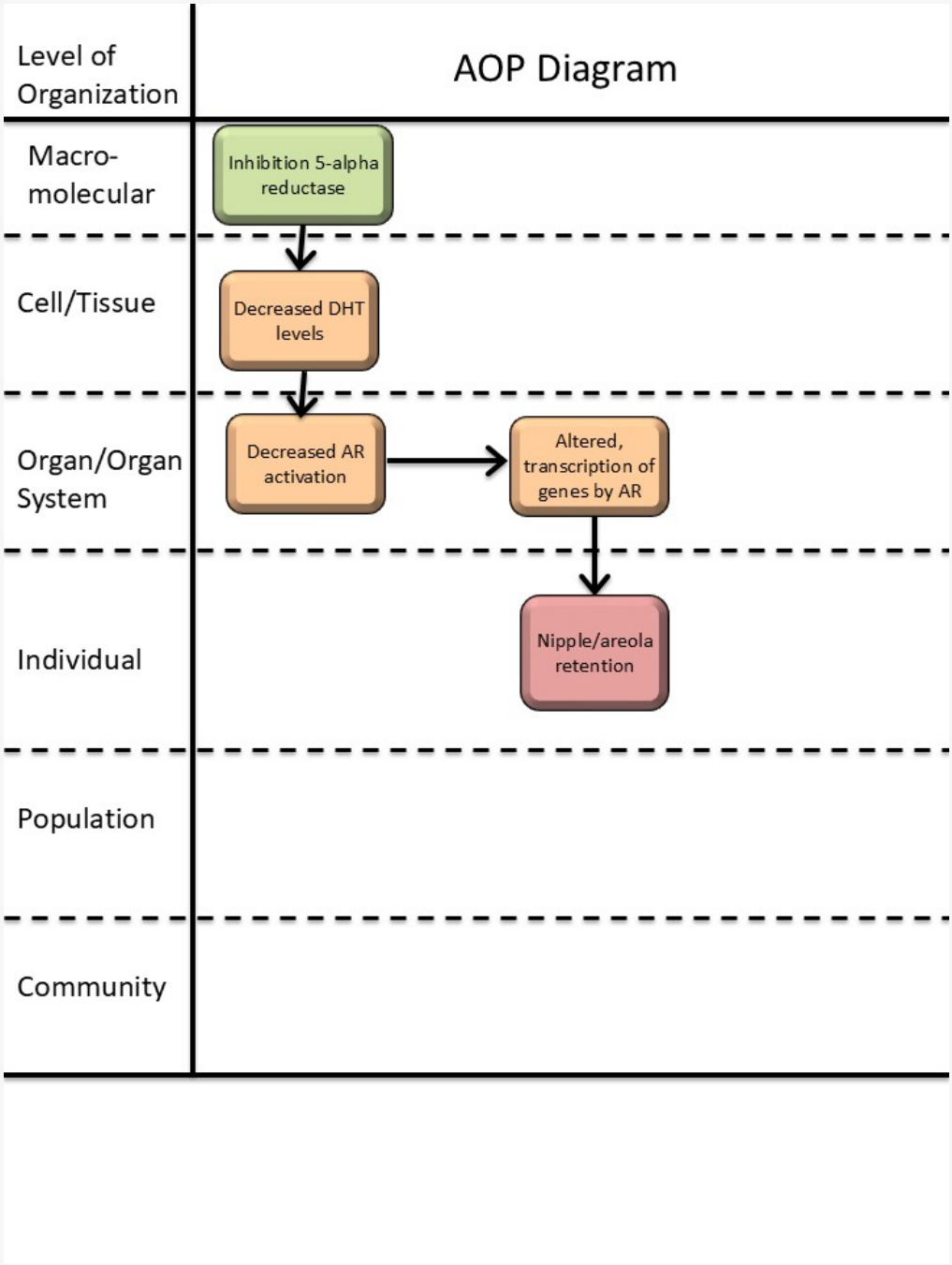
AOP ID and Title:

AOP 576: 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring
Short Title: 5α-reductase inhibition leading to nipple retention

Graphical Representation



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Abstract

This AOP links inhibition of 5α-reductase during fetal life with increased nipple/areola retention (NR) in male rodent offspring. NR, measured around 2 weeks postpartum, is a marker for disrupted masculinization of male rodents (primarily investigated in laboratory rats and mice) and is associated with general feminization of male offspring.

5α-reductase is an enzyme that converts testosterone to dihydrotestosterone (DHT). In normal male reproductive development, DHT activates the androgen receptor (AR) in many peripheral reproductive tissues to drive differentiation of the male phenotype, including regression of nipple anlagen in male rats and mice. While testosterone also acts directly at the AR, DHT is 5-10 times more potent and in tissues peripheral to the testes, conversion to DHT is necessary for proper masculinization (Amato et al., 2022; Davey & Grossmann, 2016).

This AOP delineates the evidence that inhibition of 5α-reductase reduces DHT levels and consequently AR activation, causing retention of nipples in male rodents. The AOP is supported by *in vitro* experiments upstream of AR activation and by *in vivo* studies downstream of AR activation. 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 each of the 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 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 and vertebrates more broadly due to the conserved nature of the AR and its implication in sexual differentiation across species.

Background

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 in male (rodent) offspring’), and AOP 575 (‘Decreased testosterone synthesis leading to increased nipple retention 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 identification and assessment of endocrine disruptors and to inform predictive toxicology, identification of knowledge gaps for investigation and method development.

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Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1617	Inhibition, 5α-reductase	Inhibition, 5α-reductase
	KE	1613	Decrease, dihydrotestosterone (DHT) levels	Decrease, DHT level
	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
Inhibition, 5α-reductase	adjacent	Decrease, dihydrotestosterone (DHT) levels	High	
Decrease, dihydrotestosterone (DHT) levels	adjacent	Decrease, androgen receptor activation	High	
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	
Decrease, androgen receptor activation	non-adjacent	Nipple retention (NR), increased	High	

Stressors

Name	Evidence
Finasteride	

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 KER 3348 (Decrease, AR activation, leads to increased nipple retention) is considered only directly applicable to male rodent (current evidence primarily from rats and mice) during fetal life, restricting the applicability of the AOP. NR is specific to animals with sexual dimorphism in the number of nipples, a feature most prominently investigated in laboratory rats and mice. It is, however, 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. In the males of mice and rats, the nipple anlagen are programmed during fetal development by androgens to regress, leading to no visible nipples in males postnatally, while females 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, inconsistencies and contradictory evidence

MIE-1617 Inhibition, 5α-reductase **HIGH:** This MIE is usually measured in vitro, whereas the downstream events in the AOP are usually measured in vivo. Canonical knowledge of normal male reproductive development provides strong support for essentiality, along with 5α-reductase knockout models and models using exposure to 5α-reductase inhibitors.	**Biological plausibility provides strong support for the essentiality of this event, as DHT, produced by 5α-reductase, is a ligand of the AR and a primary driver of normal regression of nipple anlagen in male fetuses** (Imperato-McGinley et al., 1986)**.** **Indirect evidence of impact of inhibition of 5α-reductase (MIE-1617) in vitro on AR activity in vitro:** • Finasteride, a specific inhibitor of 5α-reductase, can decrease proliferation of prostate cancer cells in vitro, a proxy read-out of AR activity (Bologna et al., 1995). **Direct evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on decreased DHT levels (KE-1613):** • Lack of 5α-reductase type 2 activity by e.g. inhibitor or KO decrease DHT levels locally in tissues and blood. This is demonstrated in humans, rats, monkeys, and mice (Robitaille & Langlois, 2020). **Indirect evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on decreased DHT levels (KE-1613):** • Men with androgenic alopecia treated with finasteride or dutasteride presented with decreased DHT levels in serum (Clark et al., 2004; Drake et al., 1999). **Direct evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on increased nipple retention (AO-1786):** • Exposure to the 5α-reductase inhibitors leads to increased retention of nipples in male offspring after in utero exposure (Christiansen et al., 2009; Imperato-McGinley et al., 1986).	

KE-1613 Decreased, DHT levels **HIGH:** Canonical knowledge of normal male reproductive development provides strong support for essentiality, along with rescue studies specifically demonstrating how DHT is essential for normal regression of nipple anlagen in male offspring.	**Biological plausibility provides strong support for the essentiality of this event. Androgens are AR ligands and main drivers for the regression of nipple anlagen in male offspring (Goldman et al., 1976), with DHT playing an important role** (Imperato-McGinley et al., 1986)**.** **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vivo (KE-1614):** • Androgen deprivation is used as treatment for prostate cancer, including 5α-reductase inhibitors, to reduce DHT levels and cancer growth (Aggarwal et al., 2010). **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vitro:** • Increasing concentrations of DHT lead to increasing AR activation in vitro in AR reporter gene assays (OECD, 2023; Williams et al., 2017). **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vivo:** • 5α-reductase 2 deficiency is an autosomal recessive condition in which 46,XY subjects with bilateral testes and normal testosterone production have impaired virilization during fetal life due to diminished DHT (Mendonca et al., 2016). **Direct evidence of impact of decreased DHT levels (KE-1613) on increased nipple retention (AO-1786).** • Nipple formation is inhibited in female rat fetuses exposed to DHT during gestation (Goldman et al., 1976). • Exposure to the 5α-reductase inhibitor 390 MSD leads to increased retention of nipples in male rats after in utero exposure, whereas simultaneous exposure to DHT reverses the effects (Imperato-McGinley et al., 1986).	

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 (see 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 proved 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
MIE-1617	***	**		High
KE-1613	***	**		High
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

The confidence in each of the 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-1880 Inhibition, 5α-reductase leads to a decrease, DHT levels	High	High (canonical)	It is well established that 5α-reductase converts testosterone to DHT. *In vitro, in vivo* and human studies with 5α-reductase inhibitors have shown that the stressors dose-dependently decrease formation of DHT.
KER-1935 Decrease, DHT levels leads to a decrease, AR activation	High	High (canonical)	It is well established that DHT 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.
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.
KER-3348 Decrease, AR activation leads to increase, nipple retention	High	High	It is well established that activation of AR drives 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. The 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.

Quantitative Consideration

The quantitative understanding of the AOP is limited. A key difficulty lies in the challenge of extrapolating from *in vitro* to *in vivo* events since these cannot be captured within the same experimental framework. Specifically, MIE-1617 is evaluated *in vitro*, while both KE-1613 (decrease, DHT levels'), KE-1614 (decrease, AR activation') and the AO (Increase, NR) are *in vivo*

endpoints. It should be noted that KE-1614 pertains to AR activation *in vivo* - currently lacking viable methods for direct measurement.

For *in vivo* to *in vivo* KERs like KER-1935 ('Decrease, DHT level leads to Decrease, AR activation') and KER-2124 ('Decrease, AR activation leads to Altered, Transcription of genes by the AR'), there is not enough data to define a quantitative relationship, and such a relationship will differ between biological systems (species, tissue, cell type, life stage etc).

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 mammals and other vertebrates in the environment.
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) and vertebrates more broadly due to the conserved nature of the AR and its implication in sexual differentiation across species (Ogino et al., 2023).

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Appendix 1

List of MIEs in this AOP

Event: 1617: Inhibition, 5α-reductase

Short Name: Inhibition, 5α-reductase

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	MolecularInitiatingEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:120 - Inhibition of 5α-reductase leading to Leydig cell tumors (in rat)	MolecularInitiatingEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	MolecularInitiatingEvent
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Essentially the reaction performed by the isozymes is the same, but the enzyme is differentially expressed in the body. 5α-reductase type 1 is mainly linked to the production of neurosteroids, 5α-reductase type 2 is mainly involved in production of 5α-DHT, whereas 5α-reductase type 3 is involved in N-glycosylation (Robitaille & Langlois, 2020).

The expression profile of the three 5α-reductase isoforms depends on the developmental stage, the tissue of interest, and the disease state of the tissue. The enzymes have been identified in, for instance, non-genital and genital skin, scalp, prostate, liver, seminal vesicle, epididymis, testis, ovary, kidney, exocrine pancreas, and brain (Azzouni, 2012, Uhlen 2015).

5α-reductase is well-conserved, all primary species in Eukaryota contain all three isoforms (from plant, amoeba, yeast to vertebrates) (Azzouni, 2012) and the enzymes are expressed in both males and females (Langlois, 2010, Uhlen 2015).

Key Event Description

This KE describes the inhibition of 5α-reductases (3-oxo-5α-steroid 4-dehydrogenases). These enzymes are widely expressed in tissues of both sexes and responsible for conversion of steroid hormones.

There are three isozymes: 5α-reductase type 1, 2, and 3. The substrates for 5α-reductases are 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21

steroids such as testosterone, progesterone, androstenedione, epi-testosterone, cortisol, aldosterone, and deoxycorticosterone. The enzymatic reaction leads to an irreversible breakage of the double bond between carbon 4 and 5 and subsequent insertion of a hydride anion at carbon 5 and insertion of a proton at carbon 4. The reaction is aided by the cofactor NADPH. The substrate affinity and reaction velocity differ depending on the combination of substrate and enzyme isoform, for instance 5α-reductase type 2 has a higher substrate affinity for testosterone than the type 1 isoform of the enzyme, and the enzymatic reaction occurs at a higher velocity under optimal conditions. Likewise, inhibitors of 5α-reductase may exhibit differential effects depending on isoforms (Azzouni et al., 2012).

How it is Measured or Detected

There is currently (as of 2023) no OECD test guideline for the measurement of 5α-reductase inhibition.

Assessing the ability of chemicals to inhibit the activity of 5α-reductase is challenging, but has been assessed using transfected cell lines. This has been demonstrated in HEK-293 cells stably transfected with human 5α-reductase type 1, 2, and 3 (Yamana et al., 2010), in CHO cells stably transfected with human 5α-reductase type 1 and 2 (Thigpens et al., 1993), and COS cells transfected with human and rat 5α-reductase with unspecified isoforms (Andersson & Russell, 1990). The transfected cells are typically used as intact cells or cell homogenates. Further, 5α-reductase 1 and 2 has been successfully expressed and isolated from *Escherichia coli* with subsequent functionality allowing for examination of enzyme inhibition (Peng et al., 2020). The availability of the stably transfected cell lines and the isolated enzymes to the scientific community is unknown.

The output of the above methods could be decreased dihydrotestosterone (DHT) with increasing test chemical concentrations. Other substrates exist for the different isoforms that could be used to assess the enzymatic inhibition (Peng et al., 2020). The use of radiolabeled steroids has historic and continued use for 5α-reductase inhibition examination (Andersson & Russell, 1990; Peng et al., 2020; Thigpens et al., 1993; Yamana et al., 2010); however, alternative methods are available, such as conventional ELISA kits or advanced analytical methods such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

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List of Key Events in the AOP

Event: 1613: Decrease, dihydrotestosterone (DHT) levels

Short Name: Decrease, DHT level

Key Event Component

Process	Object	Action
hormone biosynthetic process	17beta-Hydroxy-2-oxa-5alpha-androstan-3-one	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 ID and Name	Event Type
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:527 - Decreased, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) leads to Hypospadias, increased	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) 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
All life stages	Moderate

Sex Applicability

Sex	Evidence
Mixed	High

This KE is applicable to both sexes, across developmental stages and adulthood, in many different tissues and across mammals.

In both humans and rodents, DHT is important for the *in utero* differentiation and growth of the prostate and male external genitalia (Azzouni et al., 2012; Gerald & Raj, 2022). Besides its critical role in development, DHT also induces growth of facial and body hair during puberty in humans (Azzouni et al., 2012).

In mammals, the role of DHT in females is less established (Swerdloff et al., 2017), however studies suggest that androgens are important in e.g. bone metabolism and growth, as well as female reproduction from follicle development to parturition (Hammes & Levin, 2019).

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

Dihydrotestosterone (DHT) is an endogenous steroid hormone and a potent androgen. The level of DHT in tissue or blood is dependent on several factors, such as the synthesis, uptake/release, metabolism, and elimination from the system, which again can be dependent on biological compartment and developmental stage.

DHT is primarily synthesized from testosterone (T) via the irreversible enzymatic reaction facilitated by 5α-Reductases (5α-REDs) (Swerdloff et al., 2017). Different isoforms of this enzyme are differentially expressed in specific tissues (e.g. prostate, skin, liver, and hair follicles) at different developmental stages, and depending on disease status (Azzouni et al., 2012; Uhlén et al., 2015), which ultimately affects the local production of DHT.

An alternative (“backdoor”) pathway , exists for DHT formation that is independent of T and androstenedione as precursors. While first discovered in marsupials, the physiological importance of this pathway has now also been established in other mammals including humans (Renfree and Shaw, 2023). This pathway relies on the conversion of progesterone (P) or 17-OH-P to androsterone and then androstanediol through several enzymatic reactions and finally, the conversion of androstanediol into DHT probably by HSD17B6 (Miller & Auchus, 2019; Naamneh Elzenaty et al., 2022). The “backdoor” synthesis pathway is a result of an interplay between placenta, adrenal gland, and liver during fetal life (Miller & Auchus, 2019).

The conversion of T to DHT by 5α-RED in peripheral tissue is mainly responsible for the circulating levels of DHT, though some

tissues express enzymes needed for further metabolism of DHT consequently leading to little release and contribution to circulating levels (Swerdloff et al.).

The initial conversion of DHT into inactive steroids is primarily through 3α-hydroxysteroid dehydrogenase (3α-HSD) and 3β-HSD in liver, intestine, skin, and androgen-sensitive tissues. The subsequent conjugation is mainly mediated by uridine 5´-diphospho (UDP)-glucuronyltransferase 2 (UGT2) leading to biliary and urinary elimination from the system. Conjugation also occurs locally to control levels of highly potent androgens (Swerdloff et al., 2017).

Disruption of any of the aforementioned processes may lead to decreased DHT levels, either systemically or at tissue level.

How it is Measured or Detected

Several methods exist for DHT identification and quantification, such as conventional immunoassay methods (ELISA or RIA) and advanced analytical methods as liquid chromatography tandem mass spectrometry (LC-MS/MS). The methods can have differences in detection and quantification limits, which should be considered depending on the DHT levels in the sample of interest. Further, the origin of the sample (e.g. cell culture, tissue, or blood) will have implications for the sample preparation.

Conventional immunoassays have limitations in that they can overestimate the levels of DHT compared to levels determined by gas chromatography mass spectrometry and liquid chromatography tandem mass spectrometry (Hsing et al., 2007; Shiraishi et al., 2008). This overestimation may be explained by lack of specificity of the DHT antibody used in the RIA and cross-reactivity with T in samples (Swerdloff et al., 2017).

Test guideline no. 456 (OECD 2023) uses a cell line, NCI-H295, capable of producing DHT at low levels. The test guideline is not validated for this hormone. Measurement of DHT levels in these cells require low detection and quantification limits. Any effect on DHT can be a result of many upstream molecular events that are specific for the NCI-H295 cells, and which may differ in other models for steroidogenesis.

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Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

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

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Key Event Description

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

The AR is a nuclear transcription factor with canonical AR activation regulated by the binding of the androgens such as testosterone or dihydrotestosterone (DHT). Thus, AR activity can be decreased by reduced levels of steroidal ligands (testosterone, DHT) or the presence of compounds interfering with ligand binding to the receptor (Davey & Grossmann, 2016; Gao et al., 2005).

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

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

How it is Measured or Detected

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

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

Short Name: Altered, Transcription of genes by the AR

Key Event Component

Process	Object	Action
regulation of gene expression	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent

AOP ID and Name	Event Type
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:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	KeyEvent

Stressors

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

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

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

Key Event Description

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

The Androgen Receptor and its function

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

Altered transcription of genes by the AR as a Key Event

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

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

How it is Measured or Detected

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

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

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List of Adverse Outcomes in this AOP

Event: 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

Term	Scientific Term	Evidence	Links

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.

Reproducibility of NR results is challenged by the measure being a visual assessment prone to a degree of subjectivity. Thus, NR should be assessed and scored blinded to exposure groups and ideally be performed by the same person(s) within the same study.

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: 1880: Inhibition, 5α-reductase leads to Decrease, DHT level

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of 5α-reductase leading to impaired fecundity in female fish	adjacent	High	High
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	High
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	adjacent		
5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	

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 KE is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across

mammalian taxa. It is, however, acknowledged that this 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

This key event relationship (KER) links inhibition of 5α-reductase activity to decreased dihydrotestosterone (DHT) levels.

There are three isozymes of 5α-reductase: type 1, 2, and 35α-reductase type 2 is mainly involved in the synthesis of 5α-DHT from testosterone (T) (Robitaille & Langlois, 2020), although 5α-reductase type 1 can also facilitate this reaction, but with lower affinity for T (Nikolaou et al., 2021). The type 1 isoform is also involved in the alternative ('backdoor') pathway for DHT formation, facilitating the conversion of progesterone or 17OH-progesterone to dihydroprogesterone or 5α-pregnan-17α-ol-3,20-dione, respectively, whereafter several subsequent reactions will ultimately lead to the formation of DHT (Miller & Auchus, 2019). The quantitative importance of the alternative pathway remains unclear (Alemany, 2022). The type 1 and type 2 isoforms of 5α-reductase are the primary focus of this KER.

The direct conversion of T to 5α-DHT mainly takes place in the target tissue(Robitaille & Langlois, 2020). In mammals, the type 1 isoform is found in the scalp and other peripheral tissues (Miller & Auchus, 2011), such as liver, skin, prostate (Azzouni et al., 2012), bone, ovaries, and adipose tissue(Nikolaou et al., 2021). The type 2 isoform is expressed mainly in male reproductive tissues (Miller & Auchus, 2011), but also in liver, scalp and skin(Nikolaou et al., 2021). The expression level of both isoforms depend on the developmental stage and the tissue.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is considered high.

5α-reductase can catalyze the conversion of T to DHT. The substrates for 5α-reductases are 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21 steroids such as testosterone and progesterone. The enzymatic reaction leads to an irreversible breakage of the double bond between carbon 4 and 5 and subsequent insertion of a hydride anion at carbon 5 and insertion of a proton at carbon 4. The reaction is aided by the cofactor NADPH (Azzouni et al., 2012). By inhibiting this enzyme, the described catalyzed reaction will be inhibited leading to a decrease in DHT levels.

In both humans and rodents, DHT is important for the*in utero* differentiation and growth of the prostate and male external genitalia. Besides its critical role during fetal development, DHT also induces growth of facial and body hair during puberty in humans (Azzouni et al., 2012).

Empirical Evidence

The empirical evidence for this KER is considered high

Dose concordance

Several inhibitors of 5α-reductases have been developed for pharmacological uses. Inhibition of the enzymatic conversion of radiolabeled substrate has been illustrated (Table 1) and data display dose-concordance, with increasing concentrations of inhibitor leading to lower 5α-reductase product formation. These studies at large rely on conversion of radiolabeled substrate and hence serve as an indirect measurement.

Table 1: Dose concordance from selected in vitro test systems

Test system	Model description	Stressor	Effect	Reference
HEK-293 cells	Cells stably transfected human 5α-reductase type 1 and 2 used to measure conversion of [^{14}C]labeled steroids	Finasteride	Type 1: IC_{50} = 106.9 µM Type 2: IC_{50} = 14.3 µM	(Yamana et al., 2010)
		Dutasteride	Type 1: IC_{50} = 8.7 µM Type 2: IC_{50} = 57 µM	

COS cells	Cell homogenates from transfected cells with human and rat 5α-reductase (unknown isoform) used to measure conversion of radiolabeled testosterone	Finasteride	Human: IC_{50} ≈ 1 μM K_i = 340-620 nM Rat: IC_{50} ≈ 0.1 μM K_i = 3-5 nM	(Andersson & Russell, 1990)
		4-MA	Human: IC_{50} ≈ 0.1 μM K_i = 7-8 nM Rat: IC_{50} ≈ 0.1 μM K_i = 5-7 nM	
CHO cells	Stably transfected with human 5α-reductase type 1 and 2	Finasteride	Type 1: K_i = 325 nM Type 2: K_i = 12 nM	(Thigpens et al., 1993)
		4-MA	Type 1: K_i = 8 nM Type 2: K_i = 4 nM	
Isolated enzyme	Human 5α-reductase type 1 and 2 used to measure conversion of radiolabeled substrate of both isoforms	Finasteride	Type 1: K_i = > 200 nM Type 2: K_i = 0.45 nM	(Peng et al., 2020)
		Dutasteride	Type 1: K_i = 39 nM Type 2: K_i = 1.1 nM	

These in vitro studies clearly show effects on the enzymatic reaction induced by 5α-reductases in a concentration dependent manner (Andersson & Russell, 1990; Thigpens et al., 1993; Yamana et al., 2010).

In the intact organism, when 5α-reductase type 2 activity is lacking through e.g. inhibitor treatment or knockout, this will results in decreased 5α-DHT locally in the tissues, but also in blood (Robitaille & Langlois, 2020). This has been demonstrated in humans, rats, monkeys, and mice (Robitaille et al. 2020).

Finasteride is a specific inhibitor of 5α-reductase type 2(Russell & Wilson, 1994). Men with androgenic alopecia were treated with increasing concentrations of finasteride and presented with decreased DHT levels in biopsies from scalp, as well as a decrease in serum DHT levels with dose dependency being most apparent in serum, up to about 70% decrease (Drake et al., 1999). Likewise, men treated with dutasteride exhibited a clear dose dependent decrease in serum DHT after 24 weeks treatment with a maximum efficacy of about 98% (Clark et al., 2004).

Other evidence

The phenotype of males with deficiency in 5α-reductases are typically born with ambiguous external genitalia. They also present with small prostate, minimal facial hair and acne, or temporal hair loss. Comparison of affected individuals to non-affected individuals in regard to T/DHT ratio, conversion of infused radioactive T, and ratios of urinary metabolites of 5α-reductase and 5β-reductase concluded that these phenotypic characteristics were due to 5α-reductase defects that resulted in less conversion of T to DHT (Okeigwe et al. 2014). Mutations in the 5α-reductase gene can result in boys being born with moderate to severe undervirilization phenotypes (Elzenaty 2022).

Quantitative Understanding of the Linkage

Inhibitors of 5α-reductase are important for the prevention and treatment of many diseases. There are several compounds that have been developed for pharmaceutical purposes and they can target the different isoforms with different affinity. Examples of inhibitors are finasteride and dutasteride. Finasteride mainly has specificity for the type 2 isoform, whereas dutasteride inhibits both type 1 and 2 isoforms (Miller & Auchus, 2011).

These differences in isoform specificity reflects in the effects on DHT serum levels, hence the broader specificity of dutasteride leads to > 90% decrease in patients with benign prostatic hyperplasia, in comparison to 70% with

finasteride administration (Nikolaou et al., 2021).

Response-response relationship

Enzyme inhibition can occur in different ways e.g. both competitive and noncompetitive. The inhibition model depends on the specific inhibitor and hence a generic quantitative response-response relationship is difficult to derive.

Time-scale

An inhibition of 5α-reductases would lead to an immediate change in DHT levels at the molecular level. However, the time-scale for systemic effects on hormone levels are challenging to estimate.

Known Feedforward/Feedback loops influencing this KER

Androgens can regulate gene expression of 5α-reductases (Andersson et al., 1989; Berman & Russell, 1993).

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Relationship: 1935: Decrease, DHT level leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

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
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	
5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	adjacent		
5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	

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

KER1935 is assessed applicable to mammals, as DHT 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

KER1935 is assessed applicable to both sexes, as DHT activates AR in both males and females.

Life-stage applicability

KER1935 is considered applicable to developmental and adult life stages, as DHT-mediated AR activation is relevant from the AR is expressed.

Key Event Relationship Description

Dihydrotestosterone (DHT) is a primary ligand for the Androgen receptor (AR), a nuclear receptor and transcription factor. DHT is an endogenous sex hormone that is synthesized from e.g. testosterone by the enzyme 5α-reductase in different tissues and organs (Davey & Grossmann, 2016; Marks, 2004). In the absence of ligand (e.g. DHT) the AR is localized in the cytoplasm in complex with molecular chaperones. Upon ligand binding, AR is activated, translocated into the nucleus, and dimerizes to carry out its 'genomic function' (Davey & Grossmann, 2016). Hence, AR transcriptional function is directly dependent on the presence of ligands, with DHT being a more potent AR activator than testosterone (Grino et al, 1990). Reduced levels of DHT may thus lead to reduced AR activation. Besides its genomic actions, the AR can also mediate rapid, non-genomic second messenger signaling (Davey and Grossmann, 2016). Decreased DHT levels that lead to reduced AR activation can thus entail downstream effects on both genomic and non-genomic signaling.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of KER1935 is considered high.

The activation of AR is dependent on binding of ligands (though a few cases of ligand-independent AR activation has been shown, see *uncertainties and inconsistencies*), primarily testosterone and DHT in mammals (Davey and Grossmann, 2016; Schuppe et al., 2020). Without ligand activation, the AR will remain in the cytoplasm associated with heat-shock and other chaperones and not be able to carry out its canonical ('genomic') function. Upon androgen binding, the AR undergoes a conformational change, chaperones dissociate, and a nuclear localization signal is exposed. The androgen/AR complex can

now translocate to the nucleus, dimerize and bind AR response elements to regulate target gene expression (Davey and Grossmann, 2016; Eder et al., 2001). AR transcriptional activity and specificity is regulated by co-activators and co-repressors in a cell-specific manner (Heinlein and Chang, 2002).

The requirement for androgens binding to the AR for transcriptional activity has been extensively studied and proven and is generally considered textbook knowledge. The OECD test guideline no. 458 uses DHT as the reference chemical for testing androgen receptor activation *in vitro* (OECD, 2020). In the absence of DHT during development caused by 5α-reductase deficiency (i.e. still in the presence of testosterone) male fetuses fail to masculinize properly. This is evidenced by, for instance, individuals with congenital 5α-reductase deficiency conditions (Costa et al., 2012); conditions not limited to humans (Robitaille and Langlois, 2020), testifying to the importance of specifically DHT for AR activation and subsequent masculinization of certain reproductive tissues.

Binding of testosterone or DHT has differential effects in different tissues. E.g. in the developing mammalian male; testosterone 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; Robitaille and Langlois, 2020).

Empirical Evidence

The empirical support for KER1935 is considered high.

Dose concordance:

- Increasing concentrations of DHT lead to increasing AR activation *in vitro* in AR reporter gene assays (OECD, 2020; Williams et al., 2017).

Indirect (supporting) evidence:

- In cell lines where proliferation can be induced by androgens (such as prostate cancer cells) proliferation can be used as a readout for AR-activation. Finasteride, a 5α-reductase inhibitor, dose-dependently decreases AR-mediated prostate cancer cell line proliferation (Bologna et al., 1995). 0.001 μM finasteride decreased the growth rate with 44%, 0.1 μM decreased the growth rate with 80%.
- Specific events of masculinization during development are dependent on AR activation by DHT, including the development and length of the perineum which can be measured as the anogenital distance (AGD, (Schwartz et al., 2019)). E.g. a dose-dependent effect of rat *in utero* exposure to the 5α-reductase inhibitor finasteride was observed on the length of the AGD, where 0.01 mg/kg bw/day finasteride reduced the AGD measured at pup day 1 by 8%, whereas 1 mg/kg bw/day reduced the AGD by 23% (Bowman et al., 2003).

Other evidence:

- Male individuals with congenital 5α-reductase deficiency (absence of DHT) fail to masculinize properly (Costa et al., 2012).
- A major driver of prostate cancer growth is AR activation (Davey and Grossmann, 2016; Huggins and Hodges, 1941). Androgen deprivation is used as treatment including 5α-reductase inhibitors to reduce DHT levels (Aggarwal et al., 2010).

Uncertainties and Inconsistencies

Ligand-independent actions of the AR have been identified. To what extent and of which biological consequences is not well defined (Bennesch and Picard, 2015).

It should be noted, that in tissues, that are not DHT-dependent but rather respond to T, a decrease in DHT level may not influence AR activation significantly in that specific tissue.

Quantitative Understanding of the Linkage

Response-response relationship

There is a positive dose-response relationship between increasing concentrations of DHT and AR activation (Dalton et al., 1998; OECD, 2020). 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

Upon DHT binding to the AR, a conformational change that brings the amino (N) and carboxy (C) termini into close proximity occurs with a $t_{1/2}$ of approximately 3.5 minutes, around 6 minutes later the AR dimerizes as shown in transfected HeLa cells (Schaufele et al., 2005). Addition of 5 nM DHT to the culture medium of ‘AR-resistant’ transfected prostatic cancer cells resulted in a rapid (from 15 minutes, maximal at 30 minutes) nuclear translocation of the AR with minimal residual cytosolic expression (Nightingale et al., 2003). 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)

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)
Androgen deficiency syndrome	Low circulating testosterone levels due to primary (testicular) or secondary (pituitary-hypothalamic) hypogonadism	Reduced levels of circulating testosterone, precurser of DHT	(Bhasin et al., 2010)
Castration	Removal of testicles	Reduced levels of circulating testosterone, precurser of DHT	(Krotkiewski et al., 1980)

Known Feedforward/Feedback loops influencing this KER

Androgens have been shown to upregulate and downregulate AR expression as well as 5α-reductase expression, but for 5α-reductase, each isoform in each tissue is differently regulated by androgens and can display sexual dimorphism (Lee and Chang, 2003; Robitaille and Langlois, 2020). The quantitative impact of such adaptive expression changes is unknown.

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

AOPs Referencing Relationship

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

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

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

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

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

Empirical Evidence

The empirical evidence for this KER is considered high

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

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

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

Uncertainties and Inconsistencies

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

Quantitative Understanding of the Linkage

Response-response relationship

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

Time-scale

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

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
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: 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
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, fetal masculinization and 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), from gestational day (GD) 16-20 in rats, 14.5-16.5 in mice and presumably gestation weeks (GWs) 8-14 in humans (Amato et al., 2022; 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 the conversion of testosterone to DHT, 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 decreased 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 (6djoma9gmj_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, 6djoma9gmj_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 post-natally. 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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