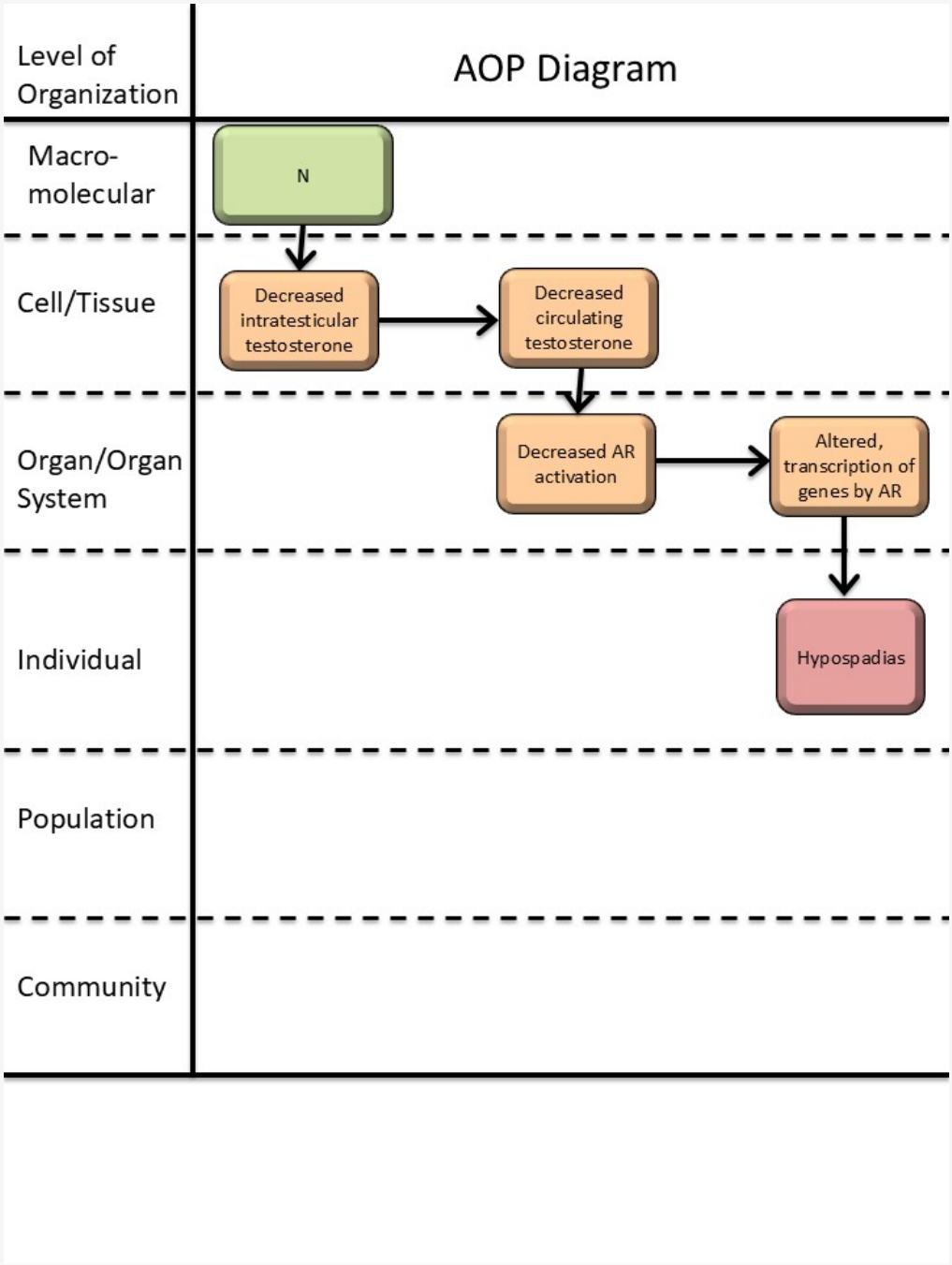
AOP ID and Title:

AOP 570: Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring
Short Title: Decreased testosterone synthesis leading to hypospadias

Graphical Representation



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Status

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Abstract

This AOP links *in utero* decreased intratesticular testosterone levels with hypospadias in male offspring. Hypospadias is a common reproductive disorder with a prevalence of up to ~1/125 newborn boys, though with high inter-country variability (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 a genital abnormality in OECD test guidelines (TG) for developmental and reproductive toxicity (TG 414, 416, 421/422, and 443; (OECD, 2001, 2016b, 2016a, 2018a, 2018b)), as both a measurement of adverse reproductive effects and a direct clinical adverse outcome.

Testosterone is one of the two main steroid sex hormones essential for male reproductive development. Testosterone is primarily, but not exclusively, produced in the testes and then secreted into the circulation. In peripheral reproductive tissues, testosterone is either converted to dihydrotestosterone (DHT) or directly activates the androgen receptor (AR). DHT is more potent than testosterone in activating the AR, and activation of AR by either androgen initiates differentiation of the male phenotype, including development of the penis (Amato et al., 2022; Davey & Grossmann, 2016). This AOP delineates the evidence that decreasing testicular testosterone production lowers circulating testosterone levels and consequently AR activation, thereby disrupting penis development and causing hypospadias. In this AOP, the first KE is not considered an MIE, as testicular testosterone production can be obstructed by various routes. The AOP does not discriminate whether the reduction in AR activation is due to direct lack of testosterone at the AR or due to decreased conversion of testosterone to DHT, as there is not sufficient information on this. The AOP is supported by *in vitro* experiments upstream of AR activation and by *in vivo* and human case studies downstream of AR activation. Downstream of a reduction in AR activation, the molecular mechanisms of hypospadias development are not fully delineated, 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.

AOP Development Strategy

Context

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-477 (‘Androgen receptor antagonism 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.

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Strategy

The OECD AOP Developer’s Handbook was followed alongside pragmatic approaches (Svingen et al., 2021).

KEs and upstream KER-3448 (‘decrease, intratesticular testosterone levels leads to decrease, circulation testosterone levels’), KER-2131 (‘decrease, circulating testosterone levels leads to decrease, AR activation’), and KER-2124 (‘decrease, AR activation leads to altered, transcription of genes by AR’) were considered canonical knowledge and part of an upstream anti-androgenic network developed using mainly key review articles (Draskau et al., 2024; Svingen et al., 2025). The non-adjacent KER-3488, KER-3350, and KER-2828 linking decreased intratesticular testosterone, circulating testosterone, and AR activation, respectively, with hypospadias were developed using a systematic weight-of-evidence approach, following methodology outlined in (Holmer et al., 2024). Articles were retrieved by literature searches in PubMed and Web of Science and extensive screening using pre-defined inclusion and exclusion criteria. Evaluation of methodological reliability of *in vivo* animal studies was performed using the Science in Risk Assessment and Policy (SciRAP) online tool. For KER-3488 and KER-3350 regarding testosterone levels, articles were included if there was a decrease in fetal testosterone levels and hypospadias was assessed in male offspring. For KER-2828, there are currently no *in vivo* methods to measure AR activation in mammals, and instead six chemicals with known anti-androgenic mechanisms-of-action were chosen for the empirical evidence for this KER. To supplement the *in vivo* toxicity studies, human case studies and epidemiologic studies were included in the KERs. These studies were not systematically evaluated for reliability but served as supporting evidence.

Regarding the inclusion of KEs and KERs, the rationale for the upstream anti-androgenic network is detailed in (Draskau et al., 2024). KE-2298 (‘decrease, intratesticular testosterone levels’) was added to discriminate between the large difference in testosterone levels between testes and circulation (Coviello et al., 2004; McLachlan et al., 2002; Turner et al., 1984). The link between the upstream network, more specifically KE-286 (‘altered, transcription of genes by AR’), and AO-2082 (‘hypospadias’) likely contains a tissue-specific KE that has not been developed, as sufficient evidence is not yet available. Thus, for now, the most evidence for the link of the anti-androgenic network on hypospadias is captured by KER-2828.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	KE	2298	Decrease, intratesticular testosterone levels	Decrease, intratesticular testosterone
	KE	1690	Decrease, circulating testosterone levels	Decrease, circulating testosterone levels
	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
Decrease, intratesticular testosterone levels	adjacent	Decrease, circulating testosterone levels	High	
Decrease, circulating testosterone levels	adjacent	Decrease, androgen receptor activation	High	
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	
Decrease, intratesticular testosterone levels	non-adjacent	Hypospadias, increased	Moderate	
Decrease, circulating testosterone levels	non-adjacent	Hypospadias, increased	Low	
Decrease, androgen receptor activation	non-adjacent	Hypospadias, increased	High	

Stressors

Name	Evidence
Dibutyl phthalate	
Di(2-ethylhexyl) phthalate	

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 KE-2298 (‘decrease, intratesticular testosterone levels’) and KER-3488 (‘decrease, intratesticular testosterone levels leads to hypospadias’), KER-3350 (‘Decrease, circulating testosterone levels leads to hypospadias’), and KER-2828 (‘decrease AR activation leads to hypospadias’). By definition, the testis is the primary sex organ in males, and the term hypospadias is mainly used for describing malformation of the male and not female external genitalia. The genital tubercle is programmed by androgens to differentiate into a penis in fetal life in 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 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 happens in the fetal life stage, but the AO is best detected postnatally. Specifically, the masculinization programming window (~ gestational day (GD)16-20 in rat, presumably gestational weeks (GW) 8-14 in humans) is the primary fetal window of applicability, but effects outside of this window in fetal life, after androgen production has started, cannot be excluded. Regarding taxonomic applicability, hypospadias has mainly been identified in rodents (rats and mice) 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 as well, 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 reduced testosterone levels, though this likely reflects lack of fetal endocrine data from these species.

Essentiality of the Key Events

Event	Evidence	Uncertainties and inconsistencies
KE-2298 Decrease, intratesticular testosterone levels (moderate)	Biological plausibility provides strong support for the essentiality of this event as the testes are the main sites of testosterone production in male mammals, and testosterone is a ligand for the AR and one of the primary drivers of penis development. Experimental evidence with phthalates lowering intratesticular testosterone supports the essentiality (see KE-3488) Human case studies indirectly support the essentiality as mutations in steroidogenesis enzymes and gonadal dysgenesis are associated with low circulating testosterone levels and hypospadias (as listed in Table 4, KER-2828)	In the human studies, testosterone levels were only measured postnatally and not in fetal life. In humans, studies have indicated a role of placental-derived androgens for androgen availability, as placental abnormalities have been associated with increased hypospadias risk.

KE-1690 Decrease, circulating testosterone levels (moderate)	Biological plausibility provides strong support for the essentiality of this event. Circulating testosterone is both a direct ligand for the AR in target tissue and serves as the primary substrate for DHT production in peripheral tissues. Thus, it is one of the primary drivers of penis development Human case studies support the essentiality as low circulating testosterone levels have been associated with hypospadias (as listed in Table 4 in KER-2828).	In human case studies, testosterone levels were only measured postnatally and not in fetal life. As hypospadias is a congenital malformation, it cannot be “reversed” by testosterone treatment.
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 presented in Table 4 in KER-2828).	
KE-286 Altered, transcription of genes by AR (moderate)	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. Genomic AR signaling is necessary and possibly sufficient to drive penis differentiation (Cunha et al., 2021) 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, though these alone are not sufficient for penis differentiation –and genomic AR signaling is necessary for penis development (Cunha et al., 2021; Leung & Sadar, 2017).

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
KE-2298		**	*	Moderate
KE-1690		***	*	Moderate
KE-1614	**			Moderate
KE-286	**	*		Moderate

Weight of Evidence Summary

The confidence in each of the KERs comprising the AOP is 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-3448 Decrease, intratesticular testosterone levels leads to decrease, circulating testosterone levels	High	High (canonical)	It is well established that testes are the primary testosterone-producing organs in male mammals. *In vivo* studies have shown that exposure to substances that lower intratesticular testosterone also lower circulating testosterone levels (Svingen et al., 2025).
KER-2131 Decrease, circulating testosterone levels leads to decrease, AR activation	High	High (canonical)	It is well established that testosterone activates the AR. Direct evidence for this KER is not possible since KE-1614 can currently not be measured and is considered an *in vivo* effect. Indirect evidence using proxy read-outs of AR activation, either *in vitro* or *in vivo* strongly supports the relationship (Draskau et al., 2024).
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-3488 Decrease, intratesticular testosterone leads to hypospadias	High	Moderate	It is well established that testicular testosterone is one of the primary drivers of penis development. *In vivo* animal studies support that reductions in fetal testicular testosterone can cause hypospadias in male offspring. One study supports dose concordance, where diisocytol caused reduced ex vivo testosterone production in rats at a dose of 0.1 g/kg bw/day, while hypospadias was observed in male offspring at 1 g/kg bw/day (Saillenfait et al., 2013).
KER-3350 Decrease, circulating testosterone levels leads to hypospadias	High	Low	Normal masculinization of the genital tubercle requires sufficient circulating testosterone, which must reach the tissue and be locally converted to DHT in order to activate AR-dependent developmental pathways. *In vivo* evidence for this KER is sparse, but human case studies of subjects with low testosterone levels (postnatally) and associated hypospadias support the KER.

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 temporal concordance for the KER. It should be recognized that the upstream KE-1614 cannot currently be measured directly (*in vivo*). Instead, empirical evidence was therefore collected for substances known to affect upstream events. This limitation is not considered to lower the strength of the evidence in this case.

Quantitative Consideration

The quantitative understanding of this AOP is judged as low.

A model for phthalate-induced malformations has been developed which aims to predict the frequency of hypospadias related to a phthalate's reduction in *ex vivo* testosterone production. The model predicted that a 60% reduction in testosterone levels would induce hypospadias, although the predictivity of this model was not good when tested for one phthalate (Earl Gray et al., 2024).

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Appendix 1

List of Key Events in the AOP

Event: 2298: Decrease, intratesticular testosterone levels

Short Name: Decrease, intratesticular testosterone

Event Component

Process	Object	Action
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
testis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence

Sex Evidence

This key event (KE) is applicable to all male vertebrates with testes that produce testosterone.

Key Event Description

This KE refers to decreased testosterone biosynthesis in the testis (male); i.e. intratesticular testosterone levels. It is therefore considered distinct from KEs describing circulating testosterone levels, or levels in any other tissue or organ of vertebrate animals. It is also distinct from indirect cell-based assays measuring effects on testosterone synthesis, including *in vitro* Leydig cells.

In males, the testis is the primary site of testosterone biosynthesis via the steroidogenesis pathway – an enzymatic pathway converting cholesterol into all the downstream steroid hormones (Miller and Auchus 2010). In mammals, the Leydig cells are considered the primary site of steroidogenesis in the testis. Although generally correct, there is evidence to suggest the involvement of Sertoli cells during fetal stages in e.g. mouse and human testis, but with Leydig cells being sufficient in adult life (O'Donnell et al 2022).

Testicular testosterone synthesis is primarily regulated by the hypothalamic-pituitary-gonadal (HPG) axis, with Gonadotropin-releasing hormone (GnRH) from the hypothalamus controlling the secretion of Luteinizing hormone (LH) from the pituitary that ultimately binds to the LH receptors on Leydig cells to stimulate steroidogenesis. Notably, the timing of HPG axis activation during development varies between species. In humans, human chorionic gonadotropin (hCG) act similarly to LH and appear to be critical in stimulating testosterone synthesis in the fetal testis (Huhtaniemi 2025), whereas in the mouse, testosterone synthesis in the fetal testis appears to be independent of pituitary gonadotropins even though LH is detectable during late gestation (O'Shaughnessy et al 1998). Irrespective of testosterone being stimulated by gonadotropins or occurring *de novo*, however, it is essential for masculinization of the developing fetus, initiation of puberty, and maintain reproductive, and other, functions in adulthood.

Notably, intratesticular testosterone concentration is significantly higher than serum testosterone levels, typically ranging from 30- to 200-fold greater in mammals, including humans (Turner et al 1984; McLachlan et al 2002; Coviello et al 2004).

How it is Measured or Detected

Testosterone levels can be quantified in testis tissue, in testis homogenate, or in supernatant following culture of testes tissue or advanced *in vitro* testis models. Methods include traditional immunoassays such as ELISA and RIA, advanced techniques like LC-MS/MS, and liquid scintillation spectrometry following radiolabeling (Shiraishi et al., 2008).

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Event: 1690: Decrease, circulating testosterone levels

Short Name: Decrease, circulating testosterone levels

Event Component

Process	Object	Action
hormone biosynthetic process	testosterone	decreased
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:526 - Decreased, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) leads to Impaired, Spermatogenesis	KeyEvent
Aop:124 - HMG-CoA reductase inhibition leading to decreased fertility	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males	KeyEvent
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents	KeyEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction □in zebrafish□	KeyEvent
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	KeyEvent
Aop:120 - Inhibition of 5α-reductase leading to Leydig cell tumors (in rat)	KeyEvent
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:570 - Decreased testosterone synthesis 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:595 - Emerging OPFRS reproductive outcome pathway	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

blood

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
Male	High
Female	High

This key event (KE) is applicable to all mammals, as the synthesis and role of testosterone are evolutionarily conserved (Vitousek et al., 2018). Both sexes produce and require testosterone, which plays critical roles throughout life, from development to adulthood; albeit there are differences in life stages when testosterone exert specific effects and function (Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022). Accordingly, this KE applies to both males and females across all life stages, but life stage should be considered when embedding in AOPs.

Notably, the key enzymes involved in testosterone production first appeared in the common ancestor of amphioxus and vertebrates (Baker, 2011). This suggests that the KE has a broader domain of applicability, encompassing non-mammalian vertebrates. AOP developers are encouraged to integrate additional knowledge to expand its relevance beyond mammals to other vertebrates.

Key Event Description

Testosterone is an endogenous steroid hormone that acts by binding the androgen receptor (AR) in androgen-responsive tissues (Murashima et al., 2015). As with all steroid hormones, testosterone is produced through steroidogenesis, an enzymatic pathway converting cholesterol into all the downstream steroid hormones. Briefly, androstenedione or androstenediol is converted to testosterone by the enzymes 17β-hydroxysteroid dehydrogenase (HSD) or 3β-HSD, respectively. Testosterone can then be converted to the more potent androgen, dihydrotestosterone (DHT) by 5α-reductase, or aromatized by CYP19A1 (Aromatase) into estrogens. Testosterone secreted in blood circulation can be found free or bound to SHBG or albumin (Trost & Mulhall, 2016).

Testosterone is produced mainly by the testes (in males), ovaries (in females), and to a lesser degree in the adrenal glands. The output of testosterone from different tissues varies with life stages. During fetal development, testosterone is crucial for the differentiation of male reproductive tissues and the overall male phenotype. In adulthood, testosterone synthesis is controlled by the Hypothalamus-Pituitary-Gonadal (HPG) axis. GnRH is released from the hypothalamus inducing LH pulses secreted by the anterior pituitary. This LH surge leads to increased testosterone production, both in testes (males) and ovaries (females). If testosterone reaches low levels, this axis is once again stimulated to increase testosterone synthesis. This feedback loop is essential for maintenance of appropriate testosterone levels (Chandrashekar & Bartke, 1998; Ellis et al., 1983; Rey, 2021).

By disrupting e.g. steroidogenesis or the HPG-axis, testosterone synthesis or homeostasis may be disrupted which can lead to less testosterone being synthesized and released into circulation.

General role in biology

Androgens are essential hormones responsible for the development of the male phenotype during fetal life and for sexual maturation at puberty. In adulthood, androgens remain essential for the maintenance of male reproductive function and behavior but is also essential for female fertility. Apart from their effects on reproduction, androgens affect a wide variety of non-reproductive tissues such as skin, bone, muscle, and brain (Heemers et al 2006). Androgens, principally testosterone and DHT, exert most of their effects by interacting with the AR (Murashima et al 2015).

How it is Measured or Detected

Testosterone levels can be quantified in serum (*in vivo*), cell culture medium (*in vitro*), or tissue (*ex vivo*, *in vitro*). Methods include traditional immunoassays such as ELISA and RIA, advanced techniques like LC-MS/MS, and liquid scintillation spectrometry following radiolabeling (Shiraishi et al., 2008).

The H295R Steroidogenesis Assay (OECD TG 456) is (currently; anno 2025) primarily used to measure estradiol and testosterone production. This validated OECD test guideline uses adrenal H295R cells, with hormone levels measured in the cell culture medium (OECD, 2011). H295R adrenocortical carcinoma cells express the key enzymes and hormones of the steroidogenic pathway, enabling broad analysis of steroidogenesis disruption by quantifying hormones in the medium using LC-MS/MS. Initially designed to assess testosterone and estradiol levels, the assay now extends to additional steroid hormones, such as progesterone and pregnenolone. The U.S. EPA's ToxCast program further advanced this method, enabling high-throughput measurement of 11 steroidogenesis-related hormones (Haggard et al., 2018). While the H295R assay indirectly reflects disruptions in overall steroidogenesis (e.g., changes in testosterone levels), it does not provide mechanistic insights.

Testosterone can be measured by immunoassays and by isotope-dilution gas chromatography-mass spectrometry in serum (Taieb et al., 2003; Paduch et al., 2014). Testosterone levels may also be measured by: Fish Lifecycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500), Male pubertal assay (PP Male Assay) (US EPA OPPTS 890.1500), OECD TG 441: Hershberger Bioassay in Rats (H Assay).

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Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

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 ID and Name	Event Type
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 activation 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), as shown for the pyrethroid cypermethrin (Wang et al., 2016).

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 activation 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

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: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 ID and Name	Event Type
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 transcriptional level of known downstream target genes by RT-qPCR or other transcription analysis 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 identify 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

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

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

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI

Life Stage Applicability

Life Stage	Evidence
Perinatal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability: Several studies have shown an association in humans between *in utero* exposure to endocrine disrupting chemicals and hypospadias (Baskin et al., 2001; Kalfa et al., 2015; Mattiske & Pask, 2021). In mice and rats, *in utero* exposure to several endocrine disrupting chemicals, in particular estrogens and anti-androgens, 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 penis. 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 prevalence of hypospadias varies greatly between countries, from 1:100 to 1:500 of newborn boys (Skakkebaek et al., 2016). Several studies report increases in hypospadias prevalence, though these trends are country- and region-specific with high variability between studies (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 (TG) 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, histological 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: 3448: Decrease, intratesticular testosterone leads to Decrease, circulating testosterone levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	adjacent	High	
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

The KER is assessed applicable to mammals, as testicular testosterone synthesis is common for all mammals. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates.

Sex applicability

This KER is only applicable to males, as testes are only found in males.

Life stage applicability

This KER is applicable to all life stages. Once formed, the testes produce and secrete testosterone during fetal development and throughout postnatal life, although testosterone levels do vary between life stages (Vesper et al., 2015).

Key Event Relationship Description

This KE describes a decrease in intratesticular testosterone production leading to a decrease in circulating levels of testosterone. Intratesticular testosterone can be measured in whole testicular tissue samples or by testing *ex vivo* testicular testosterone production, and circulating testosterone is measured in plasma or serum. In males, the testes produce and secrete the majority of the circulating testosterone, with only a small contribution from the adrenal gland (Naamneh Elzenaty et al., 2022). In mammals, intratesticular testosterone levels are 30- to 100-fold higher than serum testosterone levels (Coviello et al., 2004; McLachlan et al., 2002; Turner et al., 1984). Reducing testicular testosterone will consequently lead to a reduction in circulating levels as well.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high. The testes are the primary testosterone-producing organs in male mammals and the main contributors to the circulating testosterone levels in males (Naamneh Elzenaty et al., 2022). A decrease in intratesticular testosterone will therefore lead to a decrease in secretion of testosterone and consequently lower circulating levels of testosterone.

Empirical Evidence

The empirical evidence for this KER is overall judged as **high**.

In vivo toxicity studies in rats and mice have shown that exposure to substances that lower intratesticular testosterone also lower circulating testosterone levels. This includes *in utero* exposure and measurements in fetal males (Borch J et al., 2004; Vinggaard AM et al., 2005) as well as exposure and measurements postnatally in male rodents (Hou X et al., 2020; Ji et al., 2010; Jiang XP et al., 2017)

Supporting this evidence are castration studies in male rats and monkeys, showing a marked reduction in circulating testosterone levels when removing the testes (Gomes & Jain, 1976; Perachio et al., 1977).

Lastly, in humans, males with hypogonadism or gonadal dysgenesis present with lower circulating testosterone levels (Hirose Y et al., 2007; Jones LW et al., 1970).

Dose concordance

In vivo toxicity studies support dose concordance for this KER, as exemplified below.

In pre-pubertal/pubertal male rats, chlorocholine chloride exposure (postnatal day (PND) 23-60) in three doses reduced both intratesticular and serum testosterone levels at PND60 at all doses tested (Hou X et al., 2020).

Perinatal exposure (gestational day (GD) 10-birth) of male mice to diethylhexyl phthalate (DEHP) in three doses (100, 500, and 1000 mg/kg bw/day) reduced intratesticular testosterone at 500 and 1000 mg/kg bw/day at PND1, while only 1000 mg/kg bw/day reduced serum levels of testosterone, although this was measured later, at PND56 (Xie Q et al., 2024)

In utero exposure (GD7-21) of male rats to DEHP in doses of 300 or 750 mg/kg bw/day reduced intratesticular testosterone levels at GD21, while only the high dose also reduced plasma testosterone levels (Borch J et al., 2004).

Temporal concordance

In vivo toxicity studies moderately support temporal concordance for this KER, as exemplified below.

Several studies show that a decrease in intratesticular and circulating testosterone can be measured at the same time point (Borch J et al., 2004; Hou X et al., 2020; Jiang XP et al., 2017; Vinggaard AM et al., 2005).

In utero exposure of male mice to DEHP from GD10 to birth reduced intratesticular testosterone levels at PND1 with LOAEL 500 mg/kg bw/day, and when measured at PND56, circulating testosterone levels were decreased, but with LOAEL 1000 mg/kg bw/day (Xie Q et al., 2024).

In Fisher JS et al., 2003, exposure of male rats from GD13-21 to 500 mg/kg bw/day dibutyl phthalate reduced intratesticular testosterone by ~90% (measured at GD19). When analyzing circulating testosterone levels at PND4, 10, 15, 25, and 90, only the testosterone levels on PND25 were decreased.

One study report conflicting results on the temporal concordance of this KER (Caceres et al., 2023). Here, male rats were exposed for 20 weeks from PND60 to a mixture of the phytoestrogens genistein and daidzein (combined dose of either 29 or 290 mg/kg bw/day). Intratesticular testosterone was measured every 4 weeks, while serum levels of testosterone were measured every second week. While the mixture caused a reduction of serum testosterone after 2 weeks of exposure, a reduction in intratesticular testosterone was not measured until after 8 weeks. The discrepancy might be explained by the multiple mechanisms of action of the phytoestrogens, as they, besides affecting testicular testosterone synthesis, may also influence peripheral aromatization of testosterone to estrogens (van Duursen et al., 2011).

Incidence concordance

Incidence concordance can not be evaluated for this KER.

Uncertainties and Inconsistencies

There are examples of *in vivo* studies, in which stressors exposure have caused a reduction in intratesticular testosterone levels without a reduction in circulating testosterone levels.

Quantitative Understanding of the Linkage

Time-scale

The time-scale for this KER is likely minutes or hours, as testosterone is secreted into the blood from the testes after synthesis. *In vivo*, a decrease in intratesticular and circulating testosterone can be measured at the same time, both in fetal and postnatal studies (Borch J et al., 2004; Hou X et al., 2020; Jiang XP et al., 2017; Vinggaard AM et al., 2005). *Ex vivo*, chemically-induced reduction in testicular production of testosterone can be measured in culture media after 3 hours incubation (earlier time points were not measured) (Wilson et al., 2009).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Adrenal species difference	Adrenal glands can supply weak androgen precursors, contributing to circulating androgen levels, although not substituting for testicular testosterone in masculinization processes. The magnitude and mechanism of adrenal androgen synthesis is also species dependent.		(Olson & Ristau, 2025; Pihlajoki et al., 2015)

Known Feedforward/Feedback loops influencing this KER

Testosterone is a part of the hypothalamic-pituitary-gonadal (HPG) axis, which controls testosterone synthesis in puberty and adulthood. In this axis, gonatropin-releasing hormone (GnRH) is released from the hypothalamus and stimulates release of luteinizing hormone (LH) from the pituitary. LH acts on the testes to produce and secrete testosterone. Elevated circulating testosterone levels exert negative feedback on the HPG axis (decreasing GnRH secretion) to keep testosterone levels in balance (Tilbrook & Clarke, 2001).

Importantly, there are species-specific differences in when the HPG axis is functional during development. In the mouse, fetal testosterone synthesis is independent of pituitary LH (O'Shaughnessy et al., 1998), whereas in humans, human chorionic gonadotropin (hCG) act similarly to LH and appear to be critical in stimulating testosterone synthesis in the fetal testis (Huhtaniemi, 2025).

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Relationship: 2131: Decrease, circulating testosterone levels leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	adjacent	High	High
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	adjacent	High	
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) 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

Taxonomic applicability

KER-2131 is assessed applicable to mammals, as T and AR activation are known to be related in mammals.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.

Sex applicability

KER-2131 is assessed applicable to both sexes, as T activates AR in both males and females.

Life-stage applicability

KER-2131 is considered applicable to developmental and adult life stages, as T-mediated AR activation is relevant from the AR is expressed.

Key Event Relationship Description

This key event relationship links decreased testosterone (T) levels to decreased androgen receptor (AR) activation. T is an endogenous steroid hormone important for, amongst other things, reproductive organ development and growth as well as muscle mass and spermatogenesis (Marks, 2004).T is, together with dihydrotestosterone (DHT), a primary ligand for the AR in mammals (Schuppe et al., 2020). Besides its genomic actions, the AR can also mediate rapid, non-genomic second messenger signaling (Davey & Grossmann, 2016). When T levels are reduced, less substrate is available for the AR, and hence, AR activation is decreased (Gao et al., 2005).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

AR activation is dependent on ligand binding (though a few cases of ligand-independent AR activation has been shown, see *uncertainties and inconsistencies*). T is a primary ligand for the AR, and when T levels are decreased there is less substrate for the AR, and hence, AR activation is decreased. In the male, T is primarily synthesized by the testes, and in some target tissues, T is irreversibly metabolized to the more potent metabolite DHT. T and DHT both bind to the AR, but DHT has a higher binding affinity (Gao et al., 2005). The lower binding affinity of T compared to DHT is due to the faster dissociation rate of T from the full-length AR, as T has less effective FXXLF motif binding to AF2 (Askew et al., 2007). Binding of T or DHT has different effects in different tissues. E.g. in the developing male, T is required for development of the internal sex organs (epididymis, vas deferens and the seminal vesicles), whereas DHT is crucial for development of the external sex organs (Keller et al., 1996). In the adult male, androgen action in the reproductive tissues is DHT dependent, whereas action in muscle and bone is DHT independent (Gao et al., 2005). In patients with male androgen deficiency syndrome, clinically low levels of T leads to reduced AR activation (either due to low T or DHT in target tissue), which manifests as both androgen-related symptoms (such as incomplete or delayed sexual development, loss of body hair, small or shrinking testes, low or zero sperm count) as well as anabolic-related symptoms (such as height loss, low trauma fracture, low bone mineral density, reduced muscle bulk and strength, increased body fat). All symptoms can be counteracted by treatment with T, which acts directly on the AR receptor in anabolic tissue (Bhasin et al., 2010). Similarly, removal of the testicles in weanling rats results in a feminized body composition and muscle metabolism, which is reversed by administration of T (Krotkiewski et al., 1980). As this demonstrates, the consequences of low T regarding AR activation will depend on tissue, life stage, species etc.

Empirical Evidence

The empirical evidence for this KER is considered high

Dose concordance

There is a positive dose-response relationship between increasing concentrations of T and AR activation (U.S. EPA., 2023).

Other evidence

- In male patients with androgen deficiency, treatment with T counteracts anabolic (DHT independent) related symptoms such as height loss, low trauma fracture, low bone mineral density, reduced muscle bulk and strength,

increased body fat (Bhasin et al., 2010; Katznelson et al., 1996).
- Removal of the testicles in weanling rats result in a feminized body composition and muscle metabolism, which is reversed by administration of T (Krotkiewski et al., 1980).

Uncertainties and Inconsistencies

It should be noted that measurements of circulating total testosterone may not reflect available testosterone due to some testosterone being bound to serum proteins, which may vary. Ligand-independent actions of the AR have also been identified. To what extent and of which biological significance is not well defined (Bennesch & Picard, 2015).

Quantitative Understanding of the Linkage

Response-response relationship

There is a positive dose-response relationship between increasing concentrations of T and AR activation (U.S. EPA., 2023). However, there is not enough data, or overview of the data, to define a quantitative linkage *in vivo*, and such a relationship will differ between biological systems (species, tissue, cell type).

Time-scale

AR and promoter interactions occur within 15 minutes of ligand binding, and RNA polymerase II and coactivator recruitment are then proposed to occur transiently with cycles of approximately 90 minutes (Kang et al., 2002).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression changes with aging	Tissue-specific alterations in AR activity with aging	(Supakar et al., 1993; Wu et al., 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Chamberlain et al., 1994; Tut et al., 1997)
Male androgen deficiency syndrome	Low circulating testosterone levels due to primary (testicular) or secondary (pituitary-hypothalamic) hypogonadism	Reduced levels of circulating testosterone	(Bhasin et al., 2010)
Castration	Removal of testicles	Reduced levels of circulating testosterone	(Krotkiewski et al., 1980)

Known Feedforward/Feedback loops influencing this KER

Androgens can upregulate and downregulate AR expression (Lee & Chang, 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	High	
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	adjacent	High	
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).

It should be noted that the AR-mediated transcription operates within a broader developmental context, where timing, temporal adaptation, tissue specificity, and local signaling environments, such as cofactor presence and receptor mutations, jointly determine transcriptional outcomes. While such contextual influences are acknowledged, the KER remains focused on effects of decreased AR activation on AR-mediated gene expression.

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)
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: 3488: Decrease, intratesticular testosterone leads to Hypospadias

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	non-adjacent	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

The development and differentiation of the penis is driven by androgen hormones, mainly produced by fetal testes, in all mammals. It is therefore biologically plausible that this KER is applicable to all mammals (Murashima et al., 2015). The empirical evidence in this KER provides support that reduced intratesticular testosterone levels in fetal life can cause hypospadias in rats. Studies in humans with gonadal dysgenesis and concurrent hypospadias support this KER's applicability to humans (Boehmer et al., 2001; Crone et al., 2002).

Sex applicability

This KER is applicable to males, where the testis is the primary sex organ.

Life stage applicability

The genital tubercle is programmed by androgen hormones in the masculinization programming window (GD16-20 in rats, and GW8-14 in humans), when the testes produce high levels of testosterone (Sharpe, 2020; Welsh et al., 2014). The genital tubercle starts differentiating in fetal life, and in humans the penis is fully formed at birth, where hypospadias is usually diagnosed (Yu et al., 2019). In rats and mice, penis development continues postnatally for around 20-25 days, and hypospadias is optimally diagnosed after this timepoint, although it may also be observed earlier (Schlomer et al., 2013; Sinclair et al., 2017).

Key Event Relationship Description

This non-adjacent KER describes a fetal decrease in testis testosterone leading to hypospadias in male offspring. In this KER, intratesticular testosterone levels can both be measured in whole testes homogenates or by measuring *ex vivo* testosterone production from cultured testes.

In male mammals, the testes differentiate in early fetal life and begin steroidogenesis to synthesize testosterone. Testosterone is secreted from the fetal testes for initiation of differentiation of the male reproductive tissues. Testosterone acts at the androgen receptor (AR) or is converted by 5α-reductase to the more potent androgen dihydrotestosterone (DHT). Activation of AR in the bipotential genital tubercle starts differentiation into a penis. While penis differentiation is a longer process, programming of the genital tubercle is largely constrained to a fixed period (gestational day (GD) 16-20 in rats, presumably gestational week (GW) 8-14 in humans), when testicular testosterone production is high (Sharpe, 2020; Welsh et al., 2014). Failure of proper penis differentiation can cause genital malformations, of which the most common is hypospadias, where the urethral opening is on the underside of the penis.

A decrease in intratesticular testosterone levels may therefore lead to hypospadias in male offspring.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is judged to be **high** given the canonical biological knowledge on normal reproductive

development.

Differentiation of the penis is programmed during fetal development. Once the testes have formed around GW8 in humans and GD16 in rats, they synthesize testosterone through the steroidogenesis pathway (Murashima et al., 2015). Although the adrenal glands may also produce testosterone, the testes are the main site of testosterone production (Naamneh Elzenaty et al., 2022). Testosterone is secreted from the testes and is transported to the peripheral tissues, including the genital tubercle. Testosterone may act directly on the AR or be converted to the more potent androgen DHT.

The genital tubercle is the bipotential structure that upon hormonal cues differentiates to either penis or clitoris. Both human and rodent genital tubercles express AR (C. M. Amato & Yao, 2021; Baskin et al., 2020). Upon activation of AR, the genital tubercle differentiates to a penis by elongation and formation of a central urethra which terminates at the tip of the penis (C. Amato et al., 2022). The programming of the genital tubercle happens in the masculinization programming window (GD 16-20 in rats, GW 8-14 in humans) (Welsh et al., 2014), although elongation and growth of the penis is also programmed later, at least in rats (Welsh et al., 2008). Hypospadias is one of the most common genital malformations caused by disruptions to penis development (Baskin & Ebbers, 2006; Yu et al., 2019).

Given the dependency of testosterone for penis differentiation, either through direct AR activation or conversion to DHT, it is plausible that a decrease in intratesticular testosterone will cause hypospadias.

Empirical Evidence

The empirical evidence from studies in animals for this KER is overall judged as **moderate**

From the data collection, three data sets were extracted. The data sets included different stressors causing reduced fetal levels of testosterone, all in rats (Table 2 and Appendix 2, 2s48n3874x_KER_3488_appendix_2.pdf). All studies showed concurrent hypospadias in the male offspring.

Table 2 Empirical evidence for KER 3488 LOAEL: Lowest observed adverse effect level; see Appendix 2 for specifications: 2s48n3874x_KER_3488_appendix_2.pdf

Species	Stressors(s)	Effect on upstream event (intratesticular testosterone)	Effect on downstream event hypospadias	Reference
Rat	Dibutyl phthalate	LOAEL 750 mg/kg bw/day	LOAEL 750 mg/kg bw/day	(van den Driesche et al., 2020)
Rat	Dibutyl phthalate	LOAEL 500 mg/kg bw/day	LOAEL 500 mg/kg bw/day	(Drake et al., 2009)
Rat	Diisooctyl phthalate	LOAEL 0.1 g/kg bw/day	LOAEL 1 g/kg bw/day	(Saillenfait et al., 2013)

Supporting human studies

Supporting the empirical evidence are human cases of patients with less testicular tissue (i.e. partial gonadal dysgenesis) which can cause severe hypospadias (Boehmer et al., 2001; Crone et al., 2002).

Dose concordance

One study informs and supports dose concordance for this KER. In this study, diisocytol phthalate caused reduced *ex vivo* testosterone production at a dose of 0.1 g/kg bw/day, while hypospadias was observed in male offspring at 1 g/kg bw/day (Saillenfait et al., 2013).

Temporal concordance

Empirical evidence indirectly supports temporal concordance. In all studies, the exposure window was prenatal, and low testosterone levels were detected at GD18-22, while hypospadias was assessed in adult rats. There are, however, no assessments of hypospadias at earlier timepoints to establish when the phenotype became visible.

Incidence concordance

Incidence concordance cannot be directly informed from the empirical evidence, because testosterone levels are reported as means of all values and is a continuous variable. However, given that hypospadias was not registered in all males in any of the studies, this could suggest a higher incidence of lower testosterone levels than the incidence of hypospadias.

Uncertainties and Inconsistencies

In one study (Drake et al., 2009), testosterone levels were only reduced when performing statistical analysis on individual values and not on litter means. Hypospadias was observed in 30% of males. The difference in statistical significance between litter means and individual values is an uncertainty to this study.

In (Saillenfait et al., 2013), intratesticular testosterone was only measured in *ex vivo* testes cultures, which are assumed to be a good proxy for intratesticular testosterone levels, although it should be kept in mind as an uncertainty.

Another uncertainty for this KER from the literature is the observation that humans with 5α-reductase deficiency have hypospadias due to low DHT levels despite normal or higher testosterone levels (Mendonca et al., 1996). This indicates that the effects of low testosterone may be more through reduced conversion to DHT than due to a direct loss of testosterone action on AR. To this, there is also the existence of a “backdoor pathway” to DHT in humans. This pathway in peripheral tissues (i.e. not testes) can circumvent testosterone as a precursor for DHT by synthesis of DHT is from reduction of androsterone by 17β-HSD (Miller & Auchus, 2019). This would create the possibility that testosterone is not required for DHT production and ultimately AR activation.

Quantitative Understanding of the Linkage

The quantitative understanding of this KER is low.

Response-response relationship

A model for phthalate-induced malformations has been developed which aims to predict the frequency of hypospadias related to a phthalate’s reduction in *ex vivo* testosterone production. The model predicted that a 60% reduction in testosterone levels would induce hypospadias, although the predictivity of this model was not good when tested for one phthalate (Earl Gray et al., 2024). Thus, this model should be improved and extended to include other substances.

Time-scale

The time-scale of this KER largely depends on species, but is likely weeks. In humans, the masculinization programming window is weeks long, while in rodents it is days (Sharpe, 2020; Welsh et al., 2014). Hypospadias is diagnosed at birth in humans (Yu et al., 2019) and can also be observed at birth in rodents, but as development of the penis continues after birth in rodents, hypospadias may be more optimally evaluated later in juvenile or adult male rats (Schlomer et al., 2013; Sinclair et al., 2017).

Known modulating factors

There are no known modulating factors for this KER

Known Feedforward/Feedback loops influencing this KER

Local disruption of AR activation in the genital tubercle irreversibly disrupts development, so there are no known feedback/feedforward loops for this KER.

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Relationship: 3350: Decrease, circulating testosterone levels leads to Hypospadias

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	non-adjacent	Low	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

Sexual differentiation of the penis is an androgen-driven process in mammals, and it is therefore biologically plausible that this KER is applicable to all mammals (Murashima et al., 2015). The empirical evidence in this KER provides support that hypospadias in humans is associated with reduced circulating testosterone levels in early life. The two *in vivo* studies included in the empirical evidence support the applicability to rats.

Sex applicability

The empirical evidence in this KER supports that reduced circulating testosterone is linked to hypospadias in males. Females do have circulating testosterone, but in much lower concentrations than males (Vesper et al., 2015). Moreover, the term hypospadias is mainly used for malformation of the male external genitalia.

Life stage applicability

The genital tubercle is programmed by the surge in androgen hormones during the masculinization programming window is (GD16-20 in rats and GW8-14 in humans) (Sharpe, 2020; Welsh et al., 2014). In humans, the penis is fully formed at birth (Yu et al., 2019), while penis development continues postnatally around 20-25 days in rats and mice (Schlomer et al., 2013; Sinclair et al., 2017).

Key Event Relationship Description

This non-adjacent KER describes a decrease in circulating testosterone (often measured in serum or plasma) during the fetal male masculinization programming window leading to hypospadias in male offspring.

In male mammals, testosterone along with its more potent derivative dihydrotestosterone (DHT) drives male reproductive differentiation. Produced by the fetal testes, testosterone is transported through blood to the peripheral reproductive tissues to bind the androgen receptor (AR) or be converted to DHT (Murashima et al., 2015). Activation of AR in the genital tubercle directs its differentiation to a penis, and failure of this differentiation can lead to malformations, including hypospadias where the urethra terminates on the underside of the penis. The androgen programming of the genital tubercle is largely (but not fully) constrained to the masculinization programming window (gestational days (GD) 16-20 in rats, presumably gestational weeks (GW) 8-14 in humans), when circulating testosterone levels are high (Sharpe, 2020; Welsh et al., 2014).

A decrease in circulating testosterone levels in the masculinization programming window can thus disrupt penis differentiation and cause hypospadias.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is judged to be **high** given the canonical biological knowledge on normal reproductive development.

Sexual differentiation in males, the external genitalia, is initiated and programmed in fetal life. Once the testes have formed, they synthesize testosterone through the steroidogenesis pathway and secrete it into circulation. Testosterone is transported in the blood either as free testosterone or bound to albumin or sex-hormone binding globulin. Testosterone is produced from around GD15 in fetal rats and GW8 in humans, which is also the onset of when testosterone levels can be measured in circulation. In peripheral tissues, testosterone can be converted to the more potent androgen DHT by the enzyme 5α-reductase. Both DHT and testosterone bind and activate the AR to program fetal tissues to differentiate along the male pathway (Murashima et al., 2015; Trost & Mulhall, 2016; Welsh et al., 2014).

The genital tubercle is the bipotential structure that upon hormonal cues differentiates to either penis or clitoris. Both human and rodent genital tubercles express AR (C. M. Amato & Yao, 2021; Baskin et al., 2020). Upon activation of AR, the genital tubercle differentiates to a penis by elongation and formation of a central urethra which terminates at the tip of the penis (C. Amato et al., 2022). The programming of the genital tubercle happens in the masculinization programming window (GD16-20 in rats, GW8-14 in humans) (Welsh et al., 2014), although elongation and growth of the penis is also programmed later, at least in rats (Welsh et al., 2008). Hypospadias is one of the most common genital malformations caused by disruptions to penis development (Baskin & Ebbers, 2006; Yu et al., 2019).

Given the dependency of testosterone for penis differentiation, either through direct AR activation or conversion to DHT, it is plausible that a decrease in circulating levels of testosterone will cause hypospadias.

Empirical Evidence

The empirical evidence from studies in animals for this KER is judged as **low** overall.

From the data collection, two toxicity studies were extracted which reported a decrease in circulating testosterone levels in fetal male rats and measured hypospadias in male offspring. However, both studies were classified as not reliable (category 3). Due to the lack of any reliable studies for this KER, the evidence from the two unreliable data sets is included, although they are considered weak evidence for the KER.

In this first study (Li et al., 2017), rats were exposed to 0 or 750 mg/kg bw/day dibutylphthalate (DBP) from GD13-18. At GD19, serum testosterone and hypospadias were evaluated. At this stage, 43.6% of males exposed to DBP had hypospadias upon examination. In the hypospadias group, serum testosterone levels were 3.93 nmol/mL compared to 15.74 nmol/mL in control male rats. In exposed rats without hypospadias, serum testosterone levels were 5.89 nmol/mL. This study was categorized as unreliable, mainly due to the evaluation of hypospadias being on GD19, a timepoint at which the penis is not finished differentiating. This poses an uncertainty, in particular regarding the frequency of hypospadias reported in the exposure group.

The second study (Vo et al., 2009) was considered unreliable due to a too low reliability score (64.7) with some key information not reported. In this study, no major deficiencies were noted. Rats were exposed to 0, 10, 100, or 500 mg/kg bw/day diethylhexylphthalate (DEHP) from GD11-21. At GD21, plasma testosterone levels were reduced in males in the highest exposure group (0.53 ±0.16 ng/mL) compared to control males (1.56 ±0.84 ng/mL), but not in the other dose groups.

Hypospadias was evaluated in male offspring at postnatalday 63, and hypospadias was registered in 23/100 male pups exposed *in utero* to 500 mg/kg bw/day. There was no hypospadias in the other groups.

Supporting human case studies

Supporting this KER are case studies of humans with hypospadias and a reduction in circulating testosterone levels.

Studies extracted from the literature search are summarized in Table 2.

Table 2: Human case studies of hypospadias with reductions in circulating testosterone levels (measured postnatally).

Case	**Effect on upstream event** **(circulating testosterone)**	**Effect on downstream event hypospadias**	**Reference**
Subject with mutation in *HSD17B3*	Six weeks: 2.5 nmol/L (reference range 0-6.5 nmol/L) 10 years: 3.6 nmol/L (reference range: 9.0-31.0 nmol/L)	Ambiguous genitalia / hypospadias	(Al-Sinani et al., 2015)
7 subjects (0-2 years) with perineal or penoscrotal hypospadias	Testosterone levels < 2 ng/mL after hCG stimulation. In 2 subjects, testosterone response returned to normal	Perineal or penoscrotal hypospadias	(Allen & Griffin, 1984)
18-year old subject	Basal testosterone levels 0.8 ng/mL (normal range 2.8-18.5 ng/mL). hCG stimulated testosterone levels: 0.9 ng/mL	Perineal hypospadias	(Ammini et al., 1997)
2 subjects (15 and 12 years old) with *INHA* mutations	Testosterone levels: 0.68 and 1.48 ng/mL (normal range 2.4-9.5 ng/mL)	Hypospadias	(Arslan Ates et al., 2022)
8 subjects	hCG-stimulated testosterone levels in childhood < 3 ng/mL	Proximal hypospadias	(Blanc et al., 2011)
Subject with unilateral vanishing testes syndrome	Insufficient testosterone syndrome after hCG stimulation	Severe hypospadias	(Boehmer et al., 2001)
Newborn subject	Testosterone levels: 18 ng/dL (normal range 60-570 ng/dL)	Perineal hypospadias	(Dean et al., 1984)
13 subjects (1/2-10 years)	Low basal testosterone (0-0.34 ng/mL) and poor or absent response to hCG stimulation	Penoscrotal, scrotal or perineal hypospadias	(Iyengar et al., 1986)
5 year old subject	Testosterone response to hCG stimulation: 12-17 ng/dL (normal values 667 ng/dL)	Perineal hypospadias	(Kaufman et al., 1983)
Three subjects (0-4 years) with *NR5A1* mutations	Low testosterone levels (basal 0.05-0.4 ng/mL; hCG-stimulated 0.58-0.9 ng/mL)	Penoscrotal hypospadias	(Köhler et al., 2009)

Subject with mutation in the gene encoding LH receptor	No response to hCG stimulation (0.05 ng/mL)	Severe hypospadias	(Misrahi et al., 1997)
2 subjects evaluated at puberty	Decreased testosterone	One mild and one severe hypospadias	(Moriya et al., 2010)
10-year old subject with *HSD17B3* mutation	Baseline testosterone: 59.2 ng/dL, hCG stimulated testosterone: 139.9 ng/dL (no reference values given)	Hypospadias	(Neocleous et al., 2012)
24-year old subject	Testosterone levels 0.3-0.4 µg/100 mL (no reference values given)	Severe hypospadias	(New, 1970)
6-year old subject	Low testosterone response to hCG stimulation	Perineal hypospadias	(Pang et al., 1983)
Infant subject with *CYP11A1* mutation	Low testosterone response to hCG stimulation (0.7 nmol/L), but normal serum testosterone	Penoscrotal hypospadias	(Parajes et al., 2012)
Infant subject with *LHGCR* mutation	At birth: 0.24 ng/mL 10 weeks: 0.06 ng/mL 2 years: 0.02 ng/mL (normal range 0.03-0.52 ng/mL). No response in testosterone levels after hCG stimulation	Perineal hypospadias	(Richard et al., 2011)
Subject with *WT1* mutation	Low testosterone levels: 1.02 ng/mL (no reference values given)	Glandular hypospadias	(Schumacher et al., 2008)
Subject with *NR5A1* mutation	12 hours old: 0.9 nmol/L (low) 2 days old: <0.1 nmol/L (normal) In later years, slight increase at 9 years, then normal or decreased levels during teen years	Perineal hypospadias	(Teoli et al., 2023)
Newborn with *GPC3* mutation (Simpson-Golabi-Behmel syndrome)	1 day old: 42 ng/dL (reference range 75-400 ng/dL) 20 days old: 55 ng/dL (reference range: 60-400 ng/dL) 26 days old after hCG stimulation: 130 ng/dL (reference range: 60-400 ng/dL)	Midshaft hypospadias	(Villarreal et al., 2013)
Subject with *MAMLD1* mutation	2 months: 49 ng/dL (reference level: 196 ng/dL)	Hypospadias	(Yeste et al., 2022)

Dose concordance

Dose concordance cannot be informed from the two *in vivo* studies, although the study (Vo et al., 2009) does not argue against dose concordance as the upstream and downstream events were measured at the same dose of DEHP.

Temporal concordance

The *in vivo* studies do not directly inform temporal concordance, but in (Vo et al., 2009) plasma testosterone levels were decreased at GD21, while hypospadias was diagnosed in adult rats, long after exposure was ceased. In (Li et al., 2017), hypospadias and testosterone levels were assessed/measured at the same time point (GD19), however this is known not to be an optimal time point for hypospadias diagnosis in rats as the penis is not finished developing.

Incidence concordance

The study with DBP supports incidence concordance, because the non-hypospadiac rats exposed to DBP also had lower serum testosterone levels compared to control rats at GD19 (Li et al., 2017). This is however only weak evidence, as hypospadias was not evaluated at an optimal time point.

Uncertainties and Inconsistencies

The uncertainties of the two *in vivo* studies have been discussed. Both were classified as unreliable in the evaluation of methodological reliability. Of the two studies, the study by (Li et al., 2017) is considered most uncertain due to the timepoint of hypospadias assessment. A study with *in utero* exposure to an 5α-reductase inhibitor disrupted genital tubercle development at GD19, but hypospadias was not observed at postnatal day 90, indicating that assessment prior to birth may not be true indications of postnatal outcomes (Iguchi et al., 1991). The deficiencies in (Vo et al., 2009) are less severe but overall poses an uncertainty to the study due to missing key information about study design.

The uncertainties in the human evidence mainly pertains to the fact that testosterone levels were not measured during fetal life but in newborn or juvenile males. Given that most cases involve genetic mutations, which occur early in embryonic life, it is highly likely that the effects of mutations on testosterone levels manifest early in fetal life. Another uncertainty with the human cases which include mutations is that it cannot be excluded that the hypospadias phenotype is caused by the low testosterone levels and not directly by genetic mutation.

Another uncertainty for this KER from the literature is the observation that humans with 5α-reductase deficiency have hypospadias due to low DHT levels despite normal or higher testosterone levels (Mendonca et al., 1996). This indicates that the effects of low testosterone may be more through reduced conversion to DHT than due to a direct loss of testosterone action on AR. To this, there is also the existence of a “backdoor pathway” to DHT in humans. This pathway in peripheral tissues (i.e. not testes) can circumvent testosterone as a precursor for DHT by synthesis of DHT is from reduction of androsterone by 17β-HSD (Miller & Auchus, 2019). This would create the possibility that testosterone is not required for DHT production and ultimately AR activation.

Quantitative Understanding of the Linkage

The quantitative understanding of this KER is low.

Time-scale

The time-scale for this KER depends on species but is likely weeks. Testosterone is secreted from around GW8 in humans (GD16 in rats), marking the beginning of the masculinization programming window and programming of the genital tubercle. Hypospadias is diagnosed at birth in humans (Yu et al., 2019) and can also be observed at birth in rodents, but as development of the penis continues after birth in rodents, hypospadias may be more optimally evaluated later in juvenile or adult male rats (Schlomer et al., 2013; Sinclair et al., 2017).

Known modulating factors

There are no known modulating factors for this KER.

Known Feedforward/Feedback loops influencing this KER

Local disruption of AR activation in the genital tubercle irreversibly disrupts development, so there are no known feedback/feedforward loops for this KER.

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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	High	
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	non-adjacent	High	

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 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 AR 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 (GD 16-20 in rats, around GW 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 masculinization programming window 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) window 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 gestational days (GD) 16-20, 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, which at this point expresses AR in both humans and rodents, differentiates to a penis (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 as **high.** This includes evidence from *in vivo* animal studies and supporting evidence from studies in humans. The upstream KE 'Decreased AR activation' 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 by 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 the number of 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

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

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.

Incidence Concordance

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

Uncertainties and Inconsistencies

The *in vivo* studies do not directly inform about the upstream KE, 'decrease, AR activation'. 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 activitation 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 activation) 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 activation. 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

Local disruption of AR activation in the genital tubercle irreversibly disrupts development, so there are no known feedforward/feedback loops.

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