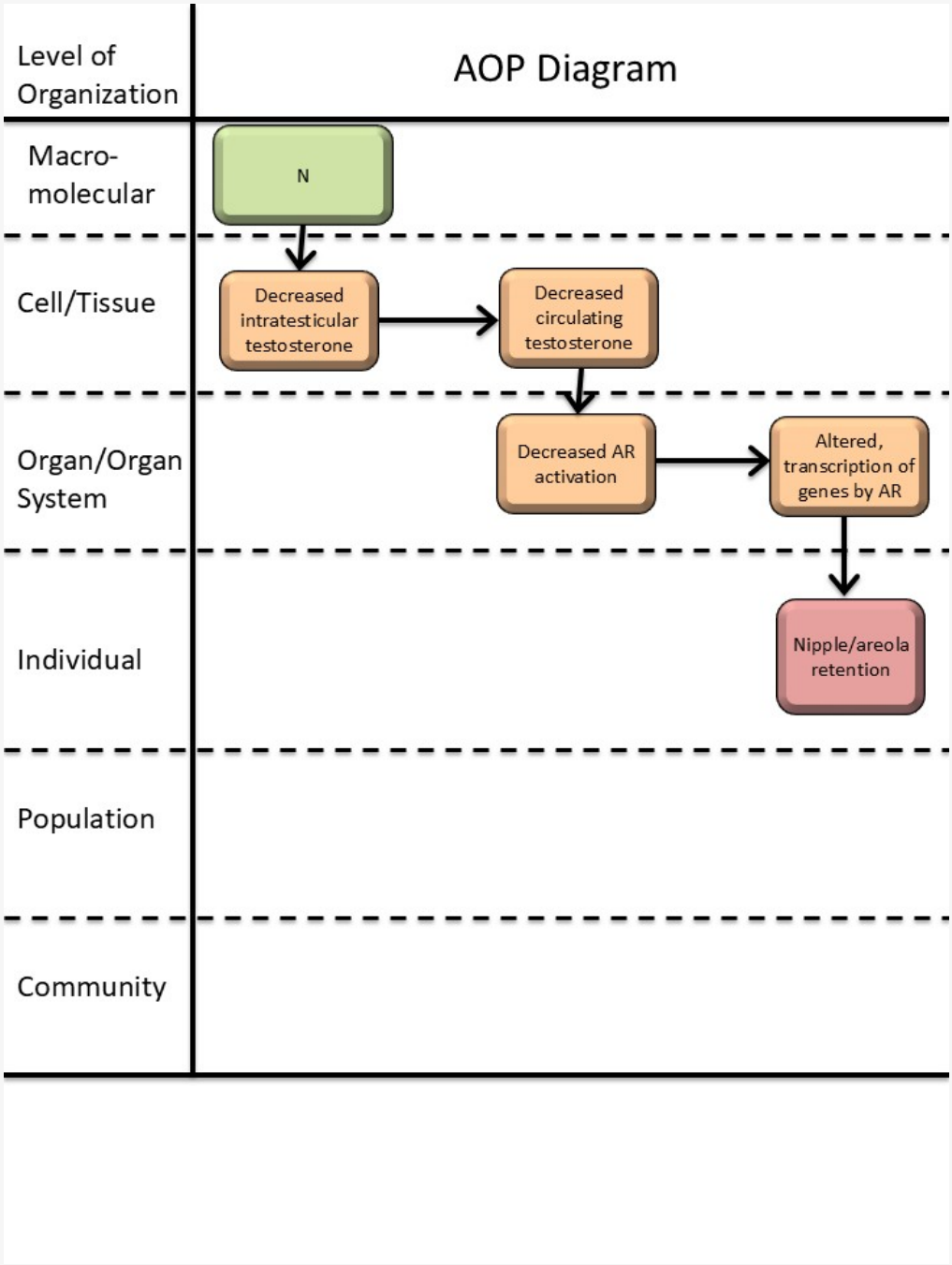
AOP ID and Title:

AOP 575: Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring
Short Title: Decreased testosterone synthesis leading to nipple retention

Graphical Representation



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Status

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Abstract

This AOP links decreased intratesticular testosterone levels during fetal development with nipple/areola retention (NR) in male rodent offspring. NR, measured around 2 weeks postpartum, is a marker for disrupted masculinization of male offspring, with data primarily from laboratory mice and rats.

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). AR is a nuclear receptor involved in the transcriptional regulation of various target genes during development and adulthood across species. AR signalling is necessary for normal masculinization of the developing fetus, and AR action in male rodents signals the nipple anlagen to regress, leaving males with no nipples.

This AOP delineates the evidence that decreasing testicular testosterone production lowers circulating testosterone levels and consequently AR activation, thereby causing retention of nipples in male rodents. In this AOP, the first KE is not considered an MIE, as testicular testosterone production can be obstructed by various mechanisms (Miller & Auchus, 2011). Moreover, the AOP does not discriminate whether the reduction in AR activation is due to a direct lack of testosterone binding AR or due to decreased conversion of testosterone to DHT, as there is not sufficient information on this distinction. Downstream of a reduction in AR activation, the molecular mechanisms of NR are unclear, highlighting a knowledge gap in this AOP and potential for further development.

The confidence in KER-3486 (‘Decrease, circulating testosterone levels’ leads to ‘Increase, nipple retention’) is moderate due to the limited empirical evidence available. The confidence in each of the remaining KERs comprising the AOP is judged as high, with both high biological plausibility and high confidence in empirical evidence. The mechanistic link between KE-286 (‘altered, transcription of genes by AR’) and AO-1786 (‘increase, nipple retention’) is not established, but given the high confidence in the KERs, the overall confidence in the AOP is judged as high.

The AOP supports the regulatory application of NR as a measure of endocrine disruption relevant for human health and the use of NR as an indicator of anti-androgenicity in environmentally relevant species. Even though NR cannot be directly translated to a human endpoint, the AOP is considered human relevant since NR is a clear readout of reduced androgen action and fetal masculinization during development and is considered an ‘adverse outcome’ in OECD test guidelines (TG 443, TG 421, TG 422). The AOP also holds utility for informing on anti-androgenicity more generally, as this modality is highly relevant across mammalian species.

AOP Development Strategy

Context

This AOP is a part of an AOP network for reduced AR activation leading to increased NR in male offspring. The other AOPs in this network are AOP-344 (‘Androgen receptor antagonism leading to increased nipple retention (NR) in male (rodent) offspring’) and AOP 576 (‘5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring’). The purpose of the AOP network is to organize the well-established evidence for anti-androgenic mechanisms-of-action leading to increased NR. It can be used in the identification and assessment of endocrine disruptors and to inform predictive toxicology, identification of knowledge gaps for investigation and method development.

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Strategy

The OECD AOP Developer’s Handbook v2.7was followed alongside pragmatic approaches (Svingen et al., 2021).

The adverse outcome, AO 1786: Increase nipple retention, was originally developed in (Pedersen et al., 2022).

Some upstream KEs and KERs were developed in Draskau et al., 2024 as part of an anti-androgenic network, using mainly key review publications, since it was considered canonical knowledge. This included KE 1690: Decrease, circulating testosterone levels; KE 1614: Decrease, AR activation and KE 286: Altered transcription of genes by the AR as well as the connecting KERs, 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 (Draskau et al., 2024).

KE 2298 (Decrease, intratesticular testosterone levels) and KER 3448, connecting this to KE 1690 (Decrease, intratesticular testosterone levels leads to decrease, circulation testosterone levels) were added as part of an AOP network, ultimately leading to the AO decreased anogenital distance (Svingen et al., 2025). This was done to discriminate between the large

difference in testosterone levels between the testes and in circulation (Coviello et al., 2004). As for other upstream KEs and KERs, this was considered canonical knowledge, and the KE and KER were developed using mainly key review publications (Draskau et al., 2024).

The non-adjacent KERs 3487, 3486, and 3348 linking reduced decreased intratesticular testosterone, circulating testosterone, and AR activation with increased NR, respectively, were developed using a systematic weight-of-evidence approach, following the methodology outlined in (Holmer et al., 2024). Publications 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 KERs 3487 and 3486 regarding testosterone levels, publications were included if there was a decrease in fetal testosterone levels and NR was assessed in male offspring. For KER 3348, 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.

The rationale for the inclusion of KEs and KERs in the upstream anti-androgenic network is detailed in (Draskau et al., 2024). The link between the upstream network, more specifically KE-286 ('altered, transcription of genes by AR'), and AO-1786 ('increase, nipple retention') 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 between the upstream anti-androgenic network and increased nipple retention is captured by KERs 3348, 3486 and 3487.

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	1786	Nipple retention (NR), increased	nipple retention, increased

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	Nipple retention (NR), increased	High	
Decrease, circulating testosterone levels	non-adjacent	Nipple retention (NR), increased	Moderate	
Decrease, androgen receptor activation	non-adjacent	Nipple retention (NR), increased	High	

Stressors

Name	Evidence
Dibutyl phthalate	

Name	Evidence
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
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	Low	NCBI

Sex Applicability

Sex	Evidence
Male	High

The upstream part of the AOP has a broad applicability domain, but the downstream KERs 3487 (‘Decrease, intratesticular testosterone levels leads to increase, nipple retention’), 3486 (‘Decrease, circulating testosterone levels leads to increase, nipple retention’), and 3348 (‘Decrease AR activation leads to increase, nipple retention’) are considered only directly applicable to male rodents (current evidence stems primarily from laboratory rats and mice) during fetal life, restricting the taxonomic applicability of the AOP. Although NR has primarily been investigated in rats and mice, it is biologically plausible that the AOP is applicable to other rodent species. The process of retention of nipples by disruption of androgen programming happens in the fetal life stage, but the AO is detected postnatally. Specifically, the MPW (~gestational days (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. In the males of these species, the nipple anlagen are programmed during fetal development by androgens to regress, leading to no visible nipples in males postnatally, while female rats and mice exhibit nipples. This AOP only contains empirical evidence for the applicability to male rats, but the AOP is considered equally applicable to male mice as these also normally exhibit nipple regression stimulated by androgens. Moreover, the AOP is relevant for other taxa, including humans, as NR in male rodents indicates a reduction in fetal masculinization. NR is therefore included as a mandatory endpoint in multiple OECD Test Guideline studies for developmental and reproductive toxicity and is considered applicable as an adverse outcome to set NOAELs and LOAELs of substances in human health risk assessments.

Essentiality of the Key Events

Event	Evidence	Uncertainties and inconsistencies

KE-2298 Decreased, intratesticular testosterone (ITT) levels **MODERATE:** Testis is the primary organ in males for testosterone synthesis and is required for serum testosterone. Studies with exposure to phthalates show reduced ITT levels and increased nipple retention.	**Biological plausibility provides strong support for the essentiality of this event, as the testes are the primary testosterone producing organs in male mammals and testosterone is a ligand of the AR and a main driver for normal regression of nipple anlagen in male offspring (Goldman et al., 1976).** **Indirect evidence of impact of decreased ITT (KE-2298) on decreased circulating T (KE-1690)** • Castrated males have significantly reduced serum T. Although at different life stage, it is highly likely same relationship exists in fetal males, with loss of testosterone from testis resulting in loss of circulating testosterone. **Indirect evidence of impact of decreased ITT (KE-2298) on increased nipple retention (AO-1786):** • Numerous rat studies evidence a relationship between reduced intratesticular testosterone levels caused by exposure to phthalates and increased nipple retention in male offspring (see empirical evidence table in KER-3487).	

KE-1690 Decreased, circulating testosterone (CT) levels **MODERATE:** CT is substrate for DHT production, also locally, and numerous studies have shown strong relationships between reduced CT and increased nipple retention.	**Biological plausibility provides strong support for the essentiality of this event. Testosterone is an AR ligand and a main driver for regression of nipple anlagen in male offspring (Goldman et al., 1976), as well as a substrate for local production of DHT (Imperato-McGinley J et al., 1986).** **Indirect evidence of the impact of decreased CT (KE-1690) on AR activity in vitro:** • Increasing concentrations of testosterone lead to increasing AR activation in vitro (U. S. EPA, 2018) (see also KER-2131). **Indirect evidence of impact of decreased CT (KE-1690) on increased nipple retention (AO-1786):** • Exposure to the phthalates DEHP and DBP during prenatal development in rats results in reduced fetal testosterone levels and increased nipple retention in male offspring. Literature review on the relationship has judged the link to be strongly evidenced (See empirical evidence in KER-3348). **Indirect evidence of the impact of decreased CT (KE-1690) on increased nipple retention (AO-1786):** • Nipple formation is inhibited in female rat fetuses exposed to testosterone during gestation (Goldman et al., 1976).	**Inconsistencies in indirect evidence of impact on the AO:** • Some inconsistencies were observed in the empirical evidence regarding increased nipple retention in male pups after in utero exposure to DEHP. However, all inconsistencies could be explained by differences in exposure doses and statistical power (See empirical evidence in KER-3348)

KE-1614 Decreased, AR activation **HIGH:** There is experimental evidence from mutant mice insensitive to androgens showing that the AR is essential for nipple retention in male offspring. There is also evidence from exposure studies in animals that substances antagonizing AR induce nipple retention in male pups.	**Biological plausibility provides strong support for the essentiality of this event, as AR activation is critical for normal regression of nipple anlagen in male embryos.** **Indirect evidence of the impact of decreased AR activation (KE-1614) on altered gene transcription by AR (KE-286):** • Exposure to known anti-androgenic chemicals induces a changed gene expression pattern, e.g. in neonatal pig ovaries (Knapczyk-Stwora et al., 2019). **Direct evidence of the impact of decreased AR activation (KE-1614) on altered gene transcription by AR (KE-286):** • Male AR KO mice have altered gene expression pattern in a broad range of organs (refer to KER-2124). **Indirect evidence of impact of decreased AR activation (KE-1614) on increased nipple retention (AO-1786):** • Rat in vivo exposure to vinclozolin, procymidone and flutamide, which are known AR antagonists, leads to increased nipple retention in offspring (see KER-3348). **Direct evidence of impact of decreased AR activation (KE-1614) on increased nipple retention (AO-1786):** • Male *Tfm* mutant mice, which are insensitive to androgens and believed to be so due to a nonfunctional androgen receptor, present with retained nipples (Kratochwil & Schwartz, 1976)	
KE-286 Altered, trans. of genes by AR **LOW:** Strongest support for essentiality comes from biological plausibility. However, exact transcriptional effects and causality remain to be fully characterized.	**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 regression of nipple anlagen in male fetuses.**	There are currently no AR-responsive genes proven to be causally involved in nipple retention, and it is known that AR can also signal through non-genomic actions (Leung & Sadar, 2017).

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
KE-2298		***		Moderate
KE-1690		***		Moderate
KE-1614	***	***		High
KE-286				Low (biological plausibility)

*Low level of evidence (some support for essentiality), ** Intermediate level of evidence (evidence for impact on one or more downstream KEs), ***High level of evidence (evidence for impact on AO).

Weight of Evidence Summary

Confidence in KER-3486 is considered moderate due to the limited empirical evidence available. The confidence in each of the remaining KERs comprising the AOP is judged as high, with both high biological plausibility and high confidence in empirical evidence. The mechanistic link between KE-286 (‘altered, transcription of genes by AR’) and AO-1786 (‘increase, nipple retention’) is not established, but given the high confidence in the KERs, the overall confidence in the AOP is judged as **high**.

KER	Biological Plausibility	Empirical Evidence	Rationale
KER-3448 Decrease, intratesticular testosterone levels leads to a 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 lowers 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-3487 Decrease, intratesticular testosterone leads to an increase, nipple retention	High	High	It is well established that testicular testosterone is one of the primary androgens responsible for the regression of nipple anlagen in male rodent fetuses *In vivo* animal studies support that reductions in fetal testicular testosterone can cause NR in male offspring. Temporal concordance is generally supported, while dose concordance is more weakly suggested.

KER-3486 Decrease, circulating testosterone levels leads to increase, nipple retention	High	Moderate	It is well established that testosterone is one of the primary androgens responsible for the regression of nipple anlagen in male rodent fetuses Two *in vivo* rat toxicity studies support the relationship and temporal concordance of the KER. Dose concordance is not informed.
KER-3348 Decrease, AR activation leads to increase, nipple retention	High	High	It is well established that activation of AR regression of nipple anlagen in males. The empirical evidence includes numerous *in vivo* toxicity studies showing that decreased AR activation leads to increased NR in male offspring, with few inconsistencies. Empirical evidence combined with theoretical considerations provide some support for dose, temporal, and incidence concordance for the KER, although this evidence is weak and indirect. 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 degree of NR related to a phthalate's reduction in *ex vivo* testosterone production. The model predicted that a 40% reduction in testosterone levels would induce NR in male rats, with increasing number of nipples as testosterone levels decrease (Gray et al., 2024).

Considerations for Potential Applications of the AOP (optional)

The AOP supports the regulatory application of NR as a measure of endocrine disruption relevant for human health and the use of NR as an indicator of anti-androgenicity in environmentally relevant species.
NR is a mandatory endpoint in multiple OECD test guidelines, including TG 443 (extended one-generation reproductive toxicity study) and TGs 421/422 (reproductive toxicity screening studies) (OECD 2025a; OECD 2025b; OECD 2025c). NR can contribute to establishing a No Observed Adverse Effect Level (NOAEL), as outlined in OECD guidance documents No. 43 and 151 (OECD 2008; OECD 2013). The ability to derive a NOAEL for increased NR in male rodent offspring, which can serve as a point of departure for determining human safety thresholds, underscores the regulatory significance of this AOP.
The AOP also holds utility for informing on anti-androgenicity more generally, as this modality is highly relevant across mammalian species (Schwartz et al., 2021).

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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
Male	High

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
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: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 ID and Name	Event Type
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: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 ID and Name	Event Type
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: 1786: Nipple retention (NR), increased

Short Name: nipple retention, increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	AdverseOutcome
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	AdverseOutcome
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rats	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	High

Sex Applicability

Sex	Evidence
Male	High

The applicability domain of NR is limited to male laboratory strains of rats and mice from birth to juvenile age.

Key Event Description

In common laboratory strains of rats and mice, females typically have 6 (rats) or 5 (mice) pairs of nipples along the bilateral milk lines. In contrast, male rats and mice do not have nipples. This is unlike e.g., humans where both sexes have 2 nipples (Schwartz et al., 2021).

In laboratory rats, high levels of dihydrotestosterone (DHT) induce regression of the nipples in males (Imperato-McGinley & Gautier, 1986; Kratochwil, 1977; Kratochwil & Schwartz, 1976). Females, in the absence of this DHT surge, retain their nipples. This relationship has also been shown in numerous rat studies with perinatal exposure to anti-androgenic chemicals (Schwartz et al., 2021). Hence, if juvenile male rats and mice possess nipples, it is considered a sign of perturbed androgen action early in life.

This KE was first published by Pedersen et al (2022).

How it is Measured or Detected

Nipple retention (NR) is visually assessed, ideally on postnatal day (PND) 12/13(OECD, 2018; Schwartz et al., 2021). However, PND 14 is also an accepted stage of examination (OECD, 2013). Depending on animal strain, the time when nipples become visible can vary, but the assessment of NR in males should be conducted when nipples are visible in their female littermates (OECD, 2013).

Nipples are detected as dark spots (or shadows) called areolae, which resemble precursors to a nipple rather than a fully developed nipple. The dark area may or may not display a nipple bud (Hass et al., 2007). Areolae typically emerge along the milk lines of the male pups corresponding to where female pups display nipples. Fur growth may challenge detection of areolae after PND 14/15. Therefore, the NR assessment should be conducted prior to excessive fur growth. Ideally, all pups in a study are assessed on the same postnatal day to minimize variation due to maturation level (OECD, 2013).

NR is occasionally observed in controls. Hence, accurate assessment of NR in controls is needed to detect substance-induced effects on masculine development (Schwartz et al., 2021). It is recommended by the OECD guidance documents 43 and 151 to record NR as a quantitative number rather than a qualitative measure (present/absent or yes/no response). This allows for more nuanced analysis of results, e.g., high control values may be recognized (OECD, 2013, 2018). Studies reporting quantitative measures of NR are therefore considered stronger in terms of weight of evidence.

A major challenge with using NR as a biomarker is the subjectivity of the measurement. In juvenile rat pups, nipples are only present as areolae, i.e., dark shadows with or without a nipple bud. This means that the experience of the personnel assessing the presence and number of areolae/nipples can influence the results. Furthermore, the results are likely prone to larger variation if several assessors are used to record NR within the same study. To minimise these sources of uncertainty, assessors must be trained to recognise areolae and not look for fully developed nipples. Moreover, the number of assessors should be limited to one or two, and they should always be blinded to exposure groups

Regulatory Significance of the AO

NR is recognized by the OECD as a relevant measure for anti-androgenic effects and is mandatory in the test guidelines Extended One Generation Reproductive Toxicity Study, TG 443 (OECD, 2018) and the two screening studies for reproductive toxicity, TGs 421/422 (OECD, 2016a, 2016b). The endpoint is also described in the guidance documents 43 (OECD, 2008) and 151 (OECD, 2013). Furthermore, NR data can be used in chemical risk assessment for setting the No Observed Adverse Effect Level (NOAEL) as stated in the OECD guidance document 151 (OECD, 2013): "*A statistically significant change in nipple retention should be evaluated similarly to an effect on AGD as both endpoints indicate an adverse effect of exposure and should be considered in setting a NOAEL*".

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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: 3487: Decrease, intratesticular testosterone leads to nipple retention, increased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	non-adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

NR is observed in male mice and rats. Male rodents (mostly investigated in laboratory rats and mice) do not have nipples, a feature that is androgen-dependent in these species with fetal androgen action impeding development of nipple anlagen. The empirical evidence supports the applicability to rats, and the KER is considered equally applicable to mice based on the biological knowledge of nipple development in this species. The KER is not directly applicable to humans, as both males and females have two nipples, and there is no known effect of androgens on their development (Schwartz et al., 2021). However, NR is a clear readout of reduced androgen action and fetal masculinization during development, which is relevant to humans and mammals in general (Schwartz et al., 2021). It is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD, 2025a, 2025b, 2025c) and considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013).

Sex applicability

This KER is only applicable to males, as the testes are male sex organs. Moreover, females usually have the maximum number of nipples (12 in rats, 10 in mice) (Schwartz et al., 2021)

Life stage applicability

The testes start producing testosterone in fetal life, around gestational day (GD) 15 in rats. Programming by androgens of the peripheral reproductive tissues, including the nipple anlagen, mainly occurs within the masculinization programming window, GD16-20 in rats. Morphological development of the mammary glands also starts in fetal life in both sexes, and upon programming by androgens, the mammary glands of males regress, causing a blockade of nipple formation (Kratochwil, 1986; Watson & Khaled, 2008). Nipples in females and retained nipples in males can first be observed postnatally, ideally at postnatal day (PND) 12-14 in rats (Schwartz et al., 2021).

Key Event Relationship Description

This non-adjacent KER describes a fetal decrease in intratesticular testosterone leading to NR in male offspring. In this KER, intratesticular testosterone includes measurements of testosterone in homogenates of testes after *in vivo* exposure to chemicals, as well as measurements of testosterone production in testes *ex vivo* from exposed animals.

In male mammals, the testes are the first sex organs to develop. Once formed, they produce testosterone by steroidogenesis. The adrenal glands have been shown to synthesize testosterone, but on a much smaller scale, and the testes are the main site of testosterone production (Naamneh Elzenaty et al., 2022). Testicular testosterone is secreted into the blood to initiate masculinization of the peripheral reproductive tissues. In rats and mice, this includes effects on the developing mammary glands, which develop sexually dimorphic. Testosterone either directly activates the AR in the mammary glands or is converted to the more potent androgen dihydrotestosterone (DHT) (Murashima et al., 2015). Activation of AR by androgens in the mammary glands causes apoptosis of epithelial cells and thus separation of the glands from the overlying epidermis. Consequently, no nipples are formed (Kratochwil, 1986). During low androgen levels, such as in female rodents, nipple development progresses to form up to 10 (mice) and 12 (rats) nipples.

As suppression of nipple development in male rats and mice is dependent on androgens, marked reductions in testicular testosterone production can thus cause nipple retention.

The KER is not directly applicable to humans, as both males and females have two nipples, and there is no known effect of androgens on their development (Schwartz et al., 2021). However, NR is a clear readout of reduced androgen action and fetal masculinization during development, which is relevant to humans (Schwartz et al., 2021). It is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD, 2025a, 2025b, 2025c) and considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013).

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 in rodents.

In common strains of rats and mice, females have 12 and 10 pairs of nipples, respectively, while males usually do not have nipples, although in rare instances control male rats may display a retained nipple (Schwartz et al., 2021). The sexual dimorphism of the mammary tissue is regulated by the androgens testosterone and DHT, during fetal life. Testosterone is produced by the fetal testes by the steroidogenesis pathway, starting from ~GD15. The testes are the primary male sex organs and produce most of the circulating testosterone, although a minor part may be contributed by other organs such as the adrenals (Naamneh Elzenaty et al., 2022). DHT is produced from testosterone in peripheral tissues by the enzyme 5α-reductase. Both testosterone and DHT activate AR in reproductive tissues to initiate masculinisation. The programming of the tissues mainly happens within the masculinization programming window (GD16-20 in rats) (Murashima et al., 2015; Welsh et al., 2014).

The mammary glands start out the same in both sexes, developing along two milk lines in early fetal life. In males, androgens activate AR in the mammary gland mesenchymal cells, and in turn, the cells activate apoptosis of epithelial cells that otherwise would contribute to the development of nipples (Kratochwil, 1986; Schwartz et al., 2021).

Given the dependency of testosterone for regression of the nipples, either through direct AR activation or conversion to DHT, it is highly plausible that a decrease in intratesticular testosterone levels will lead to nipple retention in males.

Empirical Evidence

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

From the data collection, 8 data sets were extracted. The data sets included different stressors causing reduced fetal intratesticular testosterone, all in rats (Table 2 and Appendix 2, 91ldxzzu4f_KER_3487_Appendix_2_clean.pdf). Of these 8 data sets, seven showed concurrent nipple retention.

Table 2 Empirical evidence for KER 3487 LOAEL: Lowest observed adverse effect level; NOAEL: No observed adverse effect level. See Appendix 2, for specifications.

Species	Stressors(s)	Effect on upstream event (circulating testosterone)	Effect on downstream event (NR)	Reference
Rat	Butyl benzyl phthalate	LOAEL 500 mg/kg bw/day	No effect NOAEL 500 mg/kg bw/day	(Hotchkiss et al., 2004)
Rat	Dibutyl phthalate	LOAEL 500 mg/kg bw/day	LOAEL 500 mg/kg bw/day	(Martino-Andrade et al., 2009)
Rat	Diethylhexyl phthalate	LOAEL 750 mg/kg bw/day	LOAEL 750 mg/kg bw/day	(Borch et al., 2004)
Rat	Diisonyl phthalate	LOAEL 600 mg/kg bw/day	LOAEL 750 mg/kg bw/day	(Boberg et al., 2011)
Rat	Linuron	LOAEL 75 mg/kg bw/day	LOAEL 75 mg/kg bw/day	(Hotchkiss et al., 2004)
Rat	Prochloraz	LOAEL 50 mg/kg bw/day	LOAEL 50 mg/kg bw/day	(Laier et al., 2006)
Rat	Prochloraz	LOAEL 30 mg/kg bw/day	LOAEL 30 mg/kg bw/day	(Vinggaard et al., 2005)
Rat	Tebuconazole	LOAEL 100 mg/kg bw/day	LOAEL 50 mg/kg bw/day	(Taxvig et al., 2007)

Dose concordance

The one study that did not find NR after fetal reduction in intratesticular testosterone levels only tested one dose of stressor, which therefore could be an indication of dose concordance (Hotchkiss et al., 2004).

Four datasets tested multiple doses of stressors. In two datasets, the LOAEL was the same for intratesticular testosterone and NR (Laier et al., 2006; Martino-Andrade et al., 2009). In the study by Martino-Andrade et al. (2009), rats were exposed in utero to DPB during gestation days (GD) 13 to 21. Fetal testicular testosterone levels were assessed at GD21, while NR was evaluated at postnatal day (PND) 13. At a dose of 500 mg/kg/day, both a reduction in fetal testicular testosterone and NR were observed. In contrast, at the lower dose of 100 mg/kg/day, only a slight, non-significant decrease in testosterone levels was reported, with no effect on NR. Additionally, the same publication noted a slight but non-significant reduction in fetal testicular testosterone levels following exposure to 150 mg/kg/day of DEHP, without any observed effects on NR (Martino-Andrade et al., 2009).

Further, one study found a lower LOAEL for NR than intratesticular testosterone (Taxvig et al., 2007) and another reported only dose (600 mg/kg bw/day), but not higher doses) significant for reduced intratesticular testosterone levels (Boberg et al., 2011). In both cases, there was a tendency for lower testosterone levels at other doses as well, and the inconsistency may therefore be due to low sample size and/or high variance in the testosterone data.

Overall, the data could suggest dose concordance for this KER, although the evidence for this is not strong.

Temporal concordance

The empirical evidence supports temporal concordance between the events.

NR is generally first observable in postnatal animals, while the reductions in intratesticular testosterone are measured early in fetal life during exposure. This was demonstrated with prochloraz-induced NR, which was observed at PND13, when intratesticular testosterone levels were reduced (Vinggaard AM et al., 2005). NR was also observed postnatally in studies, where exposure to the stressor was only during fetal life (Boberg et al., 2011; Hotchkiss AK et al., 2004; Martino-Andrade AJ et al., 2009; Taxvig C et al., 2007)

Incidence concordance

The data does not inform incidence concordance.

Uncertainties and Inconsistencies

In several of the studies supporting this KER, intratesticular testosterone was measured in *ex vivo* testis cultures. This means that fetal testis from animals exposed *in utero* were cultured for ~3 hours, and the culture media were then collected for testosterone measurement. This creates uncertainty in the exact intratesticular testosterone values. However, in these studies, intratesticular testosterone levels were also measured with largely similar outcomes from the methods. The large translatability is clear from the measurements in (Borch et al., 2004).

As discussed above, the study with negative results for NR might be an indication of dose concordance, as only one stressor dose was tested (Hotchkiss AK et al., 2004). The uncertainty in the study on diisonyl phthalate (Boberg et al., 2011) has also briefly been discussed. Exposure to the phthalate only reduced intratesticular testosterone in the dose of 600 mg/kg bw/day, but not 750 or 900 mg/kg bw/day. For these two higher doses, testosterone also tended to be lower, and lack of statistical significance may be explained by a low sample size.

The empirical evidence for this KER includes stressors with more than one known mechanism of action. In particular, the pesticides prochloraz and linuron are known to also be AR antagonists (Andersen et al., 2002; Lambright et al., 2000), and for these studies, it can therefore not be excluded whether the observed effect on NR is due to the chemicals lowering intratesticular testosterone levels or due to direct antagonism of the AR or a mixture of effects.

Quantitative Understanding of the Linkage

The quantitative understanding of this KER is classified as **low**.

Response-response relationship

There are no direct models for reductions in intratesticular testosterone levels and NR. A model for the phthalates has been developed, showing an induction of NR when *ex vivo* testosterone production is reduced to ~40% of control males. After this point, the number of nipples per male increases significantly as testosterone levels decrease. Other chemicals than phthalates have not been tested on this model, and it therefore does not inform of a direct relationship between intratesticular testosterone and NR (Gray et al., 2024).

Time-scale

The time scale of this KER is weeks. In rodents, the mammary glands start developing in both sexes during fetal life. However, once the testes start producing testosterone (~GD15 in rats), the androgen hormones block the further development of the nipple anlagen in males. While the programming of the tissue happens during fetal development, the development of the nipples is not finished until after birth. In females and males with retained nipples, the nipples do not appear until after birth and are optimally assessed at PND12-14, when they have emerged, but the pups have not yet developed thick fur (Schwartz et al., 2021; Welsh et al., 2014).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Rat strain		Long-Evans Hooded rats are less sensitive to NR than Sprague-Dawley rats	(Wolf et al., 1999; You et al., 1998)

Known Feedforward/Feedback loops influencing this KER

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

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Relationship: 3486: Decrease, circulating testosterone levels leads to nipple retention, increased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	non-adjacent	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic applicability

This KER is considered applicable to rodents (evidence primarily from laboratory rats and mice), where males normally lack nipples due to suppressed differentiation by high levels of androgens. The empirical evidence in this KER supports that reduction in testosterone causes NR in rats, while relevance in mice is assumed based on knowledge about developmental biology in this species. In humans, both sexes have two nipples, and there is no known androgen-driven sexual dimorphism (Schwartz et al., 2021). The KER is thus not considered directly applicable to humans. However, NR is a clear readout of reduced androgen action and fetal masculinization during development in rodents, which is relevant to humans (Schwartz et

al., 2021). It is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD, 2025a, 2025b, 2025c) and considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013).

Sex applicability

This KER is only applicable to males, as female rats and mice develop 12 and 10 nipples, respectively (Schwartz et al., 2021). Females do have circulating testosterone in fetal life, but the levels are much lower than in males (Houtsmuller et al., 1995), and do therefore not suppress nipple formation.

Life stage applicability

The programming for androgen-driven suppression of nipple development in rodents occurs during fetal life, around gestational days (GD) 16-20 in rats (Imperato-McGinley et al., 1986). In both male and female rodents, the development of the mammary glands starts in fetal life, including initial growth and subsequent sexual differentiation (Kratochwil, 1986; Watson & Khaled, 2008). The relevant timing for the investigation of NR is PND12-14 in male rat offspring when the nipples are visible in the female littermates. At this time in development, the nipples/areolas are visible through the skin without excessive fur that may interfere with the investigation (Schwartz et al., 2021).

Key Event Relationship Description

This KER describes a fetal decrease in circulating testosterone (often measured in serum or plasma) leading to NR in male rodent offspring. In rats and mice, females develop 10 and 12 nipples, respectively, with males typically displaying zero. In male rodents, testosterone is primarily produced by the fetal testes, secreted into the bloodstream, and transported to the peripheral reproductive tissues, including the preliminary mammary tissue. Testosterone can bind directly to the AR in the tissue or first being converted to DHT by 5α-reductase (Murashima et al., 2015). AR activation by androgens in mesenchymal cells of the developing mammary glands causes cell death and subsequent separation of the tissue from the epidermis, resulting in no formation of nipples (Kratochwil, 1986). In females, where androgen levels are low, nipple formation is not blocked. The dependency of androgens for suppression of nipple development in males means that reductions in circulating testosterone levels can lead to retention of nipples.

In humans, both sexes have two nipples, and there is no known androgen-driven sexual dimorphism (Schwartz et al., 2021). The KER is thus not considered directly applicable to humans, but is a clear readout of reduced androgen action and fetal masculinization during development, which is relevant to humans (Schwartz et al., 2021). It is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD, 2025a, 2025b, 2025c) and considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013).

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 in rodents.

Sexual differentiation in males, including blocking of nipple development in rodents, is programmed in fetal life. Once formed, the fetal testes synthesize testosterone through the steroidogenesis pathway. Testosterone is secreted and transported in the bloodstream either as free testosterone or bound to plasma proteins (albumin or sex-hormone binding globulin). Testosterone binds AR in peripheral tissues and can also be converted to DHT by the enzyme 5α-reductase. Binding of testosterone and DHT to AR program the fetal reproductive tissue to male differentiation (Murashima et al., 2015).

In most rodents, development of the nipples is a sexually dimorphic process. In female rats, where androgen levels are low, the nipples develop along the milk lines forming 12 nipples, which are visible around postnatal day 12-14 (Schwartz et al., 2021). In male rats, AR activation in fetal life suppresses the formation of the nipples through apoptosis of epithelial cells in the developing mammary glands. Normally, male rats do therefore not have nipples, although in rare occasions male control rats may display one or more retained nipples (Kratochwil, 1986; Schwartz et al., 2021).

Testosterone is produced from around GD15 in fetal rats and is present in circulation around the same time. Programming of the nipple tissue to regress mainly occurs within the masculinization programming window (GD16-20 in rats) (Welsh et al., 2014).

Given the dependency of testosterone for the regression of the nipples, either through direct AR activation or conversion to DHT, it is highly plausible that a decrease in circulating levels of testosterone will lead to nipple retention in males.

Empirical Evidence

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

From the data collection, two data sets were extracted. The data set included two different stressors causing reduced fetal levels of circulating testosterone in rats (Table 2 and appendix 2, 61b9o238vs_KER_3486_Appendix_2.pdf). Both data sets showed concurrent NR.

Table 2 Empirical evidence for KER 3486 LOAEL: Lowest observed adverse effect level; See appendix 2 for specifications.

Species	Stressors(s)	Effect on upstream event (circulating testosterone)	Effect on downstream event (NR)	Reference
Rat	Diethylhexyl phthalate	LOAEL 750 mg/kg bw/day	LOAEL 750 mg/kg bw/day	(Borch et al., 2004)
Rat	Prochloraz	LOAEL 30 mg/kg bw/day	LOAEL 30 mg/kg bw/day	(Vinggaard et al., 2005)

Dose concordance

The empirical evidence for this KER does not inform dose concordance, as no study uses more than one dose of stressor.

Temporal concordance

NR is generally first observable in postnatal animals, while the reductions in intratesticular testosterone are measured early in fetal life during exposure. This was demonstrated with prochloraz-induced NR, which was observed at PND13, but not when examining the males at GD21, when circulating testosterone levels were reduced (Vinggaard AM et al., 2005).

Incidence concordance

The data does not inform incidence concordance.

Uncertainties and Inconsistencies

The low number of studies retrieved in the empirical evidence collection, this KER is in itself an uncertainty, and both studies only investigated one stressor dose.

An uncertainty in the empirical evidence is that prochloraz is also known to be an AR antagonist (Andersen et al., 2002), and it can therefore not be excluded that the effects of prochloraz on NR is, at least partly, due to direct antagonism of AR and not due to the low testosterone levels.

Quantitative Understanding of the Linkage

The quantitative understanding of this KER is classified as **low**.

Response-response relationship

There is no known information on the response-response relationship for this KER.

Time-scale

The time scale of this KER is weeks. Testosterone is secreted from ~GD15 in rats, which is at the beginning of the masculinization programming window. The mammary glands start developing during fetal life as well, but cannot be observed in female rodents until weeks after birth, which is also the time at which NR can be observed in males (Schwartz et al., 2021)

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Rat strain		Long-Evans Hooded rats are less sensitive to NR than Sprague Dawley rats	(Wolf et al., 1999; You et al., 1998)

Known Feedforward/Feedback loops influencing this KER

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

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Relationship: 3348: Decrease, AR activation leads to nipple retention, increased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic

The KER is considered directly applicable to rats and mice, in which males normally have no nipples due to high levels of androgens during development, leading to regression of nipple anlagen. The empirical evidence supports the relevance to rats, whereas the relevance in mice is assumed based on knowledge about developmental biology in this species. Applicability may extend to most rodents.

While NR is not directly translatable to humans, it serves as a clear indicator of diminished androgen activity causing disrupted fetal masculinisation and sexual differentiation during development - an effect considered relevant to mammals, humans (Schwartz et al., 2021), and vertebrates more broadly (Ogino et al., 2023). NR is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD 2025a; OECD 2025b, OECD 2025c) and in OECD GD 151 considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013). NR can also be used as an indicator of anti-androgenicity in mammals and vertebrates in the environment due to the conserved nature of the AR and its implication in sexual differentiation across species (Ogino et al., 2023).

Life stage

Programming of nipple/areola regression in males occurs during a short window of sensitivity to androgens in the nipple anlagen during fetal life. This takes place in rats around embryonic days 13-15 (Imperato-McGinley et al., 1986), which is, therefore, the relevant window of exposure. The relevant timing for the investigation of NR is PND12-14 in male rat offspring when the nipples are visible in the female littermates. At this time in development, the nipples/areolas are visible through the skin without excessive fur that may interfere with the investigation (Schwartz et al., 2021). It should be mentioned that though the occurrence of nipples/areolas in male offspring is believed to be relatively stable throughout life, it may be responsive to postnatal changes. Permanent nipple/areola retention is observed in some but not all *in utero* exposure studies with antiandrogens inducing nipple/areola retention at PND 12-14. Most of the differences between studies seem explainable by the window of exposure, dose levels and methods for investigation used, but the responsiveness of nipple/areola retention to postnatal changes remains to be fully explored (Schwartz et al., 2021).

Sex

Data presented in this KER support that disruption of androgen action during fetal life can lead to increased nipple/areola retention in male rat offspring. Since female mice and rat offspring, in general, have 10 (mice) or 12 (rats) nipples at the relevant time of investigation, increased nipple/areola retention at that time point is not a relevant endpoint for females.

Key Event Relationship Description

This KER links a decrease in androgen receptor (AR) activation during fetal development to increased nipple/areola retention (NR) in male rodent offspring. It should be noted that the upstream Key Event (KE) ‘decrease, androgen receptor activation’ (KE-1614 in AOP Wiki) specifically focuses on decreased activation of the AR *in vivo*, while most methods that can be used to measure AR activity are carried out *in vitro*. Indirect information about this KE may, for example, be provided from assays showing *in vitro* AR antagonism, decreased *in vitro* or *in vivo* testosterone production/levels, or decreased *in vitro* or *in vivo* dihydrotestosterone (DHT) production/levels.

The KER is not directly applicable to humans as both sexes have two nipples, and there is no known effect of androgens on their development (Schwartz et al., 2021). However, NR is a clear readout of reduced androgen action, impaired fetal masculinisation and disrupted sexual differentiation during development, which is relevant to humans, mammals (Schwartz et al., 2021), and vertebrates more broadly (Ogino et al., 2023). It is included as a mandatory endpoint in several rodent OECD Test Guidelines (OECD, 2025a, 2025b, 2025c) and, in OECD GD 151, is considered an adverse outcome applicable to the setting of Points of Departure for use in human health risk assessment (OECD, 2013). NR can also be used as an indicator of anti-androgenicity in mammals and vertebrates in the environment due to the conserved nature of the AR and its implication in sexual differentiation across species (Ogino et al., 2023).

Evidence Supporting this KER

Biological Plausibility

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

- Sexual differentiation happens in fetal life. The testes are developed and start to produce testosterone that is converted in other tissues by the enzyme 5-alpha-reductase to the more potent androgen dihydrotestosterone (DHT). Both hormones bind and activate the nuclear receptor and transcription factor AR, which in turn drives the masculinization of the male fetus (Schwartz et al., 2021; Welsh et al., 2014).

- Fetal masculinization depends on the activation of androgen signalling during a critical time window, the masculinization programming window (MPW), which is observed around, embryonic day 14.5-17.5 in mice (Amato et al., 2022), gestational day (GD) 16-20 in rats and presumably gestation weeks (GWs) 8-14 in humans (Welsh et al., 2008).

- The fetal masculinization process involves a range of tissues and organs, including the nipple anlagen in rats and mice. In humans, both sexes have two nipples. In contrast, common laboratory mice and rats are sexually dimorphic, with females having 12 (rats) and 10 (mice) nipples and males generally having none (Mayer et al., 2008; Schwartz et al., 2021). In both male and female mouse embryos, stem cells differentiate into a mammary gland, with nipple anlagen being visible by embryonic day 11.5 (Mayer et al., 2008). In male embryos, the presence of androgen leads the nipple anlagen to regress a few days later (Kratochwil, 1977; Kratochwil & Schwartz, 1976) . The androgen responsiveness in the nipple anlagen is rather short, in mice starting late embryonic day 13, with loss of responsiveness on embryonic day 15 (Imperato-McGinley et al., 1986; Kratochwil, 1977) and thus roughly following the timing of the MPW.

- Nipple formation is inhibited in female mice and rat fetuses exposed to androgens during gestation(Goldman et al., 1976; Greene et al., 1941; Imperato-McGinley et al., 1986).

- Male *Tfm*-mutant mice, which are insensitive to androgens and believed to be so due to a nonfunctional androgen receptor, present with retained nipples (Kratochwil & Schwartz, 1976).

- Multiple mechanisms of action may potentially lead to nipple retention in male mouse and rat offspring. DHT is the main androgen responsible for nipple/areola regression through interaction with AR in the nipple anlagen (Imperato-McGinley et al., 1986). Inhibition of testosterone synthesis or inhibition of DHT to testosterone conversion, increased metabolism of androgens, or direct interference with AR activation may thus all lead to nipple/areola retention (Imperato-McGinley et al., 1986; Schwartz et al., 2021).

Empirical Evidence

The empirical support from studies in animals for this KER is judged as high overall.

It should be noted that the KE decreased AR activation (KE 1614 in AOP Wiki) specifically focuses on decreased activation of the AR *in vivo*, with no methods currently available to measure this. Examples of assays that provide indirect information about KE 1614 are described in upstream MIE/KEs.

The empirical evidence for this KER from animal studies *in vivo* is based on studies using six different substances that result in decreased AR activation by different mechanisms. Flutamide, procymidone and vinclozolin bind to the AR and inhibit the receptor activity and thereby act as AR antagonists, see MIE 26. Finasteride inhibits the 5-alpha-reductase enzyme that converts testosterone to DHT, see MIE 1617. DEHP and DBP exposure during prenatal development in rats results in reduced fetal testosterone levels, see KE-2298 and KE1690. (MIE 26, MIE 1617 and KE 1690 can be found in AOP-Wiki).

The evidence for the upstream KE is mainly based on data from *in vitro* assays (AR antagonism or 5-alpha-reductase inhibition *in vitro*), whereas the evidence for the downstream KE is based on *in vivo* studies, and there is generally no evidence for both KEs from the same study. However, decreased testosterone levels can be measured *in vivo*, and (Howdeshell et al., 2007; Martino-Andrade et al., 2009) measured the effect of developmental phthalate exposure on both testosterone levels and nipple/areola retention (see the section about “Dose concordance”).

The empirical evidence for the six substances is summarised in Table 3.

Table 3. Summary of empirical evidence for decreased androgen receptor activation, leading to increased nipple/areola retention. References for the studies supporting the empirical evidence are found in the section “Evidence for decreased AR activation (KE 1614) by flutamide, procymidone, and vinclozolin, finasteride, DEHP and DBP” and in Table 4 in Appendix 2 (5yw914oi7s_KER_3348_Appendix_2.pdf).

Stressor(s)	**Upstream effect (decreased AR activation)**	**Downstream effect (Increased nipple/areola retention)**
Flutamide	AR antagonism in *in vitro* assay, receptor binding and transactivation assays	Increased NR in males after prenatal exposure in studies in rat

Procymidone	AR antagonism in *in vitro* assay receptor binding and transactivation assays	Increased NR in males after prenatal exposure in studies in rat
Vinclozolin	AR antagonism in *in vitro* assay receptor binding and transactivation assays	Increased NR in males after prenatal exposure in studies in rat
Finasteride	Inhibition of 5-alpha-reductase enzyme in *in vitro* assays	Increased NR in males after prenatal exposure in studies in rat
DEHP	Reduced production of testosterone in fetal testis measured in *ex vivo* testis assays, reduced testosterone levels in testis, and reduced fetal plasma or serum testosterone levels	Increased NR in males after prenatal exposure in studies in rat
DBP	Reduced production of testosterone in fetal testis measured in ex vivo testis assays and reduced testosterone levels in fetal testis	Increased NR in males after prenatal exposure in studies in rat

From Table 3, it can be deduced that fetal exposure to substances known to decrease androgen receptor activation through antagonism of the AR (vinclozolin, procymidone, flutamide), inhibition of testosterone synthesis (DEHP, DBP) or inhibition of the conversion of testosterone to DHT (finasteride), results in increased nipple/areola retention in rat male offspring.

Evidence for decreased AR activation (KE 1614) by flutamide, procymidone, vinclozolin, finasteride, DEHP and DBP.

Flutamide, a pharmaceutical, binds the AR and inhibits its activity, thereby acting as an AR antagonist. It has been used as an antiandrogen for the treatment of prostate cancer and is used as a reference chemical for antiandrogenic activity in the AR transactivation assays in the OECD test guideline No 458 (Goldspiel & Kohler, 1990; Labrie, 1993; OECD, 2023; Simard et al., 1986)

Procymidone and vinclozolin are fungicides that have been shown to be AR antagonists. Procymidone binds to the AR and inhibits the agonist binding, as shown in AR binding assays using rat prostate cytosol (Hosokawa et al., 1993) or AR transfected cells (Ostby et al., 1999). Procymidone also inhibits agonist activated transcription in AR reporter assays (Hass et al., 2012; Kojima et al., 2004; Orton et al., 2011; Ostby et al., 1999; Scholze et al., 2020). Vinclozolin binds to the AR and inhibits the agonist binding, as shown in AR binding assays using rat epididymis cytosol (Kelce & Wilson, 1997) or AR transfected cells (Wong et al., 1995). Vinclozolin also inhibits agonist activated transcription in AR reporter assays (Euling, 2002; Kojima et al., 2004; Molina-Molina et al., 2006; Orton et al., 2011; Scholze et al., 2020; Shimamura et al., 2002; Wong et al., 1995).

Finasteride is a pharmaceutical that inhibits the 5-alpha-reductase enzyme that converts testosterone to DHT. Finasteride is used to treat benign prostatic hypertrophy (Andersson & Russell, 1990; Stoner, 1990; Wood & Rittmaster, 1994).

Prenatal exposure to DEHP in rats has been shown to reduce the production of testosterone in fetal testis measured in *ex vivo* testis assays, and to reduce testosterone levels in testis and in fetal plasma and serum (Borch et al., 2006; Borch J et al., 2004; Culty et al., 2008; Hannas et al., 2011, 2012; Howdeshell et al., 2007; Klinefelter et al., 2012; Parks, 2000; VO et al., 2009; Wilson et al., 2004, 2007). Conversely, prenatal DEHP exposure did not result in any effects on testosterone levels in the testis at PND1 in one study by Andrade et al. (2006) (Andrade et al., 2006). Similar to DEHP, prenatal exposure to DBP has been shown to reduce the production of testosterone in fetal rat testis measured in ex vivo testis studies (Howdeshell et al., 2007; Wilson et al., 2004) and reduce testosterone levels in the fetal rat testis (Martino-Andrade et al., 2009). The precise underlying mechanism for these effects of DEHP and DPB is presently unknown.

Evidence for increased nipple/areola retention in males (AO-1786) by prenatal exposure to flutamide, procymidone, vinclozolin, finasteride, DEHP and DBP.

All datasets that were used for the weight of evidence assessment were judged as reliable without or with restriction. The majority of datasets assessed showed an increased nipple/areola retention in male offspring after gestational exposure. The conclusion was that the level of confidence was strong for all six substances. The studies are summarised in Table 4 in Appendix 2, 5yw914oi7s_KER_3348_Appendix_2.pdf

Dose concordance

Dose concordance is challenging to assess for this KER since *in vivo* AR activity is currently not possible to measure but can only be inferred indirectly by measures of upstream events. In some studies, fetal (testicular) testosterone levels during, or close to, the fetal masculinization programming window are measured in a subset of animals exposed similarly to those investigated for NR postnatally. Such information may inform on dose concordance if more doses are included.

In a rat in utero exposure study (GD13-21) with DPB and DEHP, testosterone levels in the fetal testes were investigated at GD21, and NR was investigated at PND13 (Martino-Andrade et al., 2009). For DBP, both reduced testosterone levels in fetal testes and NR were observed at 500 mg/kg/d, whereas no effect on NR and only a slight non-significant reduction of testosterone was observed at the lower dose (100 mg/kg/d). For DEHP, a slight but non-significant decrease in testosterone levels in fetal rat testis was observed after exposure to 150 mg/kg/d DEHP, with no effects on nipple/areola retention.

Such data could suggest dose concordance for this part of the KER, although the evidence for this is not strong.

Temporal concordance

Temporal concordance can only be considered from a theoretical perspective since the downstream event, increased NR, is a result of disruption to normal regression of nipple anlagen in male rodents induced during a short window of gestational development (in mice of approximately 2 days) but usually measured at PND12-14 in rats. Earlier than this, the areolae are not yet visible through the skin and later than this, the animals grow fur and need to be shaved for proper examination. This is supported by several of the studies in the empirical evidence, where the test substance was administered during a short period during gestation and nipple retention was observed postnatally.

Based on current knowledge, it is understood that the upstream event – decreased AR activation *in vivo* – takes place minutes to hours after exposure to an anti-androgenic substance. If a substance decreases AR activation through inhibition of the AR, the upstream event is expected to happen immediately after exposure. If a substance decreases androgen receptor activation through inhibition of testosterone synthesis, the upstream event is expected to happen minutes to hours after the exposure.

Uncertainties and Inconsistencies

For DEHP and DBP, there were some inconsistencies in the empirical evidence, but they could be explained by differences in study designs and uncertainties in measurements (see Appendix 1). Some uncertainty is imposed by the poorly supported dose-concordance. However, the dose-concordance is well supported by the current understanding of biological processes.

Quantitative Understanding of the Linkage

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

Response-response relationship

The difficulties in extrapolating potency from *in vitro* to *in vivo* studies were exemplified by a comparison of the effects of pyrifluquinazon and bisphenol C *in vitro* and in utero. *In vitro*, bisphenol C antagonized the androgen receptor with a much higher potency than pyrifluquinazon, but *in vivo* the potencies were reversed with pyrifluquinazon exposure leading to NR at lower exposure levels than bisphenol C (Gray et al., 2019).

Time-scale

AR activation operates on a time-scale of minutes. The AR is a ligand-activated nuclear receptor and transcription factor. Upon ligand binding a conformational change and subsequent dimerization of the AR takes place within 3-6 minutes (Schaufele et al., 2005). Nuclear translocation (Nightingale et al., 2003) 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).

For the downstream event, the time-scale for observing a measurable effect on nipple/areola retention is closer to days and weeks, depending on species. For instance, in mice the nipple anlage are responsive to androgen action at embryonic day 13-15, while a sexual dimorphism of the nipples/areolas can first be observed after birth (Imperato-McGinley et al., 1986) .

Known modulating factors

A well established modulating factor for androgen action is genetic variations in the AR, which decrease the function of the receptor. For example, longer CAG repeat lengths have been associated with decreased AR activation (Chamberlain et al., 1994; Tut et al., 1997). Rat strain is another important modulating factor, with studies showing that the Long-Evans Hooded rat is less sensitive to nipple/areola retention than the Sprague-Dawley rat (Wolf et al., 1999; You et al., 1998)

Known Feedforward/Feedback loops influencing this KER

Not relevant for this KER.

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