouse and human PXRs. DEHP induced adipogenesis in murine pluripotent mesenchymal cells, although the mechanism was not identified it shows that DEHP/DEHP metabolites can affect cell lineage commitment of stem cells. MEHP is a partial PPARγ agonist in adipocytes and promotes adipogenesis in preadipocytes.

HPA-axis: Prenatal exposure of DEHP caused changes of the adrenal gland structure with implications of long-term effects on blood pressure control. Studies over three generations of mice suggest that changes in expression of hormones of the HPA-axis lead to impaired stress response in future generations due to epigenetic modifications induced by phthalates.

HPG-axis: Perinatal exposure of DEHP in mice caused a dose-dependent loss of germ cells and Sertoli cells in testes from 21-day old pups and could be detected already in midgestation embryos. Prenatal exposure of low levels of DEHP caused reduced anogenital distance in the one-day old pups of both sexes. Human data indicate that major stressful life events may mask effects of early ED exposure on anogenital distance in male infants. Only baby boys born by mothers without stressful life events during pregnancy exhibited a positive association between prenatal first trimester urine DEHP and anogenital distance at birth.

Foetal exposure: programming and epigenetics: Phthalates are ubiquitous and are detected in maternal urine throughout pregnancy. DEHP metabolites are detected in cord blood and in new-born infant stools, both indicative of foetal exposure of phthalates. In a prospective mother-child cohort there was inverse correlation between maternal blood MEHP quartiles at late second early third trimester and cord blood levels of cortisol, cortisone levels and glucocorticoid/adrenal androgen ratio.

Other: Animal studies show that developmental exposure of MEHP or DEHP caused increased fat mass and impaired glucose tolerance in the adult offspring. The increased fat mass was mainly due to increased fat cell size and not fat cell number, which is a marker for metabolic dysfunction in adipose tissue. Perinatal DEHP exposure

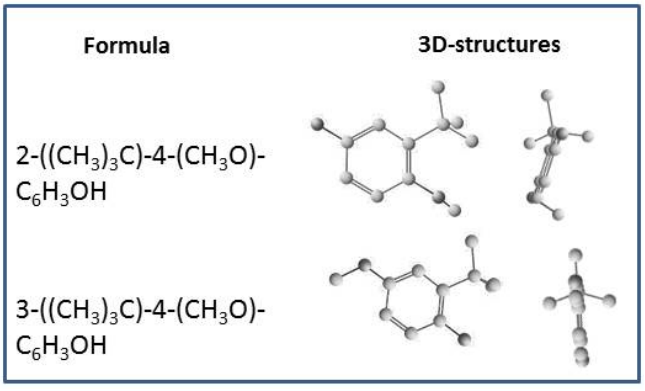
in animals affected the regulation of aldosterone from the adrenal gland, and may therefore affect long-term regulation of blood pressure.

In analogy, human data show a positive correlation between urine phthalate metabolite levels and impaired glucose tolerance, reduced insulin sensitivity (increased serum insulin) and increased insulin resistance (increased serum insulin and blood glucose). However, one study reported that although dietary intake was the main source to total phthalate exposure, intake of carbonated soft drinks was a major contributor. This positive association of a western dietary pattern and obesity with total phthalate content in urine suggests that phthalate exposure is co-associated with other lifestyle factors that increase the risk for chronic disease. Data from the NHANES showed that urine levels of DEHP and DINP metabolites were positively associated with systolic blood pressure in American children and adolescents. A cross-sectional study of Swedish 70-year old subjects found no association between urinary phthalate levels and the Framing risk score for CVD. This discrepancy between American children and Swedish elderly could both reflect differences in dietary habits and differences in cardiometabolic risk factors with age.

3.3 Antioxidants: BHA and BHT

Butylated hydroxyanisole, BHA (consists of two isomers: 2- and-3-tert-butyl-4-methoxyphenol isomers; CAS no 25013-16-5; E320)

Figure 10: Molecular structure of BHA



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=6932, https://pubchem.ncbi.nlm.nih.gov/compound/6932 (accessed 7 May 2018).

National Center for Biotechnology Information. PubChem Compound Database; CID=8456, https://pubchem.ncbi.nlm.nih.gov/compound/8456 (accessed 7 May 2018).

Since 1947, BHA has been added to edible fats and fat-containing foods for its antioxidant properties. In Europe, the use of BHA, as an antioxidant food additive or in food packaging materials, is permitted in several foods such as stock/bouillon cubes, dehydrated soups and dehydrated meats, either alone or combined with other antioxidants. The maximum limit is set to 200 mg/kg expressed on the fat content of

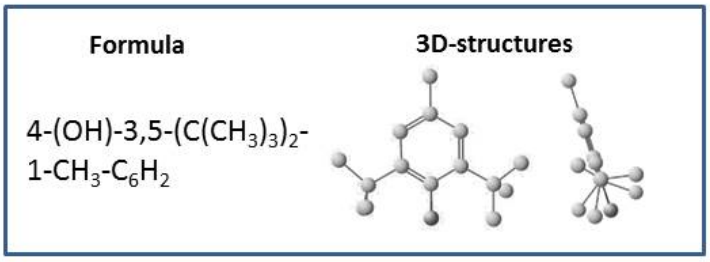
the product, and refers to the combined content of gallate (3, 4, 5,-trihydroxybenzoate), BHA and BHT according to the directive 2006/52/EC. BHA is also used in animal feed and in pharmaceuticals (*e.g.* cholesterol-lowering drugs lovastatin, and simvastatin).

EFSA: in 2011 the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) established a new acceptable daily intake (ADI) of 1.0 mg/kg bw/day based on a NOAEL of 100 mg/kg bw/day for growth retardation, increased mortality and behavioural effects in rat pups at higher dose levels, and using an uncertainty factor of 100 [207].

ECHA: BHA is on the CoRAP (Community rolling action plan) due to its putative role as an endocrine disruptor.

Butylated hydroxytolouene, BHT (2,6-di-tert-butyl-4-methylphenol; CAS no 128-37-0; E321)

Figure 11: Molecular structure of BHT



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=31404, https://pubchem.ncbi.nlm.nih.gov/compound/31404 (accessed 7 May 2018).

EFSA: in 2011 the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials (CEF Panel) derived an ADI of 0.25 mg/kg bw/day from a NOAEL of 25 mg/kg bw/day from two separate 2-generation chronic studies in Wistar rats (effects on litter size, sex ratio and pup, and adult hepatocellular carcinoma in one study; induction of hepatic Cyp2b1 expression and thyroid histopathology in the other) [208].

ECHA: the DNEL for oral exposure is set to 250 µg/kg bw/day.

3.3.1 Criteria for literature search

The PubMed search yielded an initial 209 items, filtered for humans or rat/mice. Excluding articles written in other languages than English left a final of 183 references.

Table 7: PubMed search 8 December 2016 BHA or BHT and endocrine action

Search	Query	Items found
#3	#1 and #2	209
#2	(butylated hydroxyanisole) OR butylated hydroxytoluene	4,271
#1	(((((((endocrine) OR endocrinol*) OR estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

3.3.2 Metabolites/metabolism oral route

Heat degradation of BHA produces tert-butylhydroquinone (TBHQ) which can be oxidized to tert-butylbenzoquinone, whereas photo-degradation, perhaps more relevant in food packaging materials, produces reactive oxygen species. Absorption, distribution, metabolism and excretion (ADME) studies of BHA have been performed in rats, rabbits, dogs, monkeys and humans.[54] A pharmacokinetic study in adult male Sprague Dawley rats that were administered a single oral dose of Me-14C-3-BHA (270 mg/kg bw) by gavage showed that 41% of the radioactivity was detected in urine and 53% in faeces within 48 hours. The urine content of free BHA and TBHQ were lower the first day after ingestion of BHA (0.5 mg/kg bw) compared with day 3 and 7 urine content in healthy male volunteers. This suggests an induction or inhibition of BHA-specific phase I and II enzymes, or a bioaccumulation of BHA and/or its metabolites [209]. The plasma profiles of BHT in man after a single oral dose of 0.5 mg/kg bw followed closely that of BHA [210]. These data show that BHA and BHT are metabolized fairly rapidly after a single dose but continuous exposure changes the pharmacokinetics with higher circulating levels of BHA and its metabolite TBHQ in circulation.

3.3.3 The HPA-axis

BHA activated the glucocorticoid receptor in the MDA-kb2 reporter cell line [211], with EC50 of 0.1 nM and a maximal 1.7-fold induction at a concentration of 0.5 µM [212]. Using the same cell line, BHA inhibited DHT-induction of AR with an IC50 of 4 nM and maximum inhibition of 0.7-fold at 50 µM. The potency of BHA inhibition of HSD11B1 and HSD11B2 enzyme activities was investigated in human tissues [213]. BHA acted as a selective competitive inhibitor of HSD11B2 with IC50 of 13.99 and 69.25 µM in the rat and human, respectively. This inhibition would cause an increase in the conversion of cortisol from cortisone in tissues with high HSD11B2 expression; kidney, testes and placenta [214]. These two studies show that BHA can interfere with glucocorticoid signalling either by activating glucocorticoid receptor-induced transcription or by inhibiting the deactivation of cortisol.

3.3.4 The HPT-axis

BHA induced thyroid hormone receptor in a dose-dependent manner in the rat pituitary GH3 cell line with an EC50 of 0.16 µM and a maximal induction of more than 13-fold at concentration above 100 µM [212]. At higher concentrations BHA inhibited activation of thyroid hormone receptor induced by T3 with an IC50 of 409 µM. Serum T4 levels were reduced in the male rats that were administered 5 x BHA NOAEL by gavage for 7 weeks [215].

[54] http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2011.2392/epdf

3.3.5 HPG-axis

Using an ER-negative human osteoblast cell line transfected with either ERα or ERβ, it was shown that BHA acted as agonist to both ER receptors [216]. The potency of BHA in the ERα was however weaker compared to that of ERβ. Furthermore, the effects of BHA was additive to E2 on both receptors, suggesting that even small concentrations of BHA could enhance endogenous oestrogen effects in the organism. Female CD-1 mice were fed a BHA-containing diet (0.75% w/w; approx. 750 mg/kg bw/day) for three weeks that resulted in an enhanced hepatic phase II metabolism of E2 and oestrone [217]. Consequently, serum E2 levels and E2 effects on uterus weight were reduced in BHA-fed ovariectomized CD-1 mice. The *in vitro* studies show that BHA activated both ERα and ERβ, and enhanced hepatic metabolism of E2 *in vivo* which would reduce E2 systemic effects.

Both BHA and BHT were weak inhibitors of DHT activation of AR in human cell-based reporter assays [218,219]. There were no effects on androgen-dependent accessory sex organ weights in 51 day old castrated males treated with BHA (250 mg/kg bw/day) alone or in combination with testosterone propionate for 10 days [220]. Male and female Sprague-Dawley rats were administered BHA by gavage (0, 10, 100 and 500 mg/kg bw/day; 0, 0.1, 1 and 5 x BHA NOAEL) for 7 and 10 weeks, respectively [215]. In the female rats exposed to BHA, the relative weights of liver and adrenal glands were increased at the highest dose, 5 x BHA NOAEL. In the males, liver, adrenals and thyroid relative weights were increased, whereas spleen relative weight was decreased in the group administered 5 x BHA NOAEL for 7 weeks. There was no significant effect on body weight in either sex. Serum testosterone levels were reduced in the male rats administered 1 and 5 x BHA NOAEL.

3.3.6 Foetal exposure: programming and epigenetics

Male and female Sprague-Dawley rats were given BHA by gavage (0, 10, 100 and 500 mg/kg bw/day; 0, 0.1, 1, 5 x BHA NOAEL) 2 weeks before mating until conception; the pregnant dams continued being administered BHA throughout gestation and lactation [215]. Fertility was decreased at the highest dose BHA, but there were no treatment effects on litter size or pup sex ratio. The body weight, relative liver and brain weights were lower in the 3-week old offspring from the dams administered 5 x BHA NOAEL compared with control offspring. At the high dose BHA-exposed group, sexual maturation was delayed in both males and females. The offspring were administered BHA by gavage after weaning at the same dose as they had been exposed to perinatally. At 13 weeks of age, the female rats exposed to the 5 x BHA NOAEL dose had increased liver and adrenal gland relative weights, and decreased vagina relative weights vs. control. All groups of 13-week old male offspring exposed to BHA had changes in sperm morphology. Further, the male 13-week old offspring exposed to 1 x or 5 x BHA NOAEL had decreased spleen and epididymis relative weights. Serum T_4 was reduced and serum cholesterol levels were increased in the females, but not males, from the 5 x BHA NOAEL group.

3.3.7 Other

3.3.7.1 Obesity/Type 2 diabetes

The potency of BHT to uncouple the mitochondrial respiration chain was recently highlighted due to its unusual dose response curve [221]. Data showed a marked uncoupling in isolated rat liver mitochondria of BHT at 2 pM but only very modest effects on uncoupling at 2 µM. The BHT effects were partly dependent on adenine nucleotide translocase (ANT) that shuttles ADP into and ATP out from mitochondria. The authors concluded that the wide range by which BHT could uncouple mitochondria was via (at least) two different mechanisms; a medium-capacity and high-affinity uncoupling through ANT at low BHT concentrations, and a high-capacity and low-affinity uncoupling through other pathways at high BHT concentrations.

3.3.7.2 Vascular diseases

The BHA metabolite TBHQ has been suggested to prevent stress-induced cytotoxicity in neurons. However, experimental stroke in mice exposed to TBHQ resulted in reduced survival. The TBHQ-exposure inhibited mitochondrial function in cerebrovascular endothelial cells, which compromised the integrity of the blood-brain-barrier. [222].

3.3.7.3 Cancers

Cytotoxicity and apoptosis was investigated using two human immortalized cancer cell lines, HL-60 and HCL-2 [223]. The most potent cytotoxic effect was with treatment of BHA and BHT together (BHA+BHT), followed by BHT and the lowest effect was by BHA. The order between BHA and BHT was reversed in activation of caspases, particularly CASP3, where the order was BHA+BHT > BHA > BHT. Using a cell-necrotic model system both BHA and BHT (both 100 µM) affected cell death but acted via different mechanisms [224]. BHA did not inhibit the cytotoxicity, but caused a shift from necrosis to apoptosis, whereas BHT did not affect the mode of action. Putative cytotoxic and genotoxic effects of TBHQ were studied in A549 lung cancer cells and HUVEC (human umbilical vein endothelial cells) [225]. At 0.5 mM TBHQ inhibited the growth rate of both cancer and normal cells by inducing apoptosis via chromatin and DNA fragmentation. In rats, co-treatment with E2 and BHA (0.7% w/w in feed; approx. 5.6 x BHA NOAEL)[55] increased survival by reducing tumour incidence and delaying tumour development [226].

3.3.8 Summary

In comparison with BPA and phthalates less is known about mode and mechanism-of-actions of the antioxidants BHA and BHT.

Metabolism: BHA and BHT are metabolized fairly rapidly after a single dose but continuous exposure increases levels of BHA and its metabolite TBHQ in circulation.

Mechanisms of action: Cell studies show that BHA affect glucocorticoid signalling both by activating glucocorticoid receptor-mediated transcription and by inhibiting the enzyme responsible for deactivating cortisol. BHA activated thyroid hormone receptor-

[55] See http://www.tera.org/Tools/ratmousevalues.pdfs

dependent gene expression but was less potent in inhibiting T_3 activation of thyroid hormone receptor. BHA activated both ERs and experiments in mice showed that BHA enhanced hepatic oestrogen metabolism, which would reduce oestrogen levels in circulation and thereby counteract oestrogen systemic effects. BHA and BHT were weak antagonists to dihydrotestosterone-induced activation of the androgen receptor in human transfected cells.

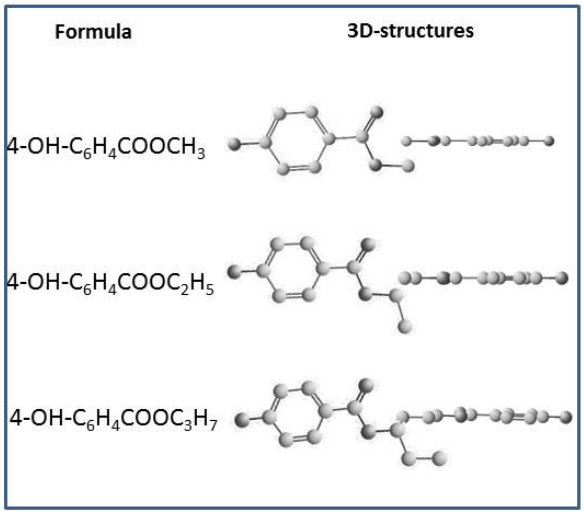
HPG-axis: Sperm morphology was affected in Sprague Dawley rats by exposure of low BHA (0.1 x NOAEL) from preconception up to 13 weeks postnatal age.

Other: TBHQ exposure in mice reduced survival after experimental stroke by interfering with the integrity of the blood-brain-barrier. BHA increased survival in a rat breast cancer model by inducing apoptosis of the cancer cells and *in vitro* study reported that TBHQ suppressed cellular growth rate by inducing apoptosis.

3.4 Parabens

Methyl-, ethyl- and propyl-4-hydroxybenzoate (E218, CAS 99-76-3; E214, CAS 120-47-8; E216, CAS 94-13-3)

Figure 12: Molecular structures of methyl-, ethyl- and propyl-paraben



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=7456, https://pubchem.ncbi.nlm.nih.gov/compound/7456 (accessed 7 May 2018).

National Center for Biotechnology Information. PubChem Compound Database; CID=8434, https://pubchem.ncbi.nlm.nih.gov/compound/8434 (accessed 7 May 2018).

National Center for Biotechnology Information. PubChem Compound Database; CID=7175, https://pubchem.ncbi.nlm.nih.gov/compound/7175 (accessed 7 May 2018).

Parabens are esters of 4-hydroxybenzoic acid and were first introduced in the mid-1920s as preservatives in pharmaceutical products. They are used as antimicrobial preservatives and are active against a broad spectrum of microorganisms. The exact mechanisms how parabens inhibit bacterial growth are not clear but it involves

disruption of membrane transport by interaction with cell plasma membrane phosphatidylcholines [227,228], inhibition of DNA/RNA synthesis [229] and/or inhibition of key enzymes such as phosphotransferases [230]. The endocrine disrupting and adipogenic potencies of parabens are generally increased with increased size of the alkyl moiety. Since parabens are used in cosmetics, foods and industrial products, the exposure of this non-persistent phenol is widespread and through multiple routes.

EFSA: In 2004 the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (the AFC Panel) decided that propylparaben should be excluded from the previous allocation on a full group ADI of 0-10 mg/kg bw/day for the sum of methyl- and ethyl-and propyl-p-OH-benzoic acid esters and their sodium salts[56] made by the joint FAO/WHO Expert Committee on Food Additives (JECFA).[57] Based on a reproductive dietary toxicity study on methyl- and ethylparaben in the rat a NOAEL was set to 1000 mg/kg bw/day. JECFA agreed with the EFSA decision in 2007.

ECHA: the DNEL for oral exposure is set at 1.04 mg/kg bw/day, 15 mg/kg bw/day and 4.08 mg/kg bw/day, for methyl-, ethyl- and propylparaben, respectively.

3.4.1 Criteria for literature search

The PubMed search yielded an initial 135 items, filtered for humans or rat/mice. Excluding articles written in other languages than English left a final of 132 references.

Table 8: PubMed search 8 December 2016 parabens and endocrine action

Search	Query	Items found
#3	#1 and #2	135
#2	(((((methylparaben) OR ethylparaben) OR propylparaben) OR methyl p-hydroxybenzoic acid) OR ethyl p-hydroxybenzoic acid) OR propyl p-hydroxybenzoic acid	934
#1	(((((((endocrine) OR endocrinol*) OR estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

3.4.2 Metabolites/metabolism oral route

In vitro metabolism of parabens has been determined in human plasma and liver microsomes [231]. Methyl- and ethylparaben were stable in human plasma during 24 hour incubation at 37C, whereas the concentration of propylparaben was reduced by 50% after 24 hour incubation due to enzymatic hydrolysis. Incubation with human liver microsomes caused rapid hydrolysis of the parabens with decreasing rate of hydrolysis with increased alkyl chain length. The parabens were also glucuronidated by hepatic phase II metabolism. Results from analyses of methyl-, ethyl- and propylparabens in urine spots over time to assess within-person-variability showed that single urine spots can be used to assess long-term exposure of these parabens [232]. In another study with aim to evaluate variability of EDs in urine overtime, three spot urine samples at about

[56] Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a Request from the Commission related to para hydroxybenzoates E 214-219. The EFSA Journal (2004) 83, 1-26.
[57] JECFA, 17th Report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization Technical Report Series 539 (1974).

gestational week 17, 23, and 29 were analyzed [233]. They found that the intraclass correlation coefficients (ICC) were small to moderate; the highest was obtained for propylparaben with an ICC of 0.60. A third study, analyzing repeated spot urine samples for nine environmental phenols at three time-points (weeks 17, 23 and 33) during pregnancy also reported moderate ICC ranging between 0.48 and 0.62 [234]. Taken together these studies show that alkylparabens are hydrolyzed by the liver at a rate that is inversely related to alkyl-chain length. Parabens can also be glucuronidated in phase II metabolism. Repeated measures of urine samples show a fair reproducibility within the subject over time.

3.4.3 The HPA-axis

Using the MDA-kb2 cell line that expresses the glucocorticoid receptor with a stably transfected reporter gene, it was shown that propylparaben and DEHP, individually (1 µM) and in combination (10 nM each), activated glucocorticoid receptor-mediated gene expression [235]. The authors concluded that risk assessment of compounds should take mixture effects into account. The group had previously published data showing that methyl- and ethylparaben are agonists to the glucocorticoid receptor [236].

3.4.4 The HPT-axis

Analyses of serum thyroid measures (free and total T_3 and T_4, TSH thyroglobin) and urinary levels of methyl-, ethyl- and propylparabens in the NHANES 2007-08 survey showed that age was positively associated with serum ethylparaben concentrations, and BMI was inversely associated with all three serum paraben concentrations [237]. All three parabens were negatively correlated to thyroid hormone levels in adult females but not in males.

3.4.5 The HPG-axis

The association between parabens with fertility was investigated in a prospective study based in New York [238]. Data showed that the preconception urinary concentration of methyl and ethyl paraben in women but not in their male partners were associated with an increase in time-to-pregnancy of 37 and 33%, respectively. In contrast, there was no association between urinary concentrations of methyl- and propylparaben and outcome in i*n vitro* fertilization in a Boston-based prospective study [239].

3.4.6 Foetal exposure: programming and epigenetics

Methyl-, ethyl and propylparaben were detected in 95%, 60% and 83%, respectively, of urine samples obtained from 200 Danish women taken at gestational week 8-30 [240]. Concentrations of urine methyl- and propylparaben had high correlation and the authors suggested that common exposure source and/or exposure associated with lifestyle could explain this co-dependence. Other studies have shown that urinary parabens drop with 25-45% during pregnancy [241] and that use of cosmetics, lotions and fragrances increase paraben concentration in urine [242]. These data indicate the impact of other sources of parabens than food/food-contact materials on paraben exposure in humans.

Paraben content in spot urine from the second to the third trimester and cord blood was investigated in a multi-ethnic New York cohort [243]. Methyl- and propylparaben were detected in all spot urine samples, and made up to more than half of the total paraben concentration. Ethylparaben was detected in 74% of all samples. Thirty-eight umbilical cord blood samples from 36 women (two twin deliveries) were analyzed for parabens. Methyl- and ethylparabens were most abundant in cord blood, detected in over 95% and 76.3% of the samples, respectively, whereas propylparaben was detected only in about half of the samples [243]. Analyses of parabens in maternal blood and paired cord blood from 46 mother-baby pairs showed that methylparaben crossed the placenta [244]. Placentas from twelve full-term singleton pregnancies were collected and tissue samples from the maternal side was dissected out and frozen [245]. The samples were analyzed for five parabens and two UV-filters. The results reveal a high inter-individual variability in pattern of detected parabens and UV filters. Methylparaben was detected in all samples and was the most prevalent of the analyzed compounds. In a cohort of pregnant women undergoing amniocentesis at Mount Sinai Medical Center (NY, U.S.), ethyl- and propylparaben were detected in 99% of maternal urine samples (n=71), whereas the detection in amniotic fluid the detection was 42% and 58%, respectively [234]. The authors concluded that due to the low detection, amniotic fluid may not be suitable for monitoring foetal exposure to nonpersistent phenols.

Paraben content in blood, urine and seminal fluid were analyzed in young healthy Danish men [246]. Methylparaben was detected with highest amounts in urine followed by propyl- and ethylparaben. Methyl- and propylparaben were present in most serum samples, whereas other parabens was only detected in a few samples. All three parabens were detected in several of the seminal samples albeit with low median levels. As in urine, methylparaben was present at the highest concentration in serum and in seminal fluid followed by propylparaben.

3.4.7 Other

3.4.7.1 Obesity/Type 2 diabetes

Peroxisome proliferator activating receptor gamma (PPARγ): using a human osteoblast cell line, transfected with PPARγ and reporter gene, showed that methyl-, ethyl- and propylparaben acted as PPARγ agonists [247]. Propylparaben (>100 µM) induced adipocyte differentiation in the mouse 3T3-L1 fibroblast cell line, whereas methyl- and ethylparabens enhanced the effects of insulin on adipocyte differentiation [247]. These effects by parabens were mediated by the PPARγ pathway since they were blocked by a specific PPARγ antagonist (T0070907). This suggests that parabens can promote body fat storage. Another study showed that the ability of parabens to induce adipocyte differentiation in 3T3-L1 fibroblasts was enhanced with increased number of carbons in the linear alkyl side chain of the paraben [248]. Parabens also dose-dependently induced key adipogenic genes; *Pparg*, *Cebpa*, *Fabp4*, *Fas*, adiponectin and leptin [248]. Propylparaben increased ROS and disrupted energy metabolism in HepG2 cells leading to apoptosis probably due to mitochondrial dysfunction [249].

C57BL mice administered methylparaben by gavage (100 mg/kg/day; 1 x methyl- and ethylparaben NOAEL) after weaning had increased body fat and serum leptin levels compared with placebo-treated control mice [250]. The methylparaben treated animals also had reduced levels of serum markers for bone formation suggesting a disturbed bone metabolism.

3.4.7.2 Cardiovascular diseases

Already in the 1980's it was proposed that parabens could have vasoactive properties. Using organs baths of dog brain basilar artery rings it was shown that methyl- and propylparabens caused vasorelaxation via effects on the vascular smooth muscle cells [251]. Later it was shown propylparaben inhibited voltage-gated Na+- channels and increased cell survival in a model for ischemia-reperfusion injury in adult rat cardiomyocytes [252].

3.4.7.3 Cancers

Treatment with methyl- and propylparabens at 20 nM increased expression levels of *ESR1* and *ESR2* mRNA and corresponding proteins in MCF7 (cancer) and MCF10A (non-cancer) breast cells [253]. Methyl- and propylparaben also increased the PGR protein expression in both cell lines. The authors concluded that parabens can be viewed as putative contributors to initiation (MCF10A) and progression (MCF7) of breast cancer. Furthermore, methyl- and propylparaben increased gene and protein expression of the *GPER1* gene and protein expression in these cell lines [254]. Taken together, the data points to that parabens are acting on multiple oestrogen pathways. In a recent review on parabens and cancer in human breast epithelial cells it was pointed out that parabens are detected present in 99% of human breast tissue biopsies, they possess estrogenic activity and stimulate proliferation of human breast cancer cells at relevant concentrations measured in breast [255]. Furthermore, they can counteract the inhibitory effects of 4-hydroxy-tamoxifen. Finally, methylparaben has been shown in human breast epithelial cells to increase mTOR, a key regulator of energy metabolism. Particularly methyl- and propylparaben was bioaccumulated in the tissue that may reflect the higher production and usage of these parabens. The content of methyl-, ethyl- and propylparaben content was analyzed in 30 human ovarian tissues (15 ovarian cancer and 15 benign tissues) [256]. They found that the paraben content was doubled in the ovarian malignant compared with benign tissue.

3.4.8 Summary

The main focus for parabens, showing both oestrogenic and anti-androgenic effects, has been on ovarian and breast cancer since parabens are accumulated in these tissues.

Metabolism: Alkylparabens are hydrolyzed by the liver at a rate that is inversely related to alkyl-chain length. Parabens are glucuronidated in hepatic phase II metabolism. Repeated measures of paraben content in urine show a fair reproducibility within a subject. However, urinary paraben concentrations generally drop with up to 25% in pregnancy. This suggests that other sources of parabens than in food or food-contact materials such as the use of cosmetics and personal hygiene products

contribute substantially to urinary paraben concentrations. This confounding factor complicates the interpretation of clinical observational studies and may explain the discrepancies between different studies

Mechanisms of action: Propylparaben activated glucocorticoid receptor-mediated transcription in transfected cells. Parabens acted as PPARγ agonists in the mouse preadipocyte 3T3-L1 cell line by inducing adipocyte differentiation and in a human osteoblast cell line by the activation of a reporter gene. Parabens caused mitochondrial dysfunction by uncoupling oxidative phosphorylation and enhancing ROS production in mitochondria from hepatocytes.

HPG-axis: Reduced fertility in women was associated with higher urinary concentrations of methyl- and ethylparabens in a prospective study. However, outcome of *in vitro* fertilization was not affected by urinary concentrations of parabens in the women.

Foetal exposure: programming and epigenetics: Parabens can be detected in seminal fluid, cord blood and placentas. Ethyl- and propylparabens were detected only in about half of the amniotic samples in humans due to low levels. Programming studies in rats showed that early exposure of methylparaben caused structural changes of the mammary gland that were consistent with an increased risk for breast cancer.

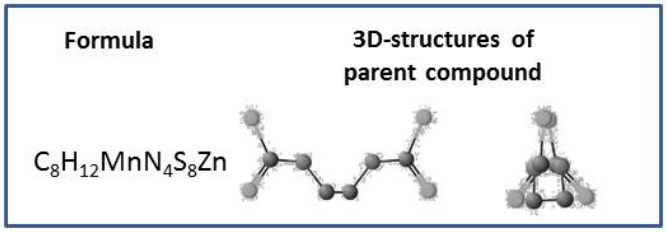
Other: The main focus on paraben effects on health has been on breast and ovarian cancers, *i.e.* tissues where these parabens accumulate. Malignant ovarian tumours had twice the content of paraben as benign tumours. Paraben content in breast cancer tumours have not been measured, however there was no association with paraben content in adjacent tissue and tumour location.

3.5 Dithiocarbamate fungicides

Dithiocarbamates are a group of systemic fungicides with protective action against several fungal diseases, and their mode of action is through inactivation of sulfhydryl groups in amino acids, proteins and enzymes, which inhibits the metabolism of lipids, respiration and production of ATP in fungi. The first dithiocarbamate fungicide patent was filed in 1934 and dithiocarbamates are still used in agriculture practice [257]. However, the approvals for these dithiocarbamates in the EU will expire during 2017 and 2018 and are therefore currently being reviewed. The dithiocarbamate moiety is highly reactive and interferes with nitrogen and carbon cycling in soil bacteria [258].

Mancozeb (CAS 8018-01-7), manganese ethylene-bis-(dithiocarbamate) (polymeric) complex with zinc salt

Figure 13: Molecular structure of mancozeb



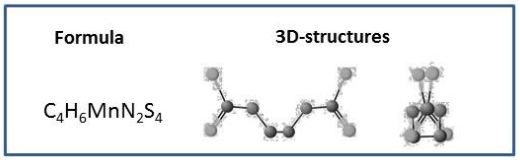
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=3034368, https://pubchem.ncbi.nlm.nih.gov/compound/3034368 (accessed 7 May 2018).

Mancozeb is approved in Denmark, Finland and Sweden but not in Iceland or Norway. In Sweden it is restricted to potato, garlic and onion cultivation.

EU pesticide database: According to Dir. 2005/72/EC the ADI for mancozeb is set at 0.05 mg/kg bw/day, based on two years rat study and using a safety factor of 100. The acute reference dose (ARfD) is set at 0.6 mg/kg bw, based on teratogenicity NOAEL of 60 mg/kg bw/day. Target organ in short-term toxicity studies in rats is the thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy). Target organ in long-term toxicity studies in rats are thyroid (inhibition of thyroid peroxidase, hypertrophy/hyperplasia) and retina (retinopathy at high doses). Reproductive toxicity: decreased rat pup weight at parentally toxic dose level; malformations at high doses (rats); embryonal/foetal toxicity (delayed ossification, abortions) at lower maternally toxic doses in rats and rabbits. Mancozeb has the following hazard statement codes: H317 - skin sensitization 1; H361d - reproductive toxicity 2.

Maneb (CAS 12427-38-2), manganese ethylene-bis-(dithiocarbamate) (polymeric)

Figure 14: Molecular structure of maneb



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=3032581, https://pubchem.ncbi.nlm.nih.gov/compound/3032581 (accessed 7 May 2018).

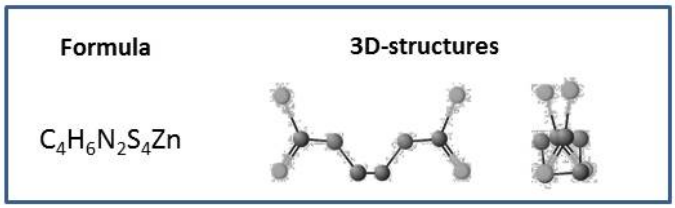
Maneb is approved for agricultural use in Denmark and Finland but not in Iceland, Norway or Sweden.

EU pesticide database: According to Dir. 2005/72/EU the ADI for maneb is set at 0.05 mg/kg bw/day, based on a multi-generation study in rats and supported by a 90-day study in rats plus including a safety factor of 100. The ARfD is 0.2 mg/kg bw, based on the NOEL of 20 mg/kg bw/d teratogenicity in rats. Target organ in short-term toxicity studies in the rat is thyroid (inhibition of thyroid peroxidase,

hyperplasia/hypertrophy). Target organs in long-term toxicity studies: thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy) in the rat and liver in mice. Reproductive toxicity: decreased pup body weight gain at parentally toxic dose levels; malformations at high dose levels (rats); embryonal/foetal toxicity (delayed ossification, reduced foetal weight, resorptions) at lower maternally toxic dose levels (rats, rabbits). Maneb has the following hazard statement codes: H317 -skin sensitization 1; H319 - serious eye damage/eye irritation 2A; H332 - acute toxicity 4, inhalation; H361d - reproductive toxicity 2.

Metiram (CAS 9006-42-2), zinc ammoniate ethylene-bis-(dithiocarbamate) - poly(ethylenethiuram disulphide)

Figure 15: Molecular structure of metiram



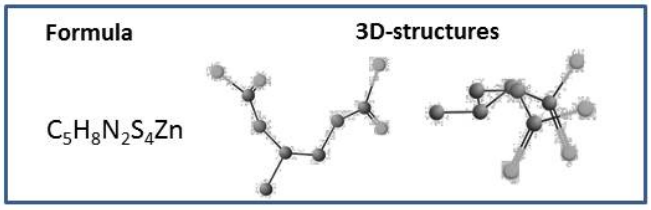
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=5284484, https://pubchem.ncbi.nlm.nih.gov/compound/5284484 (accessed 7 May 2018).

Metiram is approved for agricultural use in the EU but not in any of the Nordic countries. Metiram is commonly used in the production of grapevines, apples, tomatoes, potatoes as well as other crops.

EU pesticides database: According to Dir. 2005/72/EU the ADI for metiram is set at 0.03 mg/kg bw/day, and ARfD is not applicable. Target organs in short-term toxicity studies in rats are: the thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy), liver (increased weight) and skeletal muscle (atrophy of hindlimb muscles). Target organs in long-term toxicity studies in rats are: thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy) and skeletal muscle (atrophy of hindlimb muscles). Reproductive toxicity: no reproductive toxic effects at parentally toxic dose levels; decreased litter size and foetal weights at maternally toxic dose levels (rabbit).

Propineb (CAS 12071-83-9) polymeric Zn propylene-bis-(dithiocarbamate)

Figure 16: Molecular structure of propineb



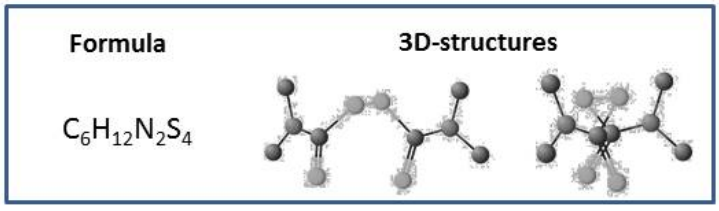
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=3032571, https://pubchem.ncbi.nlm.nih.gov/compound/3032571 (accessed 7 May 2018).

Propineb is approved in the EU for agricultural use but not in any of the Nordic countries.

EU pesticide database: According to Dir. 2003/39/EU the ADI for propineb is set at 0.007 mg/kg bw/day, based on chronic rat studies and a safety factor of 100. The ARfD is set at 0.01 mg/kg bw, based on developmental toxicity studies in the rat. Target organs in short-term toxicity studies are: skeletal muscle (muscular damage no neurotoxic activity), liver (disturbance in hepatocyte permeability) and thyroid in rats and chickens; spleen weights only observed in orally treated dogs. Target organ in long-term toxicity studies in rats is: thyroid (enlarged thyroids and decreased protein-bound iodine levels at dose of 5 mg/kg bw/day). Reproductive toxicity: in rats the teratogenic dose range overlapped with the toxic dose range of the dams; in rabbits no evidence of embryonal/foetal toxicity even in the presence of maternal toxicity.; reduced gestation rates and numbers of pups were observed at doses which also caused maternal toxicity in a three-generation study in rats. Propineb has the following hazard statement codes: H317 – skin sensitization 1; H332 acute toxicity 4, inhalation; H373 – specific target organ toxicity, repeated exposure 2; H400 – hazardous to the aquatic environment, acute toxicity.

Thiram (CAS 137-26-8), tetramethylthiuram disulphide or bis-(dimethylthiocarbamoyl) disulphide

Figure 17: Molecular structure of thiram



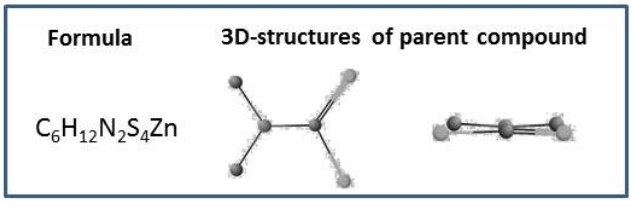
Source: National Center for Biotechnology Information. PubChem Compound Database; CID=5455, https://pubchem.ncbi.nlm.nih.gov/compound/5455 (accessed 7 May 2018).

Thiram is approved in the EU and among the Nordic countries only Denmark allows thiram for agricultural use.

EU pesticide database: According to Dir. 2003/39/EU the ADI for thiram is set at 0.01 mg/kg bw/day, based on two-year studies in rats and supported by findings in dogs and using a safety factor of 100. The ARfD is set at 0.6 mg/kg bw, based on acute neurotoxicity studies in rats and using a safety factor of 100. Target organs in short-term toxicity studies are: liver (dog) and non-glandular stomach (rat). Target organs in long-term toxicity studies are: liver, thyroid (C-cell hyperplasia), pituitary/hypothalamus function (reduced LH surge) in rats; retina in mice and dogs. Reproductive toxicology: pup body weights were reduced at maternal toxic doses; developmental delay in the rat. Thiram has the following hazard statement codes: acute toxicity, oral (H302), skin corrosion/irritation (H315), skin sensitization (H317), serious eye damage/eye irritation 2A (H319), acute toxicity 4, inhalation (H332), specific target organ toxicity, repeated exposure 2 (H373), hazardous to the aquatic environment, acute toxicity (H400) and hazardous to the aquatic environment, chronic toxicity (H410).

Ziram (CAS 137-30-4), zinc bis-(dimethyldithiocarbamate)

Figure 18: Molecular structure of ziram



Source: National Center for Biotechnology Information. PubChem Compound Database; CID=8722, https://pubchem.ncbi.nlm.nih.gov/compound/8722 (accessed 7 May 2018).

Ziram is not authorized for agricultural use in any of the Nordic countries.

EU pesticide database: According to Dir. 2003/39/EU the ADI for ziram is 0.006 mg/kg bw/day, based on two-year studies in rats and using a safety factor of 100. The ARfD is 0.08 mg/kg bw, based on developmental studies in rats. Target organs identified in short-term toxicity studies are: liver (all animals); red blood cells and associated hypocalcaemia (rat, dog); non-glandular stomach (rat, mouse). Target organs in long-term toxicity studies in rats are: liver, thyroid, stomach, adrenals, muscle and nervous tissue (rat) and urinary bladder (mouse). Reproduction toxicology: decreased pup weight at parental toxic doses; diaphragm thinning with liver protrusion. Ziram has the following hazard statement codes: acute toxicity, oral (H302), skin sensitization (H317), serious eye damage/eye irritation 1 (H318), acute toxicity, inhalation 1&2 (H330), specific target organ toxicity, single exposure; Respiratory tract irritation (H335), specific target organ toxicity, repeated exposure 2 (H373), hazardous to the aquatic environment, acute toxicity (H400) and hazardous to the aquatic environment, chronic toxicity (H410).

3.5.1 Criteria for literature search

The PubMed search criteria are shown in Table 5 below. Excluding references written in other languages than English left a final of 131 references. Of these 131 research papers 58 were excluded because they were out of the scope of this overview, for example; exposure via air or skin (n=5); aquatic species (n=4); other research fields (n=4) or too general (n=3). However, the major reason for exclusion was that full text could not be accessed or was not available (n=46). Forty-six research papers remained for the more detailed evaluation. The majority of papers were based on *in vivo* animal experiments at doses exceeding the NOAELs.

Table 9: PubMed search 7 December 2016 Dithiocarbamates and endocrine action

Search	Query	Items found
#3	#1 AND #2	131
#2	(((((mancozeb) OR maneb) OR metiram) OR propineb) OR thiram) OR ziram Filters: English	1,225
#1	(((((((endocrine) OR endocrinol*) OR estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

The aim of this overview is to report putative endocrine effects below the already established critical toxicological effects, and therefore doses below NOAEL or ADI are of interest. Additional references were selected from the reference lists or citations of the selected research papers.

3.5.2 Metabolites/metabolism oral route

Mancozeb (EU Commission): Plasma peak concentration after oral dose was detected after 1–2 hours in mice and 3–6 hours in rats; the majority of the dose was excreted within 24 hours. The highest residue levels were found in the thyroid. Main toxicological compounds are the parent compound and the major metabolite ethylene thiourea (ETU).

Maneb (EU Commission): Similar to mancozeb, maneb is rapidly absorbed and excreted; the thyroid is the main target organ. The parent compound and ETU show the highest toxic properties.

Metiram (EU Commission): The properties of metiram overlap those of mancozeb and maneb. Sixty % of absorbed metiram is excreted within 48 h; the thyroid and the kidneys are the main target organs. The parent compound and ETU show the highest toxic properties.

Propineb (EU Commission): Propineb is absorbed and excreted within 48 h; the thyroid is the main target organ. Degradation of probineb produces propylenethiourea (PTU) and propylendiamine (PDA). PDA is secreted in urine and faeces, whereas PTU appeared to be further metabolized to propyleneurea (PU) and N-formyl-PDA. The parent compound and propylene thiourea (PTU) show the highest toxic properties.

Thiram (EU Commission): Thiram exhibits slower rate of absorption and excretion; about 80–95% is absorbed/excreted within 96 hours. No evidence of accumulation. The main target organs are liver, retina. The parent compound and metabolites show highest toxic properties.

Ziram (EU commission): Absorbed ziram exhibits a wide distribution with highest residue levels in excretory organs and highly vascularized tissues (liver). There is a risk for accumulation since the T1/2 elimination exceeds 24 h; 64–85% of absorbed ziram is excreted within 168 hours. Target organs: liver, thyroid, stomach, adrenals, muscle and nervous tissue.

3.5.3 The HPA-axis

Human lung epithelial cells exposed to 50 µM thiram reduced dexamethasone-induced expression of two glucocorticoid-sensitive genes [259]. Thiram also reduced the activity the HSD11B2 enzyme, which inactivates cortisol to cortisone, and decreased the binding of dexamethasone to the glucocorticoid receptor. Another study later confirmed that thiram irreversibly inhibited HSD11B2 in the human kidney epithelial cell line HEK293 with an IC50 of 132 nM [260]. Maneb was less potent than thiram with an IC50 of 753 nM. This can be compared with the Km (enzyme kinetics; Michaelis-Menten constant for the substrate concentration that is half of the Vmax rate) for cortisol of 26 nM and the Km for corticosterone of 77 nM.[58] The interference with the glucocorticoid axis by thiram at both the receptor level and inhibition of cortisol conversion to cortisone has been confirmed in *ex vivo* studies of brain slices, colon and kidney [261,262].

3.5.4 The HPT-axis

Mancozeb and ETU induction of thyroid tumours in rats and mice at a exposure range of 3.5 – 30.9 mg/kg bw/day were associated with decreased serum T4 and T3 levels, and increased serum TSH concentrations [263]. Further, both mancozeb and ETU inhibited thyroperoxidase and reduced iodide uptake into the thyroid gland, and was associated with increased thyroid cell growth (both hyperplastic and hypertrophic).

The risk of hypothyroidism and hyperthyroidism in female spouses (n =16,529) to subjects enrolled in the Agricultural Health Study in 1993–1997 (Iowa and North Carolina, US) was investigated [264]. Pesticide exposure and thyroid disease were assessed by both the Spouse Enrolment Questionnaire and a computer-assisted follow-up telephone interview. Prevalence of self-reported clinically diagnosed thyroid disease was 12.5%, and prevalence of hypothyroidism and hyperthyroidism was 6.9% and 2.1%, respectively. Maneb/mancozeb were the only pesticide group that were associated with hyperthyroidism (ORadj = 2.3 (1.2, 4.4) as well as hypothyroidism ORadj = 2.2 (1.5, 3.3).

3.5.5 The HPG-axis

Mancozeb at 10 µM inhibited AR-activation in transfected cells and blocked agonist-induced AR activation in a concentration-dependent manner with an IC50 of 2.7 µM [265]. There were no effects on ER or aromatase activity. Mancozeb treatment at low doses (100 ng/ml and 10 ng/ml, respectively) in mouse and human

[58] The UniProt Knowledge database; http://www.uniprot.org/uniprot/P80365

primary ovarian granulosa cells caused cell morphology changes, increased cell migration and reduced p53 protein levels [266]. The same group also showed that mancozeb at 10 ng/ml suppressed cellular levels of reduced and oxidized glutathione, which was followed by a reduction in the mitochondria membrane potential and of cellular energy (ATP) levels [267].

3.5.6 Foetal exposure: programming and epigenetics

3.5.6.1 Epidemiology

Women (n=105,403) and men (n=131,243) from a farmer's family study in Norway (born in 1925–1971) and their 300,805 children available for analyses (born in 1952–1991) were identified using the national registers [268]. These data were cross-linked with national agricultural censuses, 1969–1989 and the population register. Mancozeb exposure was reflected by the combination of potato farming, pesticide use, and meteorologically based fungal forecasts. They found a moderate association between mancozeb exposure and neural tube defects in the offspring but not between mancozeb exposure and thyroid cancer in the adults.

3.5.6.2 Animal studies

Rat dams were dosed daily with different pesticides or vehicle by gavage, from gestational day 7 to 21 and from the day after birth to lactation day 16 [269]. Mancozeb was administered alone at 6.25 or 25 mg/kg bw/day or in pesticide mix with four other pesticides containing 2.08, 4.16 or 6.25 mg mancozeb/kg bw/day. The low dose of 6.25 mg mancozeb/kg bw/day is below the NOAEL and 25 mg mancozeb/kg bw/day is well above. There were no significant effects on weight gain during gestation, gestation length, pre- or perinatal loss in mancozeb-treated groups compared to control. In the pups, there were no effects on postnatal weights; nipple-retention; in the mancozeb-treated groups. In another study, they investigated the effect on male reproductive organs in the male offspring [270]. There were no differences in male reproductive organs weights in the 16 day-old or the adult offspring from the mancozeb-treated dams on either low or high dose. The thyroid gland in the offspring from dams at the higher mancozeb dose (25 mg m/kg bw/day) had structural changes that might suggest thyroid hyperactivity.

Pregnant rats received four pesticide mixtures composed of five different chemicals in increasing doses during gestation and lactation [271]. The five pesticides were; procymidone, epoxyconazole, tebuconazole, mancozeb and prochloraz. The dose of mancozeb in the lowest pesticide mix was 6.25 mg/kg bw/day, similar to the reproductive NOAEL of 7 mg/kg bw/day.[59] The doses of the other compounds in the lowest pesticide mix were: 3.75, 8.75, 12.5 and 12.5 mg/kg bw/day for epoxyconazole, prochloraz, procymidone and tebuconazole, respectively. The mixtures were administered by gavage from gestational day 7 to the day before expected birth and from lactation day 1 to 13. The 13-day old male offspring from the low pesticide mix group had reduced prostate and epididymis weight compared with control pups but

[59] SANCO/4058/2001 – rev. 4.4 July 2009.

there were no differences in testes weight. However, at 13 days of age the anogenital distance was reduced in the male offspring, and increased in the female offspring from dams exposed to the low pesticide mix. Further, the incidence of hypospadias was increased by 40% in the male pups from these dams. However, the observed effects should not necessarily be attributed to mancozeb since the other pesticides are also well-known EDs.

To investigate putative effects of pesticides on hypothalamic control of puberty and control of the HPG-axis, hypothalamic expression of Kiss1 was studied [272]. Rat dams were administered ethinyl-E2 (5, 15 or 50 µg/kg/day) or pesticides daily by gavage from gestational day 7 to 21, and lactation day 1 to 13 (Study 1) or 16 (Study 2). The pesticides were given alone or in a mixture, and mancozeb was one of the pesticides. Neither perinatal exposure of ethinyl-E2 nor pesticides affected hypothalamic expression of Kiss1.

Pregnant Wistar rats were administered 0, 50 or 100 mg mancozeb/kg bw/day by gavage from gestation day 7 to lactation day 16 [273]. There were no effects of mancozeb on the offspring reproductive organ weights, nor did the offspring differ from the controls in the behavioural tests. In this study, a moderate lowering of maternal T_4 levels induced by mancozeb during gestation did not affect behaviour or learning in the offspring.

Lactating Swiss albino dams were administered vehicle, mancozeb, imidacloprid or mancozeb+imidacloprid at 0.5% of the LD50 of respective pesticide (40 mg, 0.655 mg, 0.40+0.655 mg/kg bw/day, respectively) during lactation [274]. Body weights were higher in 21-day old pups suckling dams exposed to both pesticides compared to control. There were no effects in pups suckling dams given either mancozeb or imidacloprid during lactation. The difference in body weight was maintained in the mancozeb+imidacloprid exposed offspring throughout the study length (7 weeks of age). The dams given both pesticides had increased plasma prolactin and TSH levels, and decreased plasma T_3 and T_4 levels. Similar increase in plasma prolactin and TSH levels, with suppressed plasma T_3 and T_4 levels was observed in their 7-week offspring. Levels of plasma total cholesterol, LDL-cholesterol and triglycerides were higher in the offspring from dams treated with both pesticides. *In silico* simulation predicted that both mancozeb and imidacloprid could bind to both thyroid receptors, whereas only mancozeb would bind to PPARγ.

3.5.7 Other

3.5.7.1 Obesity/Type 2 diabetes

Low concentrations (0.1 to 10 ppm, approximately 0.1–10 ng/ml) of mancozeb caused increased intracellular lipid storage and cytoplasmic lactate dehydrogenase and cytochrome C levels in HepG2 cells, a human liver cell line [275]. This suggests that mancozeb can cause adverse effects on mitochondrial function even at low concentrations.

3.5.7.2 Inflammation/infection

Kongsbak *et al.*. used a systems toxicology approach to identify the mode-of-action of mancozeb [276]. Extracting data from the ChemProt 2.0 and CTD databases, they identified that mancozeb was associated with increased inflammatory processes.

The three following studies all point in the same direction; that the mancozeb acts on the innate immune system. This may seem to be out of scope for this review on endocrine disruption, however, the endocrine and immune systems are closely connected. Mancozeb, but not its main metabolite ETU, induced a dose- and time-dependent inhibition of LPS-induced TNF release at low concentrations (1–100 µg/ml) in a human myelocytic cell line. Further, mancozeb blocked TNF release by binding to the NF-k B protein [277]. A study in a mouse macrophage cell line showed that ziram (100 µM; NOAEL rat 0.56 mg/kg bw/day) inhibited LPS-induced activation of the NF-k B pathway [278]. This pathway is an important part of the innate immune system and its ability to fight off bacterial infections.

3.5.7.3 Cancers

Thiram induced breaks in single-stranded DNA at doses above 30 µM (7.2 µg/ml) in rat testicular cells, and in human testicular cells at doses above 100 µM [279]. This placed thiram as one of the most toxic for testicular cells of the 15 tested chemicals known to be toxic for reproduction.

Mancozeb and ETU at doses below 30.9 mg/kg bw/day (approx. 0.5 x NOAEL for mancozeb) caused formation of thyroid tumours in rats, and in rats and mice, respectively [263]. ETU also caused tumour growth in liver and pituitary in mice.

Sprague-Dawley rats (n=75 per sex and treatment group) were given mancozeb (0, 10, 100, 500, 1000 ppm) in the feed from 8 weeks of age for 104 weeks [280]. The lowest dose, 10 ppm, translates to about 0.7 and 0.8 mg/kg bw/day for males and females, respectively, applying the default values for food intake and body weight for the Sprague-Dawley rat strain in chronic studies.[60] This dose is ten times lower than the NOAEL. The age of tumour formation was late, occurring after 112 weeks, corresponding to about 60 human years [281]. Dose-related increases in total malignant tumours were detected in both males and females in all of the mancozeb-treated groups. Particularly, osteosarcomas and lymphatic tissue tumours were increased even in the group treated with the lowest mancozeb dose.

3.5.8 Summary

There was some difficulty in finding papers addressing mode-and-mechanisms of actions of the dithiocarabamate fungicides in publically available databases. This may be due to the fact that these compounds are regulated and that many of the studies that investigate adverse effects are intellectual property of the companies.

Metabolism: Mancozeb and maneb are rapidly (within 24 h) absorbed and excreted; propineb and metiram are in an intermediate (about 48 h) intermediate range

[60] U.S. EPA 1988. Recommendations for and Documentation of Biological Values for Use in Risk Assessment. EPA/600/6-87/008; http://www.tera.org/Tools/ratmousevalues.pdf

of absorption and excretion; thiram is slowly (about 96 h) absorbed and excreted, and for ziram there is a risk for bioaccumulation (half-life > 24 h, 196 h)

Mode and mechanisms of action: Mancozeb, maneb, metiram and propineb are thyroid peroxidase inhibitors, causing growth (hypertrophic and hyperplastic) of the thyroid gland. *In vitro* and *ex vivo* studies show that thiram affects glucocorticoid signalling by increasing ligand concentration via HSD11B2, and also by inhibiting ligand binding to the receptor. Although it appears that these effects could cancel each other out, the tissue distribution of HSD11B2 and the glucocorticoid receptor differs. The HSD11B2 protein is highly expressed in kidney, small intestine, salivary gland, colon, placenta and appendix. The glucocorticoid receptor on the other hand is expressed in most tissues, but reported to be expressed at higher levels in the cerebellum, skeletal muscle, adipose tissue, thyroid and placenta. Consequently, the net effect would be tissue-/cell-specific. Since glucocorticoids are anti-inflammatory this also ties in with that mancozeb blocks NF-kB-signalling in immune cells and is predicted to increase inflammatory processes.

Foetal exposure & programming: There were no effects of perinatal (2 weeks before and 2 weeks after birth) exposure of mancozeb (< NOAL or 3 x NOAEL) in rat dams on gestational length, litter size or pup weights, anogenital distance or male gonad development. Using the same protocol, there were no effects on hypothalamic control of puberty and HPG-axis in the offspring. There were also no effects of perinatal mancozeb exposure (about 1 and 2 x NOAEL) in rats on the offspring reproductive organ weights or in behavioural tests.

Human data: A Norwegian study reported a moderate association between mancozeb use in farming and neural tube defects in the farmer's children. Increased risk for thyroid tumour formation after exposure of mancozeb, maneb, metiram and propineb are presumably induced by their metabolites, ETU and PTU. Thiram and ziram on the other hand primarily had toxic effects in the liver, retina and other organs, suggesting different mode-of-action.

3.6 Genistein, a naturally occurring phytoestrogen

Figure 19: Molecular structures of genistin, genistein and s-equol

Compound	Formula	3D-structure
Genistin	$C_{21}H_{20}O_{10}$	
Genistein	$C_{15}H_{10}O_5$	
S-Equol	$C_{15}H_{14}O_3$	

Source: National Center for Biotechnology Information. PubChem Compound Database; CID=5281377, 5280961 and 91469.

Some compounds that are naturally present in food also affect endogenous pathways, including the endocrine system. One group of chemicals found in plants are known as phytoestrogens, which initially were identified due to their oestrogen-like effects in sheep grazing clover field. The sheep developed infertility and difficulties during labour and the symptoms were later attributed to the high content of isoflavones, including genistein, in clover. Genistin, a glucose-conjugated genistein, is the naturally occurring and predominant form in plants (for structure see above). Genistin is cleaved by bacterial β-glucosidases to the aglycone genistein that is the bioavailable and more lipophilic form. Genistein is formed in foods through fermentation of soybean (*e.g.* miso, soy sauce/paste, and tempeh) [282] or in the colon by specific bacteria in the gut microbiota [283].

The clinical interest in genistein is generated from epidemiological studies suggesting that the lower incidence of CVD, breast and prostate cancers in Asian countries could be explained by their diets which are rich in soybeans. More recently, concerns have been raised about the increasing soy content in infant feeding formulae that could cause too early activation of oestrogen pathways in the developing infant.

It is generally accepted that genistein can bind to both ERα and to ERβ but with a higher affinity to the latter. Genistein is also known to be a protein tyrosine kinase (PTK) inhibitor and may therefore interfere with several other cell surface receptor-mediated signalling cascades. Daidzein, the other major phytoestrogen found in soybeans, acts as an agonist to the oestrogen receptors but lacks PTK inhibitor activity. Hence, it is important to keep in mind that genistein is pluripotent and affects several pathways in addition to oestrogen-associated signalling.

3.6.1 Criteria for literature search

The PubMed search yielded an initial 6526 items, filtered for humans or rat/mice. Excluding items published before 2010-01-01 gave 1612 hits, and further exclusion of duplicate references and articles written in other languages than English gave final of 1363 references.

Table 10: PubMed search 8 December 2016 Genistein and endocrine action published after 31 December 2009

Search	Query	Items found
#8	#5 AND #7	1,612
#7	#6 Filters: Publication date from 2010/01/01 to 2017/12/31	898
#6	#2 Filters: Humans	3,606
#5	#4 Filters: Publication date from 2010/01/01 to 2017/12/31	714
#4	#2 AND #3	2,920
#3	((rat OR rats)) OR (mouse OR mice)	2,935,513
#2	#1 AND genistein	7,163
#1	((((((((endocrine) OR endocrinol*) OR estrogen*) OR estradiol) OR testosterone) OR dihydrotestosterone) OR androgen) OR thyroid) OR steroid*	1,289,000

Quite a few references were not relevant for this overview such as derivatives of genistein as putative drugs, focus on other phytoestrogens and/or other research fields. Reviews were included and additional references were selected from the reference lists or citations of the selected research papers.

3.6.2 Metabolites/metabolism oral route

Genistein, and other isoflavones, have a typical biphasic absorption pattern with an early plasma peak after 1–2 hours of ingestion and a second larger peak that occurs later, up to 8–9 hours after ingestion [284]. This pattern is consistent with an early absorption in the small intestine and a later absorption phase in the colon after bacterial fermentation. The bioavailability of ingested genistein is age-dependent and is higher in infants and prepubertal children compared to adults [284].

Genistein is extensively metabolized in humans by intestinal phase II metabolism, forming β-glucuronides and sulphate esters (for review see [285]). The half-life of oral genistein from pharmacokinetic studies in mice has been reported to be over 24 hours after a high oral dose. The long half-life may however be caused by coprophagy, a known behaviour in rodents to eat faeces to extract nutrients that have been formed in the lower gut. Indeed, another study in mice using soy protein isolate (SPI) containing 20 times lower amount than the study above (1.16 mg genistein/kg bw), reported t1/2 to 57 min for genistein aglycone and 74 min for total genistein [286]. In humans, about 75% of genistein detected in plasma was conjugated (glucuronide or sulphate), and 25% was in the free aglycone form [287].

S-equol is a metabolite derived from dadzein and genistein and produced by colon bacteria [283]. Only the S-equol enantiomer is produced in humans and it has been identified as a potent ERβ agonist [288]. There is a debate whether there are differences between populations in the ability to produce S-equol due to differences in dietary habits and consequently gut microbiota. It has been estimated that about 30% of Western omnivores and 60% of Western vegetarians and Asians can produce S-equol [289]. However, others have shown by repeated measures over an observation period of 2.5 years that subjects spontaneously changed from S-equol-producing to non-producing and vice versa [284].

3.6.3 The HPA-axis

Primary adrenocortical cells were collected during the luteal or follicular phase of the oestrous cycle from sexually mature pigs [290]. Genistein (10 µM) suppressed both basal and ACTH-stimulated *in vitro* secretion of cortisol and corticosterone regardless of which phase of the oestrous cycle the cells were collected from. Genistein dose-dependently inhibited secretion of cortisol, testosterone and aldosterone in an immortalized human adrenal cortex cell line (NCI-H295R cells) with IC50 values of 0.4 µM, 0.6 µM and 2.2 µM, respectively [291]. There was no effect on secretion of oestrogen. Genistein antagonized glucocorticoid-induced gene expression in Ishikawa cells, a human uterine endometrial cell line [292] (see further below in Section 3.6.6), and inhibited HSD11B1 in rat adipose tissue and liver microsomes (see further Section 3.6.7).

Post-weaning male Wistar rats treated with 40 mg/kg bw/day dose genistein for three weeks showed increased adrenal gland weight and decreased serum corticosterone levels [293].

3.6.4 The HPT-axis

Earlier studies in infants and children suggested that high flavonoid intake combined with iodine-deficiency caused enlargement of the thyroid gland [294]. Recent studies in adult men and postmenopausal women suggest that there are no effects of genistein on the HPT-axis (TSH, free T_3 and T_4, thyroglobulin or antibodies) in iodine-replete subjects with normal thyroid function. These negative findings do not however exclude that there may be adverse effects of genistein in certain subgroups or that other phytoestrogens, such as daidzein may affect the HPT-axis [295].

3.6.5 The HPG-axis

The aromatase knockout (*Arko*) mouse is characterized by undetectable plasma oestrogen levels and elevated plasma testosterone, LH and FSH levels. This lack of endogenous oestrogen combined with that both oestrogen receptors are intact makes the *Arko* mouse ideal to investigate putative oestrogen-like effects of a compound *in vivo*. Female *Arko* or wild-type mice were fed a genistein diet resulting in exposure of 70 mg/kg bw/day for three days [296]. In parallel, groups of mice were fed diets containing one of three ER-agonists (ethinyl-E_2, propylpyrazole-triol: PPT, or diarylpropionitrile: DPN). Dietary exposure of ethinyl-E_2 and PPT increased uterine weights but there were no uterotrophic effects of genistein or DPN. All four diets lowered serum FSH-levels in the *Arko* mice to the levels found in the wild-type mice. However, genistein, in contrast to DPN, but similar to ethinyl-E_2 and PPT also reduced circulating LH levels comparable to that detected in the wild-type mice. There were however little or no effects of genistein treatment on gene expression of known oestrogen-induced genes in the *Arko* uterus. This may reflect a higher dependence on ERα-mediated signalling in the uterus whereas hypothalamic-pituitary regulation of LH and FSH is also via the ERβ. The metabolite S-equol preferentially bound to the ERβ and also antagonized the action of DHT *in vivo* [297].

Genistein dose-dependently inhibited dexamethasone-induced gene expression in a human uterine cell line (Ishikawa) at concentrations from 1 nM to 1 µM [292]. Microarray analyses showed that only about 33% of the genes that were regulated by genistein overlapped with E_2-regulated genes. A group of genes that were coregulated by genistein and dexamethasone were dependent on intact signalling by both the glucocorticoid receptor and ERα. The authors concluded that in comparison to endogenous cyclic E_2, dietary genistein not only changed the pattern of stimuli over time to oestrogen-responsive cells but also activated different sets of genes than E_2.

3.6.6 Foetal exposure: programming and epigenetics

3.6.6.1 Prenatal exposure

Pregnant Sprague-Dawley rats were administered a single dose of genistein by gavage (20, 34, 75 mg genistein/kg bw) at gestational day 20. Two hours later, conjugated genistein and genistein aglycone was detected in foetal circulation (70% and 30%, respectively) and in foetal brain (10% and 90%, respectively) [298]. In another study, pregnant Sprague Dawley rats were administered genistein by gavage (0, 4 or 40 mg genistein/kg bw) from gestational day 5 to 19 [299]. Plasma genistein in the dams peaked after 1 hour with genistein-glucuronide as the dominant form. In placenta, genistein aglycone was the predominant form and peaked at 6 hours (approx. 100 pmoles/g tissue) and 4 hours (1500 pmoles/g tissue) after 4 or 40 mg genistein/kg bw, respectively. Twelve hours after the gavage genistein was no longer detected in the placenta. Both glucuronide- and sulphide-conjugated forms of genistein was detected at about equal levels in foetal plasma and increased with time after gavage in the foetal circulation. The elimination half-life, t1/2, was prolonged with about 50 % for the two conjugated forms in foetal circulation compared with maternal t1/2.

Samples collected from *ex vivo*-perfusion of human placentas with 10 ng genistein/ml (37 nM) showed that transfer of genistein aglycone gradually increased over time, after 3 h, 22% of the genistein aglycone was recovered from the foetal compartment [300]. Further, about 12% of the genistein in the foetal compartment was conjugated and hence formed in the placenta. Genistein was also bioaccumulated in the placenta since 30% of the genistein was not recovered. The data suggest that human foetuses are exposed to genistein.

The levels of isoflavones in different soya-based infant formulas are varied and in five formulas the range was 32 to 50 mg isoflavones per litre of formula see review by Dinsdale [301]. Consequently, infants fed these formulas were exposed to 6–12 mg isoflavones/kg bw/day during the first months of life, which is about 10 times higher than adults consuming a soy-rich diet (approx. 0.70 mg isoflavones/kg bw/day). Further, the serum isoflavone levels in these infants were calculated to be 13,000 to 22,000 times greater than serum E2 levels detected in infant circulation.

3.6.6.2 Animal studies: effects of perinatal genistein

Female Sprague Dawley rats were fed phytoestrogen-free diet supplemented with genistein (0, 25, 250 mg/kg diet) two weeks before mating until weaning of the pups at 21 days of age [302].These doses was shown previously to result in similar serum genistein levels as medium to high consumers of soy protein. Further, the high dose caused developmental changes in mammary glands of the female offspring that were consistent with reduced susceptibility to breast cancer. Male offspring that were weaned to the same genistein diet as their dams were fed showed a dose-dependent decrease in chemically induced prostate tumour formation [303]. Pregnant Sprague Dawley dams were fed phytoestrogen-free diets with either casein or soy protein isolates as protein source from gestational day 4 to weaning at 21 days [304]. The pups were maintained on the same diet as their dams. At 50 days of age, the female offspring from the soy protein isolate group had lower body weights, adipose tissue fat mass

(retroperitoneal) and mammary gland terminal end buds than the casein-fed controls. Further, decreased lipogenesis associated with reduced expression of lipogenic genes and increased expression of HSD11B1 was observed both *in vitro* (the 3T3-L1 cell line) and *in vivo* (mammary gland). The same group later identified that the mode-of-action by genistein in reducing breast cancer risk was by increasing Pten (Phosphatase and tensin homologue deleted on chromosome ten) protein expression and nuclear localisation in mammary epithelial cells resulting in cell cycle arrest [305].

The agouti mouse is used to study the nutritional effects on epigenetic regulation and adult phenotype simply by change in coat colour of the offspring from yellow to brown. The yellow fur is caused by constitutive expression of the agouti gene in the hair follicles and all cell types and is associated with adult-onset obesity, diabetes and increased cancer. Female agouti viable yellow (*Avy*) mice were fed phytoestrogen-free diet ± genistein (250 mg/kg diet) two weeks before mating and throughout pregnancy and lactation [306]. Perinatal exposure to genistein shifted the coat colour toward brown in the offspring due to increased DNA methylation upstream of the *Avy* gene. DNA methylation in the germ layers from three tissues (brain, kidney, and liver) was correlated, indicating that genistein-induced DNA methylation occurred in early embryonic development. Further the hypermethylation of *Avy* gene was maintained during post-weaning development causing suppressed *Avy* gene expression and protection from obesity in the adult offspring.

3.6.7 Other

3.6.7.1 Obesity/type 2 diabetes

HSD11B1 converts inactive glucocorticoids to the active form, and is highly expressed in adipose tissue and liver. Increased HSD11B1 activity causes increased intracellular levels of glucocorticoids, which is a mechanism proposed to contribute to the development of visceral obesity in humans and an increased risk for developing insulin resistance. Genistein inhibited HSD11B1 activity in a dose-and time-dependent manner in rat liver and mesenteric adipose tissue microsomes. The IC50-value was about 20 µM in both tissues [307]. There was no effect on HSD11B2 activity by genistein, the enzyme primarily responsible for deactivation of glucocorticoids. Further genistein-induced inhibition of HSD11B1 occurred within 10–20 min and was not affected by co-treatment with the oestrogen receptor agonists tamoxifen and fulvestrant. These data suggest protective effect of genistein on development of visceral obesity and that these effects were not mediated by ERα/ERβ.

The effects of genistein on the pancreas and insulin-secreting β-cells were reviewed by Gilbert [308]. Intake of soy isoflavone supplements improved glycaemic control in both humans and diabetic rodent models. This was attributed to a protective effects on β-cell mass and function via mechanisms that involved both ERs and inhibition of PTKs. In contrast, in a prospective multi-ethnic study from Hawaii (n=75,344, 14 years follow-up) there was a weak increased risk for developing diabetes with higher intake of soy products in over-weight and obese men and native Hawaiian and Caucasian, but not Japanese, women [309].

3.6.7.2 Cardiovascular disease

A meta-analysis of eleven randomized control trials, four parallel studies and seven cross-over design studies (publication dates between 1990 and 2006) found that soy isoflavones lowered serum total and LDL cholesterol levels, whereas HDL cholesterol and triacylglycerol levels were not affected [310]. In postmenopausal women, a high dietary intake of soy protein/isoflavones is associated with protection of cardiac function [311].

The type of soy food product appears to affect CVD outcome. In the Japanese Takayama prospective study (n= 13,355 men and 15,724 women; 16 years follow-up) there was a decreased CVD mortality in subjects with the highest *natto* consumption but not soy protein/isoflavone intake from other types of food products [312]. *Natto* is a Japanese fermented soy product that is often eaten for breakfast. The highest quartile intake of total soy protein and of *natto* was also associated with decreased mortality on stroke, and for natto also decreased mortality in ischaemic heart disease. This cardioprotective effect from *natto* consumption may however be due to the presence of *natto*-kinase, a protein produced by the bacteria during the *natto* fermentation [313]. This may explain the discrepancy with the results obtained from a Chinese prospective cohort study (27,954 men and 35,303 women; 19 years follow-up) where there was no protective effects of soy protein/isoflavone consumption (plain tofu, dessert tofu and soybean drink) and CVD mortality.[61] In fact, there was a slightly higher risk for CVD mortality with higher soy consumption in men but not in women. Similar findings were reported from the Shanghai Men's Health study (n=55,474; mean follow-up 5.4 years) where soy protein intake was positively and dose-dependently associated with increased risk for fatal and non-fatal incidence of coronary heart disease (CHD) [314]. Further, soy intake was positively correlated with plasma interleukin-8 and plasminogen-activator inhibitor 1 (PAI-1) levels. PAI-1 is important in the regulation of fibrinolysis, and elevated PAI-1 levels predisposes to thrombosis. These data suggest an association between soy protein intake in men with increased plasma PAI-1 levels and increased CHD risk.

The positive effects of dietary soy isoflavones in humans in maintaining endothelial function, arterial elasticity/stiffness and subsequently lowering systolic and diastolic blood pressure have largely been attributed to equol, the metabolite of dadzein and genistein after fermentation by specific bacteria in colon, for review see [315].

3.6.7.3 Cancer

It has been common knowledge that soy proteins/isoflavones have protective effects against development of breast cancer. Meta-analysis of 35 clinical studies of pre- and postmenopausal women reported a protective effect of soy isoflavones on incidence of breast cancer in Asian but not Western women [316]. Further, a systematic review

[61] Adjusted for age (continuous), sex, interview year (1993–1995, 1996–1998), dialect group, cigarette smoking, alcohol consumption, level of education (none, primary school, secondary school or more), physical activity level (<0.5, 0.5–3.4, ≥3.5 h/wk), BMI (<20.0, 20.0–23.9, 24.0–27.9, ≥28.0 kg/m2), baseline history of comorbidities (diabetes, hypertension, CHD, and stroke as 4 adjusting factors) and total energy intake (continuous), and dietary intakes of fiber, SFAs, MUFAs, and omega-3 and ω-6 PUFAs (quartiles).

of 131 research articles (40 random controlled trials, 11 uncontrolled trials, and 80 observational studies) also reported that soy protein may protect against development of breast cancer, especially in Asian populations, and less protective in breast cancer recurrence [317]. It is possible that there are genetic variations in the Asian population which confer the protective effects of soy isoflavone. Alternatively, an early introduction of dietary soy isoflavones may be necessary to affect the developing mammary gland and/or epigenetic modification by isoflavones of DNA in the developing foetus/infant.

There was no association between pre-diagnostic plasma genistein levels and prostate risk in the prospective case-control European Prospective Investigation into Cancer and Nutrition (EPIC; n=1605 cases; 1697 controls) with average time to diagnosis of prostate cancer of 5.4 years after blood sampling [318]. There were also no effects on serum hormone or prostate-specific antigen (PSA) levels after six weeks supplementation of isoflavones compared with control in a double-blind, placebo-controlled randomized trial (n=42 isoflavones; n=44 placebo) [319]. However, *in vivo* treatment with genistein in a mouse xenograft model using patient-derived prostate cancer cell line increased metastasis in lymph nodes and secondary tissues in a dose-dependent manner [320].

3.6.8 Summary

Metabolism: Genistin, the glucose-conjugated form of genistein which is the naturally occurring phytoestrogen in plants, typically exhibit a two-phase absorption pattern after ingestion. An early peak of genistein in circulation occurs after 1–2 hours in plasma and a later peak is detected after more than 8 hours due to hydrolysis of genistin by colon microbiota.

Mechanism-of-action: It is generally accepted that genistein can bind and activate both ERs but has a higher affinity to the ERβ. Genistein is also a known protein tyrosine kinase inhibitor and may therefore interfere with several other cell surface receptor-mediated signalling cascades. Hence, it is important to keep in mind that genistein affects several pathways in addition to oestrogen-associated signalling. Central oestrogen signalling was affected in an oestrogen-deficient mouse model, fed a genistein diet for three days. Normalization of plasma FSH and LH levels after genistein feeding, suggests an effect on hypothalamus-pituitary via ERβ *in vivo*. There were no effects in the uterus by genistein, which is dependent on ERα signalling. Indeed, E2 and genistein regulated different sets of genes in a uterine cell line with only 33% overlap. Agouti mouse dams fed genistein during pregnancy and lactation had offspring with leaner phenotype which was associated with hypermethylation of DNA. Similar results were obtained in a study in rats where adult female offspring had lower body weight and adipose tissue mass than controls.

In vitro studies showed that genistein suppressed glucocorticoid secretion from adrenocortical cells and also directly inhibited glucocorticoid-induced gene expression. Genistein treatment also inhibited enzyme activation of cortisol from cortiscosterone in liver and adipose tissue which would lower the local concentrations of glucocorticoids.

Foetal exposure & programming: Genistein can translocate through the placenta in the aglycone form. In rats, both glucuronidated and sulphide-conjugated genistein is detected in foetal circulation with a 50% increased half-life compared with half-life in maternal circulation. Infants fed soy-based infant formulas receive doses at 6–12 mg isoflavones/kg bw/day during the first months of life. This dose is about ten times higher than adults consuming a soy-rich diet of about 0.70 mg isoflavones/kg bw/day and over 13,000 times higher than serum E2 levels at this age.

Human data: Genistein intake was positively associated with an enlarged thyroid gland in iodine-deficient infants and children. However, there was no association with genistein intake and thyroid status in iodine-sufficient euthyroid adults. *In vitro* cell studies and *in vivo* animal studies point to protective effects of genistein in preserving pancreatic β-cell mass and function. However, a prospective study from Hawaii found an increased risk of diabetes with higher intake of soy products in Caucasian/native Hawaiian ethnic subgroups but not in a subgroup of Japanese origin which may suggest consumption of different types of soy foods.

Adult offspring to rat dams fed dietary genistein throughout pregnancy and lactation were less susceptible to induction of mammary gland and prostate cancer. In humans, a protective effect of soy isoflavones on incidence of breast cancer in Asian but not Western women is likely. The reason why is not clear but may involve epigenetic modifications. The effect of dietary intake of soy protein on CVD was also dependent on sex and type of soy food product. Soy protein consumption in postmenopausal women had cardioprotective effects whereas there is a slight but significant increase in CVD mortality and morbidity risk in men.

4. Summary and conclusions

There are difficulties in identifying consistent adverse health effects of specific EDs in human observational studies. For example, the urinary content of EDs with a short half-life (< 24 h) is likely to reflect the ED content of the last meal, particularly when analyzing spot urine samples. The risk of contamination from the sampling equipment (*e.g.* BPA and phthalates) of stored biobank samples, commonly used in epidemiological or population studies, adds to the sample noise. There are also discussions of the impact of variables that are not controlled; for example the fact that humans are free-living and presumably are continuously exposed to a mixture of EDs or bioactive compounds in food. It was shown that urine total phthalate content in Australian men was co-associated with other lifestyle risk factors, such an unhealthy diet pattern, smoking and low physical activity, all known to predispose to metabolic disease. Further, confounding factors such as major life stressors during pregnancy was shown to mask the effects of phthalate exposure on development of male genitalia in baby boys.

However, cell and animal studies have shown that EDs can interfere with endocrine systems. These EDs are likely to act on multiple cellular pathways, mostly as weak agonists or weak antagonists to hormone receptors. Effects of EDs would therefore be

dependent on the endogenous levels of hormones, where high hormone levels would block ED effects on the receptors and conversely low hormone levels would be permissive to the action of the ED. In particular, there is a growing awareness that exposure of EDs during sensitive windows in foetal development will affect health outcome later in adult life. This is known as foetal programming and is of high concern when discussing ED effects on human health. However, foetal exposure to EDs during sensitive windows is particularly difficult to assess since it is ethically only possible to use surrogate measures of foetal exposure such as analyses of maternal urine. These maternal samples are often spot urine samples, collected at various time points during pregnancy and investigating different end-points in the infant/child. Studies in animals are therefore essential to identify effects of EDs during perinatal development in order to prevent and protect pregnant women from exposure to such substances.

Other emerging areas of investigation of ED effects are:

- The HPA-axis – the adrenal gland and related areas of the hypothalamus (BPA, DEHP)
- HPG-axis – BPA and sperm quality
- The cardiovascular system (BPA, DEHP, DINP)
- Obesity and glucose handling (BPA, DEHP)
- Mechanisms via other nuclear receptors and hormone non-nuclear receptors.

Below, there are summaries of potential mechanisms for the substances covered in this report. These substances can be present in foods and all of them have been suggested as potential EDs.

Bisphenol A

Metabolism: BPA is rapidly conjugated in the liver via phase II metabolism and excreted in urine with a half-life of 4–5 hours. BPA has therefore a low potential for bioaccumulation.

Mode or mechanisms of action: It has previously been established that the main mode of action of BPA is as an oestrogen agonist. Therefore, earlier studies focussed on adverse effects by BPA on female reproductive health, in particular effects on ovarian function. Later research show that BPA interferes with intracellular Ca^{2+} homeostasis via ERβ, the nonnuclear mER/GPER, direct action on Ca-channels in the plasma cell membrane or sarcoplasmic reticulum and down-stream on Ca-dependent enzymes. *In vitro* studies show that BPA exposure enhanced adipogenesis in human stem cells via ERα-activation. BPA enhanced adipogenesis in human primary adipocytes via the thyroid hormone receptor, SREBF1, and mTOR pathways. Cultures of intra-abdominal human adipose tissue biopsies with BPA induced HSD11B1 activity, the enzyme that converts inactive cortisone to active cortisol.

Foetal exposure & programming: BPA has been detected in human milk, placenta, amniotic fluid, foetal circulation and foetal tissues. Two studies in C57BL mice and

Wistar rats reported changes in adrenal gland development and increased plasma glucocorticoid levels after maternal exposure to BPA levels below NOAEL via the diet. Glucocorticoid signalling was suppressed in the CNS in the adult rat offspring. In contrast, a large study in Sprague-Dawley rat offspring from dams given BPA by gavage at doses below NOAEL found no effects on adrenal gland development, plasma glucocorticoid levels or stress responses. Animal studies show that developmental exposure to low doses of BPA resulted in increased body weights and impaired glucose handling, which further deteriorated by high fat feeding. BPA appeared to affect the commitment and/or recruitment of adipocyte precursor cells causing reduction in adipocyte number and increase in adipocyte cell size, which is known to reduce insulin sensitivity of the fat cell.

Human data: The human data, based on spot urine samples, is in general difficult to interpret since it measures recent BPA exposure due to the short half-life of BPA. Infants with low-birth weight had higher placental BPA concentration than normal birth weight infants, indicating a negative association between BPA exposure and foetal growth. Another study reported that 2-fold higher maternal plasma conjugated BPA levels at first term was associated with lower birth weight and 2-fold higher levels at delivery was associated with longer gestation, both in female babies only. According to the Barker hypothesis, a low birth weight is associated with increased risk to develop non-communicable disease in adult age. Further, meta-analysis of cohort data showed that subjects with high u-BPA concentrations are more likely to have diabetes, central obesity and high blood pressure. The question is whether high u-BPA levels is a surrogate marker for poor lifestyle choices, such as high consumption of soft drinks with high sugar content from plastic bottles containing BPA.

Phthalates (DEHP, BBP, DBP, DINP, DIDP)

It has previously been established that phthalates have anti-androgenic effects and focus has been on male reproductive function, including developmental effects on male reproductive organs and effects on sperm function and fertility.

Metabolism: Absorption and metabolism of phthalates in mammals is rapid and phthalate metabolites have half-lives of 12 to 48 hours. Therefore exposure estimates in humans from spot urine samples are likely to reflect what was consumed in the last meal, causing large variations in exposure estimates.

Mode or mechanisms of action: In general, the mechanism-of-action of phthalates is focussed on the PPARα and PPARγ pathways. DEHP and its metabolites can activate different pathways in cells of the reproductive organs; MEHP acted as a partial antagonist to both PPARα and PPARγ in rat ovarian granulosa cells and DEHP acted as a partial ER-agonist in human endometrial cells; DEHP induced apoptosis via Bcl2 in mouse spermatocytes. In liver, both DEHP and DBP activated mouse and human hepatic PPARα and CAR; their metabolites, MEHP and MBzP, respectively, activated mouse and human PXRs. MEHP acted as a partial PPARγ agonist in adipocytes. DEHP induced mouse pluripotent mesenchymal cells to differentiate to adipocytes.

Foetal exposure & programming: Phthalates are ubiquitous and are detected in maternal urine throughout pregnancy. DEHP metabolites are detected in cord blood and in new-born infant stools, both indicative of foetal exposure of phthalates. In rats, prenatal exposure of DEHP at levels below NOAEL caused changes in adrenal gland structure with implications of long-term effects on blood pressure control. Studies over three generations of mice suggest that changes in expression of hormones of the HPA-axis lead to impaired stress response in future generations due to epigenetic modifications. Perinatal exposure of DEHP in mice caused a dose-dependent loss of germ cells and Sertoli cells in testes from 21-day old pups and could be detected already in midgestation embryos. Prenatal exposure of low levels of DEHP caused reduced anogenital distance in the one-day old pups of both sexes. Human data indicate that major stressful life events may mask effects of early ED exposure on anogenital distance in male infants. Only baby boys born by mothers without stressful life events during pregnancy exhibited a positive association between prenatal first trimester urine DEHP and anogenital distance at birth.

Human data: Studies on the effect of phthalate exposure on female fertility studies, measured as time-to-pregnancy, vary. There was inverse correlation between maternal blood MEHP quartiles at late second early third trimester and cord blood levels of cortisol, cortisone levels and glucocorticoid/adrenal androgen ratio in a prospective mother-child cohort. Human data show positive correlations between urine phthalate metabolite levels and impaired glucose tolerance, reduced insulin sensitivity (increased serum insulin) and increased insulin resistance (increased serum insulin and blood glucose). However, one study reported that although dietary intake was the main source to total phthalate exposure, intake of carbonated soft drinks was a major contributor. This positive association of a western dietary pattern and obesity with total phthalate content in urine suggests that phthalate exposure is co-associated with other lifestyle factors that increase the risk for chronic disease.

BHA – butylated hydroxyanisole and BHT – butylated hydroxytoluene

In comparison with BPA and phthalates less is known about mode and mechanism of actions of the antioxidants BHA and BHT.

Metabolism: BHA and BHT are metabolized fairly rapidly after a single dose but continuous exposure increases levels of BHA and its metabolite TBHQ in circulation.

Mode or mechanisms of action: In comparison with BPA and phthalates less is known about mode and mechanism-of-actions of the antioxidants BHA and BHT. Cell studies show that BHA affect glucocorticoid signalling both by activating glucocorticoid receptor-mediated transcription and by inhibiting the enzyme responsible for deactivating cortisol. BHA activated thyroid hormone receptor-dependent gene expression. BHA can activate both ERs. BHA and BHT were weak antagonists to dihydrotestosterone-induced activation of the androgen receptor in human transfected cells.

Foetal exposure & programming: Sperm morphology was affected in male Sprague Dawley rat offspring after exposure of low BHA (0.1 x NOAEL) from preconception up to 13 weeks postnatal age.

Human data: No studies were identified

Parabens (methyl-/ethyl-/propyl-p-OH-benzoates)

The main focus for parabens, showing both oestrogenic and anti-androgenic effects, has been on ovarian and breast cancer since parabens are accumulated in these tissues.

Metabolism: Alkylparabens are hydrolyzed by the liver at a rate that is inversely related to alkyl-chain length. Parabens are glucuronidated in hepatic phase II metabolism. Repeated measures of paraben content in urine show a fair reproducibility within a subject with the exception of pregnancy when urinary paraben concentrations generally drop with up to 25%. Therefore other sources of parabens than in food such as the use of cosmetics and personal hygiene products contribute significantly to paraben found in urine.

Mode or mechanisms of action: Propylparaben activated glucocorticoid receptor-mediated transcription in transfected cells. Parabens acted as PPARγ agonists in the mouse preadipocyte 3T3-L1 cell line by inducing adipocyte differentiation and in a human osteoblast cell line by the activation of a reporter gene. Parabens uncoupled oxidative phosphorylation and enhancing ROS production in mitochondria in a hepatocyte cell line.

Foetal exposure & programming: Methylparaben was the most prevalent of these three parabens and have been detected in human placenta and cord blood, which indicates foetal exposure. The second most common was ethylparaben followed by propylparaben.

Human data: Methyl-, ethyl and propylparaben were detected in 95%, 60% and 83%, respectively, in urine from Danish pregnant women (gestational week 8–30). Urine methyl- and propylparaben concentrations were highly correlated, suggesting a common source of exposure. Reduced fertility in women was associated with higher urinary concentrations of methyl- and ethylparabens in a prospective study. However, outcome of *in vitro* fertilization was not affected by urinary concentrations of parabens in the women. Methylparaben was detected at highest levels in urine, followed by propyl- and ethylparaben in young healthy Danish men. Similar pattern was detected in blood albeit at lower levels, and in seminal fluid. The main focus on paraben effects on health has been on breast and ovarian cancers, *i.e.* tissues where these parabens accumulate. Malignant ovarian tumours had twice the content of paraben as benign tumours. Paraben content in breast cancer tumours have not been measured, however there was no association with paraben content in adjacent tissue and tumour location.

Dithiocarbamate pesticides

There was some difficulty in finding papers addressing mode-and-mechanisms of actions of the dithiocarabamate fungicides in publically available databases. This may be due to the fact that these compounds are regulated and that many of the studies that investigate adverse effects are intellectual property of the companies.

Metabolism: The rate of absorption and excretion of these compounds are in order mancozeb, metiram (24 h)< metiram, propineb (about 48 h)< thiram (96 h) < ziram (>> 96h).

Mode or mechanisms of action: Mancozeb, maneb, metiram and propineb are thyroid peroxidase inhibitors, causing growth (hypertrophic and hyperplastic) of the thyroid gland. Thiram affects glucocorticoid signalling by increasing ligand concentration via HSD11B2 and also by inhibiting glucocorticoid binding to the receptor. Although it appears that these effects could cancel each other out, the tissue distribution of HSD11B2 and the glucocorticoid receptor differs. Consequently, the net effect would be tissue-/cell-specific. Since glucocorticoids are anti-inflammatory this is in line with that mancozeb blocks NF-kB-signalling in immune cells and is predicted to increase inflammatory processes.

Foetal exposure & programming: No adverse effects have been reported in rat pups after perinatal mancozeb exposure at levels around current NOAEL. The parameters included gestational length, litter size/pup weights, anogenital distance, male gonad development and maturation of the HPG-axis.

Human data: Increased risk for thyroid tumour formation presumably induced via their metabolites, ETU and PTU. Thiram and ziram on the other hand primarily had toxic effects in the liver, retina and other organs, suggesting different mode-of-action.

Genistein

Metabolism: Genistin, the glucose-conjugated form of genistein which is the naturally occurring phytoestrogen in plants, typically exhibit a two-phase absorption pattern after ingestion. An early peak of genistein in circulation occurs after 1–2 hours in plasma and a later peak is detected after more than 8 hours due to hydrolysis of genistin by colon microbiota.

Mode or mechanisms of action: It is generally accepted that genistein can bind and activate both ERs but has a higher affinity to the ERβ. Genistein is also a known protein tyrosine kinase inhibitor and may therefore interfere with several other cell surface receptor-mediated signalling cascades. Animal studies show that genistein acts on the hypothalamus-pituitary via ERβ and had no effects in the uteri growth, a known ERα-dependent parameter. Indeed, E2 and genistein regulated different sets of genes in a uterine cell line with only 33% overlap. Epigenetic effects of genistein have been reported in agouti mouse dams fed genistein during pregnancy and lactation. The offspring had leaner phenotype which was associated with hypermethylation of DNA. Similar results were obtained in a study in rats where adult female offspring had lower body weight and adipose tissue mass than controls. *In vitro* studies showed that genistein suppressed glucocorticoid secretion from adrenocortical cells and also

directly inhibited glucocorticoid-induced gene expression. Genistein treatment also inhibited enzyme activation of cortisol from cortiscosterone in liver and adipose tissue which would lower the local concentrations of glucocorticoids.

Foetal exposure & programming: Genistein can translocate through the placenta in the aglycone form. In rats, both glucuronidated and sulphide-conjugated genistein is detected in foetal circulation with an increased half-life by 50% compared with half-life in maternal circulation. Infants fed soy-based infant formulas receive doses at 6–12 mg isoflavones/kg bw/day during the first months of life. This dose is about ten times higher than adults consuming a soy-rich diet of about 0.70 mg isoflavones/kg bw/day and over 13,000 times higher than serum E2 levels at this age. Adult offspring to rat dams fed dietary genistein throughout pregnancy and lactation were less susceptible to induction of mammary gland and prostate cancer. *In vitro* and *in vivo* animal studies point to protective effects of genistein in preserving pancreatic β-cell mass and function.

Human data: Genistein intake was positively associated with an enlarged thyroid gland in iodine-deficient infants and children. There was no association with genistein intake and thyroid status in iodine-sufficient euthyroid adults. A prospective study from Hawaii found an increased risk of diabetes with higher intake of soy products in Caucasian/native Hawaiian ethnic subgroups but not in a subgroup of Japanese origin which may suggest consumption of different types of soy foods. Soy protein consumption in postmenopausal women had cardioprotective effects whereas there is a slight but significant increase in CVD mortality and morbidity risk in men. Intake of soy isoflavones has a protective effect on the incidence of breast cancer in Asian but not Western women. The reason why is not clear but may involve epigenetic modifications.

5. Future considerations

5.1 General

We are exposed to a mixture of EDs and other substances in real life. This means in theory that multiple pathways could be stimulated simultaneously with unpredictable outcome.

Some clinical studies have pointed out that the effects of the studies were co-associated with other life style factors such as soft drink consumption.

5.2 Considerations for Endocrinologists

Replacements, hazard *vs* risk.

There are also concerns on what to replace the EDs with. Novel compounds with little or no information on short-/long-term effects on eco-systems and human/animal health may not be a good option.

5.3 Considerations for Toxicologists

The concept of non-linear dose response.

Several layers of complexity: which end-point(s) do we use?

One nuclear receptor – several outcomes 1: partial agonism/recruitment of co-activators/repressors yields differences in outcome.

Endocrine systems change throughout the 24-h day which means that depending on when the stimulus is presented it may not have any or little effect at that time-point. Similarly, some endocrine systems have longer than 24-h intervals, such as the menstrual cycle with 4-week interval. Oestrogen mimetics may be swamped by endogenous E2, giving no effect or presented.

References

1. WHO-UNEP The World Health Organization and the United Nations Environment Programme (2012) State of the science of endocrine disrupting chemicals – 2012. An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme (UNEP) and WHO.
2. Li S, Hursting SD, Davis BJ, McLachlan JA & JC Barrett et al.. (2003) Environmental exposure, DNA methylation, and gene regulation: lessons from diethylstilbesterol-induced cancers. Ann N Y Acad Sci, 983: 161–9.
3. Dietert RR (2014) Developmental Immunotoxicity, Perinatal Programming, and Noncommunicable Diseases: Focus on Human Studies. Adv Med, 2014: 867805.
4. Stel J & J Legler (2015) The Role of Epigenetics in the Latent Effects of Early Life Exposure to Obesogenic Endocrine Disrupting Chemicals. Endocrinology, 156: 3466–72.
5. Barker DJ (2004) The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci, 359: 1359–66.
6. Barker DJ, Osmond C, Golding J, Kuh D & ME Wadsworth et al.. (1989) Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ*, 298: 564–7.
7. Gore AC, Chappell VA, Fenton SE, Flaws JA & A Nadal et al.. (2015) EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev*, 36: E1–E150.
8. Tata JR (2005) One hundred years of hormones. *A new name sparked multidisciplinary research in endocrinology, which shed light on chemical communication in multicellular organisms*, 6: 490–96.
9. McEwen BS, Gray JD & C Nasca (2015) 60 years of neuroendocrinology: redefining neuroendocrinology: stress, sex and cognitive and emotional regulation. *J Endocrinol*, 226: T67–83.
10. Fitzsimons CP, Herbert J, Schouten M, Meijer OC & PJ Lucassen et al.. (2016) Circadian and ultradian glucocorticoid rhythmicity: Implications for the effects of glucocorticoids on neural stem cells and adult hippocampal neurogenesis. *Frontiers in neuroendocrinology*, 41: 44–58.
11. Bianco AC & BW Kim (2006) Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest*, 116: 2571–9.
12. Farooqi IS & S O'Rahilly (2014) 20 YEARS OF LEPTIN: Human disorders of leptin action. *Journal of Endocrinology*, 223: T63–T70.
13. Fisher DA (1996) Physiological variations in thyroid hormones: physiological and pathophysiological considerations. *Clin Chem*, 42: 135–9.
14. Maes M, Mommen K, Hendrickx D, Peeters D & P D'Hondt et al.. (1997) Components of biological variation, including seasonality, in blood concentrations of TSH, TT_3, FT_4, PRL, cortisol and testosterone in healthy volunteers. *Clin Endocrinol* (Oxf), 46: 587–98.
15. Roef G, Taes Y, Toye K, Goemaere S & T Fiers et al.. (2013) Heredity and lifestyle in the determination of between–subject variation in thyroid hormone levels in euthyroid men. *Eur J Endocrinol*, 169: 835–44.
16. Shin YL (2016) An update on the genetic causes of central precocious puberty. *Ann Pediatr Endocrinol Metab,* 21: 66–9.
17. Chou SH & C Mantzoros (2014) 20 YEARS OF LEPTIN: Role of leptin in human reproductive disorders. *Journal of Endocrinology*, 223: T49–T62.
18. Tng EL (2015) Kisspeptin signalling and its roles in humans. *Singapore Med J*, 56: 649–56.

19. Barker DJ, Winter PD, Osmond C, Margetts B & SJ Simmonds et al.. (1989) Weight in infancy and death from ischaemic heart disease. *Lancet*, 2: 577–80.

20. Harris A & J Seckl (2011) Glucocorticoids, prenatal stress and the programming of disease. *Hormones and Behavior*, 59: 279–89.

21. Chango A & IP Pogribny (2015) Considering Maternal Dietary Modulators for Epigenetic Regulation and Programming of the Fetal Epigenome. *Nutrients*, 7: 2748–70.

22. Yamada S, Samtani RR, Lee ES, Lockett E & C Uwabe et al.. (2010) Developmental Atlas of the Early First Trimester Human Embryo. *Developmental Dynamics*, 239: 1585–95.

23. Xue L, Cai JY, Ma J, Huang Z & MX Guo et al.. (2013) Global expression profiling reveals genetic programs underlying the developmental divergence between mouse and human embryogenesis. *BMC Genomics*, 14: 568.

24. Attig L, Larcher T, Gertler A, Abdennebi–Najar L & J Djiane et al.. (2011) Postnatal leptin is necessary for maturation of numerous organs in newborn rats. *Organogenesis*, 7: 88–94.

25. Attig L, Brisard D, Larcher T, Mickiewicz M & P Guilloteau et al.. (2013) Postnatal leptin promotes organ maturation and development in IUGR piglets. *PLoS One*, 8: e64616.

26. Rog-Zielinska EA, Richardson RV, Denvir MA & KE Chapman et al.. (2014) Glucocorticoids and foetal heart maturation; implications for prematurity and foetal programming. *Journal of Molecular Endocrinology*, 52: R125–R35.

27. Gamble KL, Berry R, Frank SJ & ME Young et al.. (2014) Circadian clock control of endocrine factors. *Nat Rev Endocrinol*, 10: 466–75.

28. Van Cauter E, Polonsky KS & AJ Scheen (1997) Roles of circadian rhythmicity and sleep in human glucose regulation. *Endocr Rev*, 18: 716–38.

29. Dawson MI & Z Xia (2012) The retinoid X receptors and their ligands. *Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids,* 1821: 21–56.

30. Psarra AMG, Solakidi S & CE Sekeris (2006) The mitochondrion as a primary site of action of steroid and thyroid hormones: Presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells. *Molecular and Cellular Endocrinology*, 246: 21–33.

31. Chen JQ, Cammarata PR, Baines CP & JD Yager et al.. (2009) Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *Biochim Biophys Acta*, 1793: 1540–70.

32. Stirone C, Boroujerdi A, Duckles SP & DN Krause et al.. (2004) Estrogen Receptor Activation of Phosphoinositide-3 Kinase, Akt, and Nitric Oxide Signaling in Cerebral Blood Vessels: Rapid and Long-Term Effects. *Molecular Pharmacology*, 67: 105.

33. Farach-Carson MC & PJ Davis (2003) Steroid Hormone Interactions with Target Cells: Cross Talk between Membrane and Nuclear Pathways. *Journal of Pharmacology and Experimental Therapeutics*, 307: 839.

34. Pupo M, Pisano A, Lappano R, Santolla MF & EM De Francesco et al.. (2012) Bisphenol A Induces Gene Expression Changes and Proliferative Effects through GPER in Breast Cancer Cells and Cancer-Associated Fibroblasts. *Environ Health Perspect*, 120: 1177–82.

35. Hammes SR & PJ Davis (2015) Overlapping nongenomic and genomic actions of thyroid hormone and steroids. *Best Pract Res Clin Endocrinol Metab*, 29: 581–93.

36. Kojetin DJ & TP Burris (2013) Small Molecule Modulation of Nuclear Receptor Conformational Dynamics: Implications for Function and Drug Discovery. *Molecular Pharmacology*, 83: 1–8.

37. WHO (2000) Obesity: Preventing and managing the global epidemic. Report of a WHO Consultation. *WHO Technical Report Series*: 894.

38. Lee DE, Kehlenbrink S, Lee H, Hawkins M & JS Yudkin et al. (2009) Getting the message across: mechanisms of physiological cross talk by adipose tissue. *Am J Physiol Endocrinol Metab*, 296: E1210-29.

39. Marino L & FR Jornayvaz (2015) Endocrine causes of nonalcoholic fatty liver disease. *World Journal of Gastroenterology : WJG*, 21: 11053-76.

40. Chung GE, Kim D, Kim W, Yim JY & MJ Park et al. (2012) Non-alcoholic fatty liver disease across the spectrum of hypothyroidism. *J Hepatol*, 57: 150–6.

41. Sinha RA, Singh BK & PM Yen (2014) Thyroid hormone regulation of hepatic lipid and carbohydrate metabolism. *Trends in Endocrinology & Metabolism*, 25: 538–45.

42. Santini F, Marzullo P, Rotondi M, Ceccarini G & L Pagano et al. (2014) Mechanisms in endocrinology: The crosstalk between thyroid gland and adipose tissue: signal integration in health and disease. *European Journal of Endocrinology*, 171: R137–R52.

43. Geens T, Aerts D, Berthot C, Bourguignon J-P & L Goeyens et al. (2012) A review of dietary and non-dietary exposure to bisphenol-A. *Food and Chemical Toxicology*, 50: 3725–40.

44. Gyllenhammar I, Tröger R, Glynn A, Rosén J & K-E Hellenäs et al. (2014) Serum levels of unconjugated bisphenol A are below 0.2 ng/ml in Swedish nursing women when contamination is minimized. *Environment International*, 64: 56–60.

45. Gyllenhammar I, Glynn A, Jonsson BA, Lindh CH & PO Darnerud et al. (2017) Diverging temporal trends of human exposure to bisphenols and plastizisers, such as phthalates, caused by substitution of legacy EDCs? *Environ Res*, 153: 48–54.

46. KEMI (2017) Bisfenoler – en kartläggning och analys. Rapport från ett deluppdrag inom Handlingsplanen för en giftfri vardag. *The Swedish Chemicals Agency*. Rapport: 5/17.

47. Tyl RW, Myers CB, Marr MC, Thomas BF & AR Keimowitz et al. (2002) Three-Generation Reproductive Toxicity Study of Dietary Bisphenol A in CD Sprague-Dawley Rats. *Toxicological Sciences,* 68: 121–46.

48. Tyl RW, Myers CB, Marr MC, Sloan CS & NP Castillo et al. (2008) Two-Generation Reproductive Toxicity Study of Dietary Bisphenol A in CD-1 (Swiss) Mice. *Toxicological Sciences*, 104: 362–84.

49. EFSA FIP FIaP (2015) Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. *EFSA Journal*, 13: 3978.

50. Reagan-Shaw S, Nihal M &N Ahmad (2008) Dose translation from animal to human studies revisited. *FASEB J*, 22: 659–61.

51. Thayer KA, Doerge DR, Hunt D, Schurman SH & NC Twaddle et al. (2015) Pharmacokinetics of bisphenol A in humans following a single oral administration. *Environ Int*, 83: 107–15.

52. Vandenberg LN, Hauser R, Marcus M, Olea N & WV Welshons et al. (2007) Human exposure to bisphenol A (BPA). *Reprod Toxicol*, 24: 139–77.

53. Fisher JW, Twaddle NC, Vanlandingham M & DR Doerge et al. (2011) Pharmacokinetic modeling: Prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans. *Toxicology and Applied Pharmacology*, 257: 122–36.

54. Oishi K, Sato T, Yokoi W, Yoshida Y & M Ito et al. (2008) Effect of Probiotics, Bifidobacterium breve and Lactobacillus casei, on Bisphenol A Exposure in Rats. *Bioscience, Biotechnology, and Biochemistry,* 72: 1409–15.

55. Verner MA, Magher T & S Haddad (2010) High concentrations of commonly used drugs can inhibit the in vitro glucuronidation of bisphenol A and nonylphenol in rats. *Xenobiotica*, 40: 83–92.

56. Yalcin EB, Kulkarni SR, Slitt AL & R King et al. (2016) Bisphenol A sulfonation is impaired in metabolic and liver disease. *Toxicol Appl Pharmacol*, 292: 75–84.

57. Sui Y, Ai N, Park S-H, Rios-Pilier J & JT Perkins (2012) Bisphenol A and Its Analogues Activate Human Pregnane X Receptor. *Environmental Health Perspectives*, 120: 399–405.

58. Mazur CS, Marchitti SA, Dimova M, Kenneke JF & A Lumen (2012) Human and Rat ABC Transporter Efflux of Bisphenol A and Bisphenol A Glucuronide: Interspecies Comparison and Implications for Pharmacokinetic Assessment. *Toxicological Sciences*, 128: 317–25.

59. Calafat AM, Weuve J, Ye X, Jia LT & H Hu (2009) Exposure to Bisphenol A and Other Phenols in Neonatal Intensive Care Unit Premature Infants. *Environmental Health Perspectives*, 117: 639–44.

60. Bushnik T, Haines D, Levallois P, Levesque J & J Van Oostdam et al. (2010) Lead and bisphenol A concentrations in the Canadian population. *Health Rep*, 21: 7–18.

61. LaKind JS, Levesque J, Dumas P, Bryan S & J Clarke et al. (2012) Comparing United States and Canadian population exposures from National Biomonitoring Surveys: Bisphenol A intake as a case study. *Journal of Exposure Science & Environmental Epidemiology*, 22: 219–26.

62. Medwid S, Guan H & K Yang (2016) Prenatal exposure to bisphenol A disrupts adrenal steroidogenesis in adult mouse offspring. *Environ Toxicol Pharmacol*, 43: 203–8.

63. Poimenova A, Markaki E, Rahiotis C & E Kitrak (2010) Corticosterone-regulated actions in the rat brain are affected by perinatal exposure to low dose of bisphenol A. *Neuroscience, 167: 741–9.*

64. Panagiotidou E, Zerva S, Mitsiou DJ, Alexis MN & E Kitrake (2014) Perinatal exposure to low-dose bisphenol A affects the neuroendocrine stress response in rats. *J Endocrinol*, 220: 207–18.

65. Kitraki E, Nalvarte I, Alavian-Ghavanini A & J Ruegg et al. (2015) Developmental exposure to bisphenol A alters expression and DNA methylation of Fkbp5, an important regulator of the stress response. *Mol Cell Endocrinol*, 417: 191–9.

66. Gioiosa L, Palanza P, Parmigiani S & FS vom Saal et al. (2015) Risk Evaluation of Endocrine-Disrupting Chemicals: Effects of Developmental Exposure to Low Doses of Bisphenol A on Behavior and Physiology in Mice (Mus musculus). *Dose-Response*, 13: 1559325815610760.

67. Gioiosa L, Fissore E, Ghirardelli G, Parmigiani S & P Palanza et al. (2007) Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. *Hormones and Behavior*, 52: 307–16.

68. Ferguson SA, Law CD & GE Kissling (2014) Developmental Treatment with Ethinyl Estradiol, but Not Bisphenol A, Causes Alterations in Sexually Dimorphic Behaviors in Male and Female Sprague Dawley Rats. *Toxicological Sciences*, 140: 374–92.

69. Rebuli ME, Camacho L, Adonay ME, Reif DM & DL Aylor et al. (2015) Impact of Low-Dose Oral Exposure to Bisphenol A (BPA) on Juvenile and Adult Rat Exploratory and Anxiety Behavior: A CLARITY-BPA Consortium Study. *Toxicological Sciences*, 148: 341–54.

70. Braun JM, Kalkbrenner AE, Calafat AM, Yolton K & X Ye et al. (2011) Impact of early-life bisphenol A exposure on behavior and executive function in children. *Pediatrics,* 128: 873–82.

71. Evans SF, Kobrosly RW, Barrett ES, Thurston SW & AM Calafat et al. (2014) Prenatal bisphenol A exposure and maternally reported behavior in boys and girls. *NeuroToxicology*, 45: 91–9.

72. Harley KG, Gunier RB, Kogut K, Johnson C & A Bradman et al. (2013) Prenatal and Early Childhood Bisphenol A Concentrations and Behavior in School-Aged Children. *Environmental Research*, 126: 43–50.

73. Perera F, Nolte EL, Wang Y, Margolis AE & AM Calafat et al. (2016) Bisphenol A exposure and symptoms of anxiety and depression among inner city children at 10–12 years of age. *Environ Res*, 151: 195–202.

74. Perez-Lobato R, Mustieles V, Calvente I, Jimenez-Diaz I & R Ramos et al. (2016) Exposure to bisphenol A and behavior in school-age children. *NeuroToxicology*, 53: 12–19.

75. Giesbrecht GF, Liu J, Ejaredar M, Dewey D & N Letourneau et al. (2016) Urinary bisphenol A is associated with dysregulation of HPA-axis function in pregnant women: Findings from the APrON cohort study. *Environ Res,* 151: 689–97.

76. Vitku J, Heracek J, Sosvorova L, Hampl R & T Chlupacova et al. (2016) Associations of bisphenol A and polychlorinated biphenyls with spermatogenesis and steroidogenesis in two biological fluids from men attending an infertility clinic. *Environ Int*, 89–90: 166–73.

77. Moriyama K, Tagami T, Akamizu T, Usui T & M Saijo et al. (2002) Thyroid Hormone Action Is Disrupted by Bisphenol A as an Antagonist. *The Journal of Clinical Endocrinology & Metabolism*, 87: 5185–90.

78. Sheng Z-G, Tang Y, Liu Y-X, Yuan Y & B-Q Zhao et al. (2012) Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. *Toxicology and Applied Pharmacology*, 259: 133–42.

79. Somogyi V, Horvath TL, Toth I, Bartha T & LV Frenyo (2016) Bisphenol A influences oestrogen- and thyroid hormone-regulated thyroid hormone receptor expression in rat cerebellar cell culture. *Acta Vet Hung*, 64: 497–513.

80. Ahmed RG (2016) Maternal bisphenol A alters fetal endocrine system: Thyroid adipokine dysfunction. *Food Chem Toxicol*, 95: 168–74.

81. Meeker JD, Calafat AM & R Hauser (2010) Urinary bisphenol A concentrations in relation to serum thyroid and reproductive hormone levels in men from an infertility clinic. *Environ Sci Technol*, 44: 1458–63.

82. Aker AM, Watkins DJ, Johns LE, Ferguson KK & OP Soldin (2016) Phenols and parabens in relation to reproductive and thyroid hormones in pregnant women. *Environ Res*, 151: 30–37.

83. Chevrier J, Gunier RB, Bradman A, Holland NT & AM Calafat et al. (2013) Maternal Urinary Bisphenol A during Pregnancy and Maternal and Neonatal Thyroid Function in the CHAMACOS Study. *Environmental Health Perspectives*, 121: 138–44.

84. Romano ME, Webster GM, Vuong AM, Thomas Zoeller R & A Chen et al. (2015) Gestational urinary bisphenol A and maternal and newborn thyroid hormone concentrations: The HOME Study. *Environmental Research*, 138: 453–60.

85. Wang N, Zhou Y, Fu C, Wang H & P Huang et al. (2015) Influence of Bisphenol A on Thyroid Volume and Structure Independent of Iodine in School Children. *PLoS One*, 10: e0141248.

86. Park C, Choi W, Hwang M, Lee Y & S Kim et al. (2015) Associations between urinary phthalate metabolites and bisphenol A levels, and serum thyroid hormones among the Korean adult population – Korean National Environmental Health Survey (KoNEHS) 2012–2014. *Science of The Total Environment.*

87. Andrianou XD, Gangler S, Piciu A, Charisiadis P & C Zira et al. (2016) Human Exposures to Bisphenol A, Bisphenol F and Chlorinated Bisphenol A Derivatives and Thyroid Function. *PLoS One,* 11: e0155237.

88. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC & SH Safe et al. (1998) Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β. *Endocrinology*, 139: 4252–63.

89. Gutendorf B & J Westendorf (2001) Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology*, 166: 79–89.

90. Sengupta S, Obiorah I, Maximov PY, Curpan R & VC Jordan et al. (2013) Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *British Journal of Pharmacology*, 169: 167–78.

91. Li Y, Burns KA, Arao Y, Luh CJ & KS Korach et al. (2012) Differential Estrogenic Actions of Endocrine-Disrupting Chemicals Bisphenol A, Bisphenol AF, and Zearalenone through Estrogen Receptor α and β in Vitro. *Environmental Health Perspectives*, 120: 1029–35.

92. Aldad TS, Rahmani N, Leranth C & HS Taylor et al. (2011) Bisphenol-A (BPA) Exposure Alters Endometrial Progesterone Receptor Expression in the Non-human Primate. *Fertility and sterility,* 96: 175–79.

93. Okada H, Tokunaga T, Liu X, Takayanagi S & A Matsushima et al. (2008) Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-Related Receptor-γ. *Environmental Health Perspectives*, 116: 32–38.

94. Lee HJ, Chattopadhyay S, Gong E-Y, Ahn RS & K Lee et al. (2003) Antiandrogenic Effects of Bisphenol A and Nonylphenol on the Function of Androgen Receptor. *Toxicological Sciences*, 75: 40–46.

95. Zhang X, Chang H, Wiseman S, He Y & E Higley et al. (2011) Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol Sci,* 121: 320–7.

96. Moral R, Wang R, Russo IH, Lamartiniere CA & J Pereira et al. (2008) Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. *Journal of Endocrinology*, 196: 101–12.

97. Abdel-Maksoud FM, Leasor KR, Butzen K, Braden TD & BT Akingbemi et al. (2015) Prenatal Exposures of Male Rats to the Environmental Chemicals Bisphenol A and Di(2-Ethylhexyl) Phthalate Impact the Sexual Differentiation Process. *Endocrinology*, 156: 4672–83.

98. Nanjappa MK, Ahuja M, Dhanasekaran M, Coleman ES & TD Braden et al. (2014) Bisphenol A regulation of testicular endocrine function in male rats is affected by diet. *Toxicol Lett*, 225: 479–87.

99. Delclos KB, Camacho L, Lewis SM, Vanlandingham MM & JR Latendresse et al. (2014) Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. *Toxicological Sciences*, 139: 174–97.

100. Arambula SE, Belcher SM, Planchart A, Turner SD & HB Patisaul et al. (2016) Impact of Low Dose Oral Exposure to Bisphenol A (BPA) on the Neonatal Rat Hypothalamic and Hippocampal Transcriptome: A CLARITY-BPA Consortium Study. *Endocrinology,* 157: 3856–72.

101. Cao J, Rebuli ME, Rogers J, Todd KL & SM Leyrer et al. (2013) Prenatal bisphenol A exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala. *Toxicol Sci,* 133: 157–73.

102. Dolinoy DC (2008) The agouti mouse model: an epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome. *Nutrition reviews*, 66: S7–11.

103. Anderson OS, Nahar MS, Faulk C, Jones TR & C Liao et al. (2012) Epigenetic Responses Following Maternal Dietary Exposure to Physiologically Relevant Levels of Bisphenol A. *Environ Mol Mutagen*, 53: 334–42.

104. Jiang X, Chen HQ, Cui ZH, Yin L & WL Zhang et al. (2016) Low-dose and combined effects of oral exposure to bisphenol A and diethylstilbestrol on the male reproductive system in adult Sprague-Dawley rats. *Environ Toxicol Pharmacol*, 43: 94–102.

105. Lathi RB, Liebert CA, Brookfield KF, Taylor JA & FS Saal et al. (2014) Conjugated Bisphenol A (BPA) in maternal serum in relation to miscarriage risk. *Fertility and sterility*, 102: 123–28.

106. Sugiura-Ogasawara M, Ozaki Y, Sonta S-i, Makino T & K Suzumori et al. (2005) Exposure to bisphenol A is associated with recurrent miscarriage. *Human Reproduction*, 20: 2325–29.

107. Lassen TH, Frederiksen H, Jensen TK, Petersen JH, Joensen UN, Joensen UN., Main KM.

Skakkebaek NE, Juul A, Jorgensen N & AM Andersson (2014) Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. *Environ Health Perspect*, 122: 478–84.

108. La Rocca C, Tait S, Guerranti C, Busani L & F Ciardo et al.. (2014) Exposure to Endocrine Disrupters and Nuclear Receptor Gene Expression in Infertile and Fertile Women from Different Italian Areas. *International Journal of Environmental Research and Public Health*, 11: 10146–64.

109. La Rocca C, Tait S, Guerranti C, Busani L & F Ciardo et al.. (2015) Exposure to Endocrine Disruptors and Nuclear Receptors Gene Expression in Infertile and Fertile Men from Italian Areas with Different Environmental Features. *International Journal of Environmental Research and Public Health*, 12: 12426–45.

110. Liang H, Xu W, Chen J, Shi H & J Zhu , et al.. (2017) The Association between Exposure to Environmental Bisphenol A and Gonadotropic Hormone Levels among Men. *PLoS One*, 12: e0169217.

111. Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y & Y Taketani (2002) Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum Reprod,* 17: 2839–41.

112. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP & MD Mitchell (2010) Transfer of bisphenol A across the human placenta. *American Journal of Obstetrics and Gynecology*, 202: 393.e1-93.e7.

113. Schönfelder G, Wittfoht W, Hopp H, Talsness CE & M Paul, et al.. (2002) Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environmental Health Perspectives*, 110: A703–A07.

114. Hines EP, Mendola P, von Ehrenstein OS, Ye X & AM Calafat et al.. (2015) Concentrations of environmental phenols and parabens in milk, urine and serum of lactating North Carolina women. *Reproductive Toxicology*, 54: 120–28.

115. Migeot V, Dupuis A, Cariot A, Albouy-Llaty M & F Pierre et al.. (2013) Bisphenol A and Its Chlorinated Derivatives in Human Colostrum. *Environmental science & technology*, 47: 13791–97.

116. Fernandez MF, Arrebola JP, Taoufiki J, Navalón A & O Ballesteros, et al.. (2007) Bisphenol-A and chlorinated derivatives in adipose tissue of women. *Reproductive Toxicology*, 24: 259–64.

117. Morck TJ, Sorda G, Bechi N, Rasmussen BS & JB Nielsen, et al.. (2010) Placental transport and in vitro effects of Bisphenol A. *Reprod Toxicol*, 30: 131–7.

118. Gauderat G, Picard-Hagen N, Toutain P-L, Corbel T & C Viguié, et al.. (2016) Bisphenol A glucuronide deconjugation is a determining factor of fetal exposure to bisphenol A. *Environment International*, 86: 52–59.

119. Troisi J, Mikelson C, Richards S, Symes S & D Adair, et al.. (2014) Placental concentrations of bisphenol A and birth weight from births in the Southeastern U.S. *Placenta*, 35: 947–52.

120. Veiga-Lopez A, Kannan K, Liao C, Ye W & SE Domino et al.. (2015) Gender-Specific Effects on Gestational Length and Birth Weight by Early Pregnancy BPA Exposure. *J Clin Endocrinol Metab*, 100: E1394–403.

121. Nahar MS, Liao C, Kannan K & DC Dolinoy (2013) Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. *Journal of Biochemical and Molecular Toxicology*, 27: 116–23.

122. Nahar MS, Liao C, Kannan K, Harris C & DC Dolinoy (2015) In utero bisphenol A concentration, metabolism, and global DNA methylation across matched placenta, kidney, and liver in the human fetus. *Chemosphere*, 124: 54–60.

123. Faulk C, Kim JH, Jones TR, McEachin RC & MS Nahar, et al.. (2015) Bisphenol A-associated alterations in genome-wide DNA methylation and gene expression patterns reveal sequence-dependent and non-monotonic effects in human fetal liver. *Environmental epigenetics*, 1: dvv006.

124. Wei J, Lin Y, Li Y, Ying C & J Chen, et al.. (2011) Perinatal Exposure to Bisphenol A at Reference Dose Predisposes Offspring to Metabolic Syndrome in Adult Rats on a High-Fat Diet. *Endocrinology*, 152: 3049–61.

125. Xia W, Jiang Y, Li Y, Wan Y & J Liu, et al.. (2014) Early-Life Exposure to Bisphenol A Induces Liver Injury in Rats Involvement of Mitochondria-Mediated Apoptosis. *PLoS One*, 9: e90443.

126. Jiang Y, Xia W, Zhu Y, Li X & D Wang , et al.. (2014) Mitochondrial dysfunction in early life resulted from perinatal bisphenol A exposure contributes to hepatic steatosis in rat offspring. *Toxicology Letters*, 228: 85–92.

127. Biemann R, Navarrete Santos A, Navarrete Santos A, Riemann D & J Knelangen et al.. (2012) Endocrine disrupting chemicals affect the adipogenic differentiation of mesenchymal stem cells in distinct ontogenetic windows. *Biochemical and Biophysical Research Communications*, 417: 747–52.

128. Angle BM, Do RP, Ponzi D, Stahlhut RW & BE Drury, et al.. (2013) Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): Evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. *Reproductive toxicology* (Elmsford, NY), 42: 10.1016/j.reprotox.2013.07.017.

129. Johnson SA, Painter MS, Javurek AB, Ellersieck MR & CE Wiedmeyer , et al.. (2015) Sex-dependent effects of developmental exposure to bisphenol A and ethinyl estradiol on metabolic parameters and voluntary physical activity. *J Dev Orig Health Dis*, 6: 539–52.

130. Ding S, Fan Y, Zhao N, Yang H & X Ye, et al.. (2014) High-fat diet aggravates glucose homeostasis disorder caused by chronic exposure to bisphenol A. *Journal of Endocrinology*, 221: 167–79.

131. Ohlstein JF, Strong AL, McLachlan JA, Gimble JM & ME Burow et al.. (2014) Bisphenol A enhances adipogenic differentiation of human adipose stromal/stem cells. Journal of *Molecular Endocrinology*, 53: 345–53.

132. Boucher JG, Husain M, Rowan-Carroll A, Williams A & CL Yauk, et al.. (2014) Identification of mechanisms of action of bisphenol a-induced human preadipocyte differentiation by transcriptional profiling. *Obesity*, 22: 2333–43.

133. Wang J, Sun B, Hou M, Pan X & X Li (2013) The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11beta-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. *Int J Obes* (Lond), 37: 999–1005.

134. Bastos Sales L, Kamstra JH, Cenijn PH, van Rijt LS & T Hamers , et al.. (2013) Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicology in Vitro*, 27: 1634–43.

135. Soriano S, Alonso-Magdalena P, García-Arévalo M, Novials A & SJ Muhammed et al.. (2012) Rapid Insulinotropic Action of Low Doses of Bisphenol-A on Mouse and Human Islets of Langerhans: *Role of Estrogen Receptor β. PLoS One*, 7: e31109.

136. Fang C, Ning B, Waqar AB, Niimi M & S Li, et al.. (2014) Bisphenol A Exposure Enhances Atherosclerosis in WHHL Rabbits. *PLoS One*, 9: e110977.

137. Ding S, Zuo X, Fan Y, Li H & N Zhao N et al.. (2016) Environmentally Relevant Dose of Bisphenol A Does Not Affect Lipid Metabolism and Has No Synergetic or Antagonistic Effects on Genistein's Beneficial Roles on Lipid Metabolism. *PLoS One*, 11: e0155352.

138. Savastano S, Tarantino G, D'Esposito V, Passaretti F & S Cabaro , et al.. (2015) Bisphenol-A plasma levels are related to inflammatory markers, visceral obesity and insulin-resistance: a cross-sectional study on adult male population. *Journal of Translational Medicine*, 13: 169.

139. Yan S, Chen Y, Dong M, Song W & SM Belcher, et al.. (2011) Bisphenol A and 17β-Estradiol Promote Arrhythmia in the Female Heart via Alteration of Calcium Handling. *PLoS One*, 6: e25455.

140. Liang Q, Gao X, Chen Y, Hong K & H-S Wang (2014) Cellular Mechanism of the Nonmonotonic Dose Response of Bisphenol A in Rat Cardiac Myocytes. *Environmental Health Perspectives*, 122: 601–08.

141. Gao X, Liang Q, Chen Y & H-S Wang (2013) Molecular Mechanisms Underlying the Rapid Arrhythmogenic Action of Bisphenol A in Female Rat Hearts. *Endocrinology*, 154: 4607–17.

142. Saura M, Marquez S, Reventun P, Olea-Herrero N & MI Arenas et al.. (2014) Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS. *The FASEB Journal*, 28: 4719–28.

143. Sui Y, Park SH, Helsley RN, Sunkara M & FJ Gonzalez, et al.. (2014) Bisphenol A Increases Atherosclerosis in Pregnane X Receptor-Humanized ApoE Deficient Mice. Journal of the American Heart Association: *Cardiovascular and Cerebrovascular Disease*, 3: e000492.

144. Xiong Q, Liu X, Shen Y, Yu P & S Chen, et al.. (2015) Elevated Serum Bisphenol A Level in Patients with Dilated Cardiomyopathy. *International Journal of Environmental Research and Public Health*, 12: 5329–37.

145. Ranciere F, Lyons J, Loh V, Botton J & T Galloway et al.. (2015) Bisphenol A and the risk of cardiometabolic disorders: a systematic review with meta-analysis of the epidemiological evidence. *Environmental Health*, 14: 46.

146. Koch HM, Rossbach B, Drexler H & J Angerer (2003) Internal exposure of the general population to DEHP and other phthalates—determination of secondary and primary phthalate monoester metabolites in urine. *Environmental Research*, 93: 177–85.

147. Lorber M & AM Calafat (2012) Dose Reconstruction of Di(2-ethylhexyl) Phthalate Using a Simple Pharmacokinetic Model. *Environmental Health Perspectives*, 120: 1705–10.

148. Koch HM & AM Calafat (2009) Human body burdens of chemicals used in plastic manufacture. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364: 2063–78.

149. Anderson WAC, Castle L, Hird S, Jeffery J & MJ Scotter (2011) A twenty-volunteer study using deuterium labelling to determine the kinetics and fractional excretion of primary and

secondary urinary metabolites of di-2-ethylhexylphthalate and di-iso-nonylphthalate. *Food and Chemical Toxicology,* 49: 2022–29.

150. Martinez-Arguelles DB, Campioli E, Lienhart C, Fan J & M Culty, et al.. (2014) In utero exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate induces long-term changes in gene expression in the adult male adrenal gland. *Endocrinology*, 155: 1667–78.

151. Lee S, Martinez-Arguelles DB, Campioli E & V Papadopoulos (2016) Fetal Exposure to Low Levels of the Plasticizer DEHP Predisposes the Adult Male Adrenal Gland for Endocrine Disruption. *Endocrinology*, en20161604.

152. Wang D-C, Chen T-J, Lin M-L, Jhong Y-C & S-C Chen (2014) Exercise prevents the increased anxiety-like behavior in lactational di-(2-ethylhexyl) phthalate-exposed female rats in late adolescence by improving the regulation of hypothalamus-pituitary-adrenal axis. *Hormones and Behavior*, 66: 674–84..

153. Wang R, Xu X & Q Zhu (2016) Pubertal exposure to di-(2-ethylhexyl) phthalate influences social behavior and dopamine receptor D2 of adult female mice. *Chemosphere,* 144: 1771–79.

154. Farzanehfar V, Naderi N, Kobarfard F & M Faizi (2016) Determination of dibutyl phthalate neurobehavioral toxicity in mice. *Food and Chemical Toxicology*, 94: 221–26.

155. Ma P, Liu X, Wu J, Yan B & Y Zhang et al.. (2015) Cognitive deficits and anxiety induced by diisononyl phthalate in mice and the neuroprotective effects of melatonin. *Scientific Reports*, 5: 14676.

156. Yan B, Guo J, Liu X, Li J & X Yang, et al.. (2016) Oxidative stress mediates dibutyl phthalateinduced anxiety-like behavior in Kunming mice. *Environ Toxicol Pharmacol*, 45: 45–51.

157. Araki A, Mitsui T, Goudarzi H, Nakajima T & C Miyashita , et al.. (2017) Prenatal di(2-ethylhexyl) phthalate exposure and disruption of adrenal androgens and glucocorticoids levels in cord blood: The Hokkaido Study. *Sci Total Environ*, 581–582: 297–304.

158. Hansen JF, Brorson MM, Boas M, Frederiksen H & CH Nielsen, et al.. (2016) Phthalates Are Metabolised by Primary Thyroid Cell Cultures but Have Limited Influence on Selected Thyroid Cell Functions In Vitro. *PLoS One*, 11: e0151192.

159. Johns LE, Ferguson KK, McElrath TF, Mukherjee B & JD Meeker (2016) Associations between Repeated Measures of Maternal Urinary Phthalate Metabolites and Thyroid Hormone Parameters during Pregnancy. *Environ Health Perspect*, 124: 1808–15.

160. Kuo FC, Su SW, Wu CF, Huang MC & J Shiea et al.. (2015) Relationship of urinary phthalate metabolites with serum thyroid hormones in pregnant women and their newborns: a prospective birth cohort in Taiwan. *PLoS One*, 10: e0123884.

161. Minatoya M, Naka jima S, Sasaki S, Araki A & C Miyashita et al.. (2016) Effects of prenatal phthalate exposure on thyroid hormone levels, mental and psychomotor development of infants: The Hokkaido Study on Environment and Children's Health. *Science of The Total Environment*, 565: 1037–43.

162. de Cock M, de Boer MR, Lamoree M, Legler J & M van de Bor (2014) First Year Growth in Relation to Prenatal Exposure to Endocrine Disruptors — A Dutch Prospective Cohort Study. *International Journal of Environmental Research and Public Health,* 11: 7001–21.

163. Huang HB, Pan WH, Chang JW, Chiang HC & YL Guo et al.. (2017) Does exposure to phthalates influence thyroid function and growth hormone homeostasis? The Taiwan Environmental Survey for Toxicants (TEST) 2013. *Environ Res*, 153: 63–72.

164. Lovekamp-Swan T, Jetten AM & BJ Davis (2003) Dual activation of PPARα and PPARγ by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells. *Molecular and Cellular Endocrinology,* 201: 133–41.

165. Cho YJ, Park SB & M Han (2015) Di-(2-ethylhexyl)-phthalate induces oxidative stress in human endometrial stromal cells in vitro. *Mol Cell Endocrinol*, 407: 9–17.

166. Zhu L, Lu J, Tang X, Fu G & P Duan et al.. (2016) Di-(2-ethylhexyl) phthalate induces apoptosis of GC-2spd cells via TR4/Bcl-2 pathway. *Environmental Toxicology and Pharmacology,* 44: 18–24.

167. Wang Y, Yang Q, Liu W, Yu M & Z Zhang et al.. (2016) Di(2-Ethylhexyl) Phthalate Exposure In Utero Damages Sertoli Cell Differentiation Via Disturbance of Sex Determination Pathway in Fetal and Postnatal Mice. *Toxicol Sci*, 152: 53–61.

168. Araki A, Mitsui T, Miyashita C, Nakajima T & H Naito et al.. (2014) Association between maternal exposure to di(2-ethylhexyl) phthalate and reproductive hormone levels in fetal blood: the Hokkaido study on environment and children's health. *PLoS One*, 9: e109039.

169. Specht IO, Bonde JP, Toft G, Lindh CH & BA Jonsson et al.. (2015) Serum phthalate levels and time to pregnancy in couples from Greenland, Poland and Ukraine. *PLoS One*, 10: e0120070.

170. Thomsen AM, Riis AH, Olsen J, Jonsson BA & CH Lindh et al.. (2016) Female exposure to phthalates and time to pregnancy: a first pregnancy planner study. *Hum Reprod*.

171. Adibi JJ, Hauser R, Williams PL, Whyatt RM & AM Calafat et al.. (2009) Maternal Urinary Metabolites of Di-(2-Ethylhexyl) Phthalate in Relation to the Timing of Labor in a US Multicenter Pregnancy Cohort Study. *American Journal of Epidemiology*, 169: 1015–24.

172. Sathyanarayana S, Barrett E, Nguyen R, Redmon B & W Haaland W, et al.. (2016) First Trimester Phthalate Exposure and Infant Birth Weight in the Infant Development and Environment Study. *International Journal of Environmental Research and Public Health*, 13.

173. Li LX, Chen L, Meng XZ, Chen BH & SQ Chen et al.. (2013) Exposure levels of environmental endocrine disruptors in mother-newborn pairs in China and their placental transfer characteristics. *PLoS One*, 8: e62526.

174. Huang P-C, Kuo P-L, Chou Y-Y, Lin S-J & C-C Lee (2009) Association between prenatal exposure to phthalates and the health of newborns. *Environment International*, 35: 14–20.

175. Adibi JJ, Whyatt RM, Hauser R, Bhat HK & BJ Davis, et al.. (2010) Transcriptional Biomarkers of Steroidogenesis and Trophoblast Differentiation in the Placenta in Relation to Prenatal Phthalate Exposure. *Environmental Health Perspectives*, 118: 291–96.

176. LaRocca J, Binder AM, McElrath TF & KB Michels (2015) First-Trimester Urine Concentrations of Phthalate Metabolites and Phenols and Placenta miRNA Expression in a Cohort of U.S. Women. *Environ Health Perspect* 2015 Jun 19, E-publ.

177. Magenta A, Dellambra E, Ciarapica R & MC Capogrossi (2016) Oxidative stress, microRNAs and cytosolic calcium homeostasis. *Cell Calcium*, 60: 207–17.

178. Vo TT, Jung EM, Dang VH, Jung K & J Baek et al.. (2009) Differential effects of flutamide and di-(2-ethylhexyl) phthalate on male reproductive organs in a rat model. *J Reprod Dev*, 55: 400–11.

179. Jones S, Boisvert A, Duong TB, Francois S & P Thrane et al.. (2014) Disruption of rat testis development following combined in utero exposure to the phytoestrogen genistein and antiandrogenic plasticizer di-(2-ethylhexyl) phthalate. *Biol Reprod*, 91: 64.

180. Jones S, Boisvert A, Francois S, Zhang L & M Culty (2015) In utero exposure to di-(2-ethylhexyl) phthalate induces testicular effects in neonatal rats that are antagonized by genistein cotreatment. *Biol Reprod*, 93: 92.

181. Quinnies KM, Harris EP, Snyder RW, Sumner SS & EF Rissman (2017) Direct and transgenerational effects of low doses of perinatal di-(2-ethylhexyl) phthalate (DEHP) on social behaviors in mice. *PLoS One*, 12: e0171977.

182. Hao C, Cheng X, Xia H & X Ma (2012) The endocrine disruptor mono-(2-ethylhexyl) phthalate promotes adipocyte differentiation and induces obesity in mice. *Biosci Rep*, 32: 619–29.

183. Schmidt JS, Schaedlich K, Fiandanese N, Pocar P & B Fischer (2012) Effects of di(2-ethylhexyl) phthalate (DEHP) on female fertility and adipogenesis in C3H/N mice. *Environ Health Perspect*, 120: 1123–9.

184. Lin Y, Wei J, Li Y, Chen J & Z Zhou et al.. (2011) Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat. *Am J Physiol Endocrinol Metab*, 301: E527–38.

185. Marie C, Vendittelli F & M-P Sauvant-Rochat (2015) Obstetrical outcomes and biomarkers to assess exposure to phthalates: A review. *Environment International*, 83: 116–36.

186. Bornehag CG, Carlstedt F, Jonsson BA, Lindh CH & TK Jensen et al.. (2015) Prenatal phthalate exposures and anogenital distance in Swedish boys. *Environ Health Perspect*, 123: 101–7.

187. Swan SH (2008) Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. *Environmental Research*, 108: 177–84.

188. Swan SH, Sathyanarayana S, Barrett ES, Janssen S & F Liu et al.. (2015) First trimester phthalate exposure and anogenital distance in newborns. *Hum Reprod*, 30: 963–72.

189. Barrett ES, Parlett LE, Sathyanarayana S, Redmon JB & RH Nguyen et al.. (2016) Prenatal Stress as a Modifier of Associations between Phthalate Exposure and Reproductive Development: results from a Multicentre Pregnancy Cohort Study. *Paediatr Perinat Epidemiol*, 30: 105–14.

190. Barrett ES & SH Swan (2015) Stress and Androgen Activity During Fetal Development. *Endocrinology*, 156: 3435–41.

191. Doyle TJ, Bowman JL, Windell VL, McLean DJ & KH Kim (2013) Transgenerational Effects of Di-(2-ethylhexyl) Phthalate on Testicular Germ Cell Associations and Spermatogonial Stem Cells in Mice. *Biology of Reproduction*, 88: 112, 1–15.

192. Quinnies KM, Doyle TJ, Kim KH & EF Rissman (2015) Transgenerational Effects of Di-(2-Ethylhexyl) Phthalate (DEHP) on Stress Hormones and Behavior. *Endocrinology*, 156: 3077–83.

193. Lapinskas PJ, Brown S, Leesnitzer LM, Blanchard S & C Swanson et al.. (2005) Role of PPARα in mediating the effects of phthalates and metabolites in the liver. *Toxicology*, 207: 149–63.

194. Bility MT, Thompson JT, McKee RH, David RM & JH Butala et al.. (2004) Activation of Mouse and Human Peroxisome Proliferator-Activated Receptors (PPARs) by Phthalate Monoesters. *Toxicological Sciences*, 82: 170–82.

195. Hurst CH & DJ Waxman (2003) Activation of PPARα and PPARγ by Environmental Phthalate Monoesters. *Toxicological Sciences*, 74: 297–308.

196. Feige JN, Gelman L, Rossi D, Zoete V & R Métivier et al.. (2007) The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor gamma modulator that promotes adipogenesis. *J Biol Chem*, 282: 19152–66.

197. Ito Y, Nakamura T, Yanagiba Y, Ramdhan DH & N Yamagishi et al.. (2012) Plasticizers May Activate Human Hepatic Peroxisome Proliferator-Activated Receptor α Less Than That of a Mouse but May Activate Constitutive Androstane Receptor in Liver. *PPAR Research*, 2012: 201284.

198. Hurst CH & DJ Waxman (2004) Environmental phthalate monoesters activate pregnane X receptor-mediated transcription. *Toxicology and Applied Pharmacology*, 199: 266–74

199. Zhang J, Ali HI, Bedi YS & M Choudhury (2015) The plasticizer BBP selectively inhibits epigenetic regulator sirtuins. *Toxicology*, 338: 130–41.

200. Trasande L, Spanier AJ, Sathyanarayana S, Attina TM & J Blustein (2013) Urinary Phthalates and Increased Insulin Resistance in Adolescents. *Pediatrics*, 132: e646–e55.

201. Attina TM & L Trasande (2015) Association of Exposure to Di-2-Ethylhexylphthalate Replacements With Increased Insulin Resistance in Adolescents From NHANES 2009-2012. J *Clin Endocrinol Metab,* 100: 2640–50.

202. James-Todd TM, Meeker JD, Huang T, Hauser R & KK Ferguson et al.. (2016) Pregnancy urinary phthalate metabolite concentrations and gestational diabetes risk factors. *Environ Int*, 96: 118–26.

203. Bai PY, Wittert GA, Taylor AW, Martin SA & RW Milne et al.. (2015) The association of socio-demographic status, lifestyle factors and dietary patterns with total urinary phthalates in Australian men. *PLoS One,* 10: e0122140.

204. Dirinck E, Dirtu AC, Geens T, Covaci A & L Van Gaal et al.. (2015) Urinary phthalate metabolites are associated with insulin resistance in obese subjects. *Environmental Research*, 137: 419–23.

205. Trasande L & TM Attina (2015) Association of Exposure to Di-2-Ethylhexylphthalate Replacements With Increased Blood Pressure in Children and Adolescents. *Hypertension,* 66: 301–08.

206. Olsén L, Lind L & PM Lind (2012) Associations between circulating levels of bisphenol A and phthalate metabolites and coronary risk in the elderly. *Ecotoxicology and Environmental Safety,* 80: 179–83.

207. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). Scientific Opinion on the re-evaluation of butylated hydroxyanisole–BHA (E 320) as a food additive. *EFSA Journal* (2011), 9: 2392.

208. EFSA Panel on Food Additives Nutrient Sources added to, Food (2012). Scientific Opinion on the re-evaluation of butylated hydroxytoluene BHT (E 321) as a food additive. *EFSA Journal*, 10: 2588.

209. Verhagen H, Thijssen HH, ten Hoor F & JC Kleinjans (1989) Disposition of single oral doses of butylated hydroxyanisole in man and rat. *Food Chem Toxicol*, 27: 151–8.

210. Verhagen H, Beckers HH, Comuth PA, Maas LM & F ten Hoor et al.. (1989) Disposition of single oral doses of butylated hydroxytoluene in man and rat. *Food Chem Toxicol*, 27: 765–72.

211. Wilson VS, Bobseine K, Lambright CR & LE, Jr Gray (2002) A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicol Sci,* 66: 69–81.

212. Klopcic I & M Sollner Dolenc (2017) Endocrine activity of AVB, 2MR, BHA and their mixtures. *Toxicol Sci.*

213. Li L, Wu Y, Wu X, Wang H & Y Bai et al.. (2016) Butylated Hydroxyanisole Potently Inhibits Rat and Human 11beta-Hydroxysteroid Dehydrogenase Type 2. *Pharmacology*, 97: 10–7.

214. Vitku J, Starka L, Bicikova M, Hill M & J Heracek et al.. (2016) Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. *The Journal of Steroid Biochemistry and Molecular Biology*, 155, Part B: 207–16.

215. Jeong S-H, Kim B-Y, Kang H-G, Ku H-O & J-H Cho (2005) Effects of butylated hydroxyanisole on the development and functions of reproductive system in rats. *Toxicology,* 208: 49–62.

216. ter Veld MGR, Schouten B, Louisse J, van Es DS & PT van der Saag et al.. (2006) Estrogenic Potency of Food-Packaging-Associated Plasticizers and Antioxidants As Detected in ERα and ERβ Reporter Gene Cell Lines. *Journal of Agricultural and Food Chemistry*, 54: 4407–16.

217. Zhu BT, Lech J, Rosen RT & AH Conney AH (1997) Effect of Dietary 2(3)-tert-Butyl-4-hydroxyanisole on the Metabolism and Action of Estradiol and Estrone in Female CD-1 Mice. *Cancer Research*, 57: 2419–27.

218. Schrader TJ & GM Cooke (2000) Examination of Selected Food Additives and Organochlorine Food Contaminants for Androgenic Activity in Vitro. *Toxicological Sciences*, 53: 278–88.

219. Pop A, Drugan T, Gutleb AC, Lupu D & J Cherfan et al.. (2016) Individual and combined in vitro (anti)androgenic effects of certain food additives and cosmetic preservatives. *Toxicology in Vitro*, 32: 269–77.

220. Kang HG, Jeong SH, Cho JH, Kim DG & JM Park et al.. (2005) Evaluation of estrogenic and androgenic activity of butylated hydroxyanisole in immature female and castrated rats. *Toxicology*, 213: 147–56.

221. Lou P-H, Hansen Birgit S, Olsen Preben H, Tullin S & P Murphy Michael et al.. (2007) Mitochondrial uncouplers with an extraordinary dynamic range. *The Biochemical Journal*, 407: 129–40.

222. Sun J, Hu H, Ren X & JW Simpkins (2016) Tert-butylhydroquinone compromises survival in murine experimental stroke. *Neurotoxicology and Teratology*, 54: 15–21.

223. Saito M, Sakagami H & S Fujisawa (2003) Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). *Anticancer Res*, 23: 4693–701.

224. Festjens N, Kalai M, Smet J, Meeus A & R Van Coster et al.. (2005) Butylated hydroxyanisole is more than a reactive oxygen species scavenger. *Cell Death Differ*, 13: 166–69.

225. Eskandani M, Hamishehkar H & J Ezzati Nazhad Dolatabadi (2014) Cytotoxicity and DNA damage properties of tert-butylhydroquinone (TBHQ) food additive. *Food Chemistry*, 153: 315–20.

226. Singh B, Mense SM, Remotti F, Liu X & HK Bhat (2009) Antioxidant Butylated Hydroxyanisole Inhibits Estrogen-Induced Breast Carcinogenesis in Female ACI Rats. *Journal of Biochemical and Molecular Toxicology*, 23: 202–11.

227. Freese E, Sheu CW & E Galliers (1973) Function of lipophilic acids as antimicrobial food additives. *Nature*, 241: 321–5.

228. Flasinski M, Gawrys M, Broniatowski M & P Wydro (2016) Studies on the interactions between parabens and lipid membrane components in monolayers at the air/aqueous solution interface. *Biochim Biophys Acta*, 1858: 836–44.

229. Nes IF & T Eklund (1983) The effect of parabens on DNA, RNA and protein synthesis in Escherichia coli and Bacillus subtilis. *J Appl Bacteriol*, 54: 237–42.

230. Ma Y & RE Marquis (1996) Irreversible paraben inhibition of glycolysis by Streptococcus mutans GS-5. *Letters in Applied Microbiology*, 23: 329–33.

231. Abbas S, Greige-Gerges H, Karam N, Piet MH & P Netter et al. (2010) Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. *Drug Metab Pharmacokinet*, 25: 568–77.

232. Dewalque L, Pirard C, Vandepaer S & C Charlier (2015) Temporal variability of urinary concentrations of phthalate metabolites, parabens and benzophenone-3 in a Belgian adult population. *Environ Res*, 142: 414–23.

233. Guidry VT, Longnecker MP, Aase H, Eggesbø M & P Zeiner et al.. (2015) Measurement of Total and Free Urinary Phenol and Paraben Concentrations over the Course of Pregnancy: Assessing Reliability and Contamination of Specimens in the Norwegian Mother and Child Cohort Study. *Environmental Health Perspectives*, 123: 705–11.

234. Philippat C, Wolff MS, Calafat AM, Ye X & R Bausell et al.. (2013) Prenatal Exposure to Environmental Phenols: Concentrations in Amniotic Fluid and Variability in Urinary Concentrations during Pregnancy. *Environmental Health Perspectives*, 121: 1225–31.

235. Klopčič I, Kolšek K& MS Dolenc (2015) Glucocorticoid-like activity of propylparaben, butylparaben, diethylhexyl phthalate and tetramethrin mixtures studied in the MDA-kb2 cell line. *Toxicology Letters,* 232: 376–83.

236. Kolšek K, Gobec M, Mlinarič Raščan I & M Sollner Dolenc (2015) Screening of bisphenol A, triclosan and paraben analogues as modulators of the glucocorticoid and androgen receptor activities. *Toxicology in Vitro*, 29: 8–15.

237. Koeppe ES, Ferguson KK, Colacino JA & JD Meeker (2013) Relationship between Urinary Triclosan and Paraben Concentrations and Serum Thyroid Measures in NHANES 2007–2008. *The Science of the total environment*, 445–446: 299–305.

238. Smarr MM, Sundaram R, Honda M, Kannan K & GM Buck Louis (2016) Urinary Concentrations of Parabens and Other Antimicrobial Chemicals and Their Association with Couples' Fecundity. *Environ Health Perspect*.

239. Minguez-Alarcon L, Chiu YH, Messerlian C, Williams PL & ME Sabatini et al.. (2016) Urinary paraben concentrations and in vitro fertilization outcomes among women from a fertility clinic. *Fertil Steril*, 105: 714–21.

240. Tefre de Renzy-Martin K, Frederiksen H, Christensen JS, Boye Kyhl H & AM Andersson et al.. (2014) Current exposure of 200 pregnant Danish women to phthalates, parabens and phenols. *Reproduction,* 147: 443–53.

241. Smith KW, Braun JM, Williams PL, Ehrlich S & KF Correia et al.. (2012) Predictors and Variability of Urinary Paraben Concentrations in Men and Women, Including before and during Pregnancy. *Environmental Health Perspectives*, 120: 1538–43.

242. Braun JM, Kalkbrenner AE, Calafat AM, Bernert JT & X Ye et al.. (2011) Variability and Predictors of Urinary Bisphenol A Concentrations during Pregnancy. *Environmental Health Perspectives*, 119: 131–37.

243. Pycke BFG, Geer LA, Dalloul M, Abulafia O & RU Halden (2015) Maternal and fetal exposure to parabens in a multiethnic urban U.S. population. *Environment International*, 84: 193–200.

244. Towers CV, Terry PD, Lewis D, Howard B & W Chambers et al.. (2015) Transplacental passage of antimicrobial paraben preservatives. *J Expo Sci Environ Epidemiol*, 25: 604–7.

245. Valle-Sistac J, Molins-Delgado D, Díaz M, Ibáñez L& D Barceló et al.. (2016) Determination of parabens and benzophenone-type UV filters in human placenta. First description of the existence of benzyl paraben and benzophenone-4. *Environment International*, 88: 243–49.

246. Frederiksen H, Jorgensen N & AM Andersson AM (2011) Parabens in urine, serum and seminal plasma from healthy Danish men determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J Expo Sci Environ Epidemiol*, 21: 262–71.

247. Pereira-Fernandes A, Demaegdt H, Vandermeiren K, Hectors TLM & PG Jorens et al.. (2013) Evaluation of a Screening System for Obesogenic Compounds: Screening of Endocrine Disrupting Compounds and Evaluation of the PPAR Dependency of the Effect. *PLoS One*, 8: e77481.

248. Hu P, Chen X, Whitener RJ, Boder ET & JO Jones et al.. (2013) Effects of Parabens on Adipocyte Differentiation. *Toxicological Sciences*, 131: 56–70.

249. Szelag S, Zablocka A, Trzeciak K, Drozd A & I Baranowska-Bosiacka et al.. (2016) Propylparaben-induced disruption of energy metabolism in human HepG2 cell line leads to increased synthesis of superoxide anions and apoptosis. *Toxicol In Vitro*, 31: 30–4.

250. Hu P, Kennedy RC, Chen X, Zhang J & CL Shen et al.. (2016) Differential effects on adiposity and serum marker of bone formation by post-weaning exposure to methylparaben and butylparaben. *Environ Sci Pollut Res Int*, 23: 21957–68.

251. Hamilton JT, Zhou Y & AW Gelb (1990) Paraben preservatives but not succinylcholine are cerebral vasodilators in vitro. *Anesthesiology*, 73: 1252–7.

252. Ji X, Xu Z, Criswell HE & PG Boysen (2004) Propyl paraben inhibits voltage-dependent sodium channels and protects cardiomyocytes from ischemia-reperfusion injury. *Life Sciences,* 74: 3043–52.

253. Wróbel AM & EŁ Gregoraszczuk (2014) Actions of methyl-, propyl- and butylparaben on estrogen receptor-α and -β and the progesterone receptor in MCF-7 cancer cells and non-cancerous MCF-10A cells. *Toxicology Letters*, 230: 375–81.

254. Wróbel AM & EŁ Gregoraszczuk (2015) Action of methyl-, propyl- and butylparaben on GPR30 gene and protein expression, cAMP levels and activation of ERK1/2 and PI3K/Akt signaling pathways in MCF-7 breast cancer cells and MCF-10A non-transformed breast epithelial cells. *Toxicology Letters*, 238: 110–16.

255. Darbre PD & PW Harvey (2014) Parabens can enable hallmarks and characteristics of cancer in human breast epithelial cells: a review of the literature with reference to new exposure data and regulatory status. *Journal of Applied Toxicology*, 34: 925–38.

256. Sajid M, Basheer C, Narasimhan K, Choolani M & HK Lee (2015) Application of microwave-assisted micro-solid-phase extraction for determination of parabens in human ovarian cancer tissues. *J Chromatogr B Analyt Technol Biomed Life Sci*, 1000: 192–8.

257. Morton V & T Staub (2008) A Short History of Fungicides. *APSnet Features online, doi: 10.1094/APSnetFeature-2008–0308.*

258. Yang C, Hamel C, Vujanovic V & Y Gan (2011) Fungicide: Modes of Action and Possible Impact on Nontarget Microorganisms. *ISRN Ecology*, 2011: 8.

259. Garbrecht MR, Krozowski ZS, Snyder JM & TJ Schmidt (2006) Reduction of glucocorticoid receptor ligand binding by the 11-beta hydroxysteroid dehydrogenase type 2 inhibitor, Thiram. *Steroids*, 71: 895–901.

260. Atanasov AG, Tam S, Rocken JM, Baker ME & A Odermatt (2003) Inhibition of 11 beta-hydroxysteroid dehydrogenase type 2 by dithiocarbamates. *Biochem Biophys Res Commun*, 308: 257–62.

261. Katz A, Heiblum R, Meidan R & B Robinzon (2008) Corticosterone oxidative neutralization by 11-β hydroxysteroid dehydrogenases in kidney and colon of the domestic fowl. *General and Comparative Endocrinology*, 155: 814–20.

262. Katz A, Mirzatoni A, Zhen Y & BA Schlinger (2008) Sex differences in cell proliferation and glucocorticoid responsiveness in the zebra finch brain. *Eur J Neurosci*, 28: 99–106.

263. Hurley PM (1998) Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. *Environ Health Perspect*, 106: 437–45.

264. Goldner WS, Sandler DP, Yu F, Hoppin JA & F Kamel et al.. (2010) Pesticide use and thyroid disease among women in the Agricultural Health Study. *Am J Epidemiol*, 171: 455–64.

265. Kjeldsen LS, Ghisari M & EC Bonefeld-Jorgensen (2013) Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. *Toxicol Appl Pharmacol,* 272: 453–64.

266. Paro R, Tiboni GM, Buccione R, Rossi G & V Cellini et al.. (2012) The fungicide mancozeb induces toxic effects on mammalian granulosa cells. *Toxicology and Applied Pharmacology*, 260: 155–61.

267. Iorio R, Castellucci A, Rossi G, Cinque B & MG Cifone et al.. (2015) Mancozeb affects mitochondrial activity, redox status and ATP production in mouse granulosa cells. *Toxicology in Vitro,* 30: 438–45.

268. Nordby KC, Andersen A, Irgens LM & P Kristensen (2005) Indicators of mancozeb exposure in relation to thyroid cancer and neural tube defects in farmers' families. *Scand J Work Environ Health,* 31: 89–96.

269. Hass U, Boberg J, Christiansen S, Jacobsen PR & AM Vinggaard et al.. (2012) Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reproductive Toxicology*, 34: 261–74.

270. Jacobsen PR, Axelstad M, Boberg J, Isling LK & S Christiansen et al.. (2012) Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reproductive Toxicology*, 34: 237–50.

271. Jacobsen PR, Christiansen S, Boberg J, Nellemann C & U Hass (2010) Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats. *Int J Androl*, 33: 434–42.

272. Overgaard A, Holst K, Mandrup KR, Boberg J & S Christiansen et al.. (2013) The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. *NeuroToxicology*, 37: 154–62.

273. Axelstad M, Boberg J, Nellemann C, Kiersgaard M & PR Jacobsen et al.. (2011) Exposure to the Widely Used Fungicide Mancozeb Causes Thyroid Hormone Disruption in Rat Dams but No Behavioral Effects in the Offspring. *Toxicological Sciences*, 120: 439–46.

274. Bhaskar R & B Mohanty (2014) Pesticides in mixture disrupt metabolic regulation: In silico and in vivo analysis of cumulative toxicity of mancozeb and imidacloprid on body weight of mice. *General and Comparative Endocrinology*, 205: 226–34.

275. Pirozzi AV, Stellavato A, La Gatta A, Lamberti M & C Schiraldi (2016) Mancozeb, a fungicide routinely used in agriculture, worsens nonalcoholic fatty liver disease in the human HepG2 cell model. *Toxicol Lett,* 249: 1–4.

276. Kongsbak K, Vinggaard AM, Hadrup N & K Audouze K (2014) A computational approach to mechanistic and predictive toxicology of pesticides. *ALTEX*, 31: 11–22.

277. Corsini E, Viviani B, Birindelli S, Gilardi F & A Torri et al.. (2006) Molecular mechanisms underlying mancozeb-induced inhibition of TNF-alpha production. *Toxicol Appl Pharmacol*, 212: 89–98.

278. Igarashi A, Ohtsu S, Muroi M & K Tanamoto (2006) Effects of possible endocrine disrupting chemicals on bacterial component-induced activation of NF-kappa *B. Biol Pharm Bull*, 29: 2120–2.

279. Bjorge C, Brunborg G, Wiger R, Holme JA, Scholz T, et al.. (1996) A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. *Reprod Toxicol*, 10: 509–19.

280. Belpoggi F, Soffritti M, Guarino M, Lambertini L & D Cevolani et al.. (2002) Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. *Ann N Y Acad Sci*, 982: 123–36.

281. Sengupta P (2013) The Laboratory Rat: Relating Its Age With Human's. International *Journal of Preventive Medicine*, 4: 624–30.

282. Barnes S, Prasain J, D'Alessandro T, Arabshahi A & N Botting et al.. (2011) The metabolism and analysis of isoflavones and other dietary polyphenols in foods and biological systems. *Food Funct*, 2: 235–44.

283. Matthies A, Loh G, Blaut M & A Braune (2012) Daidzein and genistein are converted to equol and 5-hydroxy-equol by human intestinal Slackia isoflavoniconvertens in gnotobiotic rats. *J Nutr,* 142: 40–6.

284. Franke AA, Lai JF & BM Halm (2014) Absorption, distribution, metabolism, and excretion of isoflavonoids after soy intake. *Arch Biochem Biophys*, 559: 24–8.

285. Yang Z, Kulkarni K, Zhu W & M Hu (2012) Bioavailability and Pharmacokinetics of Genistein: Mechanistic Studies on its ADME. *Anti-cancer agents in medicinal chemistry*, 12: 1264–80.

286. Andrade JE, Twaddle NC, Helferich WG & DR Doerge (2010) Absolute bioavailability of isoflavones from soy protein isolate-containing food in female Balb/c mice. *Journal of Agricultural and Food Chemistry*, 58: 4529–36.

287. Zhang Y, Hendrich S & PA Murphy PA (2003) Glucuronides are the main isoflavone metabolites in women. *J Nutr*, 133: 399–404.

288. Setchell KD, Clerici C, Lephart ED, Cole SJ & C Heenan et al.. (2005) S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr*, 81: 1072–9.

289. Rafii F (2015) The Role of Colonic Bacteria in the Metabolism of the Natural Isoflavone Daidzin to Equol. *Metabolites,* 5: 56–73.

290. Kaminska B, Ciereszko R, Kiezun M & L Dusza L (2013) In vitro effects of genistein and daidzein on the activity of adrenocortical steroidogenic enzymes in mature female pigs. *J Physiol Pharmacol*, 64: 103–8.

291. Ohlsson A, Ulleras E, Cedergreen N & A Oskarsson (2010) Mixture effects of dietary flavonoids on steroid hormone synthesis in the human adrenocortical H295R cell line. *Food Chem Toxicol*, 48: 3194–200.

292. Whirledge S, Senbanjo LT & JA Cidlowski (2015) Genistein disrupts glucocorticoid receptor signaling in human uterine endometrial Ishikawa cells. *Environ Health Perspect*, 123: 80–7.

293. Ohno S, Nakajima Y, Inoue K, Nakazawa H & S Nakajin S (2003) Genistein administration decreases serum corticosterone and testosterone levels in rats. *Life Sciences*, 74: 733–42.

294. Marini H, Polito F, Adamo EB, Bitto A & F Squadrito et al.. (2012) Update on genistein and thyroid: an overall message of safety. *Frontiers in Endocrinology*, 3: 94.

295. Hampl R, Ostatnikova D, Celec P, Putz Z & O Lapcik et al.. (2008) Short-term effect of soy consumption on thyroid hormone levels and correlation with phytoestrogen level in healthy subjects. *Endocr Regul*, 42: 53–61.

296. Bliedtner A, Zierau O, Albrecht S, Liebhaber S & G Vollmer (2010) Effects of genistein and estrogen receptor subtype-specific agonists in ArKO mice following different administration routes. *Molecular and Cellular Endocrinology*, 314: 41–52.

297. Setchell KDR & C Clerici (2010) Equol: Pharmacokinetics and Biological Actions. The *Journal of Nutrition*, 140: 1363S–68S.

298. Doerge DR, Churchwell MI, Chang HC, Newbold RR & KB Delclos (2001) Placental transfer of the soy isoflavone genistein following dietary and gavage administration to Sprague Dawley rats. *Reproductive Toxicology*, 15: 105–10.

299. Soucy NV, Parkinson HD, Sochaski MA & SJ Borghoff (2006) Kinetics of Genistein and Its Conjugated Metabolites in Pregnant Sprague-Dawley Rats Following Single and Repeated Genistein Administration. *Toxicological Sciences,* 90: 230–40.

300. Balakrishnan B, Thorstensen EB, Ponnampalam AP & MD Mitchell (2010) Transplacental Transfer and Biotransformation of Genistein in Human Placenta. *Placenta,* 31: 506–11.

301. Dinsdale EC & WE Ward (2010) Early Exposure to Soy Isoflavones and Effects on Reproductive Health: A Review of Human and Animal Studies. *Nutrients,* 2: 1156–87.

302. Fritz WA, Coward L, Wang J & CA Lamartiniere (1998) Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis*, 19: 2151–8.

303. Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J & R Mentor-Marcel et al.. (2002) Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. *J Nutr,* 132: 552S–58S.

304. Su Y, Shankar K & RC Simmen (2009) Early soy exposure via maternal diet regulates rat mammary epithelial differentiation by paracrine signaling from stromal adipocytes. *J Nutr*, 139: 945–51.

305. Rahal OM & RCM Simmen (2010) PTEN and p53 cross-regulation induced by soy isoflavone genistein promotes mammary epithelial cell cycle arrest and lobuloalveolar differentiation. *Carcinogenesis*, 31: 1491–500.

306. Dolinoy DC, Weidman JR, Waterland RA & RL Jirtle (2006) Maternal Genistein Alters Coat Color and Protects A(vy) Mouse Offspring from Obesity by Modifying the Fetal Epigenome. *Environmental Health Perspectives*, 114: 567–72.

307. Tagawa N, Kubota S, Kobayashi Y & I Kato (2015) Genistein inhibits glucocorticoid amplification in adipose tissue by suppression of 11beta-hydroxysteroid dehydrogenase type 1. *Steroids,* 93: 77–86.

308. Gilbert ER & D Liu (2013) Anti-diabetic functions of soy isoflavone genistein: mechanisms underlying effects on pancreatic β-cell function. *Food & function*, 4: 200–12.

309. Morimoto Y, Steinbrecher A, Kolonel LN & G Maskarinec (2011) Soy consumption is not protective against diabetes in Hawaii: The Multiethnic Cohort. *European journal of clinical nutrition,* 65: 279–82.

310. Taku K, Umegaki K, Sato Y, Taki Y & K Endoh et al.. (2007) Soy isoflavones lower serum total and LDL cholesterol in humans: a meta-analysis of 11 randomized controlled trials. *The American Journal of Clinical Nutrition,* 85: 1148–56.

311. Pan A, Franco OH, Ye J, Demark-Wahnefried W & X Ye et al.. (2008) Soy Protein Intake Has Sex-Specific Effects on the Risk of Metabolic Syndrome in Middle-Aged and Elderly Chinese. *The Journal of Nutrition*, 138: 2413–21.

312. Nagata C, Wada K, Tamura T, Konishi K & Y Goto et al.. (2017) Dietary soy and natto intake and cardiovascular disease mortality in Japanese adults: the Takayama study. The American *Journal of Clinical Nutrition*, 105: 426–31.

313. Weng Y, Yao J, Sparks S & KY Wang (2017) Nattokinase: An Oral Antithrombotic Agent for the Prevention of Cardiovascular Disease. *International Journal of Molecular Sciences*, 18: 523.

314. Yu D, Zhang X, Xiang Y-B, Yang G & H Li et al.. (2014) Association of soy food intake with risk and biomarkers of coronary heart disease in Chinese men. *International journal of cardiology,* 172: e285-e87.

315. Ramdath DD, Padhi EMT, Sarfaraz S, Renwick S & AM Duncan (2017) Beyond the Cholesterol-Lowering Effect of Soy Protein: A Review of the Effects of Dietary Soy and Its Constituents on Risk Factors for Cardiovascular Disease. *Nutrients*, 9: 324.

316. Chen M, Rao Y, Zheng Y, Wei S & Y Li et al.. (2014) Association between Soy Isoflavone Intake and Breast Cancer Risk for Pre- and Post-Menopausal Women: A Meta-Analysis of Epidemiological Studies. *PLoS One*, 9: e89288.

317. Fritz H, Seely D, Flower G, Skidmore B & R Fernandes et al.. (2013) Soy, Red Clover, and Isoflavones and Breast Cancer: A Systematic Review. *PLoS One*, 8: e81968.

318. Travis RC, Allen NE, Appleby PN, Price A & R Kaaks et al.. (2012) Prediagnostic concentrations of plasma genistein and prostate cancer risk in 1,605 men with prostate cancer and 1,697 matched control participants in EPIC. *Cancer Causes & Control*, 23: 1163–71.

319. Hamilton-Reeves JM, Banerjee S, Banerjee SK, Holzbeierlein JM & JB Thrasher et al.. (2013) Short-Term Soy Isoflavone Intervention in Patients with Localized Prostate Cancer: A Randomized, Double-Blind, Placebo-Controlled Trial. *PLoS One*, 8: e68331.

320. Nakamura H, Wang Y, Kurita T, Adomat H & GR Cunha et al.. (2011) Genistein Increases Epidermal Growth Factor Receptor Signaling and Promotes Tumor Progression in Advanced Human Prostate Cancer. *PLoS One*, 6: e20034.

Appendix

Table of clinical reference ranges for hormones of the HPA-, the HPT- and the HPG-axes, their receptors and tissue expression. List of Clinical Chemistry reference values (Sweden) for hormones belonging to the HPA-, HPT- and HPG-axes. Note that reference values can vary slightly between clinical labs due to that the methods used or different manufacturers of analytical kits.

Appendix table see separate Excel sheet or pdf-file.

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New EU criteria for endocrine disrupters

Endocrine disruptors (EDs) are substances that adversely affect hormone function. The effects by EDs are thought to depend on both dose and timing of exposure, especially during foetal development. The upcoming EU regulation on identification of EDs, application biocides and pesticides, will have an impact on all actors of the food chain. The consequences of a ban of EDs in foods were discussed in a Nordic workshop (Uppsala, 29-30 Nov 2016) where risk assessors and managers from Nordic food authorities, industry, trade associations, consumer organizations and researchers were represented. It was recognized by all participants that a ban was particularly challenging for the production chain since there are few viable alternatives. A harmonized EU legislation based on scientific risk assessment was preferred compared to national specific legislation as it treats all the actors equally.

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