A strategy to validate a selection of human effect biomarkers using adverse outcome pathways: Proof of concept for phthalates and reproductive effects

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ABSTRACT

Human biomonitoring measures the concentrations of environmental chemicals or their metabolites in body fluids or tissues. Complementing exposure biomarkers with mechanistically based effect biomarkers may further elucidate causal pathways between chemical exposure and adverse health outcomes. We combined information on effect biomarkers previously implemented in human observational studies with mechanisms of action reported in experimental studies and with information from published Adverse Outcome Pathways (AOPs), focusing on adverse reproductive effects of phthalate exposure. Phthalates constitute a group of chemicals that are ubiquitous in consumer products and have been related to a wide range of adverse health effects. As a result of a comprehensive literature search, we present an overview of effect biomarkers for reproductive toxicity that are substantiated by mechanistic information. The activation of several receptors, such as PPARα, PPARγ, and GR, may initiate events leading to impaired male and female fertility as well as other adverse effects of phthalate exposure. Therefore, these receptors appear as promising targets for the development of novel effect biomarkers. The proposed strategy connects the fields of epidemiology and toxicology and may strengthen the weight of evidence in observational studies that link chemical exposures to health outcomes.

1. Introduction

Human biomonitoring is a method for assessing human exposure to environmental chemicals by measuring these compounds or their metabolites in non-invasive human biological specimens such as urine, blood, hair and other tissues (NAS, 2017). Exposure biomarkers provide a measure of the actual exposure to specific environmental chemicals

by integrating all sources and exposure routes, facilitating the evaluation of relationships between exposure and adverse health effects, thus supporting policy making (Joas et al., 2017). The HBM4EU project (https://www.hbm4eu.eu) aims to coordinate and advance human biomonitoring in Europe. The project represents a joint effort of 28 countries, the European Environment Agency, and the European Commission, co-funded by Horizon 2020) (Ganzleben et al., 2017). One of

the objectives of HBM4EU is to generate scientific evidence on the causal relationships between exposure to prioritized chemical stressors and adverse health effects with public health implications. To this end, biomarkers of exposure are complemented with biomarkers of effect, which are measurable molecular, cellular, biochemical, physiologic, behavioural, structural or other alterations in an organism occurring along the temporal and mechanistic pathways connecting exposure to chemicals and an established or possible health impairment or disease (Council, 2006). According to the 'meet in the middle approach' (Vineis et al., 2013), demonstration of associations between (i) chemical exposure and disease, (ii) chemical exposure and a preclinical effect, and (iii) the preclinical effect marker and the disease strengthens the inference of a causal relationship between exposure and health outcomes (NAS, 2017). Consequently, the identification of adequate effect biomarkers requires epidemiological, experimental, and mechanistic information related to the chemical compound of interest.

The mechanistic association between effect biomarkers and health outcomes can be systematically explored by making use of information collected in Adverse Outcome Pathways (AOPs) (Escher et al., 2017; Leist et al., 2017; NAS, 2017). An AOP is a conceptual construct that describes a sequential chain of causally linked events at different levels of biological organization that lead to an adverse human or ecological health effect, based on existing knowledge. AOPs represent biologically plausible and empirically supported links between a series of Key Events (KE), which are measurable downstream changes at the molecular, cellular, tissue, organ, individual, and population level, from a Molecular Initiating Event (MIE, a KE that forms the initial point of chemical interaction) to an Adverse Outcome (AO). The connection between two key events is called a Key Event Relationship (KER) (OECD, 2017; Vinken et al., 2017). The AOP-Wiki (https://aopwiki.org) is an open-source platform that provides a detailed and searchable description of AOPs under various stages of development. The AOP concept was originally designed to support regulatory risk assessment by disclosing all existing toxicity information and identifying targets for analysis using alternative test methods. Here, we demonstrate a strategy to link effect biomarkers to MIEs and KEs of AOPs to strengthen the weight of evidence for a causal relationship between chemical exposure and adverse health outcomes, focusing on adverse reproductive effects of phthalate exposure.

Phthalates are a group of manmade chemicals that are used as solvents for cosmetics, personal care and cleaning products, pharmaceuticals, and pesticides or as plasticizers to improve flexibility of a wide variety of consumer products including medical devices, children's toys, food packaging, and building materials. Phthalates migrate from these products resulting in environmental release and human exposure via ingestion, inhalation, absorption through the skin, or parenteral medical applications. Phthalates can also pass the placental barrier. Simultaneous exposure to multiple phthalates has been observed across the lifespan and their presence was demonstrated in blood, urine, cord blood, sperm, and breast milk samples (Benjamin et al., 2017; Karaconji et al., 2017; NRC, 2008). Around 75–100% of the population is exposed to phthalates on a daily basis due to large production volumes, widespread use, and environmental contamination (Hannon and Flaws, 2015). Exposure to di(2-ethylhexyl) phthalate (DEHP), as the first and most commonly used phthalate compound in consumer products, has been studied most extensively (Karaconji et al., 2017). Phthalates (in particular diesters of 1,2-benzenedicarboxylic acid, the *o*-phthalates) have been shown to cause a variety of adverse health effects in laboratory animals. The adverse effects on development of the reproductive system of male offspring of rats, exposed during pregnancy, have particularly raised concern. Those effects are specifically related to dibutyl, butylbenzyl, dipentyl, and diethylhexyl phthalate exposures, and include androgen insufficiency, cryptorchidism, hypospadias, reproductive tract malformations (e.g. reduced anogenital distance), and infertility and decreased sperm count later in life, collectively referred to as the 'phthalate syndrome' (NRC, 2008). The phthalate syndrome

has many similarities to the testicular dysgenesis syndrome that is postulated to occur in humans. Although there are no human data that directly link this syndrome with phthalate exposure, reproductive developmental processes in rats are analogous to those in man and several human studies have reported associations of intrauterine exposure of some phthalates (particularly DEHP and dibutyl phthalate, DBP) with diverse endocrine and developmental effects similar to those in rats (Benjamin et al., 2017; EPA, 2012; Karaconji et al., 2017; Mariana et al., 2016; NRC, 2008; Radke et al., 2018; van den Driesche et al., 2017). In addition, there is accumulating evidence on a link between phthalate exposure and impaired female reproductive function, including altered estradiol levels, ovarian reserve, pubertal development, fertility, and occurrence of endometriosis (Benjamin et al., 2017; Hannon and Flaws, 2015; Karaconji et al., 2017; Minguez-Alarcon and Gaskins, 2017; NRC, 2008). Reduced ovarian reserve, a higher risk of early pregnancy loss, and lower birth and placental weight have been observed even when exposure occurred before conception (Messerlian et al., 2016a, 2016b, 2017; Mustieles et al., 2019). Female reproductive effects have received far less attention than effects in males so far, and available study results are not conclusive. Besides, human phthalate exposure has been associated with insulin resistance, obesity, altered thyroid signaling, impaired neurodevelopment, and increased risk of allergies, asthma, cardiovascular disease, and cancer (Benjamin et al., 2017; Karaconji et al., 2017; Mariana et al., 2016). Because of the potential threat to endocrine and reproductive health, some phthalate compounds are classified as priority hazardous compounds and subjected to restrictions for use in toys and cosmetics in European legislative frameworks.

The proof of concept demonstrated in this publication links several biomarkers of reproductive effects previously associated with phthalates exposure in human observational studies to mechanisms of action and events reported in experimental studies, as well as to adverse reproductive outcomes using information collected in existing AOPs. This exercise yields effect biomarkers for the family of phthalate compounds that are sufficiently substantiated with mechanistic information, and identifies novel biomarkers for early biological response which may result in adverse reproductive outcomes. These biomarkers are promising candidates for implementation in molecular epidemiological research. To the best of our knowledge, this is the first effort to strengthen the causal relationship between chemical exposure, biomarkers of effect, and adverse health outcomes in human population studies by employing AOP information.

2. Methods

A comprehensive literature search was performed in the US National Library of Medicine (NCBI Pubmed) at February 15th, 2018. Appendix A lists the search terms, including MeSH® terms and MeSH Supplementary Concepts (https://www.nlm.nih.gov/mesh). Search terms for phthalates comprised full names linked to CAS numbers and metabolites of individual parent compounds. They were combined with search terms for reproductive effects and endocrine disruption. Full text publications published in the past ten years were selected. Human observational studies were separated from experimental studies by screening of the abstracts. Human studies that did not measure a biomarker of effect or did not assess a reproductive health endpoint were omitted. Publications without abstract or not written in English, reviews, and duplicates were also removed. Biomarkers of effect that were associated with phthalate exposure were listed from the abstracts of the remaining human observational studies. The abstracts of the experimental studies were explored to identify toxicity pathways and mechanisms of action of phthalates related to the identified biomarkers of effect. This search strategy was executed by one reviewer.

To further substantiate the links between human effect biomarkers and the toxicity pathways of phthalates, the AOP-Wiki v2.2 (OECD, 2018) was consulted. 'Phthalate' was entered as search term for

stressors. Effect biomarkers from human observational studies and biological events involved in the toxicity pathways and mechanisms of action retrieved from the experimental studies were entered as search terms for KEs and KERs. AOPs were selected if three or more KEs (including MIEs) concerned effect biomarkers and/or elements of toxicity pathways identified in the literature search and AOs were related to reproductive function.

3. Results

3.1. Literature search

Appendix B shows a flow chart summarizing the results of each step of the literature search. The search including full text publications of the past 10 years resulted in 229 human observational studies and 202 experimental studies related to reproductive health effects, and 123 human studies and 203 experimental studies on endocrine disruption. After further screening of the abstracts of the human observational studies for in- and exclusion criteria and removal of duplicates, 49 publications on reproductive outcomes and 29 additional publications resulting from the endocrine disruption literature search were considered suitable for extracting information on biomarkers of effect. These biomarkers (listed in Table 1) are related to hormone levels, gene expression and polymorphisms (molecular markers), oxidative stress, reproductive function, pregnancy outcome, and hormonally mediated diseases. Population studies reporting associations between pre- or postnatal phthalate exposure and testosterone levels, pubertal development, and sperm quality and quantity were most abundant.

Experimental studies were predominantly performed in rats and mice and in a few cases in other organisms or *in vitro* cell cultures. Mechanisms of action and toxicity pathways of phthalates related to reproductive health were abstracted from 151 experimental studies on reproductive endpoints and 31 additional studies on endocrine disruption. The majority of these studies evaluated male reproductive toxicity in relation to pre- or postnatal phthalate exposure. Table 1 shows that most human effect biomarkers could be substantiated with experimental and mechanistic information. Ample experimental evidence was available for alteration of testosterone levels, reduced sperm quality and quantity, and male reproductive tract malformations. However, limited experimental support was found for the category of molecular biomarkers. The mechanisms of action proposed for phthalates in the experimental studies largely confirmed the modes of action suggested in the human literature, including the induction of cell cycle arrest, apoptosis, and oxidative stress in testicular cells, disruption of steroidogenesis and reduction of testosterone production, effects via the hypothalamic–pituitary–gonadal (HPG) axis, altered thyroid signaling, and changes in nuclear receptor gene expression and activation.

3.2. Link to AOPs

All human effect biomarkers for which experimental evidence was obtained could be linked to one or more AOPs, except for antral or secondary follicle count and hormonally mediated diseases in females. The biological effects related to these endpoints do occur as KEs in part of the retrieved AOPs; however, these AOPs are described for males. The largest number of KEs and AOPs were found for effects on testosterone production, sperm quality and quantity, and male reproductive tract malformations. Thyroid signaling, developmental toxicity, DNA hypermethylation, and pubertal development were only linked to an AOP for which the KERs were not specified in AOP-Wiki. Phthalates as a group are for instance linked as a stressor to AOP 152: '*Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity*', which includes modification of serum T4 levels, but is actually an AOP that is still under development. The relevance of this AOP for humans is not substantiated with experimental evidence in AOP-Wiki, although it is indicated that transthyretin is a

highly conserved serum binding protein.

Fig. 1 shows the AOPs (limited to AOPs with specified KERs) that are linked to different phthalates as stressors in AOP-Wiki and/or include multiple human biomarkers and elements of toxicity pathways identified in the literature search.

3.2.1. AOPs linked to phthalates as stressors

Mono (2-ethylhexyl) phthalate (MEHP), DEHP, and DBP are linked to KE 234: '*Activation of PPARα & PPARγ*' (high evidence for occurrence in humans, rats and mice according to AOP-Wiki). MEHP and DEHP are also linked to KE 227: '*Activation of PPARα*' (high evidence for humans, rats and mice) and KE 228: '*Peroxisome proliferator activated receptor promoter demethylation*' (moderate evidence for humans and rats, high evidence for mice).

3.2.2. AOPs including human biomarkers and elements of experimental toxicity pathways

PPARα activation is a MIE in AOP 18 and 51 leading to impaired male fertility through reproductive tract malformations and Leydig cell dysfunction, respectively, although there is some uncertainty on PPARα as MIE and AOP 51 is only described for rats. For all but one of the KEs in AOP 18, there is substantial evidence for a link with phthalate exposure based on experimental studies. PPARα activation may also be related to induction of oxidative stress, which is linked to apoptosis and reproductive failure in Caenorhabitis elegans in AOP 207 with strong evidence for the KERs involved. C. elegans is considered a useful model to study human genetics, developmental biology, and disease pathogenesis (Hunt, 2017; Weinhouse et al., 2018). PPARα itself may be activated by Nrf2 via FXR and SHP activation.

PPARγ activation is suggested to be the MIE in AOP 7 leading to impaired fertility in adult females via reduction of aromatase (CYP19a1) levels. While there is some uncertainty on the functional relationship between PPARγ and aromatase, there is evidence for involvement of PPARγ in female reproductive function as the AO of this AOP. Fluctuations in PPARγ expression in ovarian follicles and the stage of the estrous cycle may influence the impact of PPARγ activation on aromatase levels. PPARα and CEBPA activation may contribute to the initiation of this AOP. PPARγ has been shown to modulate enzymes involved in estradiol catabolism downstream of aromatase as well, i.e. CYP1b1 and 17-βHSD IV. In addition, activation of PPARγ is hypothesized to contribute to or synergize with AOP 18, although contradicting effects of PPARγ ligands on androgen levels and/or production in male humans and animal models have been described. PPARγ ligands may upregulate StAR, inhibit or stimulate 3β-HSD, and modulate CYP17, which are all involved in steroid hormone synthesis.

AOP 153 '*Aromatase inhibition leading to ovulation inhibition and decreased fertility in female rats*' links reduced estradiol levels due to inhibition of aromatase to altered HPG signaling resulting in a sequential decrease in kisspeptin, gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) release and a reduced number of oocytes ovulated. The relevance for humans and the KERs of this AOP have not been specified, but kisspeptin is being recognized as a crucial regulator of the onset of puberty, the regulation of sex hormone mediated secretion of gonadotropins, and the control of fertility (Skorupskaite et al., 2014; Trevisan et al., 2018).

The MIE for impaired male fertility due to decreased sperm quality and quantity in AOP 64 is glucocorticoid receptor (GR) activation. The evidence for KERs and relevance for humans were not reported for this AOP. AOP 71 '*Modulation of adult Leydig cell function subsequent to glucocorticoid activation*' describes similar events and has high evidence for humans, but in this AOP the KERs are not specified. The same is true for AOP 69 '*Modulation of adult Leydig cell function subsequent to decreased cholesterol synthesis or transport in the adult Leydig cell*' and AOP 70 '*Modulation of adult Leydig cell function subsequent to proteomic alterations in the adult Leydig cell*', that link decreased steroidogenesis in adult Leydig cells to decreased sperm quality and quantity, in the latter case

Table 1

Reproductive and endocrine effect biomarkers related to adult or prenatal phthalate exposure linked to experimental evidence, suggested mechanisms of action, and AOPs.

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Hormones				
Free and total testosterone, sex hormone-binding globulin (SHBG), free androgen index (FAI), dehydroepiandrosterone sulfate (DHEA-S), Δ4-androstenedione, 11β-HSD2 activity	Reduction of steroid and testosterone production by adverse effects on testicular Leydig cell function resulting in genital malformations in male newborns, changes in the timing of sexual maturation in both boys and girls, and reduced sperm production and quality (Chang et al., 2015; Chen et al., 2017a; Frederiksen et al., 2012; Hu et al., 2014a; Joensen et al., 2012; Meeker and Ferguson, 2014; Mouritsen et al., 2013; Nassan et al., 2018; Pan et al., 2011; Sathyanarayana et al., 2014; Watkins et al., 2014, 2017; Wen et al., 2017). Effect on fetal development by inhibition of 11β-HSD2 activity (Hu et al., 2014a).	Disrupted development and function of testicular and Leydig cells due to cell cycle arrest and apoptosis (p53, Chk1, Cdc2, CDK6, Bcl-2, Bax/Bcl2, p21(Cip1), c-kit) induced by oxidative stress (adaptive response of Nrf2 and target genes HO-1, NQO1, peroxiredoxin 6, annexin A5) (Gao et al., 2017; Jiang et al., 2017; Shen et al., 2013, 2014, 2015; Unal et al., 2016) or higher ERα and lower ERβ and AR expression (Wakui et al., 2014; Wang et al., 2013).	226	**18**
			227	19
			234	51
			240	64
			286	66
			447	67
			413	68
			446	*69*
		Reduced activity of lipid metabolism pathways and SREBP2-dependent cholesterologenesis (Johnson et al., 2011) and down-regulation of cholesterol transport and steroid synthesis and metabolism proteins (StAr and its transcription factors SF-1, GATA-4 and C/EBP-beta, Scarb1, SRB1, 3β-HSD, 3β-HSD1, 17β-HSD(3), CYP11a1, CYP17a1, CYP17a2, CYP19a, P450scc, and vimentin) resulting in reduced testicular testosterone secretion (Bello et al., 2014; Bielanowicz et al., 2016; Boberg et al., 2008; Boisvert et al., 2016; Chen et al., 2017b; Chen et al., 2013b; Clewell et al., 2010; Clewell et al., 2009; Clewell et al., 2013; Euling et al., 2013; Hannas et al., 2012; Hannas et al., 2011b; Heng et al., 2012; Hotchkiss et al., 2010; Hu et al., 2015; Hu et al., 2013b; Kilcoyne et al., 2014; Li et al., 2015a; Li et al., 2016b; Lu et al., 2016; Mitchell et al., 2012; Motohashi et al., 2016a; Motohashi et al., 2016b; Ovacik et al., 2013; Prokkola et al., 2016; Rider et al., 2010; Rodriguez-Sosa et al., 2014; Saillenfait et al., 2016; Scarano et al., 2010; Shirai et al., 2013; Sohn et al., 2016; Struve et al., 2009; van den Driesche et al., 2012b; Wakui et al., 2013; Wang et al., 2017b; Yuan et al., 2012; Zhao et al., 2010). StAR, CYP11a, and CYP17a may be inhibited via PPARα activity resulting in reduced SF1 transcription factor activity (Boberg et al., 2008; Plummer et al., 2013).	494	*70*
			495	*71*
			496	74
			1262	*212*
			1504	
			1505	
		Reduced steroidogenesis related to antiandrogenic activity by reduced AR activation (Christen et al., 2010) (not related to AR binding (Kim et al., 2010b)), the glucocorticoid pathway (Drake et al., 2009; Xiao-feng et al., 2009; Zhang et al., 2009) or altered balance in subsets of testicular macrophages and enhanced IL-1β secretion as a potent steroidogenesis repressor (Zheng et al., 2010).		
Insulin-like factor 3 (INSL3)	Reduced release due to adverse effects on testicular cell function (Chang et al., 2015, 2017; Chen et al., 2014a; Jensen et al., 2015).	Altered testicular and Leydig cell function (*see above*) (Bielanowicz et al., 2016; Chen et al., 2013b; Heng et al., 2012; Li et al., 2015a) possibly through an effect on the Leydig stem cell population (Ivell et al., 2013) or via direct effect on INSL3 production (Boberg et al., 2008; Pathirana et al., 2011).	413	**18**
			496	51
				64
				69
				70
				71 *212*

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Luteinizing Hormone (LH) Follicle Stimulating Hormone (FSH) Iinhibin B (INB)	Disruption of the hypothalamic–pituitary–gonadal axis seems negatively associated with reproductive function of adult men (Axelsson et al., 2015; Chang et al., 2015; Chen et al., 2014a; Joensen et al., 2012; Nassan et al., 2018; Pan et al., 2011; Su et al., 2014).	Sertoli cell apoptosis mediated by the PTEN/PI3K/AKT/mTOR signaling pathway (including p70S6K and 4E-BP1 protein) (Wang et al., 2017a) and altered inhibin release correlated to Sertoli cell toxicity (Erdos et al., 2013; Moody et al., 2013). Disrupted development of Leydig cells causing down-regulation of gonadotroph biomarkers including Lhb and Gnhr (Chen et al., 2017b). Altered LH and FSH levels due to disrupted testosterone production (Giribabu and Reddy, 2017; Wakui et al., 2013). Downregulation of FSH receptor causing impaired FSH-induced intracellular signaling, decreased phosphorylation of AKT and mTOR, HIF1A expression and reduced KIT ligand (KITLG) expression (Sen et al., 2015; Wang et al., 2016a).	413 414 446 1262	**18** 51 66 67 68 74 **212**
Estradiol (E2), Progesterone	Disrupted pituitary-hypothalamic feedback and sex steroid hormone synthesis which may affect interest in sexual activity and reproductive development (Barrett et al., 2014; Frederiksen et al., 2012; Joensen et al., 2012; Johns et al., 2015; Su et al., 2014; Wen et al., 2017).	Suppression of FSH-induced biological effects and inhibition of HSD3B1 and CYP19a1, disrupting progesterone and estradiol production and granulosa cell proliferation (Wang et al., 2016a; Xu et al., 2016). Disrupted estrogen levels related to ovarian aromatase expression (Boberg et al., 2008) affecting ovarian function (Sen et al., 2015).	3 219 408	**7** 153
Prolactin Triiodothyronine (T3), Thyroxine (T4), Thyroid-stimulating Hormone (TSH)	Related to male infertility (Li et al., 2011). Disruption of thyroid signaling (Dirtu et al., 2013; Meeker and Ferguson, 2011; Weng et al., 2017; Wu et al., 2013). Disrupted thyroid hormone levels during early life may increase the risk of adverse reproductive outcomes such as fetal growth, neurodevelopment and preterm birth (Boas et al., 2010; Huang et al., 2016, 2017; Johns et al., 2015; Kuo et al., 2015; Morgenstern et al., 2017; Yao et al., 2016).	Thyroid activity (Ghisari and Bonefeld-Jorgensen, 2009), TR receptor expression (Shen et al., 2011), and TR antagonism (Shi et al., 2012).		152
Molecular markers[2]				
Nuclear receptor gene expression levels (ERα, ERβ, AR, AhR, PXR, PPAR)	Enhanced expression levels of nuclear receptors (except PPARγ) related to female infertility (La Rocca et al., 2014).	AR and ER α and β gene expression were found to be either increased or decreased (Bhatia et al., 2014; Ryu et al., 2008; Wakui et al., 2014). An estrogenic mode of action in disruption of reproductive health was demonstrated by increased ERα and β expression correlated to increased vitellogenin in plasma, choriogenin L in liver, and aromatase activity in brain (Bhatia et al., 2014; Maradonna et al., 2013). Higher ERβ and lower ERβ and AR gene expression levels were related to reduced 5α-reductase type 2 and sonic hedgehog (Shh) and abnormalities in androgen-dependent organ development (Kim et al., 2010a; Wakui et al., 2014). Reduced AR expression was related to testicular stem cell apoptosis (Wang et al., 2013). Altered expression of peroxisome proliferators activated receptors PPAR α, β, γ (Boberg et al., 2008; Maradonna et al., 2013) and retinoid X receptor-gamma (RXR-r) (Ryu et al., 2008). Increased PPARγ and p-ERK1/2 levels were related to steroidogenic or spermatogenic effects in the testis (Ryu et al., 2008). Histone methylation modifiers Kdm1, Prdm2, and Ehmt1 are involved in nuclear receptor mediated transcriptional regulation in the testis (Anderson et al., 2012).	228 234 286 1028	**7** 19
Expression of metallothioneins (MTs), fatty acid transport protein 1 (FATP1) and heart fatty acid binding protein (HFABP)	Related to fetal growth and development (birth weight and gestational age) (Li et al., 2016a).			

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
DNA hypermethylation of CNPY1, IFT140, TESC, and PRDM8	Involved in effects on endocrine function (CNPY1) and male fertility (IFT140, TESC, and PRDM8) (Solomon et al., 2017).	Promotion of epigenetic transgenerational inheritance of adult onset disease by sperm DNA methylation (Manikkam et al., 2013) and disrupted embryonic development by decreased embryonic DNA methylation (Chu et al., 2013).		74
CYP17A1 and estrogen receptor α (ERα) polymorphisms	Modulation of estrogen biosynthesis and effects on the development of leiomyoma via estrogen signaling (Huang et al., 2014).			
Glutathione S-transferase M1 polymorphism	Correlated with phthalate metabolism (detoxification) and risk of adenomyosis and leiomyoma (Huang et al., 2010).			
Fas, FasL, and caspase-3 polymorphisms	Associated with increased spermatozoa apoptosis and decreased sperm concentration and sperm count (Yang et al., 2017).			
Oxidative stress				
8-hydroxy-2'-deoxyguanosine (8-OHdG) and 15-F2t isoprostane	Potential mediator of reduced fertility (Guo et al., 2014; Wu et al., 2017).	Oxidative stress (8-OHdG) was associated with DNA damage and atrophy of the testis (Shono and Taguchi, 2014). Oxidative stress was also related to testosterone levels and sperm quality and quantity (*see citations listed for the respective biomarkers*).	1279	207
8-isoprostane	Related to preterm birth (Ferguson et al., 2017).			
Reproductive function				
Pubertal development (eg. Tanner stage), Kisspeptin (KiSS)	Altered hormone concentrations and anti-androgenic action during prenatal and peripubertal exposure (Chou et al., 2009; Frederiksen et al., 2012; Hou et al., 2015; Mouritsen et al., 2013; Shi et al., 2015; Su et al., 2015; Watkins et al., 2014, 2017; Wolff et al., 2014). Kisspeptin may promote the onset of puberty (Chen et al., 2013a).	Advanced pubertal timing related to neurotransmitter kisspeptin and its receptor GPR54, affecting hypothalamic gonadotropin-releasing hormone release, and estradiol levels (Hu et al., 2013a). Delayed female sexual maturation (Dobrzynska et al., 2011) and pubertal onset and modified morphology of the mammary gland by altered expression of genes related to eg. cell signaling, proliferation and differentiation (Moral et al., 2011).		153
Testicular volume	Impact on male reproductive function (Axelsson et al., 2015).	Germ cell and testicular cell apoptosis causing atrophy and/or autophagy mediated by excessive ROS production (8-OHdG) and endoplasmic reticulum stress (translation initiation factor 2/activating transcription factor 4 (p-eIF2α/ATF4) pathway, GRP-78, ATF-6, CHOP) and altered expression of actin cytoskeleton genes (Ahbab et al., 2014; Shono and Taguchi, 2014; Spade et al., 2014; Zhang et al., 2016a, 2016b). Inhibition of proliferation of fetal testicular somatic cells (Boekelheide et al., 2009) or a non-proliferative mechanism of multinucleated germ cells (MNGs) formation (Saffarini et al., 2012; Spade et al., 2015). Modulation of androgenic activity (Macleod et al., 2010; Shen et al., 2009),	1262 1278 1505 1506	207 **212**

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Sperm concentration, count, motility, velocity, morphology, DNA damage	Disruption of reproductive hormone balance (possibly anti-androgenicity) affects testicular function and semen quantity and quality and thereby male reproductive health and fertility (Chang et al., 2017; Han et al., 2014; Liu et al., 2012a; Pant et al., 2011; Thurston et al., 2016; Wang et al., 2015, 2016b; Wirth et al., 2008). Induction of DNA damage in semen may affect male reproduction (Pant et al., 2014).	ROS increase and suppression of antioxidant activity (SOD, CAT and GPx) resulting in oxidative stress (LPO, 4-HNE) causing testicular cell apoptosis (Du et al., 2017; Giribabu and Reddy, 2017; Jiang et al., 2017; Kwack et al., 2009; Nair, 2015; Zhou et al., 2011a; Zhu et al., 2010). Adaptive increase in Nrf2/HO-1/NQO1 in SOD, AOX, glutathione-S-transferase, catalase, glutathione reductase, glutathione peroxidase, ascorbic acid, glutathione, metallothionein, malondialdehyde (MDA) have been noticed (Aly et al., 2016; Chen et al., 2011; El-Beshbishy et al., 2014; Jiang et al., 2017; Nair, 2015; Xu et al., 2014; Zhou et al., 2010, 2011a). Reduced testosterone levels, associated with downregulation of 3βHSD, 17βHSD, P450scc (Ahmad et al., 2014; Giribabu and Reddy, 2017; Giribabu et al., 2014; Pan et al., 2017; Sharpe and Skakkebaek, 2008). Decreased testosterone secretion is either caused by oxidative stress (Aly et al., 2016) or causes oxidative stress (Giribabu and Reddy, 2017). Anti-androgenic effects may also impair Sertoli cell proliferation (Auharek et al., 2010; Boberg et al., 2011; Moody et al., 2013). Involvement of the hypothalamic-pituitary-gonadal axis (upregulation of inhibin) (Aydogan Ahbab and Barlas, 2013; Moody et al., 2013; Nair, 2015) and estrogenic effects via increased G-Protein-Coupled Receptor 30 (GPR30) (Hu et al., 2013c). Increased steroidogenesis via vimentin (promotor demethylation and NF-κB activation) which regulates the secretion of progesterone has also been reported (Li et al., 2016c). Modification of Sertoli cell cytoskeleton: collapse of vimentin filaments possibly leading to disruption of Sertoli-spermatogenic cell physical interaction and induces spermatogenic cell apoptosis (Alam and Kurohmaru, 2016; Alam et al., 2010c; Bao et al., 2011; Zhu et al., 2010); loss of Sertoli cell-germ cell membrane adhesion, probably due to Sertoli cell microfilament redistribution(van den Driesche et al., 2015); changes of Sertoli cell tight junction structure associated with regulation of tight junction proteins and phosphorylation of ERK and reduced expression of Sertoli cell specific genes like transferrin, testin and occludin (Hu et al., 2014b; Kumar et al., 2015; Zhang et al., 2008). Reduced expression of testis growth and function related genes Sox9 and Dazl (Du et al., 2017) and alterations in the expression of HnRNPA2/B1 (Bao et al., 2011). Direct effects on germ cells (Dobrzynska et al., 2009; Hutchison et al., 2008a) such as alkylation of the sperm centrioles (Du et al., 2017; Lu et al., 2017), delayed sperm maturation (Alam et al., 2010a) and spermatogenic cell apoptosis due to estrogenic activity, probably by activating estrogen receptors in testis (Alam et al., 2010b).	413 446 495 520 1262 1278 1279 1515	64 66 67 68 **69** **70** 71 74 207 **212**
Antral follicle count (AFC)	Impact on ovarian reserve and reduced fecundity (Messerlian et al., 2016a).	Impaired follicular/oocyte development related to estradiol concentrations and possibly apoptosis (Bax, Bad, Bid) (Kalo et al., 2015; Sen et al., 2015). Cell cycle arrest related to decreased cyclins D2, E1, A2, and B1 and increased p21 expression, increased levels of Bid and decreased levels of Bcl2 in antral follicles (Craig et al., 2013; Ray et al., 2012).		

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Peak estradiol levels, number of mature oocytes, and proportion of cycles resulting in pregnancy in IVF treatment	Disrupted female reproductive health (Hauser et al., 2016; Minguez-Alarcon et al., 2016).	Adverse effect on oocyte growth and maturation (Santangeli et al., 2017). Reduced oocyte developmental competence related to CYC1, ATP5B, SOX2 and DNMT3b expression (Kalo and Roth, 2017).	219 405 406	**7** 153
Impaired fertility and time to pregnancy (TTP)	Interaction with the endocrine system interfering with male and female reproduction ability (Buck Louis et al., 2014; Hauser et al., 2016; Specht et al., 2015; Tranfo et al., 2012).	Endocrine disruption and altered serum levels of estradiol and progesterone in females (Xie et al., 2016; Zhou et al., 2017a, 2017b). Increased transactivation of ER and upregulated pS2 gene expression with simultaneous activation of MAPK pathway as demonstrated by increased p-ERK/ERK ratio (Kumar et al., 2014).	219 406	**7** 153
Pregnancy outcome				
Implantation and live birth in IVF and IUI treatment	Impaired reproductive health (Dodge et al., 2015).			
First trimester hCG, placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1)	Related to disrupted angiogenesis, placental development and/or function, and anogenital distance (Adibi et al., 2015; Ferguson et al., 2015).			
Missed miscarriage and clinical pregnancy loss	Interruption of maternal hormone synthesis (Mu et al., 2015; Yi et al., 2016).	Increased oxidative stress and decreased testicular antioxidant enzymes in paternal testes due to disrupted testosterone synthesis (Chu et al., 2013; Giribabu and Reddy, 2017). Altered ATPase activity in gametes and testosterone production (CYP3a, 17β-HSD-11 and 17β-HSD-12) in embryos (Howdeshell et al., 2008). Impaired developmental competency of preimplantation embryos due to increased ROS and apoptosis (cyt C) (Zhou et al., 2011b).	413 446 495 496 1262 1278 1279	**18** 64 66 67 68 74 207
Preterm birth	Placental preterm birth; delivery with presentation of preeclampsia or intrauterine growth restriction (Ferguson et al., 2014a, 2014b).	Inhibition of HSD3B1 and CYP19a1, disrupting progesterone and estradiol production for maintaining pregnancy (Xu et al., 2016).	3219 408	**7**

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Hypospadias, anorectal malformations, anogenital distance, male reproductive organ malformations	Endocrine disruption and anti-androgenic properties (Bornehag et al., 2015; Carran and Shaw, 2012; Choi et al., 2012; Huang et al., 2009).	Malformations of the reproductive tract (Martino-Andrade et al., 2009; Rider et al., 2008; Saillenfait et al., 2008, 2009a, 2009b; Scott et al., 2008) caused by reduced androgen production, anti-androgenic effects, interference with the hypothalamic-pituitary-gonadal axis (FSH and inhibin B release) (Aydogan Ahbab and Barlas, 2015; Boberg et al., 2011; Hannas et al., 2011a; Li et al., 2015b; Moody et al., 2013; Schmitt et al., 2016; Sharpe and Skakkebaek, 2008; van den Driesche et al., 2011; Yamasaki et al., 2009), and increased expression of estrogen receptor (ERα) (Kim et al., 2010a). Suppression of testosterone production and androgen signaling pathways (CYP11a1, CYP17a1, 5α-reductase type 2, Hsd3b, Scarb1, StAR, AR, Srd5a2) in testes, decreased expression of sonic hedgehog Shh and Ptched 1, Fgf8, Fgf10, Gli2, Gli3, Bmp4, Bmp7, Wnt5a, Hoxa13, Hoxd13, Fgfr2, AR, Wnt/β-catenin pathway (β-catenin, Phospho-GSK-3β), TGF-beta1 and TGF-beta receptor III in genital tubercle and terminal rectum (Boberg et al., 2008; DeBartolo et al., 2016; Jiang et al., 2011, 2015, 2016; Johnson et al., 2008; Kim et al., 2010a; Li et al., 2012b, 2015, 2016; Saillenfait et al., 2013a, 2013b; Zhang et al., 2011; Zhu et al., 2009, 2016), and genital tubercle up-regulation of GSK-3β (glycogen synthase kinase-3β) and NFκB (Zhang et al., 2011). Delay in testis and Sertoli cell development and organization and fetal testis and Leydig cell dysfunction, indicated by altered INSL3 expression (Boberg et al., 2008; Drake et al., 2009; Hutchison et al., 2008a; Hutchison et al., 2008b; Johnson et al., 2008; van den Driesche et al., 2012a). Apoptosis and autophagy mediated by the PI3K/Akt/mTOR signaling pathway in the genital tubercles (Li et al., 2017). Inhibition of retinoic acid synthesis and disruption of retinol signaling pathway (Chen and Reese, 2016).	226 240 286 310 413 446 495 348 1262 1279	**18** 19 64 66 67 68 **69** **70** **71** 74 207

Table 1 (*continued*)

Human biomarker of effect	Link of biomarker with health outcome of phthalate exposure	Experimental evidence and suggested mechanisms of action	KE	AOP[1]
Birth weight, Quetelet's index, head circumference, gestational age, embryonic malformations	Altered expression of metallothioneins related to fetal growth and development (Li et al., 2016a; Polanska et al., 2016; Zhao et al., 2014).	Developmental toxicity (Chen et al., 2014b, 2015; Gao et al., 2014; Kroese et al., 2015; Liu et al., 2009b; Robinson et al., 2010; Saillenfait et al., 2009a, 2011), abnormal embryos (disrupted cytoskeletal protein organization as a consequence of damaged centrioles in sperm by oxidative stress) (Lu et al., 2017), morphological changes (cholesterol/lipid/steroid metabolism and apoptosis pathways) (Robinson et al., 2012), sensory motor, craniofacial development, and eye formation (related to testosterone levels and thyroid profile) (Mahaboob Basha and Radha, 2017), skeletal development (impairment of lipoprotein functions) (Kim et al., 2015b), neural tube defects (inhibited succinic dehydrogenase implicated in the defective mitochondria) (Zheng et al., 2011), heart development (Nkx2.5 and T-box transcription factor 5 downregulation) (Sun and Liu, 2017), renal fibrosis (lower testosterone concentration and reduced expression of Fgf10, Fgfr2 and AR; higher expression levels of TGF-β and α-SMA) (Sun et al., 2018), mammary gland development(Manservisi et al., 2015), and gonad development (Bhatia et al., 2015). Oxidative stress, ER transactivation, AhR agonism (Mankidy et al., 2013), disruption of retinol conversion to all-trans-retinoic acid (atRA) and subsequent receptor-mediated gene regulation in stem cells (Chen and Reese, 2013), impaired thyroid signaling (TR antagonism, expression of TR, TSHα, TSHβ, RXRγ) (Shen et al., 2009, 2011; Shi et al., 2016), calcium signaling coupled to nicotinic acetylcholine receptors (Liu et al., 2009a) and changes in expression of Marcks, Pum1, Nuprl, and Penk (Pike et al., 2014).		*152*
Hormonally mediated disease				
Endometriosis and leiomyoma	Enhanced invasive and proliferative activities of endometrial cells and increased risk of hormonally-mediated disease (Kim et al., 2015a, 2016; Upson et al., 2013).	(Anti)estrogenic activity (Ghisari and Bonefeld-Jorgensen, 2009) and ER binding (Rider et al., 2009). Increase in invasive and proliferative activities: MMP-2 and 9, Erk phosphorylation, and p21-activated kinase 4 expression (Kim et al., 2015a).		
Pubertal gynecomastia	Antiandrogenic or estrogenic effects (Durmaz et al., 2010).			

1 AOP numbers in bold refer to AOPs for which evidence for humans is indicated in AOP-Wiki. AOPs in italics contain multiple KEs that relate to effect biomarkers and mechanisms of action retrieved form experimental studies, but evidence for KERs is lacking in AOP-Wiki. KEs of these AOPs are not included in the adjacent 'KE' column.

2 Genetic polymorphisms are rather a biomarker of susceptibility than a biomarker of effect. Since they are relevant for the occurrence of effects after phthalate exposure, they are included in the selection of biomarkers.
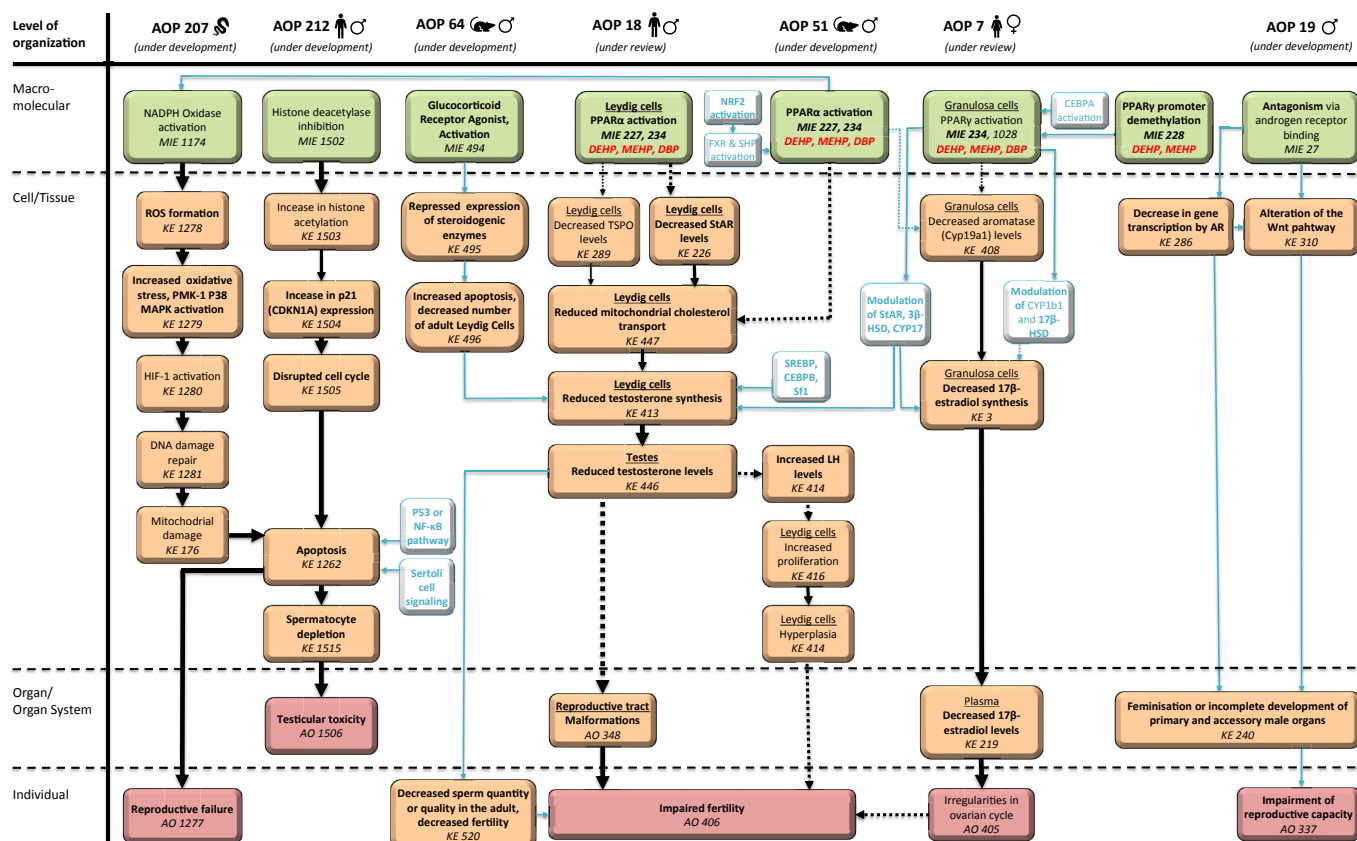
Fig. 1. Schematic representation of AOPs affected by phthalates, adapted from AOP-Wiki (OECD, 2018). MIEs are displayed in green boxes, other KEs in orange boxes, and AOs in red boxes. Numbers of MIEs and KEs affected by phthalates (displayed in red) according to the AOP-Wiki, and biological effects affected by phthalates according to experimental studies are presented in bold. The weight of the arrows reflects the strength of evidence of the KER (low, moderate, or high). Straight lines refer to adjacent events, dotted lines to non-adjacent events. Blue arrows and boxes indicate hypothesized relations or KERs without specification of evidence in AOP-Wiki. AOP 207: 'NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans' (high evidence for C. elegans); AOP 212: 'Histone deacetylase inhibition leading to testicular toxicity' (moderate evidence for humans and mice, high evidence for rats); AOP 64: 'Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility' (unspecified evidence for rats); AOP 18: 'PPARα activation *in utero* leading to impaired fertility in males' (low evidence for humans, moderate evidence for rats and mice); AOP 51: 'PPARα activation leading to impaired fertility in adult male rodents' (high evidence for rats); AOP 7: 'Aromatase (CYP19a1) reduction leading to impaired fertility in adult female' (low evidence for humans and mice, high evidence for rats); AOP 19: 'Androgen receptor antagonism leading to adverse effects in the male foetus' (unspecified evidence for mammals). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

via alterations in the cytoskeleton and apoptosis. AOP 66, 67, 68, and 74 link glucocorticoid activation, estradiol activation, proteome alterations, and hypermethylation in the fetal testis, respectively, to modulation of adult Leydig cell function and decreased sperm quality and quantity, each with high relevance for rats but without specifying the KERs.

AOP 212 describes histone deacetylase inhibition leading to apoptosis and subsequent spermatocyte depletion and testis atrophy with high evidence for all but one KER. In AOP 19, androgen receptor (AR) antagonism in mammalian fetuses is the MIE leading to disruption of male reproductive organs. The evidence for KERs and relevance for humans were not reported for this AOP.

4. Discussion

4.1. Link between phthalate exposure and adverse reproductive effects

Table 1 shows that the majority of biomarkers of reproductive effects that were associated with phthalate exposure at the epidemiological level are supported by mechanistic information from experimental studies and collected in AOPs. The identified AOPs are all under review or under development and are only to a limited extent linked to phthalates as stressors. However, partial information on KEs and

identification of high confidence KERs can already contribute to the weight of evidence of biological events following phthalate exposure, allowing the prioritization of pathways to be further investigated in experimental models or the implementation of novel biomarkers of effect in molecular epidemiological research (Escher et al., 2017; Leist et al., 2017). The paucity of experimental support for the category of molecular biomarkers is explained by the relative novelty of this type of biomarkers and the incomplete mechanistic understanding of the toxicity of phthalates, notwithstanding the large number of studies on adverse reproductive and endocrine-related health outcomes. Experimental studies and AOPs mainly point to three toxicity pathways:

I. Disruption of testosterone production and signaling due to altered cholesterol transport, reduction of steroidogenic enzymes, and apoptosis of Leydig cells, resulting in male reproductive tract malformations and impaired fertility.

The initiating events in the related AOPs are PPARα and GR activation and AR antagonism, although strong experimental support for their link to the next key event is lacking. The potential of some phthalates to activate the GR has been reported (Kolsek et al., 2014), but the role of GR activation or overexpression in reproductive effects of phthalates via impairment of steroidogenic enzymes was only demonstrated in a few of the retrieved experimental studies (Xiao-feng et al., 2009; Zhang et al., 2009). PPARα has been reported to be

activated by several phthalates in rats and humans, although humans are less responsive to PPARα activation than rats (Berger and Moller, 2002; Corton and Lapinskas, 2005; Latini et al., 2008; Mathieu-Denoncourt et al., 2015). The antiandrogenic properties of some phthalates are believed not to result from interaction with the AR but to be related to PPARα activation, although reproductive toxicity independent of PPARα has also been observed (Martinez-Arguelles et al., 2013; Mathieu-Denoncourt et al., 2015). Decreased AR signaling observed after phthalate exposure is thus potentially not mediated by AR antagonism, but rather triggered by reduced AR gene expression and/or reduced testosterone production.

Decreased testosterone levels result from apoptosis of Leydig cells and reduced testosterone synthesis due to reduced expression of cholesterol transport and steroidogenesis genes. This may be a primary effect of phthalates or a secondary effect following PPAR- or GR-dependent transcriptional changes (Mathieu-Denoncourt et al., 2015; Robinson et al., 2012). Although PPARα-induced signaling cascades are involved in fatty acid metabolism and lipid homeostasis, not all enzymes involved in testosterone production, and affected by phthalates (such as SRB1, StAR, CYP11A1, CYP17A1, CYP19, 3β-HSD, 17β-HSD), are known direct transcriptional targets of PPARα (Berger and Moller, 2002; Corton and Lapinskas, 2005; Gervois et al., 2000; KEGG, 2016; May, 2011). However, some studies indicate that their expression may be inhibited via PPARα activity (Boberg et al., 2008; Plummer et al., 2013). Translocator protein (TSPO) mediates the delivery of cholesterol to the inner mitochondrial membrane, thereby promoting steroidogenesis (Martinez-Arguelles et al., 2013). No literature reporting altered TSPO expression related to phthalate exposure or PPARα activation was identified. TSPO might therefore represent a novel biomarker of phthalates' reproductive effects.

Reduced testosterone production makes part of AOPs taking place *in utero* and in adults and has been reported to occur after both pre- and postnatal phthalate exposure in human and experimental studies. Depending on the life stage during which phthalate exposure takes place, reduced testosterone levels may lead to either reproductive tract malformations or impaired reproductive function.

II. Decreased sperm cell quantity and quality caused by reduced testosterone levels and induction of apoptosis following cell cycle arrest and oxidative stress.

The two AOPs related to apoptosis contain high KER evidence. Their initiating events, NADPH oxidase activation and histone deacetylase inhibition, did however not emerge as effects of phthalate exposure from AOP-Wiki or the literature search. This indicates that either these MIEs present novel targets of phthalates, or phthalates affect these pathways via KEs further downstream. NADPH oxidase activation has been associated with PPARα activation (Mathieu-Denoncourt et al., 2015; Rusyn et al., 2001; Sedha et al., 2015). Histone deacetylase has been reported to be involved in effects of phthalates on neuronal cell death and adipogenic effects in mesenchymal stem cells; in the latter study, involvement of PPARγ was suggested (Guida et al., 2014; Sonkar et al., 2016). In addition, phthalates may bind to histone tails thereby altering their methylation, resulting in epigenetic gene regulation (Benjamin et al., 2017). The life stage in which these AOPs occur are not specified, but are likely to be of relevance for mature males in which they may cause spermatocyte depletion.

III. Decreased aromatase levels resulting in decreased estradiol levels and impaired fertility in female adults.

AOP-Wiki states that there is low evidence for humans for this AOP, but since the pathways leading to production of ovarian hormones are similar in rodent models and humans, experimental animal data are assumed to be relevant for consideration of human risk (Latini et al., 2008). This pathway may therefore explain female reproductive toxicity related to adult exposure to phthalates, not via the estrogen receptor (ER) but via inhibition of estradiol production with PPARγ activation or promoter demethylation as an initiating event. PPARα activation may also be involved in activation of this pathway (Latini

et al., 2008; Lovekamp-Swan and Davis, 2003). PPARγ can be activated by several phthalates in rats and humans (Corton and Lapinskas, 2005; Delfosse et al., 2015). Although the link of this MIE with aromatase is not well described (Berger and Moller, 2002; Gervois et al., 2000; KEGG, 2016) there is evidence supporting this KER (Lovekamp-Swan and Davis, 2003; Xu et al., 2010) and the relation between PPARγ and adverse female reproductive outcomes. In addition, results from experimental studies show decreased transcription of genes encoding steroidogenic enzymes following phthalate exposure and correlate to limited epidemiological findings and effects observed in human ovarian cell types (Hannon and Flaws, 2015; Mathieu-Denoncourt et al., 2015). Which phthalates in particular elicit these effects, the exact impact on fertility and mechanism of action, and whether these effects occur within human exposure ranges remain to be determined (Hannon and Flaws, 2015). Reduction of aromatase levels is also a KE in AOP 153 where it results in reduced kisspeptin, GnRH, and LH levels and ovulation inhibition. The relevance for humans and KERs of this AOP have not been specified, but elements of this pathway have been associated with phthalate exposure literature (see Table 1). This AOP can thus hypothetically be linked to PPARγ activation.

Antral follicle count and female hormonally mediated diseases, which were associated with phthalate exposure in human observational studies, could not be related to any AOP. Since AOPs are a relatively new tool and have been poorly described for chronic toxicity thus far (Escher et al., 2017), the current lack of a relevant AOP does not mean that a causal relation between a biological response associated to phthalate exposure and an adverse health outcome does not exist. Increasing evidence suggests that phthalates have the ability to disrupt ovarian function and impact the ovarian reserve by intervening at different stages of folliculogenesis via disrupted follicular growth and recruitment or apoptosis (Hannon and Flaws, 2015; Vabre et al., 2017). Very few studies have investigated the effects on folliculogenesis in humans and the exact mechanisms, dose-response relation, and consequently the link to female fertility remain unclear (Hannon and Flaws, 2015). Elements of the toxicity pathways involved in effects on follicle count and female hormonally mediated diseases according to experimental studies are included in the abovementioned AOPs. Adverse effects on female reproductive organs may thus be additional AOs of these AOPs.

4.2. Biomarkers of effect

Although the exact mechanism of action and conclusive AOPs still need to be deciphered, mechanistically relevant biomarkers of effect can be derived that temporally, consistently, and plausibly fit into pathways leading to reproductive toxicity. In principle, all elements of the identified adverse outcome pathways from the point of interference by phthalates onwards can serve as biomarkers of effect. Initiating and early KEs are often well conserved between species (Escher et al., 2017) and are of use for early, preclinical observation of adverse effects. Since these biomarkers are closely linked to chemical exposure, effects may be observed during all developmental stages. Further downstream, on the other hand, adverse health effects may specifically occur in a particular window of development. Multiple biological effects of the same chemical or effects of multiple phthalates may converge towards a shared adverse outcome, thereby facilitating detection of (combined) toxicity. All potential targets of phthalates identified in this study that represent candidates for development of novel biomarkers and deserve further evaluation in experimental and epidemiological studies to verify their association with adverse health effects of specific or multiple phthalates are summarized in Table 2.

4.2.1. Early events

PPARα, PPARγ, and GR activation are initiating events in the identified AOPs and have been reported to be induced by phthalates via receptor binding and/or increased receptor gene expression; the latter

Table 2

Novel targets for development of biomarkers of effect in relation to established health effects of phthalate compounds. Potential associations between phthalate exposure, early and downstream events, and adverse health effects displayed in each row need to be further evaluated by experimental and epidemiological research.

Early events	Downstream events	Related health effects
PPARα, PPARγ, and GR activation AR expression	Steroidogenic enzyme levels TSPO	Reduced testosterone levels Male reproductive tract malformations Reduced sperm quality or quantity Metabolic disorders
NADPH oxidase activation Histone deacetylase inhibition	Oxidative stress Cell cycle arrest Apoptosis	Reduced sperm quality or quantity Disrupted folliculogenesis
PPARα, PPARγ, and GR activation ER expression	Aromatase levels and other steroidogenic enzymes Kisspeptin levels LH levels	Decreased estradiol levels Disrupted ovulation and ovarian cycle Metabolic disorders
PPARα, PPARγ, and GR activation	NFκB pathway	Altered inflammatory response Altered immune function

is potentially mediated by a positive feedback loop after receptor activation or by promotor demethylation or histone acetylation (Maradonna and Carnevali, 2018; Mathieu-Denoncourt et al., 2015; Singh and Li, 2012). This may explain why PPARγ expression was not upregulated in a study that addressed nuclear receptor gene expression related to female infertility associated with phthalate exposure (La Rocca et al., 2014). PPARs are nuclear receptors that regulate transcription of downstream genes. Target genes of PPARα and PPARγ partly overlap. PPARα regulates genes involved in fatty acid transport and metabolism and lipid and cholesterol homeostasis and is expressed in metabolically active tissues (Berger and Moller, 2002; Gervois et al., 2000). In addition, hepatic, testicular, and pancreatic cancers have been associated with activation of PPARα by phthalates, although the relevance of this effect for humans has been questioned (NRC, 2008). PPARγ regulates adipocyte differentiation, lipid metabolism, glucose uptake, insulin signaling, cellular energy homeostasis, and appetite. It also has a role in cell differentiation and carcinogenesis and may modulate the oxidative stress response by interaction with Nrf2 (Bansal et al., 2018; Berger and Moller, 2002; Kang et al., 2016; Kvandova et al., 2016). PPARγ is predominantly expressed in adipose tissue and in multiple other tissues including skeletal muscle and liver (Berger and Moller, 2002; Gervois et al., 2000). The GR is expressed in most cell types. Its activation is associated with metabolic dysregulation, as it stimulates gluconeogenesis, lipolysis, and food intake and reduces insulin signaling. PPARα, PPARγ, and GR also display anti-inflammatory effects (Berger and Moller, 2002; Kang et al., 2016). Besides, crosstalk between different nuclear receptors resulting in up- or downregulation of each other's target genes is known to occur, such as between GR and ER and thyroid receptor (TR) (Mathieu-Denoncourt et al., 2015; Miranda et al., 2013), PPAR and ER and TR (Benjamin et al., 2017; Corton and Lapinskas, 2005; Kouidhi and Clerget-Froidevaux, 2018), PPARγ and AR (Olokpa et al., 2017), and between nuclear receptors and the NF-κB pathway which is required for expression of inflammatory and immune response genes, thereby forming an important regulatory link between the endocrine and immune systems (De Bosscher et al., 2006). Nuclear receptor activation by phthalates may thus also mediate a number of known adverse health outcomes of phthalate exposure other than reproductive toxicity, including obesity, insulin resistance and diabetes, hypercholesterolemia, immune modulation, neurological function, and cancer (Benjamin et al., 2017; Berger and Moller, 2002; Delfosse et al., 2015; Ejaredar et al., 2015; Janesick and Blumberg, 2011; Sedha et al., 2015; Stojanoska et al., 2017). Application of effect biomarkers related to nuclear receptor activation and gene expression or methylation in biomonitoring studies has been very limited thus far. NADPH oxidase activation and histone deacetylase inhibition represent two other molecular initiating events of AOPs that are considered relevant for phthalates, but direct interference of phthalates with these molecular targets remains to be established.

For actual use of biomarkers of effect in human observational studies, human relevance has to be verified for different exposure windows and specificity and sensitivity need to be defined. Impairment of reproductive development and function by phthalates seems to be at least in part mediated by the activation of PPAR signaling pathways in humans in both genders and in different life stages (Corton and Lapinskas, 2005; Latini et al., 2008). Nuclear receptors can however be activated by a variety of environmental contaminants and therefore do not represent specific effects of phthalates (Gulliver, 2017; Lovekamp-Swan and Davis, 2003; Maradonna and Carnevali, 2018). They neither are specific for reproductive health effects, as discussed above. On the other hand, PPAR and GR activation may serve as an early indicator for multiple adverse effects of phthalates. This cannot be directly inferred from AOPs, as these represent a linear and unidirectional chain focused on one MIE and AO. This is regarded as a drawback of the AOP concept, as in reality biological processes are part of much more complex networks including additive, synergistic and/or antagonistic effects of chemicals, feedback loops, nuclear receptor crosstalk, and overlapping and interrelated pathways, finally affecting a broad range of physiological functions (Escher et al., 2017; Leist et al., 2017). Therefore, it would be of interest to study effects of phthalates on other nuclear receptors besides PPARs and GR as well. Although interference of phthalates with testosterone and estradiol levels is more likely to be related to PPAR and GR activation than to ER or AR binding or expression (Corton and Lapinskas, 2005), some studies have shown effects of phthalates on ER or AR activity (Hannon and Flaws, 2015; La Rocca et al., 2014; Mathieu-Denoncourt et al., 2015) and inclusion of assessment of their activation in biomonitoring studies could clarify whether the associations of PPAR and GR with reproductive toxicity are indeed present in humans and are more consistent or stronger than for other nuclear receptors.

Nuclear receptor activation can be assessed by receptor binding (for instance using reporter gene assays), receptor gene expression or epigenetic modification of the promotor region, and transcription of components of the signaling cascade (Dennis et al., 2016; Escher et al., 2017). Since PPAR and GR activation represent novel candidates for developing biomarkers of effect for phthalates, and phthalates may also interfere with downstream events, measurement of the complete signaling cascade would be interesting to further elucidate interference of phthalates with this pathway. For evaluation of differential gene expression levels, the timing of exposure and sampling and the tissue that is evaluated are of relevance. For PPARα, for example, it is known that gene expression follows a diurnal rhythm and varies between individuals, is most abundant in the liver, and the role in the phthalate syndrome is related to prenatal phthalate exposure and activation in Leydig cells (Gervois et al., 2000; NRC, 2008). Although the function and expression of PPARs may differ per tissue, their activation or upregulation in white blood cells (https://www.ncbi.nlm.nih.gov/gene/5465) may still serve as a sensor for health effects of phthalates at any

given life stage. Since gene expression changes reflect early effects of chemical exposure, they are best suited to be assessed shortly after exposure has taken place in longitudinal studies, or in cross sectional studies in continuously exposed groups.

4.2.2. Downstream events

Cell cycle arrest, apoptosis, and induction of oxidative stress are cellular processes that may mediate adverse effects of phthalates. These processes can be measured by gene expression changes as well as functional assays. In addition, 8-hydroxy-2′-deoxyguanosine (8-OHdG) excretion in urine is a classic biomarker of effect for chemicals that cause oxidative damage. These biomarkers are also not specific for phthalates neither for reproductive toxicity. Their measurement in human observational studies is nevertheless useful, as this (i) may lead to a more comprehensive mechanistic understanding of the toxicity of phthalates, and (ii) fits in the exposome concept, which integrates assessment of exposure to exogenous chemicals with subsequent endogenous generation of reactive oxygen species (ROS), oxidation products, adducts, signaling molecules formed as part of the pathway of toxicity, and markers of the adaptive cellular stress responses. The inclusion of the total biological response to a chemical stressor in exposure assessment helps to identify which exposures are biologically important and indicates the plausibility of associations between exposures and health outcomes (Dennis et al., 2016; Escher et al., 2017). Downstream events can be assessed in longitudinal studies, given that an appropriate time span between exposure and sampling is applied, or in cross sectional studies in continuously exposed groups.

Steroidogenic enzymes, cholesterol transporters and testosterone and estradiol levels are known to be affected by phthalates and represent key events further downstream in the AOPs. They are more specifically linked to pre- and postnatal phthalate exposure and represent a sensitive health endpoint for this group of chemicals. The gonadotrophin level (LH and FSH) could be an interesting biomarker as well with respect to the proposed mechanism of action via alterations in the HPG axis, even though this is supported by relatively little experimental evidence. Thyroxine has been associated with both adverse neurodevelopmental and metabolic effects of phthalates, but experimental evidence is limited for this hormone as well. Kisspeptin is an interesting novel biomarker, that was linked to phthalate exposure and reproductive outcome in only one human observational and experimental study (Chen et al., 2013a; Hu et al., 2013a), but may be an indicator of altered pubertal development due to phthalate exposure. In addition, it has been suggested to link energy homeostasis and the reproductive system (Tng, 2015; Wolfe and Hussain, 2018).

The eventual adverse reproductive outcomes of phthalate exposure are observable as male reproductive tract malformations, testicular toxicity, reduced sperm quality and quantity, altered ovarian cycle, reduced fertility, and reproductive failure. These events may associate phthalate exposure to population health impact. Anogenital distance is for instance positively correlated with fertility, sperm density, and sperm count in men, and can serve as a biomarker for male reproductive potential (Mathieu-Denoncourt et al., 2015).

4.3. Strengths and limitations of the proposed strategy

The presented strategy to comprehensively identify biomarkers of effect that are well substantiated by mechanistic data may help to infer causal relations between chemical exposure and adverse health effects by efficiently combining i. results of epidemiological studies (indicating exposure-effect biomarker and exposure-health outcome associations in

humans), ii. results of experimental studies (identifying exposure-effect biomarker, effect biomarker-health outcome, and exposure-health outcome relations), and iii. toxicological information stored in quality-controlled AOPs (representing causal effect biomarker-health outcome relations). By connecting the different fields of research, the weight of evidence for associations between chemical exposures and health outcomes is strengthened and plausible causal relations can be distinguished and prioritized for further research in epidemiological studies. In addition, novel targets for development of biomarkers of (early) biological response may be identified. Nonetheless, the strategy requires the availability of well-defined AOPs and a sufficient amount of mechanistic data relevant for human chemical exposure. Ideally, future AOP networks including feedback loops and modulatory events, instead of the unidirectional and linear AOPs currently available in the AOP-Wiki, will allow to include all biological events related to chemical exposure and health outcomes (as is illustrated by Fig. 1) (Leist et al., 2017). The approach in this proof of concept explored effect biomarkers for the family of phthalate compounds and did not *a priori* distinguish developmental stages or sexes; for specific effects of individual phthalates, a more focused search strategy would need to be applied.

5. Conclusions

Although adverse endocrine-related and reproductive health effects related to exposure to phthalates have been frequently studied, a knowledge gap still exists with respect to mechanisms of action and causal associations in humans. Literature screening in combination with collection of AOP information yielded clues to human effect biomarkers with substantial mechanistic support that may causally link phthalate exposure to adverse reproductive outcomes on the one hand, and help build the weight of evidence of phthalate toxicity in humans on the other hand. The activation of several receptors such as PPARα, PPARγ, and GR emerged as early markers for a range of health effects of phthalate exposure, thus representing promising novel biomarkers. Additional assessment of downstream events such as alteration of enzyme and hormone levels and oxidative stress, according to the exposome concept, and of interactions with other nuclear receptors would provide more insight into the mechanisms of action in humans. Our study is a first proof of concept to join epidemiological, experimental, and toxicological information in order to advance the field of human biomonitoring.

Declarations of interest

None.

Appendix A

PubMed search terms.
Phthalate exposure.
Phthalate OR Phthalates OR Diethylhexyl phthalate OR Bis(2-ethylhexyl)phthalate OR Dioctyl Phthalate OR Di-2-Ethylhexylphthalate OR Di(2-

ethylhexyl)phthalate OR Butyl benzyl phthalate OR BBPHT OR Dibutyl phthalate OR Di-n-Butyl Phthalate OR Butyl Phthalate OR Diisobutyl phthalate OR Diisononyl phthalate OR ENJ 2065 OR Diethyl phthalate OR Phthalic acid diethyl ester OR Diisodecyl phthalate OR di-isodecyl phthalate OR Di-n-octyl phthalate OR Dimethyl phthalate OR Dimethylphthalate OR Di-n-pentyl phthalate OR Diamyl Phthalate OR Dicyclohexyl phthalate OR Di-n-hexyl phthalate OR Dihexyl Phthalate OR Phthalic acid dihexyl ester OR Di(methoxyethyl) phthalate OR Diisononyl cyclohexane-1,2-dicarboxylate OR Mono-benzyl phthalate OR Monocyclohexyl phthalate OR Mono-(2-ethylhexyl) phthalate OR Monoethyl phthalate OR Mono-isobutyl phthalate OR Mono-isodecyl phthalate OR Mono(carboxy-isooctyl) phthalate OR Monobutyl phthalate OR Mono-n-octyl phthalate OR Monomethyl phthalate OR Monoisononyl-cyclohexane-1,2-dicarboxylate OR (Diethylhexyl phthalate OR Bis(2-ethylhexyl)phthalate OR Dioctyl Phthalate OR Di-2-Ethylhexylphthalate OR Di(2-ethylhexyl)phthalate [Supplementary Concept]) OR (Butyl benzyl phthalate OR BBPHT [Supplementary Concept]) OR (Dibutyl phthalate OR Di-n-Butyl Phthalate OR Butyl Phthalate [Supplementary Concept]) OR (Diisobutyl phthalate [Supplementary Concept]) OR (Diisononyl phthalate OR ENJ 2065 [Supplementary Concept]) OR (Diethyl phthalate OR Phthalic acid diethyl ester [Supplementary Concept]) OR (Diisodecyl phthalate OR di-isodecyl phthalate [Supplementary Concept]) OR (Di-n-octyl phthalate [Supplementary Concept]) OR (Dimethyl phthalate OR Dimethylphthalate [Supplementary Concept]) OR (Di-n-pentyl phthalate OR Diamyl Phthalate [Supplementary Concept]) OR (Dicyclohexyl phthalate [Supplementary Concept]) OR (Di-n-hexyl phthalate OR Dihexyl Phthalate OR Phthalic acid dihexyl ester [Supplementary Concept]) OR (Di(methoxyethyl) phthalate [Supplementary Concept]) OR (Diisononyl cyclohexane-1,2-dicarboxylate [Supplementary Concept])

Reproductive effects

Reproductive OR puberty OR pregnancy OR infertility OR semen quality OR placenta OR anogenital distance OR hypospadias OR cryptorchidism OR "Reproductive Health"[Mesh] OR "Reproductive Medicine"[Mesh] OR "Reproduction"[Mesh] OR "Reproductive Techniques, Assisted"[Mesh] OR "Infertility"[Mesh].

Endocrine disruption

"Endocrine System"[Mesh] OR "Endocrine Glands"[Mesh] OR "Endocrine System Diseases"[Mesh] OR "Hormones"[Mesh] OR "Gonadal Hormones"[Mesh] OR "Placental Hormones"[Mesh] OR "Pituitary Hormones"[Mesh] OR "Growth Hormone"[Mesh] OR "Thyroid Hormones"[Mesh] OR "Gastrointestinal Hormones"[Mesh] OR "Sex Hormone-Binding Globulin"[Mesh] OR "Adrenocorticotropic Hormone"[Mesh] OR "Adrenal Cortex Hormones"[Mesh] OR Endocrine system OR hypothyroidism OR hyperthyroidism OR adrenal.

Appendix B

Flow chart summarizing results of literature search.
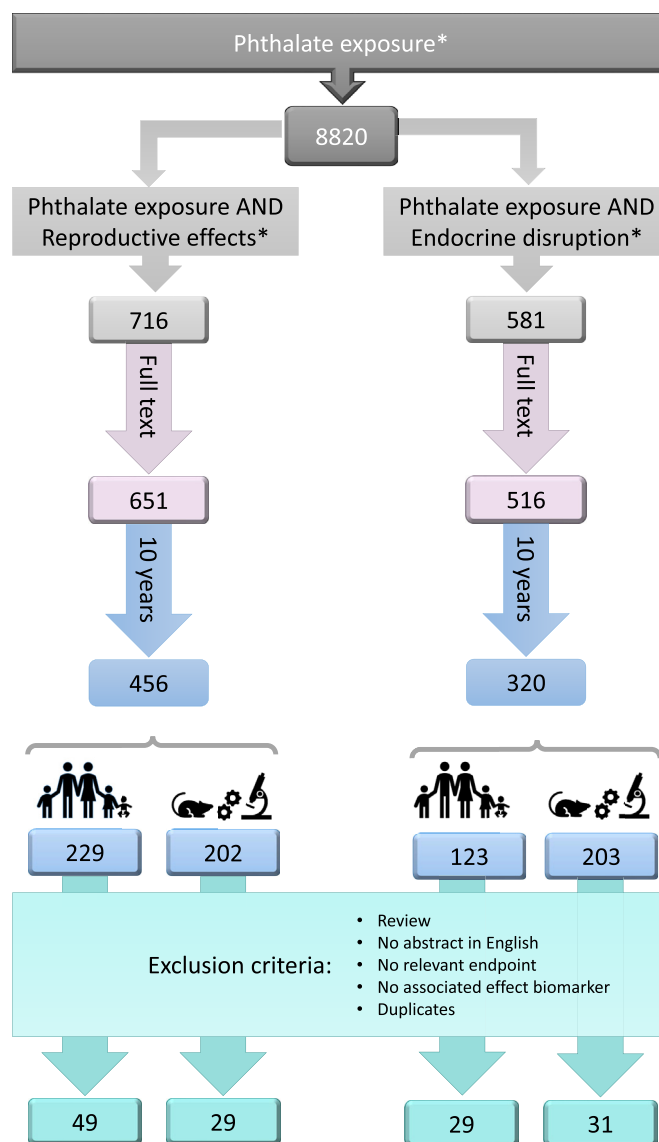
* For PubMed search terms see Appendix I

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