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Using alternative test methods to predict endocrine disruption and reproductive adverse outcomes: do we have enough knowledge?☆

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ABSTRACT

Endocrine disrupting chemicals (EDCs) are a matter of great concern. They are ubiquitous in the environment, are considered harmful to humans and wildlife, yet remain challenging to identify based on current international test guidelines and regulatory frameworks. For a compound to be identified as an EDC within the EU regulatory system, a plausible link between an endocrine mode-of-action and an adverse effect outcome in an intact organism must be established. This requires in-depth knowledge about molecular pathways regulating normal development and function in animals and humans in order to elucidate causes for disease. Although our knowledge about the role of the endocrine system in animal development and function is substantial, it remains challenging to predict endocrine-related disease outcomes in intact animals based on non-animal test data. A main reason for this is that our knowledge about mechanism-of-action are still lacking for essential causal components, coupled with the sizeable challenge of mimicking the complex multi-organ endocrine system by methodological reductionism. Herein, we highlight this challenge by drawing examples from male reproductive toxicity, which is an area that has been at the forefront of EDC research since its inception. We discuss the importance of increased focus on characterizing mechanism-of-action for EDC-induced adverse health effects. This is so we can design more robust and reliable testing strategies using non-animal test methods for predictive toxicology; both to improve chemical risk assessment in general, but also to allow for considerable reduction and replacement of animal experiments in chemicals testing of the 21st Century.

1. Introduction

Modern toxicology is dealing with the prospect of better safeguarding human health against harmful chemicals and at the same time reduce the use of animals for chemical testing (Pistollato et al., 2021). Concerning endocrine disrupting chemicals (EDCs), this remains challenging since, in many instances, we do not understand sufficiently the causal link between chemical interactions with biomolecules and effect outcomes in intact organisms. When relying on non-animal test methods to predict *in vivo* effect outcomes, *a priori* knowledge about causal relationships between what is being measured (e.g. *in vitro* or *in silico*) and the adverse outcomes (*in vivo*) should be robust; and herein lies one of the big challenges with replacing animal testing with alternative test methods. It can be difficult to predict, with reasonable accuracy, the potential adverse effects a chemical can have on human health unless we are sure we use the right alternative methods and interpret the data appropriately. Thus, there is a strong need to establish solid mechanistic knowledge of causal relationships to enable the use of correct alternative test methods for testing purposes.

Although many mechanisms-/modes-of-action have been established for endocrine disruption, it remains difficult to predict *in vivo* outcomes from alternative methods data with high precision. The reasons for this are many and obviously include complex toxicokinetic parameters (absorption, distribution, metabolism and excretion; ADME), but also sensitive windows of exposure, species-to-species differences, sex differences, quantitative understanding of dose-response relationships and so forth. It likely also includes additional mechanisms-of-action than those that are typically tested for. Currently, the most common modes-

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of-action that are considered when assessing whether a chemical has endocrine disrupting (ED) properties are estrogen, androgen, thyroid, and steroidogenesis, so-called EATS modalities (EFSA/ECHA et al., 2018; Kucheryavenko et al., 2020). It is recognized that disruption to other, non-EATS, modalities can cause adverse effects by an endocrine mode-of-action (EFSA/ECHA et al., 2018; Grignard et al., 2020; Martyniuk et al., 2021); however, the EATS modalities are the pathways for which we have the best mechanistic and causal knowledge with respect to adverse outcomes caused by endocrine disruption. They are also the modalities, or pathways, for which we have some standardized test guidelines for *in vitro* and *in vivo* testing (EFSA/ECHA et al., 2018).

This review aims to illustrate the importance of further characterizing mechanism-of-action for predicting ED properties, and we will exemplify this by focusing on male reproductive toxicity. This is because ED-related male reproductive toxicity has a long history of research with much available knowledge, as well as there being several established international test methods that are included in the international test guideline (TG) program overseen by the Organisation for Economic Co-operation and Development (OECD). As such, male reproductive toxicity can serve as an informed case, but also highlight similar challenges met for other ED modalities.

2. What defines an EDC?

Although the definition of an EDC is assumed by its descriptive naming – a chemical that can disrupt the endocrine system – it is in fact somewhat difficult to define and there is no universal agreed-upon definition(s). The most widely accepted (Solecki et al., 2017), and the one adhered to in this review, is the definition by the World Health Organization (WHO/IPCS, 2002) and the European Commission's criteria for 'endocrine disruption' applicable to pesticides and biocides, defined by the European Commission (EC, 2017; ECHA/EFSA, 2018). Here, a substance must fulfil all of three criteria to be considered an EDC:

i) it should induce an adverse effect in an intact organism or its progeny;
ii) it should do so by an endocrine mode-of-action, i.e. it alters the function(s) of the endocrine system; and
iii) the adverse effect should be a plausible consequence of an endocrine mode-of-action.

With respect to the first criterion, it is worth noting that "an intact organism" may not have to be an experimental animal. As discussed in a 2017 consensus statement on the identification of EDCs, the term "intact organisms" refers to an *in vivo* situation, which would include epidemiological and clinical observations in humans in addition to experimental animals (Solecki et al., 2017). The authors of the consensus paper also argue that it could include evidence from adequately validated alternative test systems that are predictive of effects in humans or wildlife, or from surgically or genetically modified animals included in focused experiments. Albeit, there is currently no final agreed-upon consensus on what constitute an 'intact organism' and, as recently discussed, the terminology 'intact animal' is not defined by the IPSC (Vandenberg, 2021). Thus, whether surgically altered or genetically modified animals can be regarded as 'intact organisms' is still under debate and should be better clarified by entities such as the IPSC. .

Regardless, with the aforementioned three criteria it becomes obvious that it is not sufficient for a chemical to disrupt endocrine signaling to be regarded an EDC. Nor is it sufficient to cause *in vivo* adverse outcomes that could be considered to originate from endocrine disruption. For this latter point to hold true, a biologically plausible link should also be established between the adverse outcome and an endocrine mode-of-action. Hence, mechanistic knowledge becomes very important; not only to establish a plausible link between endocrine modes-of-action and apical effect outcomes, but also to ensure that chemical substances are tested using appropriate alternative test methods to provide the required data for chemical hazard and risk assessments.

3. Test methods used for assessment of endocrine disruption relevant for male reproductive toxicity

Adverse effects on the reproductive system characteristically occur after exposure to chemicals that affect either estrogen- or androgen signaling, or the synthesis of the steroid hormones (i.e. EAS modalities). Correct levels and action of steroid hormones are important for proper development and function of both the male and the female reproductive system, thus exposure to compounds that cause endocrine disruption can lead to a range of effects on the reproductive system in both sexes (EFSA/ECHA et al., 2018; OECD, 2018b). In males, androgen signaling is required for development of the male sexual phenotype, including the reproductive system. If androgen action is inhibited during critical stages of development, the male fetus will not fully masculinize (Jorgensen et al., 2021; Welsh et al., 2008). In turn, this failure to fully masculinize can lead to various reproductive disorders that manifest either at birth or later in life, such as those included in the testicular dysgenesis syndrome (TDS): hypospadias and cryptorchidism (birth disorders); infertility and testicular cancers (late life disease) (Skakkebaek et al., 2016).

Regarding male reproductive development, a number of *in vivo* endpoints can be measured in rodent toxicity studies (OECD, 2018b), as summarized in Table 1. For instance, the anogenital distance (AGD) and nipple retention (NR) are considered biomarkers for masculinization, where both a short male AGD and NR in male offspring indicate demasculinization caused by incomplete fetal androgen action. Both effect biomarkers are often affected after prenatal exposure to anti-androgenic chemicals. So too are malformations such as hypospadias and cryptorchidism, which are both linked to perturbed androgen action, or testis function, during fetal life and these malformations are evident at birth. Fetal androgen disruption can also have consequences into adult life, not least manifesting with effects on sperm parameters. These, and other relevant endpoints that are included in OECD Test Guidelines, are listed in Table 1.

In addition to the adverse effect endpoints listed in Table 1, several additional *in vivo* endpoints can be assessed in order to obtain ED-relevant information related to test chemicals. These endpoints are not apical endpoints, but rather *in vivo* mechanistic endpoints that may provide important knowledge on modes-of-action regarding the observed adversity. As listed in Table 2, they include measurements of steroid sex hormones, as well as luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the serum, intra-testicular testosterone levels and transcriptional changes in target organs. None of these endpoints are mandatory in OECD test guidelines, and they are therefore primarily assessed in *invivo* studies from academia; however, they could be included in some of the TGs on a case-by-case basis.

Alternative test methods are constantly being developed to reduce the need for animal toxicity testing of chemical substances (Parish et al., 2020). Approaches include various *in vitro* assays, but also *in silico* methods such as physiologically-based toxicokinetic (PBTK) models and quantitative structure-activity relationship (QSAR) models (Judson et al., 2018). A great advantage of these alternative methods is their potential for high-throughput and relative low cost compared to extensive animal toxicity studies, ultimately allowing for the screening of thousands of chemicals within a reasonable timeframe. In turn, data from alternative methods can be used to prioritize compounds for *in vivo* toxicity testing, but also provide evidence for ED modes-of-action and contribute to weight of evidence in chemical risk assessments.

Currently, if using *in vitro* assays to screen for potential ED effects through the A modality, one needs to include all mechanisms that are relevant (and essential) to cause apical effect endpoints as listed in Table 1. In the case of androgen signaling, there are validated *in vitro* test guidelines under OECD (Table 3) that can be used to perform mode-of-

Table 1
Apical endpoints included in rodent toxicity studies designed to evaluate reproductive toxicity and potential ED-related male reproductive effects.

Male			
Endpoint	Life stage for investigation	Description	Endpoint included in OECD TG no.*
Anogenital distance (AGD)	Neonatally (PND 1–4) or gestational (GD 21–22 at caesarian section in TG 414)	The AGD in male rats and mice is typically twice as long as in females. A shorter male AGD indicates incomplete masculinization through inadequate androgen action during late gestation (Schwartz et al., 2019). Note: in TG 416 (the two generation study) measurement of AGD is included as an optional endpoint and is only measured in the second generation (F2 pups) if it is triggered by alterations in first generation (F1) sex ratio or timing of sexual maturation	**TG 414, TGs 421/ 422, TG 443, TG 416**
Nipple/areolae retention (NR)	Neonatally (PND 12–14) when visible in female littermates	In common laboratory rodents, the males do not have nipples, while females typically have 10 (mice) or 12 (rats). In males, nipples regress due to androgen signaling during late gestation. Thus, when males express nipples, it indicates incomplete masculinization (Schwartz et al., 2021).	**TGs 421/ 422, TG 443**
Hypospadias (misplacement of urethral opening of the penis) and cryptorchidism (undescended testicles).	Neonatally, pre-puberty, puberty & adulthood or gestational (GD 21–22 at caesarian section in TG 414)	Genital malformations such as cryptorchidism and hypospadias are linked to disrupted androgen signaling and potentially also androgen-estrogen imbalance in fetal life (Welsh et al., 2008) (Mattiske & Pask, 2021). Both malformations can be assessed in the male offspring shortly after birth and around PND 16, but hypospadias are more easily assessed after sexual maturation. In fetuses an indication of incomplete testicular descent/cryptorchidism and observation of external and internal (gonadal) sex can be assessed. Note: in TG 414 this is done in fetuses after caesarian section and in TG 416 only one pup per litter is examined for these malformations	**TG 421/ 422, TG 443, TG 414 TG 416**
Preputial separation	Puberty, male rats is examined daily for preputial separation from around 30 days of age. This consists of attempts to manually retract the prepuce with gentle pressure (Korenbrot et al., 1977).	The timing of sexual maturation is controlled by steroid hormones and can therefore be affected by exposure to EDCs. Effects may occur after exposure during perinatal life but will most likely be more marked if the exposure continues post-weaning and during the pre-pubertal and pubertal period.	**TG 416, TG 443, TG 426**
Weight of testes, prostate, epididymis, seminal vesicles and the levator ani plus bulbocavernosus (LABC) muscles.	Neonatally, pre-puberty, puberty, adulthood	Absolute and relative weights of these male reproductive organs are sensitive to androgen signaling. Anti-androgenic EDCs will typically result in decreased weights, but the effects can differ depending on the age of the animals, the examined doses, and the endocrine mechanisms of the investigated compounds. Note: In several of the TGs some reproductive organs are only optional to weigh at the selected ages	**TG 441, TG 407, TG 408, TG 416, TGs 421/ 422, TG 443**
Histological examination of male reproductive organs	Neonatally, pre-puberty, puberty, adulthood	Exposure to EDCs (especially anti-androgens) will typically cause degeneration of spermatogonia or the testis, as well as Leydig cell hyperplasia/ hypertrophy (OECD, 2018a). The effect on reproductive organ histology may differ depending on the age of the animals, the examined doses and the endocrine mechanisms of the investigated compounds. Note: in some TGs histological examination of male reproductive organs is included as optional	**TG 407, TG 408, TG 416, TG 422, TG 443, TG 451**
Sperm quality (sperm count, motility, and morphology).	Adulthood	Exposure to endocrine disrupting chemicals can affect sperm quality. Adverse effects may manifest after exposure to EDCs during perinatal life but will most likely be more marked if exposure continues throughout puberty and adulthood.	**TG 408, TG 416, TG 443**

Gestation day (GD), Postnatal day (PND), Test guideline (TG), Second generation (F2) * overview of the TG numbers can be accessed at https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.

Table 2
Mechanistic endpoints that are occasionally assessed in rodent reproductive toxicity studies. These endpoints are only optional in current OECD Test Guidelines. Consequently, they are assessed far less frequently than the adverse effect endpoints.

Endpoint	Life stage for investigation	Description
Changes in testosterone (male), estradiol (female) and progesterone (female) concentrations in serum	Pre- and neonatally, in pre-puberty, puberty & adulthood	Concentrations of steroid hormones in serum can be affected by EDC exposure. The magnitude and direction of the changes depend on the EDC being investigated, their doses, modes-of-action, as well as sex and age of the animals being assessed.
Changes in testosterone production in the testes	Neonatally	Testosterone production in fetal testes can be markedly decreased after prenatal exposure to EDC. Testosterone can be measured in both serum and in the testes.
Changes in LH & FSH concentrations in serum/tissues	Neonatally, pre-puberty, puberty, adulthood	In rodent studies, changes in gonadotrophin secretion would be measured postnatally after activation of the HPG axis. Both LH and FSH can be measured in serum and inform on systemic dysregulation.
Changes in gene expression in reproductive organs, pituitary or hypothalamus	Neonatally, pre-puberty, puberty, adulthood	Transcriptional analysis can be performed in all relevant targets and tissues to characterize potential endocrine-mediated changes to gene transcription.

Luteinizing hormone (LH), Follicle stimulating hormone (FSH), hypothalamic-pituitary-gonadal (HPG) axis.

action analysis in chemical risk assessment. The OECD validated TGs cover only a subset of relevant endpoints, and several supplementary and alternative methods to test for other modes-of-action important for male reproductive development and function are available. For example, androgen receptor (AR) binding assay, AR translocation assay, coregulatory recruitment (Kleinstreuer et al., 2017; Lynch et al., 2017), aromatase (CYP19A1) inhibition (CompTox_Chemicals_Dashboard) and 5α-reductase inhibition (Kim et al., 2021; Srivilai et al., 2016; Zheng et al., 2020) are available; albeit, they remain to be validated under OECD.

4. Challenges in predicting *in vivo* effect outcomes from non-animal test method data

Although alternative test methods are essential for both current and future testing of chemical substances, they also have certain shortcomings that should be carefully considered. Prominently, it is difficult to mimic the complexity of higher order animals using cell-based assays. This includes factors such as ADME, cell-cell and cell-organ interactions, local and systemic feedback mechanisms, but also temporal aspects. In living organisms, not least during development, molecules and cells can have different functions at different life stages. Theoretically this can be modelled by alternative methods, but it requires that we know what to model in the first place. And for this, we need a solid fundamental understanding of mechanisms-of-action.

Even with the well-characterized androgen signaling pathway in male reproductive development, predicting *in vivo* effects from *in vitro* data can be challenging. Several studies have shown that, unfortunately, *in vitro* assays do not always faithfully predict adverse *in vivo* effects. As

Table 3
Validated *in vitro* OECD test guidelines to test ED effects by EAS modalities.

Endpoint	Assay name	Description	OECD TG
Activation or inhibition of reporter gene linked to AR	Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals	Three validated methods for testing the ability of chemicals to bind to AR and lead to changes in androgen responsive genes in different cell lines: - AR-EcoScreen™ cell line - AR-CALUX cell line - 22Rv1/MMTV_GR-KO cell line All cell lines are stably transfected with an AR-responsive luciferase reporter gene.	**TG 458**
Disruption of steroidogenesis/ Altered estrogen and testosterone levels	H295R Steroidogenesis assay	One validated method for testing the ability of chemicals to affect 17β-estradiol and testosterone production in a human adrenocarcinoma cell line.	**TG 456**
Binding to ERs	Performance-based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity	Two validated methods for testing the ability of chemicals to bind to ERα. - Freyberger-Wilson *In vitro* ER binding assay based on Full length human recombinant ERα - Chemical Evaluation and Research Institute (CERI) *In vitro* ER binding assay based on Human recombinant Ligand binding domain protein	**TG 493**
Activation or inhibition of reporter gene linked to ER	Performance-based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists (ER STTA)	Two validated methods to detect substances that bind hER leading to transcription of estrogen responsive genes using different cell lines: - Stably transfected TA assay using the (h) ERα-HeLa-9903 cell line - VM7Luc ER STTA assay using a variant of MCF7 cell line Both human cell lines stably transfected with luciferase as reporter gene.	**TG 455**

Androgen receptor (AR), Estrogen receptor (ER).

depicted in Fig. 1, the androgen signaling pathway – simply put – involves several key steps, starting with testosterone synthesis by the fetal testes. After secretion, testosterone can act directly on the AR, or be converted locally to the more potent AR ligand dihydrotestosterone (DHT) to ensure masculinization of target tissues. These key steps can be measured by *in vitro* assays in order to test the potential of chemical substances to interfere with either of these processes. But even when *in vitro* studies show potent inhibition of testosterone synthesis, potent inhibition in DHT formation or potent inhibition of AR activity, we do not always observe adverse effects *in vivo*. Similarly, when we do observe adverse effects, the *in vivo* potency of chemicals can be difficult to ascertain, since studies sometimes show great variability in effect

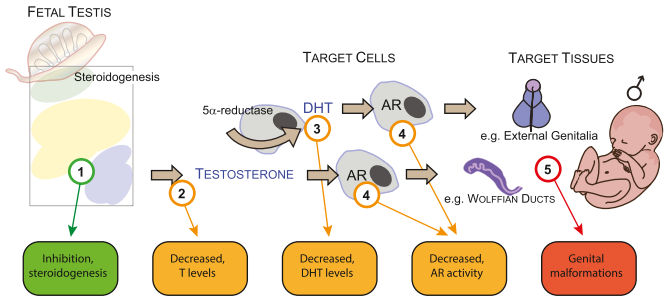

Fig. 1. The canonical androgen signaling pathway involved in male reproductive development and its vulnerability to endocrine disruption. During development, Leydig cells of the fetal testes produce testosterone by steroidogenesis. Testosterone is released into circulation and act as ligand for the androgen receptor (AR) directly in target tissues located close to the testes (e.g. Wolffian ducts). In more distant tissues (e.g. external genitalia), testosterone can be converted to the more potent AR ligand dihydrotestosterone (DHT) by the catabolizing enzyme 5α-reductase, which induces androgen-sensitive tissues to differentiate into male phenotypes. Chemicals with anti-androgenic potentials can disrupt androgen signaling at several key steps of the pathway, ultimately leading to the same adverse outcomes.

doses on same adverse effect endpoints, as for instance for the androgen-sensitive endpoints AGD and NR as previously reviewed (Schwartz et al., 2021; Schwartz et al., 2019).

To exemplify further; two recent studies clearly illustrate how *in vitro* potency for AR antagonism can fail to predict *in vivo* outcomes. In the first study, data from more than 250 studies were used to test the ability to use ED_{50} (median effective dose) values for *in vitro* AR antagonism to predict *in vivo* Hershberger assay outcomes or effects on AGD in male rats (Gray et al., 2020). Overall, the predictive power of *in vitro* AR values proved poor (less than 5% accuracy), whereas predictions from Hershberger ED50 values to male AGD had better predictive power (around 85% accuracy). The second study, from the same research team, compared the *in vitro* and *in vivo* effects of pyrifluquinazon (PFQ) and bisphenol C (BPC) as related to AR antagonism and male reproductive malformations, including AGD and NR (Gray et al., 2019). PFQ displayed weak AR antagonism *in vitro,* but induced marked effects *in vivo* with a very potent response on NR (9–10 nipples) and AGD (decreased by 33%). BPC, on the other hand, displayed strong AR antagonism *in vitro*, but induced no significant effects on either NR or AGD. The authors propose that the incorporation of toxicokinetic and toxicodynamic data into quantitative *in vitro* to *in vivo* extrapolations (QIVIVE) could improve on the predictive power of *in vitro* data, not least by improving pregnancy IVIVE models to predict fetal concentrations (Gray et al., 2019; Gray et al., 2020). And indeed, this has been shown in a separate study using data from several *in utero* rat toxicity studies on anti-androgenic chemicals. By incorporating physiologically-based toxicokinetic (PBTK) modeling, actual fetal exposure levels could be predicted with relatively high accuracy, as could a short male AGD for certain chemicals (Scholze et al., 2020). Notably, however, these improved models also failed in some instances in male AGD predictions, highlighting the fact that predictive models still need improvements despite our good basic knowledge about how androgen signaling is controlling male reproductive development. Clearly, we have some way to go before we can safely predict complex *in vivo* adverse effects from alternative test method data alone, but we are moving in the right direction.

The case of AGD can also be used to illustrate the importance of delineating spatiotemporal knowledge before devising alternative test method strategies. As already mentioned, a shortened AGD is a broad, retrospective biomarker for incomplete androgen action during fetal development and can be induced by different mechanisms-of-action and, importantly, is associated with several other male reproductive disorders (Schwartz et al., 2019; Welsh et al., 2008). As illustrated in Fig. 1, some chemicals can induce male reproductive disorders (including a short AGD) by perturbing testosterone synthesis by the fetal testes. However, as mentioned above, chemical perturbation can also occur by other mechanisms. For instance, DHT synthesis from testosterone can be blocked in target cells by chemicals inhibiting 5α-reductase activity or by directly antagonizing the AR. Alternative test methods must therefore cover all possible mechanisms underpinning the same, or related, adverse outcomes. So how does this extend to developing new test methods or strategies for adverse male reproductive effects?

When looking for new biomarkers of effects based on mechanistic knowledge, and using these to develop novel test assays, spatiotemporality must be carefully considered. Phthalates and other compounds that reduce testosterone synthesis in rat fetuses and subsequently cause adverse outcomes such as short AGD have been reported to leave a specific transcriptional footprint in the fetal testes (Gray et al., 2021; Hannas et al., 2011). In the correct context, such a footprint could potentially be used to scrutinize the ability of chemicals to inhibit testosterone synthesis and cause male reproductive disorders using, for instance, cell assays or organoids. However, other chemicals inducing male reproductive disorders do not necessarily leave the same transcriptional footprint in the testes, as they may interfere with androgen signaling at different steps of the pathway. For instance, the fungicide triticonazole can both inhibit steroidogenesis and AR activity, and fetal exposure leads to a short male AGD (Draskau et al., 2019). However, when analyzing the transcriptome of the fetal testes, very few changes are observed (Draskau et al., 2021) and thus triticonazole would not have been flagged as potentially harmful if relying on transcriptional changes to the fetal testis. When analyzing the transcriptome of the androgen-sensitive genital tissues, however, marked changes to the transcriptome is observed (Draskau et al., in preparation) This is just a single case to exemplify the importance of looking at the right place at the right time when elucidating mechanisms-of-action for apical *in vivo* endpoints and using that knowledge to develop simplified alternative methods.

Another prevailing challenge with predicting *in vivo* effect outcomes caused by EDCs is the potential involvement of non-EATS modalities in the induction of ED effects. This potential gap in knowledge and test assays was highlighted by the OECD already a decade ago (OECD, 2012) and recently reiterated in the context of developing robust testing strategies with stronger reliance on alternative test methods (Martyniuk et al., 2021). But despite this, our understanding of how non-EATS modalities may contribute remains very limited, not least regarding chemical substances evaluated under current EDC guidance document, as for instance those regulated under the European Union's REACH regulation. There are several potential non-EATS modalities and seven have been suggested to be of particular relevance to ED effects, including vitamin D, peroxisome proliferator-activated receptor (PPAR) signaling and retinoid signaling (OECD, 2012).

The retinoid signaling pathway has received increased attention within chemical risk assessment and regulation in recent years, with emerging evidence suggesting the pathway to be vulnerable to endocrine disruptors and a likely contributor to human endocrine disorders (Grignard et al., 2020; Knudsen et al., 2020; Martyniuk et al., 2021; OECD, 2012). Retinoid signaling is essential for normal development and is involved in numerous processes from axial patterning to organogenesis and reproductive development. For instance, in the gonads (testes and ovaries) of mice, the presence of retinoic acid (RA) directs the timely differentiation of germ cells by regulating meiotic entry (Spiller & Bowles, 2019). RA may also be involved in somatic development in the fetal testis, with the presence of ectopic RA preventing the production of essential masculinization factors such as anti-Müllerian hormone (AMH) and insulin-like factor 3 (INSL3) that instruct male reproductive system development, as well as steroidogenic enzymes required for testosterone synthesis (Bowles et al., 2018). Data from humans are still sparse, but RA appears to also be involved in proper development of the human testis

(Jørgensen et al., 2015). This is only one example of how non-EATS modalities can potentially contribute to adverse outcomes. Thus, any robust testing strategy relying chiefly on non-animal test data may also have to include relevant non-EATS modalities; modalities that are yet to be established for the majority of ED-related endpoints.

5. Using the adverse outcome pathway framework to aid in ED assessments of chemicals

As mentioned in section 2, the testing and assessment of chemicals for ED properties pose the challenge of establishing a plausible causal link between adverse outcomes and endocrine modes-of-action. This criterion can inflict a high workload for risk assessors, as the amount of information to be collected and analyzed can be very extensive. Building cases for plausible causal relationships also requires expert knowledge within specialized fields of research, something that can be very challenging for risk assessors that are operating across many different biological fields. To address this, and related issues, the adverse outcome pathway (AOP) framework is a promising tool that could greatly aid risk assessors, as it represents a pragmatic instrument for chemical safety assessment that can maximize the use of existing knowledge and minimalize dependence on resource-intensive testing approaches (Ankley et al., 2010). The AOP concept also aligns well with the criteria for ED identification, not least since it provides biologically plausible links between upstream events and apical adverse effect outcomes in intact organisms.

Essentially, an AOP describes a sequence of events from initiation stressor, or molecular initiating event (MIE), to an adverse outcome (AO) in an intact organism (Ankley & Edwards, 2018; OECD, 2018c). All intermediate steps between the MIE and AO are understood as key events (KEs) and only events that are essential to progress down the causal pathway should be included (OECD, 2018c; Villeneuve et al., 2014). Finally, these individual 'events' (MIE, KE, AO) are linked by key event relationships (KERs), which are units of knowledge from which causality is inferred (Svingen et al., 2021; Villeneuve et al., 2014). This way, an AOP spans multiple levels of biological organization and the content depends on the extent of biological information existing for the specific AOP. Hence, the AOP concept provides an almost fit-for-purpose framework for ED assessments as it i) defines an AO in an intact organisms, ii) it describes a mode-of-action, and iii) it establishes a biologically plausible link between the mode-of-action and the AO (Audouze et al., 2021).

Another advantage of the AOP framework is that it can help identify where we need to develop or improve non-animal test methods relevant for chemical testing and assessment. AOPs can be sufficiently described so that early KEs can be used with more certainty in predicting apical effect endpoints relevant for chemical regulation (Ankley & Edwards, 2018). In relation to human health risk assessment, AOPs can aid in the use of relatively simple screening tests to support association with adverse health effects and thereby lead to faster and more efficient evaluations of chemical substances as it potentially can avoid the need for resource-demanding animal experiments and instead use existing knowledge to leapfrog data gaps to infer causality. Finally, AOPs are also envisioned to help with mixture risk assessment, as they can be used for grouping of substances for which the available information is incomplete, as recently shown with a rat study on mixtures of EDCs with estrogenic or anti-androgenic modes-of-action (Christiansen et al., 2020).

6. Current guidance for assessing a substance for having ED properties

The reason for elucidating mechanisms-of-action, or developing alternative test methods or AOPs, is to aid in chemical risk assessment and, by so doing, better safeguard humans or the environment from harmful effects caused by exposure. This means that such endeavors only hold real value if they are implemented in international regulatory frameworks. Thus, before concluding, we will briefly touch on the current situation for testing and regulating EDCs. The fact is that EDCs are currently regulated within the European Union and many other countries, yet there is still an ongoing debate on how to best assess and possibly classify such substances. Also, agencies and industries rely on internationally recognized OECD test guidelines for implementing regulation, for instance those mentioned in previous section. Guidance documents describing how to perform these guidelines are available; for evaluating if a substance has ED properties as they relate to the aforementioned EATS modalities, the *Guidance Document 150 on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption* is the most relevant resource (OECD, 2018b).

In 2018, the EU Commission published criteria to evaluate ED properties of biocidal and plant protection products (BP/PPPs) with the aim to reduce human exposure to EDCs. Within the EU, all BP/PPPs now require, by law, to be assessed for their potential ED properties. To aid with this assessment mandate, the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) prepared an extensive guidance document for the end users (EFSA/ECHA et al., 2018). The objective of this document is to provide technical guidance for the implementation of the ED criteria. Although specifically developed for BP/PPPs, this guidance can also be adopted more broadly, also including non-BP/PPP chemical substances as recently exemplified for butylparaben (Boberg et al., 2020). Importantly, however, the actual evaluation of chemicals using this, and other, guidance documents still requires the establishment of biologically plausible links between ED modes-of-action and ED-related adverse outcomes, something that requires *a priori* knowledge about causal pathways.

Following the implementation of the ED criteria within the European regulatory framework, only a very limited number of compounds have been identified as endocrine disrupters for human health; and only limited regulatory action taken based on this identification. Since 2018 until time of writing (March 2022), only 5 REACH chemicals have been identified as substances of very high concern (SVHC) as regards their endocrine disrupting properties based on EATS modalities (ECHA, 2022; ED_List, 2022; Lynch et al., 2017). Anti-androgenic action has been shown to be the main mode-of-action leading to the adverse reproductive effects in one case only (dicyclohexy phthalate; DCHP), whereas estrogenic action has been shown to be the main mode-of-action for butylparaben, 4-MBC, Bisphenol B and dodecyl phenol (PDDP). During the same period, only one pesticide has been regulated based on its endocrine disrupting properties; Mancozeb was banned due to its thyroid hormone system disrupting properties (EFSA et al., 2019). This very limited number of chemicals regulated based on endocrine disrupting properties clearly exemplifies that the EU EDC criteria are indeed very strict and that a substantial amount of evidence is needed for regulatory action to eventuate. How these regulatory aspects will be dealt with as we move towards more reliance on alternative test methods and less on animal studies, remains to be seen.

7. Perspectives and conclusion

We possess a solid understanding of how hormones and the endocrine system regulate male reproductive development and function in animals and humans. We also have a good understanding of how many chemicals can interfere with biomolecules and regulatory pathways. We even have an extensive database on how a sizeable number of chemicals cause various male reproductive diseases in experimental animals (Brehm & Flaws, 2019; Gray et al., 2001; Schwartz et al., 2021; Schwartz et al., 2019). In humans the evidence is less clear and still debated, yet many epidemiological studies associate chemical exposures with comparative disease to those observed in animal models (Bonde et al., 2016; Kristensen et al., 2016; Rodprasert et al., 2021; Skakkebæk et al., 2022; Stukenborg et al., 2021). Nevertheless, our capacity to predict *in vivo* adversity based on alternative test method data is far from foolproof. We should, however, endeavor to improve on our ability to

predict *in vivo* effect outcomes from alternative test methods and thereby greatly reduce our reliance on animal testing for chemical risk assessment purposes. But it is a sizeable challenge, not least with respect to endocrine disruption as the endocrine system is challenging to mimic *in vitro* or *in silico*. To aid with this, we likely need to develop sophisticated testing strategies that include non-animal test methods that are very close to the *in vivo* situation, as relying on simplistic upstream key events such as nuclear receptor interactions will not suffice. We also need to ensure the assays are relevant for what we seek to protect, being it wildlife or humans.

Male reproductive toxicity potentially represents the ED field for which we have most mechanistic knowledge and the most established test assays. The challenge then for other ED fields – e.g. female reproduction, metabolic disorders, and neuroendocrinology – is obvious. Here, as with male reproduction, we foresee great advances both in establishing novel mechanistic understanding about causal molecular pathways and development of alternative test methods. For instance, human stem cell methodologies hold great promise in the context of chemical hazard identification and risk assessment and will witness significant advances in the years to come. This will include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), both of which are already used in toxicity testing. Multipotent stem cells also share many of the same characteristics as hiPSCs and ESCs and will likely be exploited further despite representing more specialized cells than pluripotent cells. For now, however, better consistency between stem cell assays and intact animals should be achieved before they can safely replace animal toxicity studies (Trosko and Chang, 2010).

With respect to endocrinology, complex assays closely resembling an *in vivo* situation may need to be complex in design, and here organoids could be of great value. Again, it has proven challenging to establish organoids, or cell based systems that can faithfully capture endocrine organs such as gonads, but recent advances show great promise, as for instance for testis organoids differentiated from human iPSCs (Knarston et al., 2020). In the interim, explant organ culture systems, both from animals and human tissues, could be a great aid in both chemical testing and for characterizing important mechanism-of-action that needs to be incorporated in testing strategies.

To conclude, modern chemical testing should move towards less reliance on animal toxicity studies for chemical hazard identification, risk assessment and international regulation; however, it should not be at a great expense for human, nor environmental, health. The fact remains that there are certain things about a complex system, such as a human being, that cannot be fully replicated or predicted by reductionist approaches. In most cases, the whole is greater than the sum of its parts. What we should aim for is to devise testing strategies that allow for adequate predictions of adversities in complex organisms. This should be a goal, but we are not fully there yet, at least not with respect to the male reproductive system and the involvement of endocrine disruption.

Author statements

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