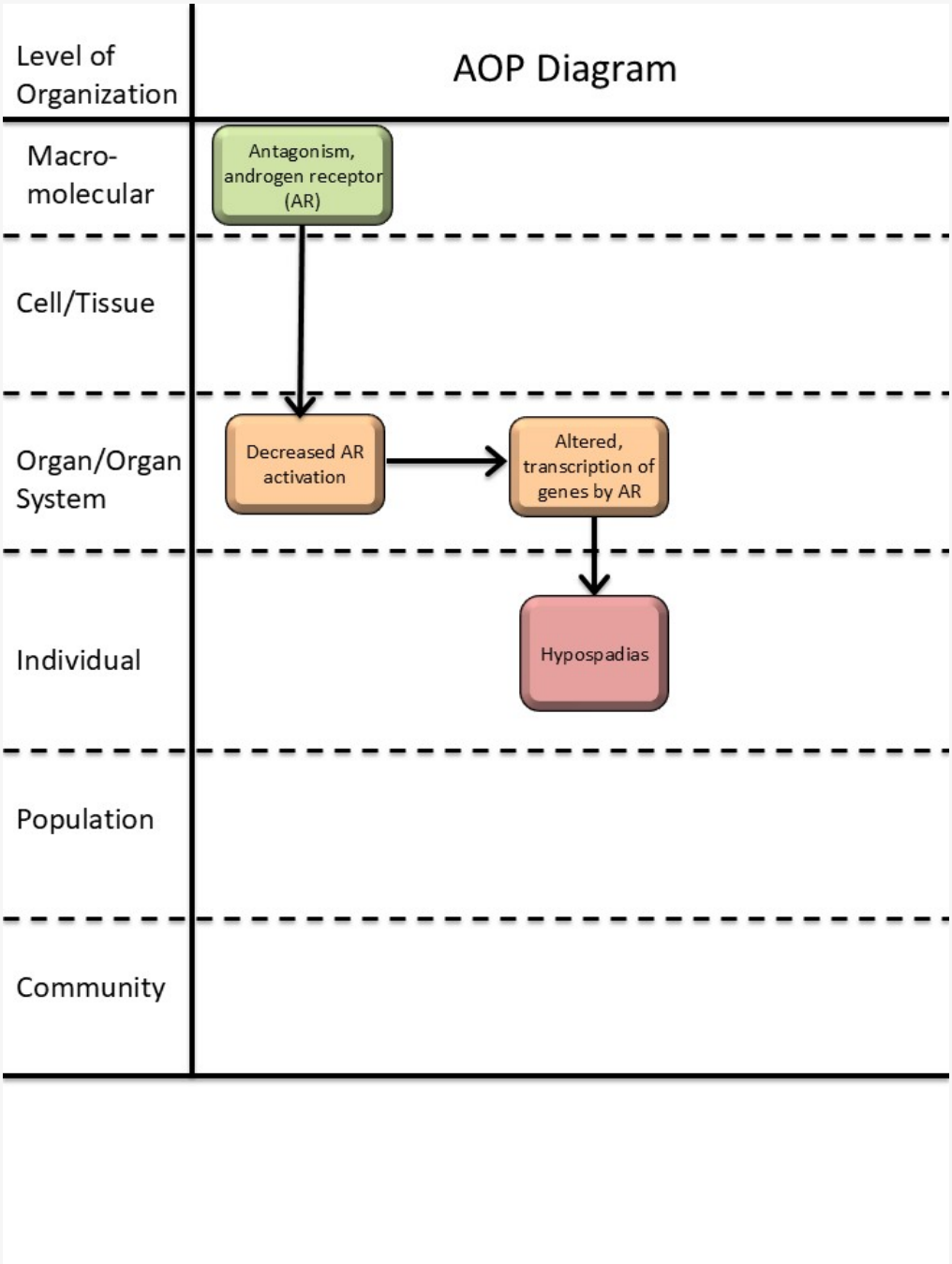
AOP ID and Title:

AOP 477: Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring
Short Title: AR antagonism leading to hypospadias

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			

Abstract

This AOP links *in utero* androgen receptor (AR) antagonism with hypospadias in male offspring. Hypospadias is a common reproductive disorder with a prevalence of up to ~1/125 newborn boys (Leunbach et al., 2025; Paulozzi, 1999). Developmental exposure to endocrine disrupting chemicals is suspected to contribute to some cases of hypospadias (Mattiske & Pask, 2021). Hypospadias can be indicative of fetal disruptions to male reproductive development, and is associated with short anogenital distance and cryptorchidism (Skakkebaek et al., 2016). Thus, hypospadias is included as an endpoint in OECD test guidelines (TG) for developmental and reproductive toxicity (TG 414, 416, 421/422, and 443; (OECD, 2016b, 2016a, 2018a, 2018b, 2021)), as both a measurement of adverse reproductive effects and a direct clinical adverse outcome. In normal male reproductive development, androgen activation of the AR plays an essential role in driving differentiation of the male phenotype, including development of the penis (Amato et al., 2022). This AOP delineates the evidence that antagonism of AR through a reduction in AR activation can disrupt penis development and cause hypospadias. Substantial evidence exists that links exposure to AR antagonists to hypospadias in *in vivo* rodent studies. Moreover, human case studies of subjects with *AR* mutations and hypospadias support the link. Downstream of a reduction in AR activation, the molecular mechanisms of hypospadias development are less clear, highlighting a knowledge gap in this AOP. Thus, the AOP has potential for inclusion of additional KEs and elaboration of molecular causality links, once these are established. Given that hypospadias is both a clinical and toxicological endpoint, this AOP is considered highly relevant in a regulatory context.

Background

This AOP is a part of an AOP network for reduced androgen receptor activation causing hypospadias in male offspring. The other AOPs in this network are AOP-570 (‘Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring’) and AOP-571 (‘5α-reductase inhibition leading to hypospadias in male (mammalian) offspring’). The purpose of the AOP network is to organize the well-established evidence for anti-androgenic mechanisms-of-action leading to hypospadias, thus informing predictive toxicology and identifying knowledge gaps for investigation and method development.

This work received funding from the European Food and Safety Authority (EFSA) under Grant agreement no. GP/EFSA/PREV/2022/01 and from the Danish Environmental Protection Agency under the Danish Center for Endocrine Disrupters (CeHoS).

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	26	Antagonism, Androgen receptor	Antagonism, Androgen receptor
	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	286	Altered, Transcription of genes by the androgen receptor	Altered, Transcription of genes by the AR
	AO	2082	Hypospadias, increased	Hypospadias

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Androgen receptor	adjacent	Decrease, androgen receptor activation	High	
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	
Decrease, androgen receptor activation	non-adjacent	Hypospadias, increased	High	

Stressors

Name	Evidence
Flutamide	
Vinclozolin	
Procymidone	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Male	High

Although the upstream part of the AOPN has a broad applicability domain, the overall AOPN is considered only applicable to male mammals during fetal life, restricted by the applicability of KER-2828 (‘Decrease, AR activation leads to hypospadias’). The term hypospadias is mainly used for describing malformation of the male, and not female, external genitalia. Some studies refer to hypospadias in females, but these have not been reported to be caused by exposure to AR antagonists, and the mechanisms behind these malformations are likely different from the mechanisms in males (Greene, 1937; Stewart et al., 2018). The genital tubercle is programmed by androgens to differentiate into a penis in fetal life during the masculinization programming window, followed by the morphological differentiation (Welsh et al., 2008). In humans, hypospadias is diagnosed at birth and can also often be observed in rodents (rats and mice) at this time point, although the rodent penis does not finish developing until a few weeks after birth (Baskin & Ebbers, 2006; Sinclair et al., 2017). The disruption to androgen programming leading to hypospadias thus takes place during fetal life, but the AO itself is best detected postnatally. Regarding taxonomic applicability, hypospadias has mainly been described in rodents and humans, and the evidence in this AOP is almost exclusively from these species. It is, however, biologically plausible that the AOP is applicable to other mammals, given the conserved role of androgens in mammalian reproductive development, and hypospadias has been observed in many domestic animal and wildlife species, albeit not coupled to AR antagonism.

Essentiality of the Key Events

Event	Evidence	Uncertainties and inconsistencies

MIE-26 Antagonism, AR (high)	Biological plausibility provides strong support for the essentiality of this event, as androgens, acting through AR, are the primary drivers of penis development *In vivo* toxicity studies in rats show that *in utero* exposure to known AR antagonists flutamide, procymidone, and vinclozolin cause hypospadias in male offspring (listed in table 3 in KER 2828) *Ex vivo* culture of genital tubercles with AR antagonist flutamide disrupted genital tubercle development, but this was rescued with addition of DHT (Petiot et al., 2005)	In the *ex vivo* study, androgens were not added to the baseline culture media, even though they are present in an *in vivo* scenario to induce penis differentiation.
KE-1614 Decrease, AR activation (moderate)	Biological plausibility provides strong support for the essentiality of this event, as AR activation is critical for normal penis development. Conditional or full knockout of *Ar* in mice results in partly or full sex-reversal of males, including a female-like urethral opening(Willingham et al., 2006; Yucel et al., 2004; Zheng et al., 2015). Human subjects with *AR* mutations may also have associated hypospadias (as listed in table 4 in KER 2828).	
KE-286 Altered, transcription of genes by AR (low)	Biological plausibility provides support for the essentiality of this event. AR is a nuclear receptor and transcription factor regulating transcription of genes, and androgens, acting through AR, are essential for normal male penis development. Known AR-responsive genes active in normal penis development have been thoroughly reviewed (Amato et al., 2022).	There are currently no AR-responsive genes proved to be causally involved in hypospadias, and it is known that the AR can also signal through non-genomic actions (Leung & Sadar, 2017).

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
MIE-26	***			High
KE-1614	**			Moderate
KE-286		*		Low

Weight of Evidence Summary

The confidence in each of the KERs comprising the AOP are judged as high, with both high biological plausibility and high confidence in the empirical evidence. The mechanistic link between KE-286 ('altered, transcription of genes by AR') and AO-2082 ('hypospadias') is not established, but given the high confidence in the KERs including the non-adjacent KER-2828 linking to the AO, the overall confidence in the AOP is judged as **high**.

KER	Biological Plausibility	Empirical Evidence	Rationale
KER-2130 Antagonism, AR leads to decrease, AR activation	High	High (canonical)	It is well established that antagonism of the AR leads to decreased AR activity, and this has been evidenced *in vitro* (Draskau et al., 2024; Pedersen et al., 2022).
KER-2124 Decrease, AR activation leads to altered, transcription of genes by AR	High	High (canonical)	It is well established that the AR regulates gene transcription. *In vivo* animal studies and human genomic profiling show tissue-specific changes to gene expression upon disruption of AR (Draskau et al., 2024).
KER-2828 Decrease, AR activation leads to hypospadias	High	High	It is well established that AR drives penis differentiation. Numerous *in vivo* toxicity studies and human case studies indirectly show that decreased AR activation leads to hypospadias, with few inconsistencies. The empirical evidence moderately supports dose, temporal, and incidence concordance for the KER.

Quantitative Consideration

The quantitative understanding of this AOP is judged as low.

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Appendix 1

List of MIEs in this AOP

Event: 26: Antagonism, Androgen receptor

Short Name: Antagonism, Androgen receptor

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	MolecularInitiatingEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	MolecularInitiatingEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	MolecularInitiatingEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	MolecularInitiatingEvent
Aop:595 - Nanoplastic effect	MolecularInitiatingEvent

Stressors

Name
Mercaptobenzole
Triticonazole
Flusilazole
Epoxiconazole
Prochloraz
Propiconazole
Tebuconazole
Flutamide
Cyproterone acetate
Vinclozolin

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

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

Sex	Evidence
Mixed	High

Both the DNA-binding and ligand-binding domains of the AR are highly evolutionary conserved, whereas the transactivation domain show more divergence which may affect AR-mediated gene regulation across species (Davey & Grossmann, 2016). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutations studies from both humans and rodents showing strong correlation for AR-dependent development and function (Walters et al, 2010).

This KE is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across mammalian taxa. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

The androgen receptor (AR) and its function

The AR is a ligand-activated transcription factor belonging to the steroid hormone nuclear receptor family (Davey & Grossmann, 2016). The AR has three domains: the N-terminal domain, the DNA-binding domain and the ligand-binding domain, with the latter being most evolutionary conserved. Testosterone (T) and the more biologically active dihydrotestosterone (DHT) are endogenous ligands for the AR (MacLean et al, 1993; MacLeod et al, 2010; Schwartz et al, 2019). In teleost fishes, 11-ketotestosterone is the second main ligand (Schuppe et al, 2020). Human AR mutations and mouse knock-out models have established a pivotal role for the AR in masculinization and spermatogenesis (Walters et al, 2010). Apart from the essential role for AR in male reproductive development and function (Walters et al, 2010), the AR is also expressed in many other tissues and organs such as bone, muscles, ovaries, and the immune system (Rana et al, 2014).

AR antagonism as Key Event

The main function of the AR is to activate gene transcription in cells. Canonical signaling occurs by ligands (androgens) binding to AR in the cytoplasm which results in translocation to the cell nucleus, receptor dimerization and binding to specific regulatory DNA sequences (Heemers & Tindall, 2007). The gene targets regulated by AR activation depends on cell/tissue type and what stage of development activation occur, and is, for instance, dependent on available co-factors. Apart from the canonical signaling pathway, AR can also initiate cytoplasmic signaling pathways with other functions than the nuclear pathway, for instance rapid change in cell function by ion transport changes (Heinlein & Chang, 2002) and association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway (Leung & Sadar, 2017).

How it is Measured or Detected

AR antagonism can be measured in vitro by transient or stable transactivation assays to evaluate nuclear receptor activation. There is already a validated test guideline for AR (ant)agonism adopted by the OECD, Test No. 458: *Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals* (OECD, 2016). This test guideline contains three different methods. More information on limitations, advantages, protocols, and availability and description of cells are given in the test guideline.

Besides these validated methods, other transiently or stably transfected reporter cell lines are available as well as yeast based systems (Campana et al, 2015; Körner et al, 2004). AR nuclear translocation can be monitored by various assays (Campana et al 2015), for example by monitoring fluorescent rat AR movement in living cells (Tyagi et al 2020), with several human AR translocation assays being commercially available; e.g. Fluorescent AR Nuclear Translocation Assay (tGFP-hAR/HEK293) or Human Androgen NHR Cell Based Antagonist Translocation LeadHunter Assay.

Additional information on AR interaction can be obtained employing competitive AR binding assays (Freyberger et al 2010, Shaw et al 2018), which can also inform on relative potency of the compounds, though not on downstream effect of the AR binding.

The recently developed AR dimerization assay provides an assay with an improved ability to measure potential stressor-mediated disruption of dimerization/activation (Lee et al, 2021).

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List of Key Events in the AOP

Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type

AOP ID and Name	Event Type
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:111 - Decrease in androgen receptor activity leading to Leydig cell tumors (in rat)	MolecularInitiatingEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) 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

This KE is considered broadly applicable across mammalian taxa as all mammals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and functions. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

This KE refers to decreased activation of the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs in vivo. It is thus considered distinct from KEs describing either blocking of AR or decreased androgen synthesis.

The AR is a nuclear transcription factor with canonical AR activation regulated by the binding of the androgens such as testosterone or dihydrotestosterone (DHT). Thus, AR activity can be decreased by reduced levels of steroidal ligands

(testosterone, DHT) or the presence of compounds interfering with ligand binding to the receptor (Davey & Grossmann, 2016; Gao et al., 2005).

In the inactive state, AR is sequestered in the cytoplasm of cells by molecular chaperones. In the classical (genomic) AR signaling pathway, AR activation causes dissociation of the chaperones, AR dimerization and translocation to the nucleus to modulate gene expression. AR binds to the androgen response element (ARE) (Davey & Grossmann, 2016; Gao et al., 2005). Notably, for transcriptional regulation the AR is closely associated with other co-factors that may differ between cells, tissues and life stages. In this way, the functional consequence of AR activation is cell- and tissue-specific. This dependency on co-factors such as the SRC proteins also means that stressors affecting recruitment of co-activators to AR can result in decreased AR activity (Heinlein & Chang, 2002).

Ligand-bound AR may also associate with cytoplasmic and membrane-bound proteins to initiate cytoplasmic signaling pathways with other functions than the nuclear pathway. Non-genomic AR signaling includes association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway. Decreased AR activity may therefore be a decrease in the genomic and/or non-genomic AR signaling pathways (Leung & Sadar, 2017).

How it is Measured or Detected

This KE specifically focuses on decreased *in vivo* activation, with most methods that can be used to measure AR activity carried out *in vitro*. They provide indirect information about the KE and are described in lower tier MIE/KEs (see for example MIE/KE-26 for AR antagonism, KE-1690 for decreased T levels and KE-1613 for decreased dihydrotestosterone levels). Assays may in the future be developed to measure AR activation in mammalian organisms.

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Event: 286: Altered, Transcription of genes by the androgen receptor

Short Name: Altered, Transcription of genes by the AR

Key Event Component

Process	Object	Action
regulation of gene expression	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent

AOP ID and Name	Event Type
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:495 - Androgen receptor activation leading to prostate cancer	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction □in zebrafish□	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	KeyEvent

Stressors

Name
Bicalutamide
Cyproterone acetate
Epoxiconazole
Flutamide
Flusilazole
Prochloraz
Propiconazole
Stressor:286 Tebuconazole
Triticonazole
Vinclozalin

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

Both the DNA-binding and ligand-binding domains of the AR are highly evolutionary conserved, whereas the transactivation domain show more divergence, which may affect AR-mediated gene regulation across species (Davey and Grossmann 2016). Despite certain inter-species differences, AR function mediated through gene expression is

highly conserved, with mutation studies from both humans and rodents showing strong correlation for AR-dependent development and function (Walters et al. 2010).

This KE is considered broadly applicable across mammalian taxa, sex and developmental stages, as all mammals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and function. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

This KE refers to transcription of genes by the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs *in vivo*. Rather than measuring individual genes, this KE aims to capture patterns of effects at transcriptome level in specific target cells/tissues. In other words, it can be replaced by specific KEs for individual adverse outcomes as information becomes available, for example the transcriptional toxicity response in prostate tissue for AO: prostate cancer, perineum tissue for AO: reduced AGD, etc. AR regulates many genes that differ between tissues and life stages and, importantly, different gene transcripts within individual cells can go in either direction since AR can act as both transcriptional activator and suppressor. Thus, the 'directionality' of the KE cannot be either reduced or increased, but instead describe an altered transcriptome.

The Androgen Receptor and its function

The AR belongs to the steroid hormone nuclear receptor family. It is a ligand-activated transcription factor with three domains: the N-terminal domain, the DNA-binding domain, and the ligand-binding domain with the latter being the most evolutionary conserved (Davey and Grossmann 2016). Androgens (such as dihydrotestosterone and testosterone) are AR ligands and act by binding to the AR in androgen-responsive tissues (Davey and Grossmann 2016). Human AR mutations and mouse knockout models have established a fundamental role for AR in masculinization and spermatogenesis (Maclean et al.; Walters et al. 2010; Rana et al. 2014). The AR is also expressed in many other tissues such as bone, muscles, ovaries and within the immune system (Rana et al. 2014).

Altered transcription of genes by the AR as a Key Event

Upon activation by ligand-binding, the AR translocates from the cytoplasm to the cell nucleus, dimerizes, binds to androgen response elements in the DNA to modulate gene transcription (Davey and Grossmann 2016). The transcriptional targets vary between cells and tissues, as well as with developmental stages and is also dependent on available co-regulators (Bevan and Parker 1999; Heemers and Tindall 2007). It should also be mentioned that the AR can work in other 'non-canonial' ways such as non-genomic signaling, and ligand-independent activation (Davey & Grossmann, 2016; Estrada et al, 2003; Jin et al, 2013).

A large number of known, and proposed, target genes of AR canonical signaling have been identified by analysis of gene expression following treatments with AR agonists (Bolton et al. 2007; Ngan et al. 2009, Jin et al. 2013).

How it is Measured or Detected

Altered transcription of genes by the AR can be measured by measuring the transcription level of known downstream target genes by RT-qPCR or other transcription analyses approaches, e.g. transcriptomics.

Since this KE aims to capture AR-mediated transcriptional patterns of effect, downstream bioinformatics analyses will typically be required to identify and compare effect footprints. Clusters of genes can be statistically associated with, for example, biological process terms or gene ontology terms relevant for AR-mediated signaling. Large transcriptomics data repositories can be used to compare transcriptional patterns between chemicals, tissues, and species (e.g. TOXsIgN (Darde et al, 2018a; Darde et al, 2018b), comparisons can be made to identified sets of AR 'biomarker' genes (e.g. as done in (Rooney et al, 2018)), and various methods can be used e.g. connectivity mapping (Keenan et al, 2019).

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List of Adverse Outcomes in this AOP

Event: 2082: Hypospadias, increased

Short Name: Hypospadias

Key Event Component

Process	Object	Action
embryonic organ development	penis	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	AdverseOutcome
Aop:527 - Decreased, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) leads to Hypospadias, increased	AdverseOutcome
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	AdverseOutcome
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	AdverseOutcome

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

Organ term

penis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
mammals	mammals		NCBI

Life Stage Applicability

Life Stage	Evidence
Perinatal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability: Numerous studies have shown an association in humans between *in utero* exposure to endocrine disrupting chemicals and hypospadias. In mice and rats, *in utero* exposure to several endocrine disrupting chemicals, in particular estrogens and antiandrogens, have been shown to cause hypospadias in male offspring at different frequencies (Mattiske & Pask, 2021). Androgen-driven development of the male external genitalia is evolutionary conserved in most mammals and, to some extent, also in other vertebrate classes (Gredler et al., 2014). Hypospadias can in principle occur in all animals that form a genital tubercle and have been observed in many domestic animal species and wildlife species.

Life stage applicability: Penis development is finished prenatally in humans, and hypospadias is diagnosed at birth (Baskin & Ebbers, 2006). In rodents, penis development is not fully completed until weeks after birth, but hypospadias may be identified in early postnatal life as well, and in some cases in late gestation (Sinclair et al., 2017).

Sex applicability: Hypospadias is primarily used in reference to malformation of the male external genitalia.

Key Event Description

Hypospadias is a malformation of the penis where the urethral opening is displaced from the tip of the glans, usually on the ventral side on the phallus. Most cases of hypospadias are milder where the urethral opening still appears on the glans proper or on the most distal part of the shaft. In more severe cases, the opening may be more proximally placed on the shaft or even as low as the scrotum or the perineum.

In addition to the misplacement of the urethral opening, hypospadias is associated with an absence of ventral prepuce, an excess of dorsal preputial tissue, and in some cases a downward curvature of the penis (chordee). Patients with hypospadias may need surgical repairment depending on severity, with more proximal hypospadias patients in most need of surgeries to achieve optimal functional and cosmetic results (Baskin, 2000; Baskin & Ebbers, 2006; Mattiske & Pask, 2021). The incidence of hypospadias varies greatly between countries, from 1:100 to 1:500 of newborn boys (Skakkebaek et al., 2016), and the global prevalence seems to be increasing (Paulozzi, 1999; Springer et al., 2016; Yu et al., 2019).

The external genitalia arise from the biphasic genital tubercle during fetal development. Androgens (testosterone and dihydrotestosterone) drive formation of the male external genitalia. In humans, the urethra develops by fusion of two endoderm-derived urethral folds. Disruption of genital tubercle differentiation results in an incomplete urethra, i.e. hypospadias. (Baskin, 2000; Baskin & Ebbers, 2006).

How it is Measured or Detected

In humans, hypospadias is diagnosed clinically by physical examination of the infant and is at first recognized by the absence of ventral prepuce and concurrent excess dorsal prepuce (Baskin, 2000). Hypospadias may be classified according to the location of the urethral meatus: Glandular, subcoronal, midshaft, penoscrotal, scrotal, and perineal (Baskin & Ebbers, 2006).

In mice and rats, macroscopic assessment of hypospadias may be performed postnatally, and several OECD test guidelines require macroscopic examination of genital abnormalities in *in vivo* toxicity studies (TG 414, 416, 421/422, 443). The guidelines do not define hypospadias or how to identify them. Fetal and neonatal identification of hypospadias may require microscopic examination for proper evaluation of the pathology. This can be done by scanning electron microscopy (Uda et al.,

2004), or by histological assessment in which the presence of the urethral opening in proximal, transverse sections (for example co-occuring with the os penis or corpus cavernosum), indicates hypospadias (Mahawong et al., 2014; Sinclair et al., 2017; Vilela et al., 2007). In a semiquantitative, histologic approach, the number of transverse sections of the penis with internalization of the urethra was related to the total length of the penis, achieving a percentage of urethral internalization. In this study, ≤89% of urethral internalization was defined as indicative of mild hypospadias (Stewart et al., 2018).

Regulatory Significance of the AO

In the OECD guidelines for developmental and reproductive toxicology, several test endpoints include examination of structural abnormalities with special attention to the organs of the reproductive system. These are: Test No. 414 ‘Prenatal Developmental Toxicity Study’ (OECD, 2018a); Test No. 416 ‘Two-Generation Reproduction Toxicity’ (OECD, 2001) and Tests No. 421/422 ‘Reproduction/Developmental Toxicity Screening Test’ (OECD, 2016a, 2016b). In Test No. 443 ‘Extended One-Generation Reproductive Toxicity Study’ (OECD, 2018b), hypospadias is specifically mentioned as a genital abnormality to note.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2130: Antagonism, Androgen receptor leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	High
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Moderate

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

This KER is applicable to mammals as AR expression and activity is highly conserved (Davey & Grossmann, 2016). AR activity is important for sexual development and reproduction in both males and females (Prizant et al., 2014; Walters et al., 2010). AR function is required during development, puberty, and adulthood. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Relationship Description

The androgen receptor (AR) is a ligand-activated steroid hormone nuclear receptor (Davey & Grossmann, 2016). In its inactive state, the AR locates to the cytoplasm (Roy et al., 2001). When activated, the AR translocates to the nucleus, dimerizes, and, together with co-regulators, binds to specific DNA regulatory sequences to regulate gene transcription (Davey & Grossmann, 2016) (Lamont and Tindall, 2010). This is considered the canonical AR signaling pathway. The AR can also activate non-genomic signalling (Jin et al., 2013). However, this KER focuses on the canonical pathway.

The two main AR ligands are the androgens testosterone (T) and the more potent dihydrotestosterone (DHT). Androgens bind to the AR to mediate downstream androgenic responses, such as male development and masculinization (Rey, 2021; Walters et al., 2010). Antagonism of the AR would decrease AR activation and therefore the downstream AR-mediated effects.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high.

The AR belongs to the steroid hormone nuclear receptor family. The AR has 3 main domains essential for its activity, the N-terminal domain, the ligand binding domain, and the DNA binding domain (Roy et al., 2001). Ligands, such as T and DHT, must bind to the ligand binding domain to activate AR allowing it to fulfill its role as a transcription factor. The binding of the ligand induces a change in AR conformation allowing it to translocate to the nucleus and congregate into a subnuclear compartment (Marcelli et al., 2006; Roy et al., 2001) homodimerize and bind to the DNA target sequences and regulate transcription of target genes. Regulation of AR target genes is greatly facilitated by numerous co-factors. Active AR signaling is essential for male reproduction and sexual development and is also crucial in several other tissues and organs such as ovaries, the immune system, bones, and muscles (Ogino et al., 2011; Prizant et al., 2014; Rey, 2021; William H. Walker, 2021).

AR antagonists can compete with or prevent in different ways AR ligand binding, thereby preventing AR activation. Antagonism of the AR can prevent translocation to the nucleus, compartmentalization, dimerization and DNA binding. Consequently, AR cannot regulate transcription of target genes and androgen signalling is disrupted. This can be observed using different AR activation assays such as AR dimerization, translocation, DNA binding or transcriptional activity assays (Brown et al., 2023; *OECD*, 2020).

Empirical Evidence

The empirical evidence for this KER is considered high

The effects of AR antagonism have been shown in many studies *in vivo* and *in vitro*.

Several stressors can act as antagonists of the AR and lead to decreased AR activation. Some of these are detailed in an AOP key event relationship report by (Pedersen et al., 2022) and shown below, exhibiting evidence of dose-concordance:

Stressors

- Cyproterone acetate: Using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld, 2005)
- Epoxiconazole: Using transiently AR-transfected CHO cells, epoxiconazole showed a LOEC of 1.6 µM and an IC50 of 10 µM (Kjærstad et al., 2010).
- Flutamide: Using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 µM (Sonneveld, 2005).
- Flusilazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.8 µM and an IC50 of 2.8 (±0.1) µM (Draskau et al., 2019).
- Prochloraz: Using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 µM and an IC50 of 13 µM (Kjærstad et al., 2010).
- Propiconazole: Using transiently AR-transfected CHO cells, propiconazole showed a LOEC of 12.5 µM and an IC50 of 18 µM (Kjærstad et al., 2010).
- Tebuconazole: Using transiently AR-transfected CHO cells, tebuconazole showed a LOEC of 3.1 µM and an IC50 of 8.1 µM (Kjærstad et al., 2010).
- Triticonazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.2 µM and an IC50 of 0.3 (±0.01) µM (Draskau et al., 2019).
- Vinclozolin: Using the AR-CALUX reporter assay in antagonism mode, vinclozolin showed an IC50of 1.0 µM(Sonneveld, 2005).”(Pedersen et al., 2022)

Other evidence:

Known AR antagonists are used for treatment of AR-sensitive cancers such as flutamide for prostate cancer (Mahler et al., 1998).

Uncertainties and Inconsistencies

Known antiandrogenic compounds like hydroxyflutamide have been shown to act as agonists when the AR carries certain mutations, therefore contributing to uncertainties (Yeh et al., 1997). Additionally, the levels of endogenous androgens (e.g., testosterone or dihydrotestosterone) and the variability in the presence and function of AR co-activators may modulate the effect of AR antagonism.

Quantitative Understanding of the Linkage

Response-response relationship

The quantitative relationship between AR antagonism and AR activation will depend on the type of antagonist.

Time-scale

Nuclear translocation in HeLa cells transfected with AR-GFP show a response within 2 hours after ligand exposure (Marcelli et al., 2006; Szafran et al., 2008). Another assay focusing on AR binding to promoters in LNCaP cells has shown that after ligand binding, AR is able to translocate and bind to the DNA sequences within 15min showing the speed of AR activation (Kang et al., 2002).

Known Feedforward/Feedback loops influencing this KER

AR antagonism can lead to increased AR transcript stability and levels as a compensatory mechanism in prostate cancer cells (Dart et al., 2020). In turn, in presence of increased AR levels, AR antagonists can exhibit agonistic activity (Chen et al., 2003).

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Relationship: 2124: Decrease, AR activation leads to Altered, Transcription of genes by the AR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Moderate
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	Low
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	adjacent		
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	adjacent		
5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	adjacent	High	

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

This KER is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across mammalian taxa. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Relationship Description

The androgen receptor (AR) is a ligand-dependent nuclear transcription factor that upon activation translocates to the nucleus, dimerizes, and binds androgen response elements (AREs) to modulate transcription of target genes (Lamont and Tindall, 2010, Roy et al. 2001). Decreased activation of the AR affects its transcription factor activity, therefore leading to altered AR-target gene expression. This KER refers to decreased AR activation and altered gene expression occurring in complex systems, such as *in vivo* and the specific effect on transcription of AR target genes will depend on species, life stage, tissue, cell type etc.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

The AR is a ligand-activated transcription factor part of the steroid hormone nuclear receptor family. Non-activated AR is found in the cytoplasm as a multiprotein complex with heat-shock proteins, immunophilins and, other chaperones (Roy et al. 2001). Upon activation through ligand binding, the AR dissociates from the protein complex, translocates to the nucleus and homodimerizes. Facilitated by co-regulators, AR can bind to DNA regions containing AREs and initiate transcription of target genes, that thus will be different in e.g. different tissues, life-stages, species etc.

Through mapping of AREs and ChIP sequencing studies, several AR target genes have been identified, mainly studied in prostate cells (Jin, Kim, and Yu 2013). Different co-regulators and ligands lead to altered expression of different sets of genes (Jin et al. 2013; Kanno et al. 2022). Alternative splicing of the AR can lead to different AR variants that also affects which genes are transcribed (Jin et al. 2013).

Apart from this canonical signaling pathway, the AR can suppress gene expression, indirectly regulate miRNA transcription, and have non-genomic effects by rapid activation of second messenger pathways in either presence or absence of a ligand (Jin et al. 2013).

Empirical Evidence

The empirical evidence for this KER is considered high

In humans, altered gene expression profiling in individuals with androgen insensitivity syndrome (AIS) can provide supporting empirical evidence (Holterhus et al. 2003; Peng et al. 2021). In rodent AR knockout (KO) models, gene expression profiling studies and gene-targeted approaches have provided information on differentially expressed genes in several organ systems including male and female reproductive, endocrine, muscular, cardiovascular and nervous systems (Denolet et al. 2006; Fan et al. 2005; Holterhus et al. 2003; Ikeda et al. 2005; Karlsson et al. 2016; MacLean et al. 2008; Rana et al. 2011; Russell et al. 2012; Shiina et al. 2006; Wang et al. 2006; Welsh et al. 2012; Willems et al. 2010; Yu et al. 2008, 2012; Zhang et al. 2006; Zhou et al. 2011).

Exposure to known antiandrogens has been shown to alter transcriptional profiles, for example of neonatal pig ovaries (Knapczyk-Stwora et al. 2019).

Dose concordance has also been observed for instance in zebrafish embryos; a dose of 50 µg/L of the AR antagonist flutamide resulted in 674 differentially expressed genes at 96 h post fertilization whereas 500 µg/L flutamide resulted in 2871 differentially expressed genes (Ayobahan et al., 2023).

Uncertainties and Inconsistencies

AR action has been reported to occur also without ligand binding. However, not much is known about the extent and biological implications of such non-canonical, ligand-independent AR activation (Bennesch and Picard 2015).

Quantitative Understanding of the Linkage

Response-response relationship

There is not enough data to define a quantitative relationship between AR activation and alteration of AR target gene transcription, and such a relationship will differ between biological systems (species, tissue, cell type, life stage etc).

Time-scale

AR and promoter interactions occur within 15 minutes of ligand binding, RNA polymerase II and coactivator recruitment are proposed to occur transiently with cycles of approximately 90 minutes in LNCaP cells (Kang et al. 2002). RNA polymerase II elongation rates in mammalian cells have been shown to range between 1.3 and 4.3 kb/min (Maiuri et al. 2011). Therefore, depending on the cell type and the half-life of the AR target gene transcripts, changes are to be expected within hours.

Known modulating factors

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

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression in aging male rats	Tissue-specific alterations in AR activity with aging	(Supakar et al. 1993; Wu, Lin, and Gore 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Tut et al. 1997; Chamberlain et al. 1994)

Known Feedforward/Feedback loops influencing this KER

AR has been hypothesized to auto-regulate its mRNA and protein levels(Mora and Mahesh 1999).

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List of Non Adjacent Key Event Relationships

Relationship: 2828: Decrease, AR activation leads to Hypospadias

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	non-adjacent	High	
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	non-adjacent		
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	non-adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

In mammals, androgens are one of the primary drivers of penis differentiation. Hypospadias has been observed in several mammals, but most frequently reported in laboratory rodents and in humans (Chang et al., 2020; S. Wang & Zheng, 2025). *In vivo* studies in rats and mice show that *in utero* exposure to anti-androgenic chemicals can cause hypospadias in male offspring (see table 3). Many human case studies report boys born with hypospadias and associated deficiency in steroid hormone synthesis, 5α-reductase activity, or androgen receptor (AR) activity (see table 4).

The biologically plausible domain of applicability may extend beyond the empirical domain because androgen-controlled development of male external genitalia is evolutionary conserved in most mammals and, to some extent, also in other vertebrate classes (Gredler et al., 2014). Hypospadias can in principle occur in all animals that form a genital tubercle and have been observed in many domestic animal species including dog (Sonne et al., 2008; Switonski et al., 2018), cat (Nowacka-Woszuk et al., 2014), cattle (Murakami, 2008), sheep (Smith et al., 2012), and horse (De Lorenzi et al., 2010) as well as in wildlife species such as polar bear (Stamper et al., 1999), giraffe (Meuffels et al., 2020), and Tamar Wallaby (Leihy et al., 2011). The observed hypospadias in these animals is not, per se, linked to anti-androgenic exposure, which has only been sparsely investigated in other species than mice, rats, and humans. One study in monkeys did show hypospadias upon oral exposure to finasteride (Prahalada et al., 1997), and bicalutamide exposure induced hypospadias in guinea pigs (S. Wang et al., 2018). A study in rabbits exposed to procymidone did not find hypospadias in males (Inawaka et al., 2010). Another study in hyenas did also not find hypospadias in males after exposure to the anti-androgen finasteride (Drea et al., 1998), but it should be noted that the hyenas have a remarkable sexual development where penile growth occur in both females and males before androgen synthesis is initiated (Cunha et al., 2014) (the studies in hyena and rabbit were identified in our evidence collection but were judged as ‘unreliable’ and therefore not included as empirical evidence).

Sex applicability

The androgen receptor is expressed in the fetal genital tubercle of both females and males (Amato & Yao, 2021; Baskin et al., 2020), but hypospadias is primarily a term used for a malformation of the penis (Baskin & Ebbers, 2006), limiting the applicability of this KER to males.

Life stage applicability

Differentiation of the penis occurs during fetal life in the masculinization programming window (MPW) (GD 16-20 in rats, around gestational weeks 8-14 in humans), when androgen production is high (Welsh et al., 2008; C. Wolf et al., 2000a). In rats, exposure to anti-androgenic chemicals outside of, or in the late part of the MPW does not cause hypospadias or only to a low degree (Clark et al., 1993; van den Driesche et al., 2017; C. Wolf et al., 2000a), while exposure in the earlier (or full) MPW causes a higher frequency of hypospadias (depending on dose and chemical) (table 3). In humans, hypospadias can be diagnosed at birth (X. Yu et al., 2019), while in rodents, some parts of penis development occur postnatally (Schlomer et al., 2013; Sinclair et al., 2017). In these species, hypospadias may be observed at birth but is optimally diagnosed and severity classified weeks later. Given that disruptions to androgen programming takes place in fetal life, even though the AO is best detected postnatally, the life stage applicability is defined as fetal life.

Key Event Relationship Description

This non-adjacent KER describes a fetal decrease in androgen receptor (AR) activation in the genital tubercle causing hypospadias in male offspring, postnatally. During fetal development, androgens induce differentiation of the bipotential genital tubercle to a penis, including closure of the urethra. Androgens signal through AR and reduced fetal AR activation can therefore disrupt penis differentiation and lead to the genital malformation hypospadias. Reduced AR activation may happen both through reduced ligand availability (testosterone or dihydrotestosterone (DHT)) and by direct antagonism of AR (Amato et al., 2022; Mattiske & Pask, 2021).

The upstream KE 'decrease, androgen receptor activation' (KE 1614) refers to the *in vivo* event of overall reduction in AR activation. In this case, it therefore refers to a reduction in AR activation in the genital tubercle. Currently, decreased AR activation in mammals is only directly measured *in vitro* and not *in vivo*. Instead, indirect assessment of this KE may come from assays measuring AR antagonism, 5α-reductase activity (the enzyme converting testosterone to DHT), or decreased androgen levels (Draskau et al., 2024).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is judged as **high**. This is largely based on canonical knowledge on normal reproductive development.

The penis originates from a sexually bipotential structure, the genital tubercle, which may differentiate to either a penis or a clitoris, depending on internal cues during fetal development. In males, the fetal testes produce large amounts of testosterone, which can subsequently be converted to the more potent androgen DHT by 5α-reductase in peripheral tissues. Testosterone and DHT both signal through AR in target tissues to initiate masculinization (Amato et al., 2022; Murashima et al., 2015). The critical developmental window for androgen programming of masculinization has been identified in rats as GD16-20, and is proposed to be gestational weeks 8-14 in humans (Sharpe, 2020; Welsh et al., 2008). As part of the masculinization process orchestrated by androgens, the genital tubercle differentiates to a penis, which at this point expresses AR in both humans and rodents (Amato & Yao, 2021; Baskin et al., 2020). This includes androgen-mediated elongation of the tubercle, formation of the prepuce, and tubular internalization of urethra, which is closed at the distal tip of the glans penis (Amato et al., 2022). Failure of full closure of the urethra can result in hypospadias, in which the urethra terminates at the ventral side of the penis instead of at the tip (Baskin & Ebbers, 2006; Cohn, 2011).

The dependency of androgens for penile development has been demonstrated in mice with conditional or full knockout of *Ar,* which results in partly or full sex-reversal of males, including a female-like urethral opening (Willingham et al., 2006; Yucel et al., 2004; Zheng et al., 2015). Similarly, female rats and mice exposed *in utero* to testosterone present with varying degrees of intersexuality, including, in some cases, a penis (Greene & Ivy, 1937; Zheng et al., 2015).

Empirical Evidence

The empirical evidence for this KER is generally judged **high.** This includes evidence from *in vivo* animal studies and evidence from studies in humans. The upstream KE 'Decreased AR activity' refers to an *in vivo* effect, for which no methods for measurement of this *in vivo* in mammals currently exist. The effects on the upstream KE were therefore indirectly informed as described in each section.

Animal studies

Effects on the upstream KE were indirectly informed by including animal studies with stressors that are known to reduce AR activity through antagonizing the AR, lowering testosterone production, or inhibiting 5α-reductase. Six stressors, with established anti-androgenic effects, were included (more detailed evaluation of these chemicals can be found in KER-2820 (Holmer et al., 2024)). Table 3 summarizes the empirical evidence and confidence level for each chemical. Details on included evidence is presented in Table 1 in Appendix 2, 9prbqyba2x_Appendix_2_KER_2828.pdf. In summary, all six substances were shown to cause hypospadias in male offspring, and the confidence level for all substances was judged as strong, as conflicting results could be explained (see the section 'Uncertainties and inconsistencies'). Thus, antagonism or AR, inhibition of 5α-reductase, or reduction in testosterone synthesis, all lead to hypospadias.

Table 3 Summary of empirical evidence for the KER – animal studies . See Table 1 in Appendix 2 (9prbqyba2x_Appendix_2_KER_2828.pdf) for details.

Chemical	Upstream effect	Downstream effect	Overall confidence
Flutamide	Androgen receptor antagonist (Simard et al., 1986).	*In utero* exposure causes hypospadias in rat and mouse	Strong
Dibutyl phthalate (DBP)	Has been shown to reduce fetal intratesticular testosterone and serum testosterone *in vivo*, but exact mechanism is unknown (Foster, 2006).	*In utero* exposure causes hypospadias in rat	Strong
Vinclozolin	AR antagonist (Kelce et al., 1994, 1997)	*In utero* exposure causes hypospadias in rat and mouse	Strong

Di(2-ethylhexyl) phthalate (DEHP)	Has been shown to reduce fetal intratesticular testosterone and serum testosterone *in vivo*, but exact mechanism is unknown (Parks et al., 2000).	*In utero* exposure causes hypospadias in rat	Strong
Procymidone	AR antagonist (Ostby et al., 1999).	*In utero* exposure causes hypospadias in rat	Strong
Finasteride	5α-reductase inhibitor, causing a reduction in DHT (Rittmaster & Wood, 1994).	*In utero* exposure causes hypospadias in rat	Strong

Supporting human evidence

Effects on the upstream KE were indirectly informed by including studies in humans with a condition (genetic or other) that would reduce or disrupt either 1) function of AR, 2) conversion of testosterone to DHT by disrupting 5α-reductase activity, or 3) production of androgen hormones. Studies measuring low testosterone levels with no underlying cause were also included (see evidence collection strategy). Table 4 lists the studies, in which these conditions were linked to hypospadias in males.

Table 4 Supporting evidence for the KER – human studies. The table lists human studies reporting hypospadias in association with an upstream defect in AR activity, grouped according to the precise effect, and how it was diagnosed (mutation, *in vitro* activity, or blood hormone and metabolite profile). SRD5A2: 5α-reductase 2; HSD17B3: 17β-hydroxysteroid dehydrogenase 3; HSD3B2: 3β-hydroxysteroid dehydrogenase 2; CYP17A1: 17α-hydroxylase. See table 2 in Appendix 2 (9prbqyba2x_Appendix_2_KER_2828.pdf) for all included references.

Effect on upstream KE	**Supporting studies**
Effects on Androgen receptor	
AR mutations	27 studies
Extended CAG repeat length in *AR*	4 studies
Reduced AR activity (e.g. low receptor binding) in *in vitro* genital skin fibroblasts	11 studies
Effects on 5α-reductase activity	
SRD5A2 mutations	30 studies
SRD5A2 deficiency, diagnosed by T/DHT-ratio and/or reduced *in vitro* 5α-reductase activity in genital skin fibroblasts	8 studies
Effects on upstream steroidogenesis enzymes	
HSD17B3 mutations	6 studies
HSD3B2 mutations	5 studies
CYP17A1 mutation	1 study
HSD17B3 deficiency, diagnosed by hormone and metabolite profile	2 studies
HSD3B2 deficiency, diagnosed by hormone and metabolite profile	4 studies
CYP17A1 deficiency, diagnosed by hormone and metabolite profile	5 studies
Other upstream effects on low testosterone	
Low testosterone due to gonadal dysgenesis or hypogonadism	7 studies
Low basal testosterone or low testosterone response to hCG stimulation. Idiopathic or rare mutations.	7 studies

Six case-control studies were extracted, all of which found a correlation between lower testosterone levels (basal or hCG-stimulated) and hypospadias (Austin et al., 2002; Okuyama et al., 1981; Raboch et al., 1976; Ratan et al., 2012; Svensson et al., 1979; Yadav et al., 2011). In two of these studies, the correlation was age-dependent (Austin et al., 2002; Raboch et al., 1976)

One epidemiologic study was extracted, which investigated the association between phthalate exposure and hypospadias risk. Western Australian women exposed through their occupation to phthalates were more likely to have sons with hypospadias (Nassar et al., 2010). It should be noted that there are reported species differences in the effects of phthalates (including DEHP and DBP) on fetal testosterone production between humans, mice, and rats, and the direct translatability of the *in vivo* evidence is uncertain (Sharpe, 2020).

Dose concordance

Direct information about dose concordance is not available because AR activity currently cannot be measured *in vivo*.

Indirect information on dose concordance can be obtained from empirical evidence. *In utero* exposure of rats to DBP caused a dose-dependent decrease in serum testosterone levels at PND70 with LOAEL 250 mg/kg bw/day. Hypospadias was observed at this stage with LOAEL 500 mg/kg bw/day (Jiang et al., 2007). It should be noted that fetal testosterone levels were not measured.

Temporal concordance

Direct information about temporal concordance is not available because AR activity currently cannot be measured *in vivo*.

Indirect information on temporal concordance can be obtained from empirical evidence. In two studies, in which rats were exposed *in utero* to 750 mg/kg bw/day DBP, intratesticular testosterone levels were reduced in fetal testes, while hypospadias was identified in adult males. Plasma levels of testosterone were also measured in adults, and testosterone levels in exposed males were not significantly different from control males (van den Driesche et al., 2017, 2020). This has also been shown in a study with 500 mg/kg bw/day DBP (Drake et al., 2009). These studies indicate temporal concordance. Another study with DBP-induced hypospadias in rats saw a dose-dependent reduction in serum testosterone levels at PND70 after *in utero* exposure to as low as 250 mg/kg bw/day from GD14-18 (Jiang et al., 2007), though fetal testosterone levels were not measured in this study.

Incidence Concordance

Direct information about dose concordance is not available because AR activity currently cannot be measured *in vivo*.

Indirect information on incidence concordance can be obtained from empirical evidence. In the dose-response study with DBP, the incidence of hypospadias was 6.8% for 500 mg/kg bw/day DBP and 41.3% for 750 mg/kg bw/day. When separating hypospadias males from exposed males without hypospadias, plasma testosterone levels were decreased in both groups, indicating that DBP reduced testosterone levels at higher incidence than hypospadias (Jiang et al., 2007). The same was seen in another study with DBP, in which serum testosterone levels at PND7 were reduced in both hypospadiac and non-malformed males exposed to 750 mg/kg bw/day DBP from GD14-18 (Jiang et al., 2016).

Uncertainties and Inconsistencies

The *in vivo* studies do not directly inform about the upstream KE, ‘decreased AR activity’. The direct concordance between the KEs can therefore not be determined from the evidence.

For flutamide, two studies reported 100% hypospadias frequencies at doses of 6.25 and 10 mg/kg bw/day (Goto et al., 2004; McIntyre et al., 2001), while another study found a frequency of 56.9% when giving 20 mg/kg bw/day (Kita et al., 2016). This might be explained by a longer exposure window in the first two studies and uncertainties in assessment of hypospadias.

For DBP, there were discrepancies in whether 250 mg/kg bw/day was LOAEL (Mylchreest et al., 1998, 1999) or NOAEL (Jiang et al., 2007) for DBP. This conflict was explained by differences in exposure windows, supported by the observation that the frequency of hypospadias at 250 mg/kg bw/day was reported as very low (Mylchreest et al., 1998, 1999).

One study with vinclozolin (Ostby J et al., 1999) and one with procymidone (Hass et al., 2012) did not find hypospadias after *in utero* exposure. In both cases, this was likely due to too low doses tested.

In most of the human studies of steroidogenesis deficiency, serum or plasma levels of testosterone were reduced at baseline and/or upon hCG stimulation (Al-Sinani et al., 2015; Ammini et al., 1997; Cara et al., 1985; Chen, Huang, et al., 2021; Dean et al., 1984; Galli-Tsinopoulou et al., 2018; Imperato-McGinley et al., 1979; Kaufman et al., 1983; Mendonca et al., 1987, 2000; Neocleous et al., 2012; New, 1970; Pang et al., 1983; Perrone et al., 1985; Rabbani et al., 2012; Sherbet et al., 2003), but in a few studies, testosterone levels were normal (Donadille et al., 2018; Kon et al., 2015; Luna et al., 2021). In these cases, the effect of these deficiencies on tissue AR activity is uncertain.

For *AR* CAG repeat length, a case-control study did not find an association with hypospadias (Radpour R et al., 2007), but this could be because the hypospadias cases included had other etiologies.

Lastly, as there are currently no universal guidelines for identification and scoring of hypospadias in rodents, there are large variations in methods of assessment, and minor cases of hypospadias may be overlooked in some studies and included in others. This poses an uncertainty in the frequency reports in the scientific evidence.

Quantitative Understanding of the Linkage

The quantitative understanding of the relationship is low. As there are currently no direct measurement methods of the upstream KE (reduced AR activity) in mammals, quantification of the relationship is difficult to assess.

Response-response relationship

A model for phthalates has been developed, aiming to predict the frequency of hypospadias in male offspring based on reductions in *ex vivo* testosterone production, an indirect indication of AR activity. In this model, hypospadias was induced from around a 60% reduction in testosterone levels. The model does not consider hypospadias severity and is only for phthalate chemicals (Earl Gray et al., 2024).

Time-scale

The time-scale of this KER depends on the species but is likely days to weeks.

AR activation happens within minutes, from ligand binding to nuclear translocation and promotor activation (Nightingale et al., 2003; Schaufele et al., 2005), while transcriptional and translational effects are observed minutes to hours later (Kang et al., 2002). AR programming of the genital tubercle occurs during fetal development in the Masculinization Programming Window (Sharpe, 2020). The time-scale for morphological effects in the tissue then depends on the species. In humans, penis development is completed prior to birth and hypospadias can be observed at birth. In rodents, penis development is not fully completed until weeks after birth, but hypospadias can often be observed earlier than this (table 3).

Known modulating factors

Modulating Factors	MF details	Effects on the KER	References
AR CAG repeat length	Extended CAG repeat length in *AR* is associated with reduced AR activity	Higher risk of hypospadias development	(Chamberlain et al., 1994)

Known Feedforward/Feedback loops influencing this KER

There are no known feedforward/feedback loops influencing this KER.

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