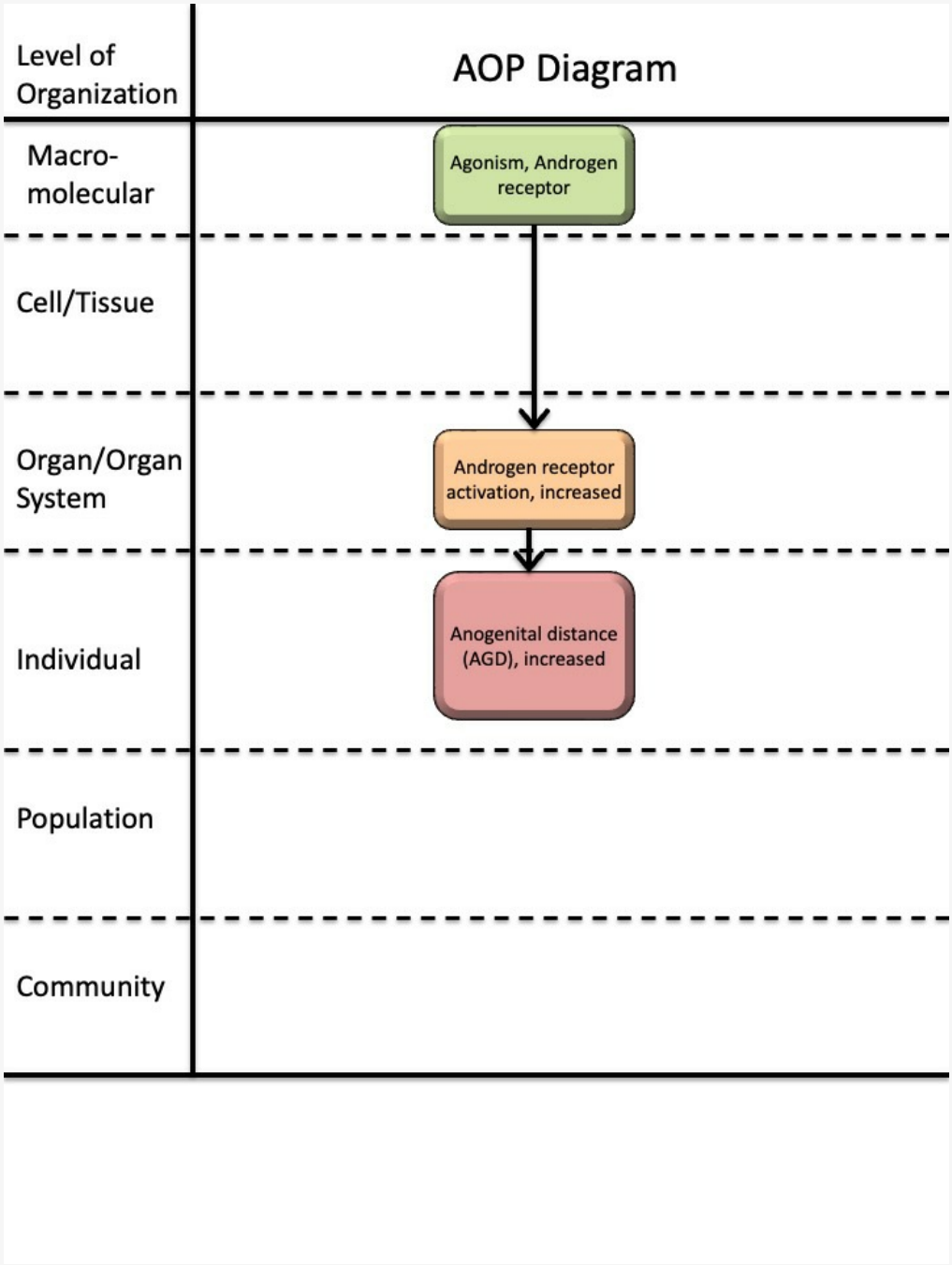
AOP ID and Title:

AOP 547: Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring
Short Title: Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring

Graphical Representation



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Status

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Abstract

The purpose of developing this AOP was to establish a framework for identifying endocrine disruptors based on the measurement of anogenital distance (AGD) in female rodents. AGD is defined as the distance between the anus and the external genitalia. In both rodents and humans, female AGD is approximately half the length of male AGD (Liu et al., 2014; Salazar-Martinez et al., 2004; Schwartz et al., 2019; Sharpe, 2020; Thankamony et al., 2016; Wise, 2024). This difference reflects androgen-dependent regulation of perineal growth during fetal development in males, whereas in females, the absence of androgens limits this growth (Ipulan et al., 2016; Schwartz et al., 2019; Sharpe, 2020; Welsh et al., 2008).

AGD is considered an apical endpoint and is included in OECD test guidelines TG414, TG416, TG421, TG422 and TG443 (OECD, 2001; OECD, 2018; OECD, 2025a; OECD, 2025b; OECD, 2025c). According to ECHA/EFSA guidance for identifying endocrine disruptors, AGD is classified as an EATS-mediated parameter and is used in the identification of endocrine disruptors (ECHA/EFSA, 2018).

The molecular initiating event (MIE) for this AOP is androgen receptor (AR) agonism, meaning the binding of a compound to the AR that activates the receptor. This leads to the first key event (KE): increased AR activation in tissues and organs in vivo, including the perineum. During fetal development in females, increased AR activation in the perineum results in increased AGD considered an adverse outcome (AO).

The overall weight of evidence supporting this AOP is high. Biological plausibility for both key event relationships (KERs) is strong, as androgen receptor mechanisms and androgenic effects on AGD are well established. Empirical evidence for the KERs is based on studies showing that AR agonists increase female AGD in prenatal rat studies.

This AOP is applicable for identifying androgenic endocrine disruptors.

AOP Development Strategy

Context

This AOP was developed as part of the European Food Safety Authority (EFSA) project (Grant Agreement No. GP/EFSA/PREV/2022/01) aimed at creating AOPs for endocrine disruptors. The purpose of this AOP is to provide a framework for identifying endocrine disruptors associated with increased AGD in female rodent offspring. Other AOPs within the project address shortened AGD in males (AOPs 305, 306, and 307).

The molecular initiating event (MIE25: Agonism, Androgen receptor) and key event (KE2274: Androgen receptor activation, increased) described in this AOP can serve as a foundation for developing additional AOPs related to adverse outcomes resulting from androgen receptor agonism.

Furthermore, this AOP can be expanded into an AOP network by incorporating pathways such as “Increased testosterone levels leading to long anogenital distance in female offspring” and “Increased dihydrotestosterone levels leading to long anogenital distance in female offspring.” These can be developed using the key events (KE2272, KE2273) and key event relationships (KER3380, KER3381) established in the EFSA-funded project.

A shortened AGD is recognized as a sensitive marker of reduced AR activity during the masculinization programming window in the male, whereas in the female a longer AGD is a marker of increased AR activity and masculinization of the female fetus in this period.

Strategy

This AOP was developed in two steps.

First, an upstream AOP network for “increased androgen activity” was constructed. The AOP-Wiki was screened to identify existing key events (KEs) related to increased androgen activity by searching KE and KER pages for the terms androgen, testosterone, dihydrotestosterone, and DHT. Two relevant KEs were identified, but no relevant KERs. KE25 was updated, while KE286 required no revision. All other KEs and KERs were newly developed following the methodology outlined in the AOP Developers’ Handbook (version 2.7): KE2272, KE2273, KE2274, KER3378, KER3379, KER3380, and KER3381.

The network reflects processes widely regarded as canonical, and evidence was primarily drawn from review articles and book chapters identified through searches in PubMed and library catalogues. For the current AOP, the following KEs from the upstream network were used: KE25, KE2274, and KER3378. The remaining KEs and KERs could be used to expand the current AOP into an AOP network as described above.

In the second step, KE2365 and KER3628 were developed.

Evidence for KE2365 was drawn mainly from review articles identified through PubMed searches. A systematic

weight-of-evidence approach was applied to collect, evaluate, extract, and integrate evidence for KER3628, as detailed on its dedicated page. Briefly, a scoping literature search was first conducted in PubMed to identify relevant model substances. Based on these findings, a targeted search was performed in PubMed and Web of Science using terms related to the model substances and AGD.

Study reliability for included publications was assessed using the Science in Risk Assessment and Policy (SciRAP) tools for in vivo studies (http://www.scirap.org), and overall confidence in the evidence for each model substance was categorized.

The AOP includes three KEs: the MIE, the AO, and one intermediate KE.

These were selected to capture the main steps in the pathway from AR agonism to increased AGD. Additional KEs could be added, such as KE286 (Altered transcription of genes by the AR) and a KE describing androgen regulation of perineal growth, which likely involves androgen-mediated development of the levator ani and bulbocavernosus (LABC) muscle complex in the perineum. However, sufficient empirical evidence for these related KERs is currently lacking.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	25	Agonism, Androgen receptor	Agonism, Androgen receptor
	KE	2274	Androgen receptor activation, increased	Androgen receptor activation, increased
	AO	2365	Anogenital distance (AGD), increased	Anogenital distance (AGD), increased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Agonism, Androgen receptor	adjacent	Androgen receptor activation, increased	High	Low
Androgen receptor activation, increased	adjacent	Anogenital distance (AGD), increased	High	Low

Stressors

Name	Evidence
17-Methyltestosterone	
Testosterone propionate	
17beta-Trenbolone	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Fetal	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	Low	NCBI

Sex Applicability

Sex	Evidence
Female	High

Life stage applicability

The life stage applicability domain for this AOP is fetal. Although AR activation occurs during fetal development, puberty, and adulthood, the regulation of AGD growth is specifically controlled by androgens during the masculinization programming window, which takes place in fetal development (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017; Welsh et al., 2008).

Taxonomic applicability

The biologically plausible taxonomic applicability domain for this AOP is mammals, as fetal masculinization is regulated by androgens across all mammalian species (Welsh et al., 2014). The empirical taxonomic applicability domain is rat and human, supported by prenatal studies in rat demonstrating that androgen receptor activation leads to a measurable increase in female offspring AGD (Armoskus et al., 2014; Guerra et al., 2014; Hotchkiss et al., 2007; Sathishkumar et al., 2011; Welsh et al., 2009; Wilson et al., 2002; Wolf et al., 2002; Wolf et al., 2004) and epidemiological studies suggesting that elevated androgen levels in women are associated with increased AGD (Mira-Escolano et al., 2014; Pan et al., 2021; Zamani et al., 2023).

Sex applicability

The sex applicability domain for this AOP is females, supported by studies showing effects of androgen activation on female AGD (Armoskus et al., 2014; Guerra et al., 2014; Hotchkiss et al., 2007; Mira-Escolano et al., 2014; Pan et al., 2021; Sathishkumar et al., 2011; Welsh et al., 2009; Wilson et al., 2002; Wolf et al., 2002; Wolf et al., 2004; Zamani et al., 2023).

Essentiality of the Key Events

KE	Evidence	Level of evidence
MIE25	Direct evidence on effect on KE2274: Studies in AR knockout mice demonstrate that AR is essential for mediating effects on the reproductive system by AR activation (De Gendt et al., 2004; Holdcraft & Braun, 2004; Matsumoto et al., 2003; Notini et al., 2005; Yeh et al., 2002). AR antagonists flutamide, procymidone and vinclozolin inhibit AR activity in vivo as shown in the OECD test guideline Hershberger assay (Browne et al., 2018). Direct evidence on effect on AO2365: Studies in AR knockout mice demonstrate that AR is essential for a longer AGD, as male AR knockout mice exhibit AGD lengths comparable to female wild-type mice (Yeh et al., 2002, Sato et al., 2004). It should be noted AGD in male mice was studied but the AO2365 is in females. Prenatal exposure to the AR antagonists flutamide, procymidone and vinclozolin results in shorter AGD in rat studies (Casto et al., 2003; Foster & Harris, 2005; Fussell et al., 2015; Goto et al., 2004; Gray et al., 1994; Hass et al., 2007; Hass et al., 2012; Inawaka et al., 2010; Kita et al., 2016; Ostby et al., 1999; McIntyre et al., 2001; Shimamura et al., 2002; Wolf et al., 2000; Yamasaki et al., 2005). It should be noted AGD in male rats was studied but the AO2365 is in females.	High. There is direct evidence from knockout and antagonist studies. There are no inconsistencies identified but it should be noted that the effect on AGD was studied in male animals.

KE2274	Direct evidence on effect on AO2365: Studies in AR knockout mice demonstrate that AR activity is essential for a longer AGD, as male AR knockout mice exhibit AGD lengths comparable to female wild-type mice (Yeh et al., 2002, Sato et al., 2004). It should be noted AGD in male mice was studied but the AO2365 is in females. Prenatal exposure to the AR antagonists flutamide, procymidone and vinclozolin results in reduced AGD in rat studies (Casto et al., 2003; Foster & Harris, 2005; Fussell et al., 2015; Goto et al., 2004; Gray et al., 1994; Hass et al., 2007; Hass et al., 2012; Inawaka et al., 2010; Kita et al., 2016; Ostby et al., 1999; McIntyre et al., 2001; Shimamura et al., 2002; Wolf et al., 2000; Yamasaki et al., 2005). It should be noted AGD in male rats was studied but the AO2365 is in females.	High. There is direct evidence from knockout and antagonist studies. There are no inconsistencies identified, but it should be noted that the effect on AGD was studied in male animals.

Weight of Evidence Summary

Assessment of biological plausibility

KER	Assessment
KER 3378: Agonism, Androgen receptor leads to Androgen receptor activation, increased	The biological plausibility of the KER is High. It is a generally recognised process, canonical knowledge, that binding of agonists to AR leads to increased AR activation in vivo (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).
KER 3628: Androgen receptor activation, increased leads to AGD, increased	The biological plausibility of the KER is High. It is a well-documented by numerous studies in rats that AR activation drives the fetal masculinization process, including the growth of the perineum, which can be measured as AGD (Liu et al., 2014; Salazar-Martinez et al, 2004; Schwartz et al., 2019; Sharpe, 2020; Thankamony et al., 2016; Welsh et al., 2008; Wise, 2024). This is supported by epidemiological studies (Mira-Escolano et al., 2014; Pan et al., 2021; Zamani et al., 2023).

Assessment of empirical evidence

KER	Assessment
KER 3378: Agonism, Androgen receptor leads to Androgen receptor activation, increased	The empirical support for the KER is High. AR agonists such as testosterone propionate, methyl testosterone and trenbolone have been identified as agonists in the US EPA Androgen receptor pathway model, which integrates in vitro assays for receptor binding, coregulator recruitment and transactivation. These stressors have also been shown to increase androgen activity in Hershberger assay and other in vivo assays (Kleinstreuer et al., 2018; Browne et al., 2018). Assessing dose and temporal concordance for this KER is challenging because the upstream KE is measured in vitro, whereas the downstream KE reflects on increased in vivo activation. There are no available data on incidence concordance.

KER 3628: Androgen receptor activation, increased leads to AGD, increased	The empirical support for the KER is High. The empirical evidence is based on four model substances: methyl testosterone, testosterone, testosterone propionate and trenbolone. These substances bind AR and act as AR agonists, as demonstrated in in vitro studies (Kleinstreuer et al., 2018; Judson et al., 2020) and androgen activity in in vivo Hershberger assays (Browne et al., 2018). Prenatal exposure to these substances in rat studies has been shown to increase AGD in female offspring, as reported in multiple studies (Armoskus et al., 2014; Guerra et al., 2014; Hotchkiss et al., 2007; Juarez et al., 1995; Kawashima et al., 1975; McCoy et al., 1992; Rhees et al., 1997; Tehrani et al., 2014; Welsh et al., 2009; Wilson et al., 2002; Wolf et al., 2002; Wolf et al., 2004; Wu et al., 2010) Assessing dose and temporal concordance for this KER is challenging because the upstream KE is measured in vitro, whereas the downstream KE is assessed in vivo. The upstream KE, increased AR activation, is expected to occur rapidly, within minutes to hours after exposure to the substance (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017). In the prenatal in vivo studies, the exposure occurred during GD15–19, with AGD measured in female offspring between GD21 and PND30. No evidence is currently available to assess incidence concordance for this KER.

Quantitative Consideration

The level of quantitative understanding of the AOP is low. No specific evidence has been identified for response-response relationships for the KERs or for the presence of feedforward or feedback loops. There is some understanding of the time-scale. Agonism of the AR leads rapidly to increased AR activity in vivo. Effects on activation of the receptor on cellular function can be seen after minutes to hours (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017). In prenatal in vivo studies, exposure to a substance that activates AR for five to six days during the masculinization window (around GD16-20 in rats) can result in increased AGD at PND2 (Hotchkiss et al., 2007; Wilson et al., 2002; Wolf et al., 2004).

Considerations for Potential Applications of the AOP (optional)

AGD is considered an apical endpoint and is included in OECD test guidelines TG414, TG416, TG421, TG422, and TG443 (OECD, 2001; OECD, 2018; OECD, 2025a; OECD, 2025b; OECD, 2025c). According to ECHA/EFSA guidance for identifying endocrine disruptors, AGD is classified as an EATS-mediated parameter and is used in the identification of endocrine-disrupting substances (ECHA/EFSA, 2018).

This AOP supports the regulatory use of increased AGD in female rodents as an indicator of endocrine disruption, specifically to identify androgenic endocrine disruptors.

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Appendix 1

List of MIEs in this AOP

Event: 25: Agonism, Androgen receptor

Short Name: Agonism, Androgen receptor

Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	MolecularInitiatingEvent
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	MolecularInitiatingEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction in zebrafish	MolecularInitiatingEvent
Aop:547 - Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring	MolecularInitiatingEvent
Aop:117 - Androgen receptor activation leading to hepatocellular adenomas and carcinomas (in mouse and rat)	MolecularInitiatingEvent
Aop:495 - Androgen receptor activation leading to prostate cancer	MolecularInitiatingEvent

Stressors

Name
17beta-Trenbolone
Spironolactone
5alpha-Dihydrotestosterone

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	High	NCBI
medaka	Oryzias latipes	High	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Taxonomic applicability: Androgen receptor orthologs are primarily limited to vertebrates (Baker 1997; Thornton 2001; Eick and Thornton 2011; Markov and Laudet 2011). Therefore, this MIE would generally be viewed as relevant to vertebrates, but not invertebrates.

Life stage applicability: Androgen receptor is expressed from the fetal period throughout adult life and activation of the androgen receptor controls sexual development during the fetal period and reproductive function as well as effects in other organs during puberty and adulthood (Dalton et al., 2010; Luetjens et al., 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017). (added Nov 2024)

Sex applicability: Androgen receptor is expressed in both males and females and have important roles for sexual development and reproduction as well as effects in other organs in both sexes (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017). (added Nov 2024)

Key Event Description

Site of action: The molecular site of action is the ligand binding domain of the AR. This particular key event specifically refers to interaction with nuclear AR. Downstream KE responses to activation of membrane ARs may be different. The cellular site of action for the molecular initiating event is undefined.

Responses at the macromolecular level: Binding of a ligand, including xenobiotics that act as AR agonists, to the cytosolic AR mediates a conformational shift that facilitates dissociation from accompanying heat shock proteins and dimerization with another AR (Prescott and Coetzee 2006; Claessens et al. 2008; Centenera et al. 2008). Homodimerization unveils a nuclear localization sequence, allowing the AR-ligand complex to translocate to the nucleus and bind to androgen-response elements (AREs) (Claessens et al. 2008; Cutress et al. 2008). This elicits recruitment of additional transcription factors and transcriptional activation of androgen-responsive genes (Heemers and Tindall 2007).

AR paralogs:

- Most vertebrates have a single gene coding for nuclear AR. However, most fish have two AR genes (AR-A, AR-B) as a result of a whole genome duplication event after the split of Acipenseriformes from teleosts but before the divergence of Osteoglossiformes (Douard et al. 2008).
- AR-B has been lost in Cypriniformes, Siluriformes, Characiformes, and Salmoniformes (Douard et al. 2008).
- In Percomorphs, AR-B has accumulated significant substitutions in the both ligand binding and DNA binding domains (Douard et al. 2008).
- Differential ligand selectivity and subcellular localization has been reported for AR paralogs in some fish species (e.g., Bain et al. 2015), but the difference is not easily generalized based on available data in the literature.

How it is Measured or Detected

Measurement/detection:

- **In vitro methods:**
  - OECD Test No. 458: Stably transfected human androgen receptor transcriptional activation assay for detection of androgen agonists and antagonists has been reviewed and validated by OECD and is well suited for detection of this key event (OECD 2016).
  - Binding to the androgen receptor can be directly measured in cell free systems based on displacement of a radio-labeled standard (generally testosterone or DHT) in a competitive binding assay (e.g., (Olsson et al. 2005; Sperry and Thomas 1999; Wilson et al. 2007; Tilley et al. 1989; Kim et al. 2010).
  - Cell based transcriptional activation assays are typically required to differentiate agonists from antagonists, in vitro. A number of reporter gene assays have been developed and used to screen chemicals for AR agonist and/or antagonist activity (e.g., (Wilson et al. 2002; van der Burg et al. 2010; Mak et al. 1999; Araki et al. 2005).
  - Expression of androgen responsive proteins like spiggin in primary cell cultures has also been used to detect AR agonist activity (Jolly et al. 2006).
  - US EPA Androgen receptor pathway model. The model includes 11 in vitro assays measuring receptor binding, coregulator recruitment, nuclear translocation, transactivation or cell proliferation. A “reduced” model that include fewer assays also exists. The output of the AR pathway model provides an AUC value for the potential of a chemical to cause AR agonism and/or AR antagonism. (EPA, 2022; Judson et al., 2020; Kleinstreuer et al., 2017). (added Nov 2024)
  - CoMPARA: Collaborative Modeling Project for Androgen Receptor Activity. The US EPA lead consortium has developed consensus computational models that can be used to predict androgen receptor binding, agonist or antagonist activity (Mansouri et al., 2020). (added Nov 2024)
- **In vivo methods**:
  - In fish, phenotypic masculinization of females has frequently been used as an indirect measurement of in vivo androgen receptor agonism.
    - Development of nuptial tubercles, a dorsal fatpad, and a characteristic banding pattern has been observed in female fathead minnows exposed to androgen agonists (Ankley et al. 2003; Jensen et al. 2006; Ankley et al. 2010; LaLone et al. 2013; OECD 2012).
    - Anal fin elongation in female western mosquitofish (*Gambusia affinis*) has similarly been viewed as evidence of AR activation (Raut et al. 2011; Sone et al. 2005).
    - In medaka, development of papillary processes, which normally only appear on the second to seventh or eighth fin aray of the anal fin, has also been used as an indirect measure of androgen receptor agonism (OECD 2012).
    - Production of the nest building glue, spiggin, in three female 3-spined sticklebacks (Gasterosteus aculeatus) has also been well documented as an indicator of androgen receptor agonism (Jakobsson et

al. 1999; Hahlbeck et al. 2004). Quantification of the spiggin protein in exposed female 3-spined stickleback or green fluorescence protein expression in a transgenic spg1-gfp medaka line (Sébillot et al. 2014) can be used to detect androgen receptor agonism.

- **High Throughput Screening**
  - Measures of AR agonism have been included in high throughput screening programs, such as US EPA's Toxcast program. Toxcast assays relevant for screening chemicals for their ability to bind and/or activate the AR include:
    - ATG_AR_TRANS A cell based assay that can differentiate agonism from antagonism
    - NVS_NR_hAR A cell free assay using recombinant human AR. Can detect binding, but cannot distinguish agonism from antagonism.
    - NVS_NR_rAR A cell free assay using recombinant rat AR. Can detect binding, but cannot distinguish agonism from antagonism.
    - OT_AR_ARELUC_AG_1440 A cell based assay that measures expression of a reporter gene under control of androgen-responsive elements. Can distinguish agonism from antagonism.
    - Tox21_AR_BLA_Agonist_ratio A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
    - Tox21_AR_LUC_MDAKB2_agonist A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
  - Assay descriptions

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List of Key Events in the AOP

Event: 2274: Androgen receptor activation, increased

Short Name: Androgen receptor activation, increased

Event Component

Process	Object	Action
regulation of androgen receptor signaling pathway	androgen receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:547 - Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Taxonomic applicability.

The AR is present in vertebrates. Mammals, birds and amphibians have one AR gene, whereas some fish species have two genes. AR activity has been studied in mammals, fish, birds and amphibians (Ogino et al., 2018). The biologically plausible domain of taxonomic applicability is vertebrates since the AR is present in vertebrates. The empirical domain of taxonomic applicability is human, rat and mice increased AR activity has been studied. The KE description focuses on mammals, but AOP developers are encouraged to expand the applicability to other species.

Life stage applicability

The AR is expressed from the fetal period throughout adult life and increased activity of the AR controls sexual

development during the fetal period and reproductive function as well as effects in other organs during puberty and adulthood (Dalton et al., 2010; Luetjens et al., 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Sex applicability

The AR is expressed in both males and females and has important roles for sexual development and reproduction as well as effects in other organs in both sexes (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Key Event Description

The androgen receptor (AR) belongs to the steroid hormone receptor family and mediates the biological effects of androgens. Increased AR activation described in this KE is occurring in complex biological systems such as tissues and organs in vivo due either to AR agonism (i.e. activation of the receptor by a compound) or to increased levels of the endogenous hormones testosterone or dihydrotestosterone (DHT). It is thus considered distinct from KEs describing either AR agonism or increased hormone levels.

In the absence of ligand, the AR resides in the cytoplasm. Upon binding of endogenous hormone or a compound acting as an agonist, the receptor is activated, forms a homodimer, translocates into the nucleus and binds to androgen-response elements and regulates target gene transcription by recruiting cofactor protein complexes. The AR can also exert rapid non-genomic action by binding to plasma membrane proteins and activating kinase signalling in the cytoplasm. Increased AR activity can have various effects in vivo, including on sexual development and reproductive function, as well as effects on other organs such as adipose tissue, bone, brain, cardiovascular system, hair, muscle and skin (Dalton et al., 2010; Davey & Grossmann, 2016; Luetjens et al., 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

How it is Measured or Detected

This KE specifically focuses on increased in vivo activation, but most methods that can be used to measure AR activity are carried out in vitro. They provide indirect information about the KE and are described in lower tier KEs (for example KE-25 for AR agonism, KE-2272 for increased testosterone levels or KE-2273 for increased dihydrotestosterone levels). In this way, this KE is a placeholder for tissue-specific responses to AR activation that will depend on the adverse outcome (AO) for which it is included.

In fish, The Rapid Androgen Disruption Activity Reporter (RADAR) assay included in OECD test guideline no. 251 can be used to measure genomic AR activity (OECD, 2022). Employing a spg1-gfp construct under control of the AR-binding promoter spiggin1 in medaka fish embryos, any stressor activating or inhibiting the androgen axis will be detected. This includes for instance stressors that agonize or antagonize AR, as well as stressors that modulate androgen synthesis or metabolism. Non-genomic AR activity cannot be detected by the RADAR assay. Similar assays may in the future be developed to measure AR activity in mammalian organisms.

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List of Adverse Outcomes in this AOP

Event: 2365: Anogenital distance (AGD), increased

Short Name: Anogenital distance (AGD), increased

Event Component

Process	Object	Action
androgen receptor signaling pathway	Musculature of female perineum	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:547 - Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring	AdverseOutcome

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

perineum

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Fetal	High

Sex Applicability

Sex	Evidence
Female	High

Taxonomic Applicability

The biologically plausible taxonomic applicability domain is mammals, as fetal masculinization is regulated by androgens across all mammalian species (Welsh, 2014). The empirical taxonomic applicability domain is based on rat, supported by prenatal studies demonstrating that exogenous androgen treatment leads to a measurable increase in female AGD (Armoskus, 2014; Guerra, 2014; Hotchkiss, 2007; Sathishkumar, 2011; Welsh, 2009; Wilson, 2002; Wolf, 2002; Wolf, 2004). In humans, epidemiological studies show that patients with polycystic ovary syndrome (PCOS) presents with a longer AGD than controls (Pan et al., 2021; Zamani, 2023). There is also some conflicting evidence that girls born to women with PCOS have longer AGD. Evidence regarding AGD in daughters of women with PCOS is mixed: Barrett (2018) reported an association, whereas Glintborg (2019) did not. Additionally, one study found that fetuses of women with PCOS exhibited longer AGD (Perlman, 2020).

Sex applicability

This KE focuses on effects on AGD in females. A long AGD in female offspring is a marker of excess (ectopic) androgen action during fetal life (Schwartz et al. 2019). A longer AGD is thus a sign of virilization of the female fetus.

Life Stage Applicability

Growth of AGD in rats is regulated by androgens during the masculinization programming window, which occurs during fetal development between GD15-18 (Welsh, 2008; Mc Leod et al, 2010).

Key Event Description

Anogenital distance (AGD)—the span between the anus and the external genitalia—is a sexually dimorphic trait observed in

both rodents and humans, where males exhibit an AGD approximately twice as long as that of females (Liu, 2014; Salazar-Martinez et al., 2004; Schwartz et al., 2019; Sharpe, 2020; Thankamony, 2016; Wise, 2024). This difference arises from the influence of androgens during fetal development, which drive the formation of secondary sexual characteristics. Presence of androgen in the male fetus drives he elongation of perineum, whereas the lack of androgen in female fetuses prevents this masculinization process (Ipulan, 2016; Schwartz et al., 2019; Sharpe, 2020; Welsh, 2008).

In rats, androgens act within a critical developmental window – around gestational days 15.5 to 18.5 (Welsh et al, 2008; MacLeod et al., 2010) - and AGD has gained recognition as a reliable proxy for assessing the intrauterine hormonal environment. In XY fetuses, insufficient androgen exposure results in a shorter AGD, while in XX fetuses, excessive androgen exposure can lead to an elongated AGD. This pattern has been observed in both human and rodent studies (Schwartz et al., 2019).

How it is Measured or Detected

In rodent studies, anogenital distance (AGD) is measured as the span between the genital papilla and the anus using a stereomicroscope equipped with a micrometer eyepiece. To account for body size, the AGD index (AGDi) is calculated by dividing AGD by the cube root of the body weight.

For statistical analysis, it is essential to treat the litter as the experimental unit, especially when multiple pups from the same litter are assessed. In such cases, statistical models are adjusted by including litter as an independent, random, and nested factor. Additionally, AGD measurements are analyzed using body weight as a covariate, in accordance with the recommendations outlined in OECD Guidance Document 151 (2013).

Regulatory Significance of the AO

Measuring the AGD is mandatory in OECD test guidelines used to test for developmental and reproductive toxicity of chemicals. Guidelines include ‘TG 443 extended one-generation study’ (OECD, 2025a), ‘TG 421/422 reproductive toxicity screening studies’ (OECD, 2025b) and ‘TG 414 developmental toxicity study’ (OECD, 2018). However, there is a huge challenge in interpreting a longer AGD in females.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 3378: Agonism, Androgen receptor leads to Androgen receptor activation, increased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Taxonomic applicability.

The AR is present in vertebrates. Mammals, birds and amphibians have one AR gene, whereas some fish species have two genes. AR activity has been studied in mammals, fish, birds and amphibians (Ogino et al., 2018). The biologically plausible domain of taxonomic applicability is vertebrates since the AR is present in vertebrates. The empirical domain of taxonomic applicability is human, rat and mice increased androgen receptor activity has been studied. The KER description focuses on mammals, but AOP developers are encouraged to expand the applicability to other species.

Life stage applicability

The AR is expressed from the fetal period throughout adult life and increased activity of the AR controls sexual development during the fetal period and reproductive function as well as effects in other organs during puberty and adulthood (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Sex applicability

The AR is expressed in both males and females and has important roles for sexual development and reproduction as well as effects in other organs in both sexes (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Key Event Relationship Description

The androgen receptor (AR) belongs to the steroid hormone receptor family and mediates the biological effects of androgens. AR agonism is the activation of the receptor by an agonist, a ligand, that binds and activates the receptor function leading to increased AR activity in tissues and organs in vivo, which results in effects on sexual development and reproductive function, as well as effects on other organs and tissues (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Evidence Supporting this KER

Biological Plausibility

The activation of the AR is a generally recognized process, i.e. canonical knowledge and the biological plausibility of the KER is considered high.

The AR belongs to the family of steroid hormone nuclear receptors. It contains three major domains essential for its activity: the N-terminal region, the ligand binding domain (LBD), and the DNA binding domain (DBD). In the absence of a ligand, the AR resides in the cytoplasm. Upon binding of an agonist such as endogenous hormones (testosterone or dihydrotestosterone) or a compound acting as an agonist, AR is activated, forms a homodimer, translocates into the nucleus and binds to androgen-response elements to regulate target gene transcription by recruiting cofactor protein complexes. Additionally, the AR can also have rapid non-genomic actions by binding to plasma membrane proteins and activating kinase signalling in the cytoplasm. AR agonism results in increased AR activity in vivo that can affect numerous biological processes, including sexual development and reproductive function, as well as effects on other organs such as adipose tissue, bone, brain, cardiovascular system, hair, muscle and skin (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

The role of AR agonism is evident in diseases, for instance in prostate cancer in men, which depends on androgen activity for growth (Naamneh Elzenaty et al., 2022), and polycystic ovary syndrome (PCOS) in women, which is associated with increased AR activity (Naamneh Elzenaty et al., 2022).

Empirical Evidence

The activation of the AR is a generally recognized process and is supported by empirical evidence for AR agonists.

Testosterone propionate, 17-methyl testosterone and trenbolone were identified as agonists in the US EPA Androgen receptor pathway model, which integrates in vitro assays for receptor binding, coregulator recruitment and

transactivation, and these stressors have also been shown to increase AR activation in Hershberger assay or other in vivo assays (Kleinstreuer et al., 2018; Browne et al., 2018).

Compounds that act as AR agonists have been used as positive controls in validation of OECD TG 458 and in validation studies by ICCVAM (OECD, 2023; ICCVAM, 2003). Compounds acting as AR agonists have been evaluated in ToxCast in vitro assays for AR (U.S. EPA, 2022).

Synthetic androgen ligands have been developed as potential drugs for the treatment of androgen related disorders; selective AR modulator are ligands acting as tissue-specific AR agonists and are being studied for effects on anemias, osteoporosis, muscle wasting and benign hyperplasia of the prostate but have not yet been approved for clinical use (Dalton & Gao, 2010; Luetjens & Weinbauer, 2012).

Uncertainties and Inconsistencies

No uncertainties or inconsistences have been identified for the KER that is based on canonical knowledge.

Quantitative Understanding of the Linkage

Response-response relationship

No specific evidence for response-response relationships has been identified for the KER.

Time-scale

Agonism of the AR results rapidly in increased AR activity in vivo. Effects on activation of the receptor on cellular function can be seen after minutes to hours (Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Known Feedforward/Feedback loops influencing this KER

No specific evidence for feedforward or feedback loops has been identified for the KER.

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Relationship: 3628: Androgen receptor activation, increased leads to Anogenital distance (AGD), increased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to long anogenital distance (AGD) in female offspring	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Fetal	High

Sex Applicability

Sex	Evidence
Female	High

Taxonomic Applicability

The biologically plausible taxonomic applicability domain is mammals, as fetal masculinization is regulated by androgens across all mammalian species (Welsh, 2014). The empirical taxonomic applicability domain is based on rat, supported by prenatal studies demonstrating that androgen receptor activation leads to a measurable increase in female AGD (Armoskus et al., 2014; Guerra et al., 2014; Hotchkiss et al., 2007; Sathishkumar et al., 2011; Welsh et al., 2009; Wilson et al., 2002; Wolf et al., 2002; Wolf et al., 2004). Additionally, epidemiological studies suggest that elevated androgen levels in women are associated with increased AGD (Mira-Escolano et al., 2014; Pan et al., 2021; Zamani et al., 2023).

Sex applicability

This KER focuses on effects on AGD in females, supported by empirical evidence from prenatal studies in rats (Armoskus et al., 2014; Guerra et al., 2014; Hotchkiss et al., 2007; Sathishkumar et al., 2011; Welsh et al., 2009; Wilson et al., 2002; Wolf et al., 2002; Wolf et al., 2004).

Life Stage Applicability

Growth of AGD in rats is regulated by androgens during the masculinization programming window, which occurs during fetal development around GD16-20 (MacLeod et al., 2010; Welsh et al., 2008).

Key Event Relationship Description

This KER describes how increased androgen receptor (AR) activation in females during fetal development can lead to an increased AGD. The AR is a member of the steroid hormone receptor family and mediates the biological effects of androgens. Increased AR activation can occur either through AR agonism (i.e., activation of the receptor by a compound) or through elevated levels of endogenous hormones such as testosterone or dihydrotestosterone (DHT) (Dalton & Gao, 2010; Davey & Grossmann, 2016; Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022; Sutinen et al., 2017).

Anogenital distance (AGD) refers to the distance between the anus and the external genitalia. In both rodents and humans, female AGD is approximately half the length of male AGD (Liu et al., 2014; Salazar-Martinez et al, 2004; Schwartz et al., 2019; Sharpe, 2020; Thankamony at al., 2016; Wise, 2024). This difference is due to androgen-dependent regulation of perineal growth during fetal development in males, whereas in females, the absence of androgens limits this growth (Ipulan at al., 2016; Schwartz at al., 2019; Sharpe, 2020; Welsh at al., 2008).

It should be noted that the upstream Key Event (KE) ‘increase, androgen receptor activation’ (KE-2274) specifically focuses on increased activation of the androgen receptor *in vivo*, while most methods that can be used to measure AR activity are carried out *in vitro*. Indirect information about this KE may for example be provided from assays showing *in vitro* AR agonism, increased *in vitro* or *in vivo* testosterone production/levels or increased *in vitro* or *in vivo* dihydrotestosterone (DHT) production/levels.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is judged to be high based on the following:

- During the masculinization programming window of fetal development, androgens drive the masculinization process in males, including growth of the perineum, which can be measured as anogenital distance (AGD), the distance between the anus and the genitalia. In female fetuses, lower androgen levels limit this growth, resulting in male AGD being approximately twice as long as female AGD (Liu et al., 2014; Salazar-Martinez et al, 2004; Schwartz at al., 2019; Sharpe, 2020; Thankamony at al., 2016; Welsh at al., 2008; Wise, 2024).
- Studies in androgen receptor (AR) knockout mice demonstrate that AR is essential for a longer AGD, as male AR knockout mice exhibit AGD lengths comparable to female wild-type mice (Yeh et al., 2002, Sato et al., 2004).
- The mechanism underlying androgen regulation of perineal growth likely involves androgen-mediated development of the levator ani and bulbocavernosus (LABC) muscle complex in the perineum. AR knockout in non-myocytic cells of the LABC leads to defective muscle formation and reduced AGD (Ipulan et al., 2016). Furthermore, differential gene expression profiles have been observed in the perineum of male and female rats (Schwartz et al., 2019)
- An association between higher testosterone levels in young women and longer AGD has been reported in a cross-sectional study (Mira-Escolano at al., 2014).
- Epidemiological studies of women with polycystic ovary syndrome (PCOS) indicate that elevated androgen levels are associated with increased AGD. Women with PCOS produce higher androgen levels (Yang & Chen, 2024), and several studies have shown longer AGD in women with PCOS (Pan at al., 2021; Zamani at al., 2023). Evidence regarding AGD in daughters of women with PCOS is mixed: Barrett at al. (2018) reported an association, whereas Glintborg at al. (2019) did not. Additionally, one study found that fetuses of women with PCOS exhibited longer AGD (Perlman et al., 2020).

Empirical Evidence

The empirical evidence for this KER is judged to be high based on the following: .

The empirical evidence is based on four model substances: methyl testosterone, testosterone, testosterone propionate and trenbolone. Evidence for the upstream KE (androgen receptor activation, increased) is derived from *in vitro* assays assessing AR agonism, as well as *in vivo* Hershberger assays that measure androgenic effects. Evidence for the downstream KE (Anogenital distance, increased) is based on prenatal *in vivo* studies measuring AGD. To date, no *in vivo* studies have measured both KEs within the same experiment.

Evidence for the upstream KE

Methyl testosterone

Methyl testosterone is synthetic androgen previously used as a pharmaceutical. It binds to AR and acts as an AR agonist *in vitro* (Kleinstreuer et al., 2018; Judson et al., 2020) and show androgen activity in vivo in Hershberger assays (Browne et al., 2018).

Testosterone

Testosterone is an endogenous androgen that binds to AR and acts as an AR agonist.

Testosterone propionate

Testosterone propionate is synthetic androgen formerly used as a pharmaceutical. It binds to AR and acts as an AR agonist *in vitro* (Kleinstreuer et al., 2018; Judson et al., 2020) and show androgen activity *in vivo* in Hershberger assays (Browne et al., 2018).

Trenbolone

Trenbolone is synthetic androgen used in veterinary medicine. It binds to AR and acts as an AR agonist *in vitro*(Kleinstreuer et al., 2018; Judson et al., 2020) and show androgen activity*in vivo* in Hershberger assays (Browne et al., 2018).

Evidence for the downstream KE

Methyl testosterone

Overall confidence in the data for methyl testosterone is weak. 2 publications including 3 datasets were analysed. Increased AGD in females was observed in all three datasets, but the datasets were classified as not reliable (Table 4).

Testosterone

Overall confidence in the data for testosterone is weak. 3 publications including 16 datasets were analysed. All datasets were classified as not reliable. Increased AGD in females was observed in 12 out of 16 datasets. The conflicting results in four datasets, i.e. no effect on AGD in females, could possibly be explained by that exposure was only during one of the days GD19, 20, 21 or 22, which is at the end of the male programming window GD16-20. Effect was observed in the same study for exposure during GD16, 17 or 18 (Table 4).

Testosterone propionate

Overall confidence in the data for testosterone propionate is moderate. 9 publications including 22 datasets were analysed. Increased AGD in females was observed in eight out of twelve datasets classified as reliable without

restriction and in both datasets classified as reliable with restriction. The conflicting results, i.e. no effect on AGD in females, could possibly be explained in three of the datasets (testosterone-propionate-3B, testosterone-propionate-8B and testosterone-propionate-8C) by that AGD was measured at later timepoint (PND24, at puberty, PND75), since an effect was observed in the same studies when AGD was measured at earlier timepoints (PND2, PND1). The conflicting result, i.e. no effect on AGD in females, in the fourth dataset (testosterone-propionate-7) cannot be explained by differences in study design. Increased AGD in females was also observed in seven out of eight datasets classified as non-reliable. The dataset, testosterone-propionate-2B, showed increased AGD but the authors report that it was not significant (Table 4).

Trenbolone

Overall confidence in the data for trenbolone is strong. 2 publications including 2 datasets were analysed. Increased AGD in females was observed in two datasets classified as reliable (Table 4).

Uncertainties and Inconsistencies

For the model substances, there were some inconsistencies in the empirical evidence, but they could mostly be explained by differences in study design.

Quantitative Understanding of the Linkage

The quantitative understanding of the KER is low. This is a consequence of it not being possible to measure the upstream and the downstream event in the same study.

Response-response relationship

No specific evidence for response-response relationships has been identified for this KER.

Time-scale

In a prenatal *in vivo* study, exposure to a substance that acts on the upstream KE (androgen receptor activation) for five to six days during the masculinization window (around GD16-20 in rats) can lead to a measurable effect on the downstream KE (increased anogenital distance) at PND2 (Hotchkiss et al., 2007; Wilson et al., 2002; Wolf et al., 2004).

Known modulating factors

No known modulating factors have been identified for this KER.

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Known Feedforward/Feedback loops influencing this KER

No specific evidence for feedforward or feedback loops has been identified for this KER.

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