TOX/2019/51

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Scoping paper on endocrine disruptors and risk assessment

Introduction

1. As discussed at the March meeting and during horizon scanning at the April meeting, the COT wished to consider the approaches that should be taken to the risk assessment of endocrine disruptors. This would include whether thresholds exist. The Committee agreed that all endocrine systems should be considered. The Committee was of the view that a subgroup should be formed to consider this. However, the first stage should be to produce a scoping paper.

2. This scoping paper briefly describes the different endocrine systems that have been listed in documents considering endocrine disruptors, and for each endocrine system gives an example of disruption by chemical(s). It briefly touches on the criteria, tests and guidance used to identify endocrine disruptors. It then summarises the considerations in recent reports and opinions relevant to the risk assessment of endocrine disruptors, i.e. whether thresholds exist for endocrine disruptors, low-dose effects, non-monotonic dose-response relationships and critical windows of susceptibility.

3. The COT is asked to consider how it would wish to take this issue forward further. Should a COT subgroup be formed, and if so what questions should the subgroup consider and advise on and what expertise should be included in the subgroup?

Endocrine systems

4. OECD (2012) describes and discusses the following endocrine systems:

- hypothalamus-pituitary-adrenocortical (HPA) axis
- hypothalamus-pituitary-gonad (HPG axis)
- somatotrophic axis
- retinoid signalling pathway
- hypothalamus-pituitary-thyroid (HPT) axis
- vitamin D signalling pathway
- peroxisome proliferator-activated receptor signalling pathway

5. Brief overviews of these endocrine systems, based on the text in OECD (2012), are provided below. A list of abbreviations and acronyms can be found on pages 32-34. In addition, EFSA (2013) referred to renal signalling and pancreatic signalling as endocrine modalities that may be affected by endocrine disruptors. Therefore, summaries are also provided on:

- the renin-angiotensin system (RAS)
- endocrine pancreas signalling

HPA axis

6. The HPA axis is primarily a regulator of metabolism. It also has stimulatory and inhibitory effects on the immune system and growth, and it has stimulatory and inhibitory effects on reproduction. Various aspects of early development and the timing of events such as puberty and reproductive organ development are regulated by glucocorticoids produced by the adrenal cortex.

7. The HPA axis responds to various stressors, allowing the body to respond metabolically to counteract the short- and long-term effects of the stressors. The HPA axis also affects cardiovascular functions, ionic regulation and memory. Due to the role of the HPA axis in metabolism, virtually all tissues in the body are affected by HPA axis hormones.

8. The main hormones of the HPA axis are corticotropin-releasing hormone (CRH), which is produced primarily in the parvocellular neurons of the parvocellular nucleus (PVN) of the hypothalamus, arginine vasopressin (AVP), which is co-localised with CRH in some PVN neurons, adrenocorticotropic hormone (ACTH), which is produced by corticotropic cells in the pituitary, and the glucocorticoids, which are steroids produced in response to ACTH in the zona fasciculata (ZF) in the adrenal cortex. In primates the principle glucocorticoid is cortisol, whereas is most other vertebrate it is corticosterone.

9. In addition, the sex steroids dehydroepiandrosterone (DHEA), DHEA sulphate (DHEAS) and androstenedione (AND) are produced by adrenal cells of the zona reticularis upon stimulation by ACTH. The fetal adrenals and the placenta also produce oestrogens (oestradiol and oestriol) under the influence of CRH.

10. The synthesis and release of glucocorticoids is subject to feedback by ACTH and glucocorticoids, primarily at the hippocampus, PVN and corticotrophic cells in the pituitary, to reduce production of CRH, AVP, ACTH and adrenal steroids. Various neurones, originating within the hypothalamus or other areas of the brain, influence the secretion of CRH and AVP into the hypothalamo-hypophysial portal system (HHPS), which are transported to the pituitary, where they stimulate the release of ACTH from the corticotropes. These neurones use noradrenalin, dopamine, 5-hydroxtryptamine and gamma-amino butyric acid, as well as CRH, as neurotransmitters. Additional

factors can influence the activity of the HPA axis, including the urocortins (particularly Ucn I and II), pituitary adenylate cyclase-activating peptide (PACAP) and various interleukins (ILs).

11. The zona glomerulosa (ZG) of the mammalian adrenal cortex produces aldosterone, the major mineralocorticoid controlling Na+/K+ balance in tetrapod vertebrates. Additionally, excess glucocorticoids also can influence Na+/K+ balance through the mineralocorticoid receptor (GR1). Secretion of aldosterone is controlled by the renin-angiotensin system (not the HPA axis); however, ACTH maintains the responsiveness of cells of the ZG cells to angiotensin II.

Example of disruption

12. Atrazine activates the HPA axis in rodents and increases circulating concentrations of ACTH, corticosterone and progesterone (OECD, 2012). This may contribute to modification of the central control of the HPG axis, which results in reproductive effects of atrazine in female rats (Fraites et al., 2009).

HPG axis

13. Reproduction in vertebrates is primarily controlled by the HPG axis. The hypothalamic neuroendocrine system regulates synthesis and release of the gonadotropins, follicle-stimulating-hormone (FSH), and luteinising hormone (LH) from the pituitary, which in turn stimulate gonadal development, particularly by inducing sex steroid synthesis. The sex steroids feed back to the hypothalamus and the pituitary, regulating gonadotropin synthesis and release. In addition, non-steroidal feedback regulation of gonadotropins by FSH-stimulated gonadal inhibins contributes to the synchronization of the HPG axis at all stages of the life cycle.

14. Hypothalamic gonadotropin-releasing hormones (GnRH) are the substances stimulating gonadotropin release from the pituitary. GnRHs are decapeptides that act via G-protein coupled receptors (gonadotropin-releasing hormone receptors, or GnRH-R). In most species, two forms of GnRH are present, one that stimulates gonadotropin release from the pituitary and one that plays a neuromodulatory role in the central nervous system (CNS). The hypothalamus forms an interface between the CNS and the endocrine system, integrating internal (e.g. nutrition, metabolism) and external factors (e.g. temperature, photoperiod, pheromones). Thus, the hypothalamus is triggered by the CNS and peripheral hormones to maintain physiological homeostasis by regulating pituitary release of tropic hormones, which control the activities of peripheral endocrine glands. Neurotransmitters modulating the activity of GnRH neurons include glutamate, γ-aminobutric acid, noradrenaline and dopamine. In mammals the recently-discovered kisspeptin/GPR54 system is critical for puberty and the regulation of reproduction; it is thought to integrate environmental cues and nutrition to the reproductive axis.

15. FSH and LH have different roles in reproduction. In females, FSH is primarily important for cyclic recruitment of follicles during the follicular phase, whereas the LH surge leads to ovulation and the luteal phase. In males, LH regulates androgen synthesis in Leydig cells, whereas FSH controls Sertoli cell activity, thereby promoting spermatogenesis in conjunction with androgens. Gonadotropins stimulate gonadal growth and development via the synthesis of sex steroids (i.e., oestrogens, androgens, and gestagens) and local growth factors. Generally, in mammals, gametogenesis is regulated by FSH, and steroidogenesis is induced by LH. Oestrogen production by the ovary involves LH stimulated testosterone synthesis in theca cells and subsequent FSH-mediated aromatization to 17β-oestradiol in granulosa cells. In the testis, testosterone synthesis in Leydig cells is stimulated by LH, whereas FSH controls Sertoli cell function.

16. The three classes of sex steroids—oestrogens, androgens, and gestagens—are primarily produced by the gonads or other reproductive tissues such as the placenta. Steroidogenesis in the gonads involves the synthesis of pregnenolone from cholesterol and the subsequent conversion to progesterone and successively to C19 androgens, which can be further aromatized by P450 aromatase (CYP19) to oestrogens. In mammals, oestradiol is the principle oestrogen, testosterone and dihydrotestosterone are the principle androgens and progesterone is the principle gestagen.

17. The action of sex steroids is mediated by nuclear receptors, which act as ligand-dependent transcription factors within the cell nucleus. In mammals, two nuclear oestrogen receptors (ERα and ERβ), one androgen receptor (AR), and two forms of progesterone receptors (PR-A and PR-B, which are encoded on the same gene locus) have been identified. In addition, the role of rapid, non-genomic signalling initiated at the cell membrane is increasingly recognized. Receptors involved in rapid oestrogen signalling include membrane-localised forms of ERα and ERβ, and possibly G-protein-coupled receptor 30 (GPR30). Rapid gestagen signalling has been attributed to membrane G-protein-coupled gestagen receptors (mPR) mPRα, mPRβ and mPRγ and membrane-localised forms of the nuclear PR. Furthermore, rapid non-genomic action of androgens is well documented.

18. The HPG axis regulates the differentiation of the sex-specific phenotype during early development. In females, oestradiol is important for reproductive processes, such as differentiation and maintenance of primary sexual characteristics and behaviour, proliferation of the endometrium, and for cyclicity of female reproductive events. In males, androgens play a pivotal role in the development of the reproductive system and phenotypic sex and are required for testicular spermatogenesis/spermiogenesis and the expression of male sexual behaviour. Although oestrogens and androgens are often considered as female or male hormones due to their sex-specific plasma profiles, ER and AR are expressed in many tissues in both sexes, and androgens are converted to oestrogens in tissues by aromatase (CYP19) in both males and females. In males, oestrogens are required for spermatogenesis, and the local aromatisation of testosterone into oestradiol is pivotal for the development of male-specific brain structures. In females, AR

knock-out revealed that androgens are required for proper ovarian function and mammary development.

19. Gestagens are also essential reproductive hormones in all vertebrates. In female mammals, progesterone is primarily produced in the corpus luteum and has key roles in the placenta in the initiation and maintenance of pregnancy and in the mammary gland. Female PR knock-out mice display reproductive dysfunctions, including impaired ovarian and uterine function, impaired mammary gland development and absence of sexual behaviour.

20. In addition to their roles in reproduction and reproductive development, the sex steroids also modulate other physiological functions, including metabolism, the immune system, the cardiovascular system and skeletal homeostasis.

Example of disruption

21. There are numerous examples of disruption of the HPG axis. One of the best-documented examples of endocrine disruption occurring in humans is the case of diethylstilboestrol (DES), which was used in the 1940–1970s during pregnancy with the aim of preventing miscarriages. In utero exposure to DES was strongly associated with rare cases of vaginal cancer and abnormalities of the reproductive tract in women and was also associated with various adverse effects on the reproductive system in men (OECD, 2012).

Somatotropic axis

22. Figure 1 summarises the somatotropic axis. The somatotropic axis is responsible for the release of growth hormone and insulin-like growth factor. These hormones regulate a variety of functions related mainly to growth, maturation and metabolism. The hypothalamus secretes growth hormone releasing hormone (GHRH) and somatostatin in a coordinated fashion. GHRH and somatostatin bind to surface receptors of the growth hormone-producing cells (somatotrophs) of the pituitary gland, where they coordinate the pattern of growth hormone release. The secretory patterns of GHRH and somatostatin are influenced by various factors including sex, age and circadian timing.

23. In rodents and humans, growth hormone secretion occurs in a pulsatile fashion. Secretory patterns in adult males are regimented, growth hormone surges occurring at regular intervals, while female secretory patterns are typically less ordered and there is generally less of a difference between peak levels and between-peak levels in females. Sex-specific secretory patterns develop at puberty and are, at least in part, regulated by sex steroids. Studies in rat have demonstrated that the male sex-specific pattern that occurs at puberty is partly programmed in the brain by a neonatal pulse in testosterone production.

24. Growth hormone binds to cell surface receptors in peripheral tissues, which initiate a phosphorylation cascade that involves the JAK/STAT

pathway. Elevated growth hormone levels result in insulin resistance, increased blood glucose, and increased lipid metabolism. Tissue responses to growth hormone are dependent upon both the amount of circulating hormone and its pattern of production and release. In the liver, growth hormone regulates CYP enzymes, primarily those involved in steroid metabolism and in the production of insulin-like growth factor-1 (IGF-1) and IGF-2. IGF-1 is the primary cell-signalling form of IGF.

25. IGF-1 is largely responsible for the growth-promoting activities of the somatotropic axis, exerting multiple effects at various tissues relating to growth. IGF-1 and/or IGF-2 contribute to spermatogenesis and/or oocytes maturation. Both IGF-1 and IGF-2 also appear to contribute to fetal development in mammals. Serum IGF-1 levels positively correlate to birth weight, and fetal IGF-1 deficiency results in low birth length.

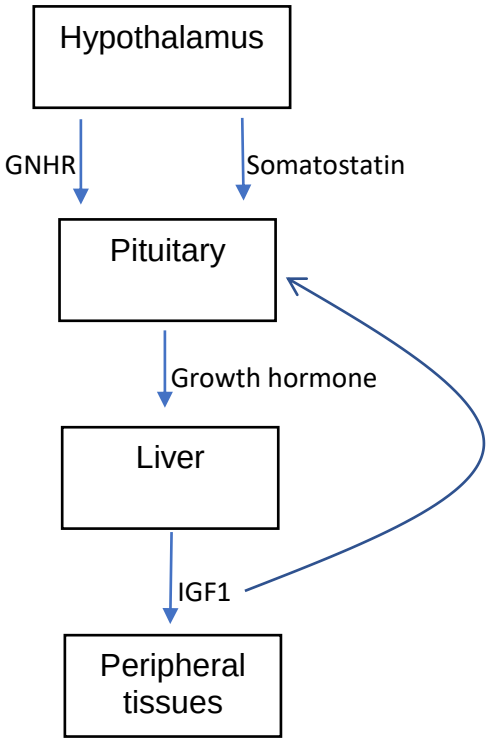

Figure 1: The somatotropic axis (OECD, 2012)

Example of disruption

26. There are no known environmental chemicals which directly disrupt the somatotropic axis, though inhibitors of the IGF-1 receptor have been developed for possible pharmaceutical use. However, many chemicals can indirectly affect the somatotropic axis by interacting with other endocrine signalling pathways that influence the somatotropic axis. For example, there is evidence that thyroid hormone may stimulate the somatotropic axis through its induction of pituitary growth hormone synthesis or through direct action on

hepatic IGF-1 synthesis. Conversely, it is possible that chemicals that suppress thyroid hormone levels may also suppress IGF-1 levels. PCBs, which have anti-thyroid effects, were reported to reduce the expression of IGF-2 levels in the livers of adult mink (*Mustela vison*).

Retinoid signalling pathway

27. Retinol is a fat-soluble vitamin (vitamin A) derived from dietary sources. It is metabolised to biologically-active retinoids through oxidative reactions catalysed by alcohol and retinol dehydrogenases. Retinoid signalling in the body is also regulated by the level of retinol and retinoic acid binding to binding proteins and the level of metabolic inactivation, mostly by members of CYP26. The retinoid compounds act as signalling molecules, regulating pleiotropic activities relating to development and differentiation in vertebrates. This activity is mediated through the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Both excess and suboptimal levels of retinoids during development result in developmental abnormalities.

Example of disruption

28. Various chemicals have been shown to affect retinoid signalling in vitro or in vivo, the mechanism being RXR activation, RXR inactivation or reductions in endogenous retinoid reserves. For example, aryl hydrocarbon receptor ligands such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) can deplete endogenous retinoid reserves. The exact mechanism is not known, but tetrachlorodibenzo-p-dioxin (TCDD) has been shown to cause loss of hepatic retinoids, presumably resulting from the mobilization of retinoids from retinyl ester stores, resulting in the increased renal excretion of polar retinoid metabolites.

HPT axis

29. Thyroid hormones are required for physiological functions including neurodevelopment, growth and cellular metabolism. Thyroid hormones are released by the thyroid gland through regulation of the HPT axis, which is controlled through a complex system of negative and positive feedback. Activation of the HPT is activated by the synthesis and release of thyrotropin-releasing hormone (TRH). TRH is produced in the hippocampus, primarily in the PVN. Multiple pathways contribute to the synthesis of TRH, including thyroid hormone signalling through feedback mechanisms, leptin and melanocortin signalling, body temperature regulation and cardiovascular physiology. Each of these affects TRH neurons, which integrate multiple inputs and provide a mechanism to establish set points for TRH production and the thyroid axis at appropriate levels, dependent upon physiological demands. HPT axis signalling is mediated through the paraventricular neurones that project to the median eminence, which is connected to the anterior pituitary gland through hypothalamic-portal vessels.

30. In mammalians, TRH is critical for the synthesis and secretion of thyroid stimulating hormone (TSH). TSH is a heterodimer consisting of α and β subunits. The α subunit is common to TSH, FSH, LH and chorionic gonadotropin (CG), while the β subunit is specific to TSH and confers specificity with the TSH receptor. TRH binds to thyrotropin-releasing hormone receptor (TRHR), a G protein-coupled receptor in the plasma membrane of the thyrotroph. This causes TRHR phosphorylation, which results in activation of the phospholipase C second messenger systems, down-stream kinases and ultimately in synthesis and release of TSH from the pituitary gland.

31. TSH is released from the anterior pituitary and binds to receptors on the cell surface of thyroid follicle cells. TSH receptors are G protein-coupled receptors, which, when activated, stimulate the adenylate cyclase and the cAMP secondary messenger kinase cascade. This includes phosphorylation of phosphokinase A (PKA) and subsequent phosphorylation of transcription factors such as cAMP-responsive element modulator (CREM) and cAMP response element binding protein (CREB), while there is also some evidence that TSH additionally activates protein kinase C (PKC) and diacylglycerol signalling pathways. The effects of receptor activation include increased uptake of iodide into the thyroid cells, iodination of tyrosyl residues on thyroglobulin, synthesis and oxidation of thyroglobulin, thyroglobulin uptake from thyroid colloid, and production of the thyroid hormones T4 and T3.

32. Iodine uptake into the thyroid gland is governed by the sodium-iodide symporter (NIS), which is located on the outer plasma membrane of the thyrocyte. The thyroid gland can concentrate iodine 20- to 40-fold. NIS gene transcription is regulated by transcription termination factors 1 and 2 (TTF1 and TTF2), and paired box gene 8 (Pax8), which are activated by PKA, which in turn is stimulated by TSH. NIS is also auto-regulated, excessive iodine accumulation supressing NIS gene expression. Iodine molecules transported into the cell are bound to tyrosine residues of thyroglobulin protein as either mono-iodothyronine or di-iodothyronine. Thyroglobulin is also under the regulatory control of TTF1, TTF2, and Pax8 within the thyrocyte and thus its production is stimulated by TSH.

33. T4 and T3 are produced through a series of peroxidation reactions that require iodide, hydrogen peroxide, the enzyme thyroperoxidase and thyroglobulin. Hydrogen peroxidase is produced through the activity of dual oxidase/thyroid oxidase (DUOX/ThOX) enzymes located at the apical pole of the thyroid follicular cells. Thyroid peroxidase (TPO) facilitates the covalent attachment of iodide to thyroxin by reducing H_2O_2 and oxidising iodine whereby the iodine binds to distinct tyrosyl residues on the thyroglobulin protein forming digoxigenin or monoiodothyronine. Two digoxigenin molecules form T4, and one digoxigenin and one mono-iodothyronine molecule form T3.

34. TSH also stimulates secretion of T4 and T3, stored in the colloid, via endocytosis into the circulation. This is mediated through activation of the TSH receptor, intracellular accumulation of cAMP, and subsequent transport, regulation, and proteolysis of thyroglobulin, resulting in the liberation of T4 and T3. In the blood stream, thyroid hormones are either bound to transport

proteins, thyroid binding globulin (TBG), transthyretin (TTR) or albumin, or they circulate freely in the plasma. The fractions circulating freely are approximately 0.5% of the total. TBG has much greater binding affinity towards thyroid hormone than do the other two thyroxine-binding proteins and it is the predominant thyroid hormone-binding plasma protein in humans (75% of serum T4 is bound to TBG in humans). However, TBG is lacking in adult rats. This is an important difference in thyroid hormone physiology between humans and rats.

35. The thyroid-binding proteins play an important role in regulating circulating levels of thyroid hormone concentration. Binding of T4 and T3 to these proteins serves as a mechanism to regulate the transport of thyroid hormone to target sites and may also provide a mechanism to control iodine clearance. Thyroid hormone levels are also controlled by three deiodinases. These deiodinases are responsible for local synthesis of T4 and T3 within the thyroid, the peripheral and local conversion of T4 to T3 (the biologically active form of thyroid hormone), breakdown of reduced T3 (rT3), and inactivation of T3. In addition to deiodination, thyroid hormones are metabolized in the liver and kidney through conjugation with sulphate or glucuronic acid.

36. At the site of action, bioactive T3 either diffuses passively across the cellular membrane or is actively transported into the cell. Thyroid hormones are lipophilic and can enter the cell by passive diffusion; however, a number of stereoselective T4 and T3 transporters have been identified, including organic ion transport proteins (OATP) and members of the monocarboxylate transporter (MCT) family.

37. Inside the cell, thyroid hormone signalling is mediated through ligand interaction with thyroid hormone receptors (TRs). TRs are nuclear hormone receptors. They are ligand-dependent transcription factors that are governed through ligand-dependent interactions, DNA-dependent interactions, and co-regulator-dependent interactions. There are multiple forms of the thyroid receptor (THα, THβ1, and THβ2), which facilitate transcriptional activation and repression of target genes through interaction with thyroid hormone response elements within the promoter/enhancer region of each gene. T3 binds to each of the TRs with near equal affinity. Both T3 and T4 have affinity for TRs, but T3 exhibits an approximately 50-fold greater affinity for TRs than does T4. There is also some evidence of selective functional activation of T3 with each receptor that may be co-regulator–dependent. TRs also exhibit temporal and tissue-specific expression patterns. Numerous genes are affected by transcriptional activation of TRs, each highly cell specific. In peripheral tissues, thyroid hormone results in TR ligand–dependent activation of genes associated with development, growth and metabolic control. In the case of negative feedback to the hypothalamus and pituitary, T3 binding to the THβ receptor results in ligand-dependent repression of gene transcription and subsequent reductions in THR and TSH levels. Additional nuclear receptors, including RXR, the TR receptor obligate heterodimerization partner, and PPARγ, also function to regulate *Trh* gene expression within the hypothalamus.

Example of disruption

38. Perchlorate, chlorate, thiocyanate, bromate and nitrate compete with iodine for binding to the sodium-iodide symporter (NIS), inhibiting the uptake of iodine into the follicular thyroid cell. Severe inhibition of iodine uptake, at levels that induce depletion of the thyroid hormone stores, can result in hypothyroidism. Chronic mild to moderate inhibition may result in sustained adaptive changes which can lead to long term effects such as the development of toxic multinodular goitre, in particular in populations with mild to moderate iodine deficiency (EFSA, 2014).

Vitamin D signalling pathway

39. Vitamin D is a steroid hormone. Its biological effects are mediated by the binding of 1α, 25-dihydroxyvitamin D3 to the vitamin D receptor (VDR). VDR is a nuclear receptor. 1α,25-dihydroxyvitamin D3 binds to the ligand-binding pocket of VDR with high affinity. This results in a conformational change in the receptor to its active form. VDR heterodimerises with RXR, and the heterodimer binds to target genes containing a canonical vitamin D response element (VDRE) within the promoter region. Co-regulatory proteins are recruited, followed by the recruitment of RNA polymerase II and the initiation of gene transcription.

40. The VDR is only found in vertebrates. In humans and rodents, 36 tissues express VDR, including tissues not associated with the classic vitamin D effects of calcium mobilisation and ion homeostasis. Recent evidence suggests that VDR signalling has additional roles to these, including in immune system function, cell proliferation, and neurodevelopment.

41. Terrestrial vertebrates obtain vitamin D from both the diet and from the photolytic conversion of 7-dehydrocholesterol to pre-vitamin D3 in the skin. 7-dehydrocholesterol is present in large quantities in the skin and is a precursor molecule in the cholesterol. 7-dehydrocholesterol absorbs UVB light in the 290–315 nm wavelength, which breaks the bond between carbons 9 and 10, creating pre-vitamin D3. Pre-vitamin D3 is thermodynamically unstable and rapidly isomerizes to vitamin D3. Factors such as latitude, sunscreen use, ethnicity, age, and nutritional status can affect vitamin D3 production in the skin. Vitamin D3 is not biologically active and must be metabolized to its active form through two hydroxylation reactions.

42. The first hydroxylation reaction takes place in the liver. Vitamin D3 circulates in the bloodstream bound principally to vitamin D binding protein (VDBP), though some is bound to albumin. In the liver, the P450 enzyme 25-hydroxylase (CYP2R1) adds a hydroxyl group to carbon 25, creating 25-hydroxyvitamin D3. CYP2R1 is highly expressed in the liver. Because this reaction reflects the vitamin D3 status of an individual, measuring serum levels of 25-hydroxyvitamin D3 is a common method of determining vitamin D status.

43. 25-Hydroxyvitamin D3 is again bound to transport proteins and is transported in the blood to the kidneys, where the second hydroxylation reaction takes place. The 25-hydroxyvitamin D3-VDBP complex is filtered out of the blood at the glomerulus, before being reabsorbed at the proximal tubules by endocytosis mediated by a surface receptor protein, megalin. Inside the cells of the proximal tubule, 25-hydroxyvitamin D3 is transported to the mitochondria, where the P540 enzyme 1α-hydroxylase (CYP27B1) adds a hydroxyl group, creating 1α,25-dihydroxyvitamin D3, the active vitamin D3 metabolite.

44. The second hydroxylation step is tightly regulated. Calcium-sensing receptors in the parathyroid gland detect when serum calcium levels are low and trigger the release of parathyroid hormone (PTH). PTH induces the expression of 1α-hydroxylase, increasing the concentration of 1α,25-dihydroxyvitamin D3. 1α,25-dihydroxyvitamin D3 binds to the VDR, activating it and initiating the transcription of genes involved in calcium uptake and distribution.

45. 1α,25-dihydroxyvitamin D3 has a role in regulating its own levels in that it suppresses the expression of 1α-hydroxylase and induces the expression of the main enzyme to catabolise it, 24-hyroxylase (CYP24A1). Most cells in the body express 24-hydroxylase, but the highest activity is in the kidney.

46. Vitamin D is required for normal bone development and growth, remodelling and maintenance. Vitamin D signalling controls the differentiation of bone-forming osteoblasts and bone-resorbing osteoclasts and the balance between these two cell types. Vitamin D regulates the actions of osteoblasts, including cell proliferation, bone matrix synthesis, mineralization, and the initiation of osteoclastogenesis. Vitamin D and the VDR are both required for the expression of transport channels and proteins required for calcium absorption from the small intestine. Vitamin D deficiencies result in the bone-softening disease, rickets, in the young and osteomalacia in adults.

47. 1α,25-dihydroxyvitamin D3 is believed to play a role in the differentiation and function of immune cells. The VDR is expressed in multiple types of immune cell, including T lymphocytes, macrophages and dendritic cells. Immune cells are capable of producing and maintaining local concentrations of 1α,25-dihydroxyvitamin D3 through the expression of both 1α-hydroxylase and 24-hydroxylase. There is evidence to suggest that a lack of vitamin D contributes to the aetiology of multiple autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and type 1 diabetes, and that sufficient vitamin D during infancy and childhood decreases the incidence of autoimmune disease in adult life.

48. The VDR and vitamin D status have been inversely associated with various cancers in some epidemiological studies, including breast, prostate and colon cancers, and UVB exposure has also been inversely associated with some cancers. Activation of the VDR by vitamin D in cancer cells has been shown to inhibit cancer cell proliferation, induce apoptosis, inhibit angiogenesis, and decrease the metastatic potential of cancer cells.

49. The VDR and CYP450 enzymes involved in the synthesis and catabolism of vitamin D are expressed in the brain, CNS and PNS. Studies have shown that gestational vitamin D deficiency results in offspring with abnormal brain development. Developmental alterations in mouse models include abnormal brain size, increased cell proliferation, decreased cortical brain thickness and altered neurotransmitter production. Vitamin D activates both tyrosine hydroxylase and choline acetyltransferase, which are important for the production of dopamine, noradrenaline, adrenaline and acetylcholine. These neurotransmitters have roles in neurobehavioural disorders such as autism, schizophrenia and ADHD. Risk factors for vitamin D deficiency, such as living in areas with little UV light exposure, have been associated with increased risks of schizophrenia, autism and other mental health disorders. In contrast, adequate levels of vitamin D may have neuroprotective effects. For example, vitamin D increases levels of nerve growth factor (NGF), which is believed to counteract neural degeneration in Alzheimer's disease. Vitamin D also helps defend the brain against oxidative degeneration by increasing the expression of γ-glutamyltranspeptidase, which is involved in the production of glutathione. Vitamin D has also been shown to protect against the neurotoxic effects of the street drug methamphetamine in rats.

50. Many cardiovascular cells express VDR and respond to 1α,25-dihydroxyvitamin D3. One such system is the renin-angiotensin system, which regulates blood pressure and electrolyte homeostasis. Renin is a protease that cleaves angiotensin I from angiotensinogen. Angiotensin I is converted to angiotensin II, which exerts its effects on multiple organs to regulate blood pressure and electrolyte balance. The production of angiotensin II is tightly regulated, and the overproduction of angiotensin II has been linked to hypertension, heart attack, and stroke. 1α,25-dihydroxyvitamin D3-bound VDR directly inhibits renin expression by binding to the VDRE in the promoter of the renin gene. In VDR-null mice, renin expression was increased, leading to hypertension, cardiac hypertrophy, and increased water intake.

51. The most common secondary bile acid in humans is lithocholic acid (LCA). LCA is metabolised in the intestine by the P450 enzyme CYP3A4, which is under the regulatory control of vitamin D, suggesting that vitamin D has an important role in LCA detoxification. LCA is formed from the primary bile acid chenodeoxycholic acid (CDCA) by bacterial dihydroxylation. LCA has been shown to cause DNA strand breaks, form DNA adducts, and inhibit DNA repair enzymes, and promotes colon cancer in animals. LCA and its major metabolites have been shown to be VDR ligands, binding to and activating VDR and inducing the expression of CYP3A4. Other bile acid receptors such as FXR and PXR can be activated by LCA, but VDR is activated at much lower concentrations. Vitamin D supplementation has inhibited colon carcinogenesis promotion by LCA and high fat diets in rats.

Example of disruption

52. A study of PCB126 in rats showed decreased serum vitamin D3 level and decreased bone mineralisation. Another study of a PCB mixture in rats

reported decreased serum 25-hydroxyvitamin D3 and 1α, 25-dihydroxyvitamin D3. The exact mechanism of PCB disruption of the vitamin D pathway is unknown but may involve AhR activation.

Peroxisome proliferator-activated receptor signalling pathway

53. Peroxisome proliferator-activated receptors (PPARs) are type II nuclear receptors and thus are typically localised to the nucleus. There are three distinct PPARs in mammals, PPARα, PPARβδ and PPARγ. All three heterodimerise with the RXR to initiate their transcription actions.

54. The ligand binding domain (LBD) of the PPRs is large, with a binding pocket of approximately 1300-1400 angstroms, and can accommodate large ligands such as fatty acids. The LBDs of PPARα and PPARγ are similar, with only small differences in their affinities for ligands. For example, the greater pharmacological sensitivity of PPARγ for the thiazolidinedione drugs is due to a one amino acid difference, and the higher affinity for saturated fatty acids of PPARα may be due to the greater lipophilicity of the binding pocket. In contrast, PPARβ/δ has a large LBD, but its pocket is much narrower.

55. PPARs are activated by fatty acids, pharmacological ligands and other xenobiotics. In turn, the PPARs regulate genes involved in fatty acid metabolism, inflammation and proliferation. PPARα is primarily expressed in the liver, intestine, kidney, heart, and adipose tissue. It controls β-oxidation in the peroxisomes and mitochondria and ω-oxidation in the endoplasmic reticulum of the liver. Its ligands reduce very low density lipoprotein (VLDL), increase high density lipoprotein (HDL) and reduce the duration of macrophage-induced inflammation. PPARβ/δ is ubiquitously expressed, but its greatest expression is in the intestinal epithelium, liver and keratinocytes. PPARβ/δ activation improves glucose tolerance and mediates cellular differentiation of skin and intestine. It also improves fatty acid catabolism in skeletal muscle. There are three isoforms of PPARγ: PPARγ1, PPARγ2 and PPARγ3. PPARγ1 is expressed in a variety of tissues at low levels. PPARγ2 and PPARγ3 are expressed in adipose, and PPARγ3 is also expressed in macrophages. PPARγ regulates adipocyte differentiation and represses inflammation.

56. Table 1 summarises the functions of PPARs.

Table 1: Summary of the functions of PPARs. From OECD (2012)

PPAR	**Activity**
PPARα	Facilitates peroxisome proliferation, liver cancer, fatty acid metabolism and developmental delay. Alters lipid homeostasis. Inhibits inflammation.
PPARβ/δ	Increases fatty acid metabolism. Facilitates skin proliferation and differentiation. Facilitates placental development.
PPARγ	Facilitates adipocyte differentiation and glucose homeostasis. Controls trophoblast invasion and placental angiogenesis. Represses inflammation.

Example of disruption

57. PFOA and PFOS activate PPARα. Reduced neonatal survival, delayed eye opening and decreased postnatal growth occurred when wild-type mice were exposed to PFOA on gestation days 1-17 but not PPARα-null mice, demonstrating that these developmental effects of PFOA in mice are mediated by PPARα. In contrast, the same authors found in another study that in utero exposure of mice to PFOS resulted in neonatal mortality and delayed eye opening in both wild type and PPARα-null mice.

58. The term “obesogen” has been coined to describe a subgroup of endocrine disruptors that are able to perturb metabolic signalling and lipid homeostasis resulting in increased weight, adipogenesis and obesity in rodent models and therefore potentially humans. Of primary interest have been chemicals that affect the PPARs, in particular PPARγ. Bisphenol A (BPA) increases PPARγ expression, and consequently alters IGF-1 expression, and perinatal exposure of rats has been reported to increase early adipogenesis.

Renin-angiotensin system

59. The following text is based on Sparks et al. (2014).

60. The renin-angiotensin system is a major control of blood pressure and fluid balance. The major biologically active hormone of this system is angiotensin II (ang II). Ang II is produced from the substrate angiotensinogen via angiotensin I (ang I) by two cleavage steps, the first catalysed by the enzyme renin and the second by angiotensin converting enzyme (ACE). Ang II binds to specific receptors, triggering effects in most systems in the body including the brain, heart, kidney, vasculature and immune system. However, the primary function of the renin-angiotensin system is in circulatory homeostasis, protecting body fluid volumes. Abnormal activation of the renin-angiotensin system can contribute to the development of hypertension, cardiac hypertrophy, and heart failure.

61. Angiotensinogen is synthesised by hepatocytes and released into the circulation after removal of a 33-amino acid signal peptide. While the liver is the main source of circulating angiotensinogen, other tissues have also been reported to synthesise it, including adipose tissue, brain, spinal cord, heart, kidney, lung, adrenal gland, large intestine, stomach, spleen, ovaries and blood vessels. It has been suggested that independent regulation of levels of Agt in individual tissue compartments may form the basis of local or tissue renin-angiotensin systems operating independently of the circulatory renin-angiotensin system. Studies in mice with genetic ablation of the Agt gene that are completely deficient in angiotensinogen have shown increased perinatal mortality, profound hypotension and abnormalities of the kidney including hydronephrosis, hypertrophic lesions of renal arteries and arterioles and an impaired ability to concentrate urine. This phenotype is virtually identical to that of mice with deficiencies of ACE, renin or with combined deletions of type

1a angiotensin (AT1A) and type 1b (AT1B) receptors, indicating that the major role of angiotensinogen is the production of Ang II.

62. Plasma angiotensinogen levels increase during pregnancy and during the administration of synthetic oestrogens such as oral contraceptive pills, and patients with preeclampsia have been shown to have higher circulating levels of the oxidized form of angiotensinogen relative to reduced angiotensinogen. Oxidised angiotensinogen is a more efficient substrate for renin. Therefore, it appears that alterations of angiotensinogen may contribute to blood pressure elevation in preeclampsia.

63. In humans, there is an excess of angiotensin in the serum and ACE is ubiquitous in the endothelium and plasma. The amount of renin in the bloodstream is the rate limiting step determining the level of ang II. Circulating renin is primarily produced in the kidney, where its production and secretion are tightly regulated at the juxtaglomerular (JG) apparatus by two separate mechanisms: a renal baroreceptor and sodium chloride (NaCl) delivery to the macula densa. Through these sensing mechanisms the level of renin in plasma can be adjusted in response to changes in blood pressure and salt balance.

64. Inbred Dahl salt hypertension sensitive (S) and inbred Dahl salt hypertension resistant (R) rats exhibit significant polymorphism in their renin genes, which have been suggested to contribute to their differences in blood pressure.

65. The biological actions of Ang II are mediated by a family of cell surface receptors. These angiotensin receptors can be divided into two pharmacological classes, AT1 and AT2, based on their differential affinities for various nonpeptide antagonists. Most of the classically recognised functions of the renin-angiotensin system are mediated by AT1 receptors, including regulation of tubuloglomerular feedback, stimulation of renal tubular sodium reabsorption, release of aldosterone from the adrenal glomerulosa, smooth muscle cell contraction and stimulation of hypothalamic thirst sensors. Studies suggest that the primary function of the AT2 receptor is to negatively modulate the actions of the AT1 receptor. For example, in contrast to the effects of AT1 receptors in the kidney to drive sodium retention, activation of renal AT2 receptors by Ang II augments natriuresis. It has been suggested that the activation of AT2 receptors stimulates bradykinin, nitric oxide and cyclic guanine monophosphate (cGMP), and these pathways may mediate actions of the receptor to promote natriuresis and blood pressure lowering. Studies with a putative specific AT2 receptor agonist suggest that AT2 receptor activation may limit progressive cardiovascular and renal damage in contrast to the actions of AT1 receptors to promote such injury.

Example of disruption

66. ACE inhibitors, which inhibit the production of Ang II, are used therapeutically in the treatment of hypertension, congestive heart failure and kidney diseases.

Pancreatic signalling pathway

67. The following text is based on Seino et al. (2010) and Chandra and Liddle (2013).

68. Insulin is a key metabolic hormone, which regulates blood glucose levels. It is secreted by pancreatic β-cells. Dysfunction of the β-cell and/or a decrease in β-cell mass are associated closely with the pathogenesis and pathophysiology of diabetes mellitus.

69. The principle mechanism of insulin secretion is glucose-stimulated insulin secretion (GSIS). Glucose is transported into the β-cells by the glucose transporter. The metabolism of glucose in the cell increases ATP production. This closes ATP-sensitive K+ channels, depolarising the cell membrane and thereby opening voltage-dependent calcium channels (VDCCs), which allows an influx of Ca2+. This Ca2+ influx triggers insulin granule exocytosis.

70. In addition to GSIS, signals which potentiate GSIS are also important for the normal regulation of insulin secretion. Incretins such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are released in response to the ingestion of nutrients from gastrointestinal endocrine L-cells and K-cells, respectively. These bind to G_s-coupled receptors on the β-cell and activate adenylyl cyclase (AC), resulting in increased intracellular concentrations of cyclic AMP (cAMP). cAMP activates both PKC and Epac2 (exchange protein activated by cAMP) to potentiate insulin secretion. In addition, the major parasympathetic neurotransmitter acetylcholine binds to G_q coupled receptors on β-cells, activating phospholipase Cβ (PLCβ). Activation of PLCβ generates the messengers 1,4,5-triphosphate (IP3) and diacyglycerol (DAG). DAG activates PKC and IP3 mobilises Ca2+ from intracellular storage sites.

71. There is evidence that a number of other neuronal, hormonal and non-hormonal factors affect pancreatic endocrine secretion as follows:

- A study conducted in rats indicated that the hypothalamus also plays an important role in glucose-stimulated insulin release GSIS. The specific mechanisms are not yet clear.

- Ghrelin, an orexigenic hormone released by gastric endocrine cells under fasting condition, counteracted the insulinotropic effects of GLP-1-mediated GSIS in isolated rat islets by preventing elevation of intracellular Ca^{2+} and cAMP levels.

- Leptin, which is secreted by adipocytes, plays a significant role in glucose homeostasis. Leptin receptors are expressed in many areas of the brain, and mice with a point mutation in the leptin receptor (db/db mice) are obese and have high plasma insulin levels and impaired glucose tolerance. Further studies in mice indicated that leptin receptor signalling in hypothalamic proopiomelanocortin (POMC) neurons is

sufficient to modulate glucose homeostasis. Independent of its regulatory role in glucose homeostasis, leptin interacts directly with receptors located on pancreatic β-cells to attenuate insulin gene expression and secretion.

- A nerve growth factor inducible (VGF) derived peptide, TLQP-21, has been shown to potentiate GSIS.

- Galanin is a small neuropeptide that is widely distributed in the nervous system. It is expressed in autonomic nerve terminals of the endocrine pancreas and negatively regulates insulin secretion.

- Melatonin receptors, MT1 and MT2, are expressed in pancreatic islet cells and mutations in these receptors have been associated with elevated glucose levels. Melatonin acting on MT1 and MT2 receptors diminishes insulin secretion by reducing second messenger signalling.

- Increasing evidence suggests that endoplasmic reticulum (ER) stress contributes to β-cell dysfunction. Studies in mouse models have shown that the ER stress protein Wolfram syndrome 1 (WFS1) is critical for β-cell function and insulin release through cAMP stimulated pathways.

- Protein restriction can negatively affect glucose regulation, and a study of leucine supplementation of mice on a high fat diet showed improved glucose tolerance and insulin secretion. Leucine down-regulated the adrenergic α2A receptor by activating mTOR ("mammalian target of rapamycin", a member of the phosphoinositol-3-kinase family), thereby augmenting insulin secretion.

- There is evidence that apolipoprotein A-IV (apoA-IV) enhances insulin secretion. ApoA-IV is synthesised and secreted by enterocytes following the ingestion of lipids. ApoA-IV similarly increased insulin secretion by isolated pancreatic islets and this effect was inhibited by KATP and Ca2+ channel blockers. ApoA-IV knockout mice have delayed glucose clearance and reduced insulin secretion, which is exacerbated by a high fat diet. Similarly, previous studies had suggested that apoA-I and apoA-II increased insulin production and secretion in a glucose-dependent manner, by activation of KATP channels and elevation of intracellular Ca2+.

- In a study in mice, exercise-induced release of cytokine interleukin-6 (IL-6) from muscles stimulated GLP-1 release, which promoted insulin secretion, causing a reduction in circulating glucose. IL-6 also acted directly on BRIN-BD11 β-cells (a pancreatic β-cell line) and isolated mouse islets to stimulate insulin release.

- Small ubiquitin-related modifier (SUMO) proteins are found in many types of cells and modify protein function by reversible attachment or detachment. In isolate mouse islets, high glucose augmented the expression of SUMO isoforms, which covalently modified the GLP-1

receptor, diminishing its trafficking to the membrane and decreasing insulin secretion.

Example of disruption

72. Inhibition of mTOR by rapamycin and activation of the adrenergic α2A receptors by clonidine supressed leucine-stimulated insulin release in a study in mice. This may explain why a retrospective analysis showed that renal transplant patients who received a combination of rapamycin and clonidine had a higher incidence of new onset of diabetes after transplantation (Chandra and Liddle, 2013).

Identification and testing of endocrine disruptors

73. An endocrine disrupter is defined by WHO/IPCS and EFSA as 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 in (sub)populations (WHO/IPCS, 2002; EFSA, 2013). Based on this definition EFSA (2013) considered that endocrine disruptors can be identified by three criteria: i) the presence of an adverse effect in an intact organism or (sub)population, ii) the presence of an endocrine activity, and iii) a plausible or demonstrated causal relationship between the endocrine activity and the adverse effect.

74. EFSA (2013) noted that most of the knowledge about endocrine disruption has been gained for substances which interact with oestrogen, androgen, or thyroid hormone systems or affect steroidogenesis in vertebrates ("EATS" modalities), and thus the HPG and HPT axes. EFSA noted that there are, as yet, no standardised mechanistic assays to detect the non-EATS modalities. However, the downstream effects of disruption of these endocrine pathways are probably detectable in some of the apical tests, i.e. some of the standard toxicology studies. They gave the example that if a chemical were to impair the functional capacity of the endocrine pancreas then degenerative changes, e.g. to the islets of Langerhans, may be detected by histopathological examination in the OECD TG 408 90-day subchronic toxicity study, and changes in blood sugar concentrations would be identified by clinical chemistry.

75. The OECD has developed a Conceptual Framework for the testing and assessment of endocrine disruptors. The Conceptual Framework (CF) provides guidance on how tools from lower levels of the CF can be used to determine which higher level tests are needed for a specific chemical, to increase evidence that it is or is not an endocrine disruptor. In summary:

- Level 1 of the CF is existing information and non-test information, which may guide the initial need for testing. This can include physicochemical properties, existing toxicology and epidemiology data, read-across, (Q)SAR etc.

- Level 2 includes selected in vitro endocrine mechanistic/mode of action tests, e.g. the ER transactivation assay (OECD TG 455).

- Level 3 includes selected in vivo endocrine mechanistic screening methods, e.g. the Hershberger assay in rodents (OECD TG 441).

- Level 4 is apical tests which include endocrine-relevant endpoints, e.g. chronic toxicity and carcinogenicity studies (OECD TG 451-3).

- Level 5 is in vivo apical tests which provide more comprehensive data over more extensive parts of the lifecycle, e.g. the extended one-generation reproductive toxicity study (OECD TG 443).

76. Table 2, adapted from EFSA (2013), provides further information on the in vivo tests at CF levels 3-5 that provide screening data and/or mechanistic information or which study apical endpoints for disrupters of the different endocrine systems.

77. EFSA noted that no single test is likely to be able to identify a chemical as an endocrine disruptor since both mechanistic and apical information is needed, and that it is the level 4 and 5 apical tests that identify the adverse effects required to conclude that a chemical is an endocrine disruptor. In terms of demonstrating a plausible or causal relationship between endocrine activity and the adverse effect, EFSA considered that evidence for this relationship should be obtained from the OECD CF or from other investigations and assessed on a weight-of-evidence (WoE) basis.

78. EFSA and ECHA jointly published guidance in 2018 on the identification of endocrine disruptors in the context of Regulation (EU) No. 528/2012 (Biocidal Products Regulation) and Regulation (EC) No. 1107/2009 (Plant Protection Products Regulation) (ECHA/EFSA, 2018). This provides guidance on the steps necessary to identify a substance as an endocrine disruptor for the purposes of these regulations. It involves conducting and documenting a mode of action analysis to establish if there is a biologically plausible link between observed adverse effects and endocrine activity, and depending on the data already available may require additional testing. It does not provide guidance on further hazard characterisation or risk assessment. The guidance mainly addresses the EATS modalities due to the availability of standardised in vitro and in vivo test guidelines and agreement on the interpretation of findings in these tests. The COT submitted comments on a draft of the guidance that was released for public consultation prior to finalisation, noting that a significant gap was the non-EATS modalities. The finalised guidance notes that although the existing knowledge for those non-EATS modalities is not as advanced as for the EATS modalities it may, in some cases, be already possible to reach a conclusion on a non-EATS endocrine modality, e.g. where data in the scientific literature provide mechanistic information which can be linked to adverse effects measured in apical tests, e.g. histopathological findings in the pancreas.

Table 2: In vivo mammalian tests listed in the OECD CF and their known or potential responses to the different endocrine axes/modalities/pathways. M = provides mechanistic information; A = provides apical test; P = potentially responsive to apical endpoints. From EFSA (2013)

Endocrine axis/modality/pathway	Assays														
	Uterotrophic assay (OECD TG 440) (CF level 3)	Hershberger assay (OECD TG 441) (CF level 3)	Enhanced 28-day study (OECD TG 407) (CF level 4)	90-day study (OECD TG 408) (CF level 4)	1-generation study (OECD TG 415) (CF level 4)	Male pubertal assay (US EPA OPPTS 890.1500) (CF level [illegible])	Female pubertal assay (US EPA OPPTS 890.1450) (CF level [illegible])	Intact adult male assay (no TG) (CF level 4)	Prenatal dev tox study (OECD TG 414) (CF level 4)	Chronic tox and carcinogenicity studies (OECD TG [illegible])	Enhanced reproductive screening assay (OECD TG [illegible])	Enhanced combined 28-day/reproductive screening (OECD TG [illegible])	Developmental neurotoxicity study (OECD TG 426) (CF [illegible])	Extended 1-generation study (OECD TG 443) (CF level 5)	2-generation study (OECD TG 416) (CF level 5)
Oestrogen	M		A	A	A		A		A	A	A	A	A	A	A
Anti-oestrogen	M		A	A	A		A		A	A	A	A	A	A	A
Androgen	M	M	A	A	A	A		A	A	A	A	A	A	A	A
Anti-androgen		M	A	A	A	A		A	A	A	A	A	A	A	A
Thyroid		M	M/A	A	A	A	A	A	A	A	A	A	A	A	A
Anti-thyroid		M	M/A	A	A	A	A	A	A	A	A	A	A	A	A
Steroidogenesis			A	A	A	A	A	A	A	A	A	A	A	A	A
HPA/corticosteroid axis			P	P	P				P	P	P	P	P	P	P
Somatotropic axis			P	P	P				P	P	P	P	P	P	P
Vitamin D signalling			P	P	P				P	P	P	P	P	P	P
Retinoid signalling			P	P	P				P	P	P	P	P	P	P
PPAR pathway			P	P	P				P	P	P	P	P	P	P
Other endocrine modalities			P	P	P				P	P	P	P	P	P	P

Considerations of the existence of thresholds

Swedish Chemicals Agency (KEMI)

79. The Swedish Chemicals Agency published a review of the scientific argumentation from 15 published papers on the existence or otherwise of thresholds for endocrine disruptors (KEMI, 2013). It was concluded that the presence of absence of a threshold can never be proven experimentally since all experiments have a limit of detection below which effects cannot be observed and no conclusion can be drawn on the shape of the dose-response relationship below that point. Thus, the scientific support for the existence of a threshold may depend on what is known about the mechanism of action.

80. The primary argument against assuming thresholds was that chemicals that act by the same mechanism as endogenous factors (hormones in this case) add to the actions of these factors and increase the response of already ongoing biological processes. This is known as the “additivity to background” argument, which has also been made to defend the non-threshold approach to genotoxic carcinogens.

81. The argument in support of a threshold was that a threshold will exist if there is no endogenous hormone, if the endogenous hormone is not causing an adverse effect, or if there is effective homeostatic control. Another argument was that the initial interaction of any toxic agent with a biological target is likely to have no threshold, but the complexity of biological systems makes non-threshold dose-response relationships unlikely for many higher endpoints such as behaviour, reproduction, organ weights and growth.

82. Overall the KEMI report concluded that conclusions on thresholds must be based on considerations of mechanisms of action. The conclusions may vary for different endpoints, and are also closely connected with considerations of what effects are considered adverse. An assumption of no threshold may be as valid or not as for genotoxic carcinogens.

Endocrine Disrupters Expert Advisory Group

83. The European Commission’s Joint Research Centre (JRC) chaired meetings of the Endocrine Disrupters Expert Advisory Group and produced a report: “Thresholds for Endocrine Disrupters and Related Uncertainties. Report of the Endocrine Disrupters Expert Advisory Group” (JRC, 2013). The Endocrine Disrupters Expert Advisory Group comprised toxicologists and ecotoxicologists with regulatory and/or endocrinology backgrounds nominated by Member State Competent Authorities for REACH and the Plant Protection Products Regulation (PPPR), relevant industry associations and non-governmental organisations. The purpose of this report was to capture the members' opinions on the likelihood of the existence of thresholds for responses to endocrine disrupters, in particular considering thresholds of adversity and the uncertainties associated with reliably estimating such

thresholds from experimental data. This report captured the range of views expressed at a one-day session devoted to this topic.

84. The report notes that the term threshold can be interpreted in three different ways: biological threshold (a dose below which an organism experiences no (adverse) effects), experimental threshold (a dose below which no (adverse) effects are observed) and mathematical threshold (a dose below which the response is zero and above which response is non-zero). True thresholds cannot be precisely determined experimentally, as opposed to experimental or practical thresholds.

85. The current risk assessment paradigm takes one of two approaches: either it is assumed that a biological threshold exists and an experimental NOAEL or BMDL is taken as a dose level at which there is a small response level and is divided by a number of uncertainty or variability factors to derive an acceptable exposure level, or in the case of genotoxic carcinogens and germ cell mutagens it is unsure whether or not a threshold exists and even if a NOAEL is available this simply reflects the limit of detection of the assay and it is considered inappropriate to derive an acceptable exposure level by applying the same methodology used for threshold effects. The latter case leads to risk management measures to remove a substance from the market or, if not possible, to reduce exposure to as low as reasonably achievable.

86. The non-threshold approach for genotoxic carcinogens has its origin in the premise that even one molecule could cause one irreversible mutation which could lead to an eventual malignant tumour.

87. Since hormones act via receptors and response is dependent on hormone (and receptor) concentration, it follows that a certain level of receptor occupancy is required before a response is produced. For exogenous substances which act as receptor agonists, the Group agreed that theoretically one molecule could activate a receptor when adding to the background level of endogenous hormone and so it could be considered that there is no threshold on this level. Some of the Group noted that this does not necessarily imply that one molecule could have an adverse effect by changing a normal response to an abnormal response, while other maintained that it could in principle produce an adverse effect if it occurred early in development by triggering a process leading to a premature change in cell differentiation.

88. Most of the group considered that thresholds of adversity are likely to exist for endocrine disrupters but may be particularly low during fetal development due to the immaturity of homeostatic mechanisms and absence of endocrine feed-back loops or immaturity of toxicokinetic defence/detoxification mechanisms as compared to adults. For these reasons some of the group considered it uncertain whether thresholds will exist for effects on development, while some considered that while thresholds for adversity may exist they may be difficult to identify with confidence based on the currently available standard tests. Others again considered that while thresholds of adversity may be lower in the developing organism and the nature of the effect may be different (severe, permanent changes in the foetus

versus less severe changes in the adult) a threshold must exist and can be identified with appropriate testing involving exposure during development and other sensitive life stages.

EFSA

89. EFSA (2013) noted that in general for toxic effects, homeostatic and cytoprotective mechanisms and the redundancy of cellular targets mean that a certain degree of interaction of the substance with the critical sites or their occupancy must be reached in order to elicit a toxicologically relevant effect. Below that level of interaction, homeostatic mechanisms would be able to counteract any perturbation caused by the exposure and no structural or functional changes would be observed. However, in certain developmental stages homeostatic capacity is limited which will affect the sensitivity of the organism.

90. EFSA also discussed the difference between endocrine modulation and endocrine disruption in terms of a threshold of adversity. Exposure to substances with endocrine activity may activate compensatory feedback mechanisms. If this modulation is temporary or within the homeostatic capacity of the endocrine system then it is not adverse; however, if the body is unable to compensate within its limits of homeostasis (e.g. at high doses or during critical periods of development), the threshold of adversity is crossed. However, a transient endocrine modulation which is simply adaptive in the adult organism can result in permanent adverse changes in the developing organism as the system responsible for the normal endocrine homeostasis of the latter may not yet be fully developed.

91. EFSA considered that it is difficult to propose at present generic criteria to determine a biological threshold between endocrine modulation and adverse effect, and therefore this must be determined at present by expert judgement on a case by case basis. In general, transient, inconsistent and minor fluctuations at the biochemical and molecular level may be considered adaptive (and therefore non-adverse), whilst sustained, consistent and permanent changes at the cell, organ or organism-level, resulting in pathology or functional impairment in vivo, as well as altered timing of development, may be considered adverse.

Danish Centre on Endocrine Disrupters (CeHoS)

92. The Danish Centre on Endocrine Disrupters (CeHoS) published a report in 2019 on the risk assessment of endocrine disrupters, which included the results of two workshops, one primarily of Danish participants and one involving international participants. A previous CeHoS report had concluded that an assumption of no threshold appears more plausible for the effects of endocrine disruptors during development than assumption of existence of a threshold.

93. This CeHoS report considered it difficult to demonstrate a threshold for an endocrine disruptor, but in the event that this was the case proposed that

additional uncertainty factors be applied in establishing a derived no-effect level (DNELs, health-based guidance values set under REACH). Additional uncertainty factors proposed included 2 for severity of effect (examples given were malformations, dystocia (obstructed labour), decreased fertility), 3 if study power is low (if only effect sizes larger than 10% can be identified), 10 where data indicate that non-monotonic dose-response relationships (NMDRs) may produce effects below the NOAEL (there was currently considered little knowledge to guide the application of this uncertainty factor and its magnitude) and 10 where using a LOAEL or $BMDL_{10}$ as the point of departure (a $BMDL_{10}$ was considered to be an effect level, similar to a LOAEL). It was considered that an additional uncertainty factor may also be required in some cases when the point of departure is a NOAEL (cases where a NOAEL might be higher than a BMDL).

94. In cases where a threshold has not been "demonstrated", it was considered that a derived minimal effect level (DMEL) could be established, similarly as for genotoxic carcinogens under REACH. Two approaches as used in REACH for genotoxic carcinogens were considered, a "large assessment factor" approach and a linear extrapolation approach. The former applies an assessment/uncertainty factor of 10,000 to a $BMDL_{10}$ is derived from the approach of considering a margin of exposure of 10,000 or greater for substances which are genotoxic and carcinogenic to be of low concern. The latter approach uses an allometric scaling factor to adjust a $BMDL_{10}$ for toxicokinetic differences between species and then extrapolates down linearly to a risk of 10^{-5} or 10^{-6}. Both approaches were demonstrated in case studies in the report for malformations caused by procymidone and di-2-ethylhexyl phthalate (DEHP).

95. The participants of the workshops considered that it is difficult or impossible to demonstrate a threshold for an endocrine disrupter and therefore recommended a "unified" approach of assuming no threshold, at least as a default. If strong evidence becomes available for the existence of a threshold then a threshold approach can be taken. Participants were divided on whether the "large assessment factor" approach or linear extrapolation should be used. BMDLs should be used where possible as they take into account the power of the data.

Considerations of low dose effects or non-monotonic dose-response relationships

Endocrine Disrupters Expert Advisory Group

96. The Endocrine Disrupters Expert Advisory Group considered "low dose" effects and non-monotonic dose response (NMDR) relationships (JRC, 2013). "Low dose" effects were considered to be effects (potentially adverse) reported at doses well below the NOAEL/NOECs established in conventional regulatory guideline toxicity studies and often at doses within the magnitude of actual or predicted levels of the exposure to the chemical. There was a lack of consensus on the evidence for such low-dose effects. Some members

considered there was sufficient evidence for low-dose effects of endocrine disrupters while others considered that the issue needs to be studied further by performing inter-laboratory comparisons using sensitive test methods before drawing any firm conclusions or incorporating such considerations into regulatory decision-making. If such low-dose effects were substantiated there was general agreement that the use of NOAELs/NOECs from conventional toxicity studies as the points of departure for risk assessments could be problematic and flawed. The Group also noted that the use of a study with greater statistical power and more sensitive endpoints may lead to a lower NOAEL/NOEC. Therefore, the sensitivity of the methods used in combination with adequately sensitive endpoints was of great importance.

97. NMDRs are defined as dose-response relationships which change direction from ascending to descending or vice versa; this can occur at any point in the dose axis. An NMDR may also be a biphasic dose-response. The Group agreed that NMDRs may exist at the pharmacodynamic and molecular level for some substances. Possible mechanisms are known (e.g. receptor saturation) or may be explained by dose-dependent targeting, e.g. dose-dependent activation or inhibition of different receptors or biological target molecules. However, not all of the members were convinced of the evidence and/or relevance of NMDRs for adverse effects at the functional level in vivo. It was suggested that an NMDR for a specific endpoint may be the output of more than one underlying mechanism operating at different dose levels, and resulting in the same endpoint being affected in opposite directions.

98. The implication of NMDRs for conventional testing is the possibility of missing effects below the NOAEL/NOEC. If NMDRs arise they do not affect the existence of a biological threshold but they may affect the ability of conventional testing to identify where a threshold may lie and therefore the ability to determine secure acceptable levels of exposure.

99. The Group considered the limitations in current experimental approaches and possible ways to overcome them. Further research may identify additional endocrine-relevant endpoints to be included in studies, and it was recommended to investigate how in vitro data (e.g. metabolomic/toxicogenomic data) might inform and focus in vivo testing with respect to what might be expected. It was also recommended that specific attention be given to sensitive windows of exposure, including during development and puberty. With respect to low dose effects and NDMRs some members proposed that current experimental designs be reconsidered, to incorporate more dose groups over a wider dose range, with an attempt to cover a more relevant part of the dose-response relationship in relation to anticipated exposure. This may conflict with aims to reduce animal testing. However, it was suggested that using the BMD approach it may be possible to increase the number of dose groups without increasing the number of animals used or at least minimise the additional use of animals implied by extending the dose range.

EFSA

100. EFSA (2013) noted that low dose effects on NMDRs are of intensive debate in the scientific community, mainly due to issues related to reproducibility. It noted the discussion at workshops, including the EFSA colloquium on low dose responses in 2012, which had failed to reach consensus on both low dose effects and non-monotonic dose response relationships. Some participants considered findings of low-dose effects and non-monotonic dose-response relationships plausible and reliable, but no consensus was reached. Therefore, this report could not conclude on whether the current test methods are adequate to fully define dose-response relationships. The available information was also insufficient to conclude that current dose response analysis in regulatory (eco)toxicology should be modified on a routine basis. However, on a case-by-case basis, if triggered by unusual findings, an extended dose response analysis could be performed in a second tier.

CeHoS

101. The CeHoS report considered the potential for NMDRs to be one of the key uncertainties in the risk assessment of endocrine disrupters (CeHoS, 2019). If a threshold approach were taken to risk assessment it was considered that additional uncertainty factors were required for endocrine disrupters. These included a proposed uncertainty factor of 10 in cases where the mode of action of the chemical indicates that lower doses than the point of departure may induce effects through an NMDR.

Considerations of windows of susceptibility

Endocrine Disrupters Expert Advisory Group

102. The Endocrine Disrupters Expert Advisory Group considered the implications of potentially greater sensitivity to hormonal action during critical period of development, e.g. male and female programming windows. The key issue was considered to be the immaturity of the homeostatic mechanisms, the immature metabolism as well as the absence of some endocrine axes during sensitive periods of fetal life (e.g. the HPA axis is not developed during the sensitive window of sexual differentiation). These facts increased the concerns regarding the existence of a threshold and its possibility, if it exists, to be identified with sufficient confidence.

103. Concern was expressed that a small change in hormone levels during development could have permanent serious consequences for the organism. Other members of the group were of the view that a threshold of adversity might be lower in the developing organism, and the consequences may be more severe (e.g. a permanent, but a threshold must exist and can be identified with appropriate testing, involving developmental exposure. Other possibly sensitive life-stages were considered to be puberty, pregnancy and menopause, for which there was considered to be less knowledge. Not

considering these life stages would increase uncertainty in terms of concluding that thresholds exist or reliably identifying thresholds.

EFSA

104. EFSA (2013) noted that in mammals the critical periods of development are conception, pregnancy, infancy, childhood and puberty. In order to avoid missing relevant effects, toxicology testing needs to address recognised periods of sensitivity and endpoint assessment needs to cover all life stages. Some Level 4 and 5 tests in the OECD conceptual framework for testing and assessment of endocrine disrupting substances do cover critical periods of development in utero and in later life stages. Levels 4 and 5 are guideline toxicology tests, including, at Level 5, the two-generation reproductive toxicity study or extended one-generation reproductive toxicity study. However, recent reviews had concluded that current mammalian tests do not cover certain endpoints that might be induced by exposure during fetal or pubertal development but emerge later in life like certain cancers (breast, prostate, testis, ovarian and endometrial) and effects on reproductive senescence.

CeHoS

105. The CeHoS (2019) report considered that the issue of sensitive windows needs to be addressed. If only data from adult exposure are available then an additional uncertainty factor would be needed to compensate for the relative insensitivity of adults compared to exposure in utero and during early postnatal life. An uncertainty factor of 10 had been considered but would not be adequate in all cases.

Summary and overview

106. At the March 2019 COT meeting, Members had noted the differing views of different scientists on whether thresholds could be identified for endocrine disruptors. This was further discussed as part of horizon scanning at the April 2019 meeting, at which the Committee agreed to consider approaches to risk assessment for endocrine disruptors. The Committee requested that all endocrine systems should be considered.

107. The following endocrine systems have been introduced and summarised in this paper:

- hypothalamus-pituitary-adrenocortical (HPA) axis
- hypothalamus-pituitary-gonad (HPG axis)
- somatotrophic axis
- retinoid signalling pathway
- hypothalamus-pituitary-thyroid (HPT) axis
- vitamin D signalling pathway
- peroxisome proliferator-activated receptor signalling pathway
- renin-angiotensin system (RAS)

- endocrine pancreas signalling

108. However, most knowledge on endocrine disruption has been obtained for substances which affect oestrogen, androgen, or thyroid hormone systems or affect steroidogenesis in vertebrates ("EATS" modalities), and thus the HPG and HPT axes. There are, as yet, no standardised mechanistic assays to detect the non-EATS modalities. However, the downstream effects of disruption of these endocrine pathways are probably detectable in some of the apical studies.

109. Endocrine disruptors are defined by WHO/IPCS as 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 in (sub)populations (WHO/IPCS, 2002), and EFSA has also accepted this definition (EFSA, 2013). Based on this, endocrine disruptors can be identified by three criteria: i) the presence of an adverse effect in an intact organism or (sub)population, ii) the presence of an endocrine activity, and iii) a plausible or demonstrated causal relationship between the endocrine activity and the adverse effect. In terms of demonstrating a plausible or causal relationship between endocrine activity and the adverse effect, EFSA considered that evidence for this relationship should be obtained from studies in the OECD Conceptual Framework (CF) for the testing and assessment of endocrine disruptors or from other investigations and assessed on a WoE basis.

110. A number of published papers, meetings and reports have considered whether thresholds exist for endocrine disruptors. It has been widely agreed that the presence of absence of a threshold can never be proven experimentally since all experiments have a limit of detection below which effects cannot be observed. Therefore, conclusions on whether a threshold or non-threshold approach should be taken to risk assessment are based on considerations of the mechanism.

111. The views of different scientists have differed with regard to the presence of thresholds for endocrine disruptors. Some have made the case of "additivity-to-background," whereby theoretically one molecule could activate a receptor when adding to the background level of an endogenous hormone, so it could be considered that there is no threshold, while others have argued that this does not necessarily mean that an adverse response would result. One argument has been that the initial interaction of a substance with a biological target is unlikely to have a threshold, but that the complexity of biological systems makes non-threshold dose-response relationships unlikely for higher endpoints such as behaviour, reproduction, organ weights and growth.

112. There was general agreement that fetal development would be particularly susceptible to endocrine disruption due to the immaturity of homeostatic mechanisms and absence of endocrine feedback loops. Some scientists considered that it is uncertain whether thresholds will exist during

development, and that if they do they would be difficult to identify using standard tests, while others considered that thresholds may be lower, and the consequences of endocrine disruption more severe following exposure during development but that thresholds will exist and can be identified by appropriate testing which involves exposure during development and other sensitive life stages.

113. EFSA (2013) discussed the difference between endocrine modulation and endocrine disruption in terms of a threshold of adversity, and noted that if modulation is temporary or within homeostatic capacity of the endocrine system it is not adverse. However, a transient endocrine modulation which is simply adaptive in an adult can result in permanent adverse changes in the developing organism as the system responsible for normal endocrine homeostasis may not yet be fully developed.

114. There is a lack of consensus on the existence of low-dose effects (below NOAELs/BMDLs from regulatory studies) and NMDRs. EFSA were unable to conclude on whether current dose response analysis in regulatory toxicology studies should be modified on a routine basis. However, they concluded that on a case-by-case basis, if triggered by unusual findings, an extended dose response analysis could be performed in a second tier. The CeHoS report proposed that, if a threshold-based approach to risk assessment is taken, an additional uncertainty factor of 10 be applied in cases where the mode of action of the chemical indicates that lower doses than the point of departure may induce effects through an NMDR.

115. Potentially sensitive life stages were generally identified as being conception, pregnancy, infancy, puberty and menopause. Not considering these life stages would increase uncertainty in terms of concluding that thresholds exist or reliably identifying thresholds. Some studies do cover critical periods of development *in utero* and in later life stages, including the two-generation reproductive toxicity study and extended one-generation reproductive toxicity study. However, current tests do not cover certain endpoints that might be induced by exposure during fetal or pubertal development but emerge later in life like certain cancers (breast, prostate, testis, ovarian and endometrial) and effects on reproductive senescence.

116. One aspect of risk assessment that has not been discussed in this scoping paper is mixtures, since, as noted by EFSA (2013), this is not specific to endocrine disruptors. However, Members may wish to note if there are any unique aspects to risk assessment of mixtures for endocrine disruptors that should be considered.

<u>Questions on which the views of the Committee are sought</u>

117. Members are invited to provide general comments on the information in this scoping paper and to consider the following questions:

i). Can any conclusions be drawn from the information in this paper on:

a) The existence or otherwise of thresholds for endocrine disruptors?

b) The implications of non-monotonic dose-response relationships for risk assessment?

c) The extent to which critical windows of susceptibility are addressed in risk assessments of endocrine disruptors?

ii). Does the Committee still consider that a COT subgroup should be formed to consider the topic of risk assessment for endocrine disruptors?

ii). *If yes*, what specific aspects should the subgroup consider and advise on? And what expertise should be included on the subgroup?

Secretariat
August 2019

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Acronyms and abbreviations

AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AND	Androstenedione
Ang I	Angiotensin I
Ang II	Angiotensin II
Apo	Apolipoprotein
AR	Androgen receptor
AT1	Angiotensin receptor type 1
AT1A	Angiotensin receptor type 1A
AT1B	Angiotensin receptor type 1B
AT2	Angiotensin receptor type 2
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
cAMP	Cyclic adenosine monophosphate
CDCA	Chenodeoxycholic acid
CF	Conceptual framework
CG	Chorionic gonadotropin
CNS	Central nervous system
CREM	cAMP-responsive element modulator
CRH	Corticotropin-releasing hormone
DAG	Diacylglycerol
DES	Diethylstilbeoestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DUOX/ThOX	Dual oxidase/thyroid oxidase
EATS	Oestrogen, androgen, thyroid and steroidogenic
Epac2	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
FSH	Follicle-stimulating hormone
FXR	Farnesoid X receptor
GHRH	Growth hormone releasing hormone
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GnRH	Gonadotropin-releasing hormone
GPR30	G-protein-coupled receptor 30
GR1	Glucocorticoid receptor 1, often called the mineralocorticoid receptor
G_q	A type of G-protein
G_s	A type of G-protein
GSIS	Glucose-stimulated insulin secretion
HDL	High density lipoprotein
HHPS	Hypothalamo-hypophysial portal system
HPA axis	Hypothalmus-pituitary-adrenocortical axis
HPG axis	Hypothalamus-pituitary-gonal axis

HPT axis	Hypothalamus-pituitary-thyroid axis
NGF	Nerve growth factor
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IL-6	Interleukin-6
IP3	1,4,5-triphosphate
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JG	Juxtaglomerular
LBD	Ligand binding domain
LCA	Lithocholic acid
LH	Luteinising hormone
MCT	Monocarboxylate transporter
mPR	Membrane G-protein-coupled gestagen receptor
MT1	Melatonin receptor 1
MT2	Melatonin receptor 2
mTOR	Mammalian target of rapamycin
NIS	Sodium-iodide symporter
NMDR	Non-monotonic dose response
OATP	Organic anion transport protein
PACAP	Pituitary adenylate cyclase-activating peptide
Pax8	Paired box gene 8
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzofurans
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanane sulphonate
PKA	Phosphokinase A
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator activated receptor
PR-A	Progesterone receptor A
PR-B	Progesterone receptor B
PTH	Parathyroid hormone
PXR	Pregnane X receptor
(Q)SAR	(Quantitative) structure-activity relationship
RAR	Retinoic acid receptor
RAS	Renin-angiotensin system
rT3	Reduced T3
RXR	Retinoid X receptor
SUMO	Small ubiquitin-related modifier
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroid binding globulin
TCDD	Tetrachlorodibenzo-p-dioxin
TG	Test Guideline (OECD)
THα	A form of the thyroid receptor
THβ1	A form of the thyroid receptor
THβ2	A form of the thyroid receptor
TLQP-21	A 21 residue neuroendocrine VGF-derived peptide

TPO	Thyroid peroxidase
TR	Thyroid hormone receptor
TRH	Thyrotropin-releasing hormone
TRHR	Thyrotropin-releasing hormone receptor
TTF1	Transcription termination factor 1
TTF2	Transcription termination factor 2
TTR	Transthyretin
TSH	Thyroid stimulating hormone
VDBP	Vitamin D binding protein
VDCC	Voltage-dependent calcium channel
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VGF	Nerve growth factor inducible
VLDL	Very low density lipoprotein
WFS1	Wolfram syndrome 1
WoE	Weight of evidence
ZF	Zona fasciculata
ZG	Zona glomerulosa