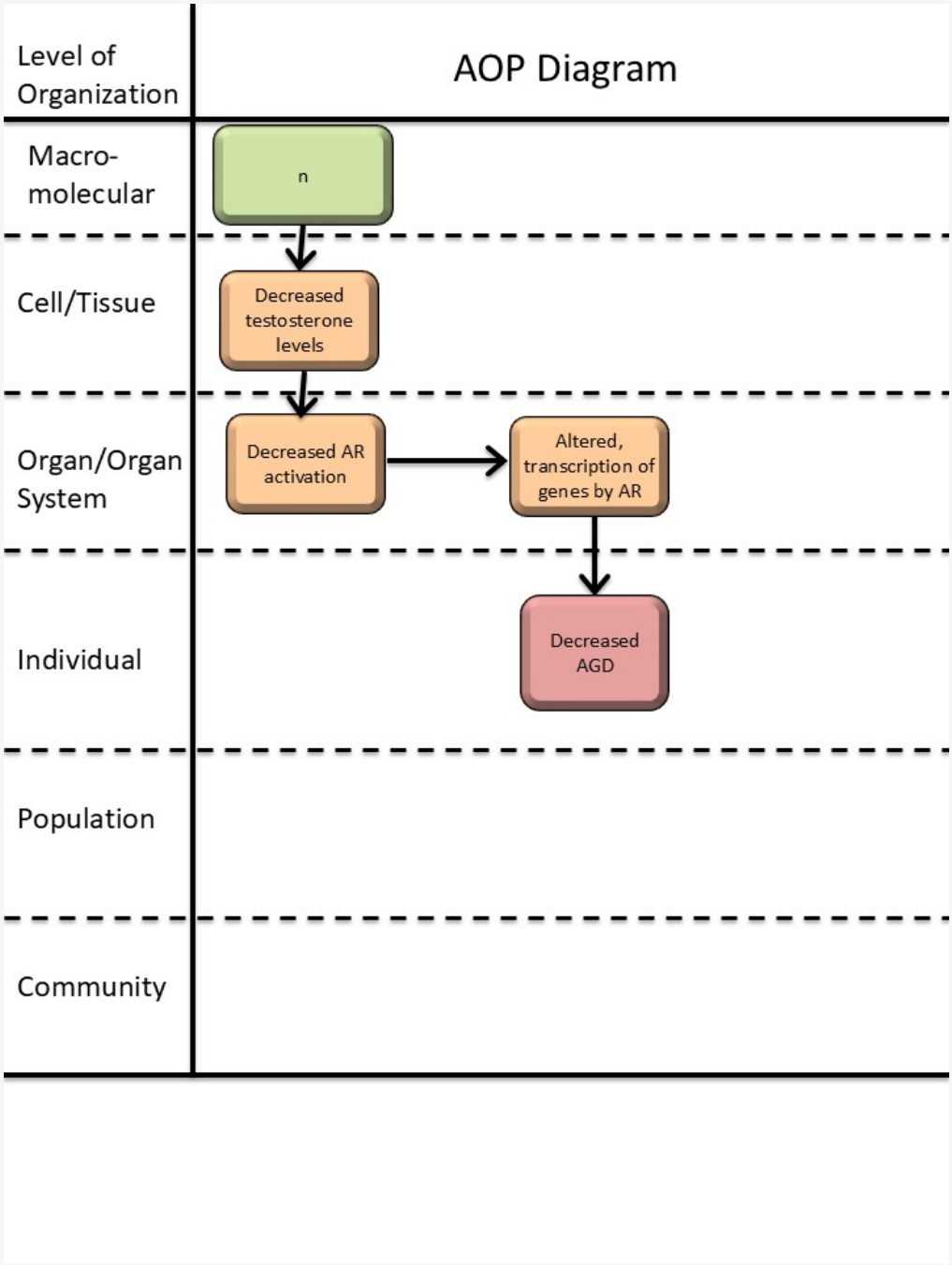
AOP ID and Title:

AOP 307: Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring
Short Title: Decreased testosterone synthesis leading to short AGD

Graphical Representation



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Status

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Abstract

This AOP links decreased testosterone levels with short anogenital distance (AGD) in male offspring. It does not yet contain an MIE, as the upstream events leading to 'reduced testosterone' synthesis in fetal testis can be many, for example by inhibiting various enzymes of the steroidogenesis pathway. The precursor molecule cholesterol is converted to testosterone via several enzymatic steps and includes, for instance, the CYP enzymes CYP11 and CYP17. Following synthesis, testosterone is released into the circulation and transported to target tissues and organs where it initiates masculinization processes, typically by binding to and activating the androgen receptor (AR) in target cells. Notably, testosterone can be converted to DHT by 5α-reductase, with DHT being a more potent AR agonist than testosterone; this testosterone-to-DHT conversion is critical during development for differentiation of male traits, including masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex (Davey and Grossmann, 2016; Keller et al, 1996; Robitaille and Langlois, 2020). The LABC complex does not develop in the absence, or low levels of, androgen signaling, as in female fetuses.

A short AGD around birth is a marker for feminization of male fetuses and is associated with male reproductive disorders, including reduced fertility in adulthood (Schwartz et al, 2019). Although a short AGD is not necessarily 'adverse' from a human health perspective, it is considered an 'adverse outcome' in OECD test guidelines; AGD measurements are mandatory in specific tests for developmental and reproductive toxicity in chemical risk assessment (TG 443, TG 421/422, TG 414), with measurement guidance provided in OECD guidance documents 43 (OECD, 2008) and 151 (OECD, 2013).

A central event in this pathway is inhibition of testosterone synthesis by fetal testes. In turn, this results in reduced circulating testosterone levels and less DHT (converted by 5α-reductase). Low DHT fails to properly activate AR in target tissues, including the developing perineal region, which leads to failure to properly masculinize the perineum/LABC complex and ultimately a short AGD.

Background

Androgen signaling is critical for male sex differentiation during fetal life and suboptimal action during critical life stages leads to under-masculinized offspring. Androgens, primarily testosterone and dihydro-testosterone (DHT), act by binding to and activating the AR is target cells. Blocking the AR basically blocks androgen signaling and masculinization of tissues and organs that otherwise should masculinize in male fetuses. One morphometric marker for reduced fetal androgen action is a shorter than normal anogenital distance.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	KE	1690	Decrease, testosterone levels	Decrease, 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	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Decrease, testosterone levels	adjacent	Decrease, androgen receptor activation	High	Moderate
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	Moderate	Low
Decrease, androgen receptor activation	non-adjacent	anogenital distance (AGD), decreased	High	Moderate

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Altered, Transcription of genes by the androgen receptor	non-adjacent	anogenital distance (AGD), decreased	Moderate	Low

Stressors

Name	Evidence
Dibutyl phthalate	High
Bis(2-ethylhexyl) phthalate	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Foetal	High
Pregnancy	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Male	High

The upstream part of the AOP, culminating at KE-286 (altered transcription of genes by the AR), has a broad applicability domain. It is built primarily on mammalian data and includes all life stages and both sexes. It could be extended to cover non-mammalian vertebrates by adding additional relevant knowledge, as previously discussed (Draskau et al, 2024). The overall applicability domain is limited by AO-1688 (decreased AGD). The AGD is strongly influenced by androgen action during critical fetal stages in mammals, with evidence from humans (Murashima et al, 2015; Thankamony et al, 2016), and from numerous gestational exposure studies in rats and mice to anti-androgenic chemicals (Gray et al, 2001; Schwartz et al, 2019). The male masculinisation programming window occurs at a developmental stage included in the applicability domain of these AOPs and corresponds to around gestational day 16-20 in rats and gestation weeks 8-14 in humans (Welsh et al, 2008). Only males are included in the applicability domain since the male AGD, but not the female AGD, is shortened by decreased androgen action (Schwartz et al, 2019).

Essentiality of the Key Events

The essentiality of each key event (KE) was evaluated, meaning that if an upstream KE is blocked or does not occur, subsequent downstream KEs or the adverse outcome (AO) are prevented or altered. Both direct and indirect evidence of essentiality were assessed according to the OECD developer's handbook, with a summary provided in Table 1.

Table 1: Essentiality assessment of KEs of AOP 307.

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

*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

Evidence for anti-androgenicity, by antagonizing the AR, is strong. In this AOP, most KERs are considered highly biologically plausible with strong empirical evidence in support of this assessment, both from human data and animal studies. The overall evidence assessment scores for each KER are summarized in the below Table:

ID	Assessment score	Rationale
KER-2131	High	It is well established that testosterone activates the AR and that decreased testosterone levels leads to decreased AR activation.
KER-2124	High	It is well established that the AR regulates gene transcription, and that decreased AR activity leads to altered gene transcription.
KER-2820	High	It is well established that decreased AR activity leads to decreased AGD in male offspring.
KER-2127	Moderate	It is highly plausible that altered gene transcription in the perineum leads to decreased AGD in male offspring.

Quantitative Consideration

The quantitative understanding between in vitro test data and in vivo is low. There is good quantitative understanding about the magnitude of reduction in explanted fetal testis testosterone production and effect on AGD (and other masculinization parameters) in rats, related to phthalate exposures. The dose-response relationship appears non-linear, with a low incidence rate of male under-virilization effects when testosterone production is reduced to around 46%, but with a steep increase in rate of malformations when testosterone is reduced by more than 75% (Earl Gray 2023; Earl Gray et al, 2024). This relationship has not been systematically evaluated for other chemicals.

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Appendix 1

List of Key Events in the AOP

Event: 1690: Decrease, testosterone levels

Short Name: Decrease, testosterone levels

Key Event Component

Process	Object	Action
hormone biosynthetic process	testosterone	decreased
	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

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
Mixed	High

This KE is applicable to mammals since the role of testosterone and its synthesis are conserved (Vitousek et al., 2018). Both sexes need, and produce, testosterone and its role is observed throughout different life stages, from development to adulthood (Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022). Therefore, this KE is also applicable to both males and females as well as throughout these life stages. Also of note, key enzymes needed for testosterone production first appear in the common ancestor of amphioxus and vertebrates (Baker 2011). Consequently, it is 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 beyond mammals to other vertebrates.

Key enzymes needed for testosterone production first appear in the common ancestor of amphioxus and vertebrates (Baker 2011). Consequently, this key event is applicable to most vertebrates, including humans.

Key Event Description

Testosterone is an endogenous steroid hormone and a potent androgen. Androgens act by binding androgen receptors in androgen-responsive tissues (Murashima et al., 2015). Testosterone and other androgens such as dihydrotestosterone (DHT) are important for reproductive development and masculinization of the fetus. Androgens are also important for bone, brain, muscle and skin health (Alemany, 2022). Just like other steroid hormones, testosterone is produced through a process known as steroidogenesis which is controlled by enzymes converting cholesterol into all of the downstream steroid hormones. In steroidogenesis, 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, DHT, by 5α-reductase, or aromatized by aromatase (CYP19A1) into estrogens. Testosterone secreted in blood circulation can be found free but more frequently is found bound to SHBG or albumin (Trost & Mulhall, 2016).

Testosterone is produced mainly by the ovaries (in females), testes (in males), and to a lesser degree in the adrenal glands. During fetal development testosterone plays a crucial role in 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. If testosterone reaches low levels, this axis is once again stimulated to provoke more testosterone synthesis. This feedback loop is essential for maintenance of appropriate testosterone levels (Chandrashekar & Bartke, 1998; Ellis et al., 1983; Rey, 2021).

Disruption of any of the aforementioned processes may result in reduced testosterone levels, such as inhibition of steroidogenic enzyme activity thereby inhibiting production of testosterone.

General role in biology

Androgens, the main male sex steroids, are the critical factors responsible for the development of the male phenotype during embryogenesis and for the achievement of sexual maturation at puberty. In adulthood, androgens remain essential for the maintenance of male reproductive function and behaviour. Apart from their effects on reproduction, androgens affect a wide variety of non-reproductive tissues such as skin, bone, muscle, and brain (Heemers,

Verhoeven, & Swinnen, 2006). Androgens, principally T and 5α-dihydrotestosterone (DHT), exert most of their effects by interacting with a specific receptor, the androgen receptor (AR), for review see (Murashima, Kishigami, Thomson, & Yamada, 2015). On the one hand, testosterone can be reduced by 5α-reductase to produce 5α dihydrotestosterone (DHT). On the other hand, testosterone can be aromatized to generate estrogens. Testosterone effects can also be classified by the age of usual occurrence, postnatal effects in both males and females are mostly dependent on the levels and duration of circulating free testosterone.

How it is Measured or Detected

Quantification of testosterone levels can be performed by various means (e.g. serum levels in vivo, cell culture medium levels in vitro, tissue ex vivo or in vitro). Traditional immunoassay methods (ELISA or RIA), and advanced instrumental techniques (e.g. LC-MS/MS) or liquid scintillation spectrometry (after radiolabeling) can be used (Shiraishi et al., 2008).

The H295R Steroidogenesis assay (OECD TG 456) is used to measure mainly the production of estradiol and testosterone. This is a validated OECD test guideline using adrenal H295R cells and hormone levels are then measured in the cell medium (OECD 2011). H295R adrenocortical carcinoma cells produce all the main enzymes and hormones of the steroidogenic pathway. Therefore, exposure to different stressors allows for broad analysis of their impact on steroidogenesis by measuring hormones in culture medium by LC-MS/MS. H295 assay was designed measure disruption to testosterone or estradiol levels but can now also be used to measure additional steroid hormones such as progesterone or pregnenolone. The U.S. EPA's ToxCast program developed a high throughput method for the H295R assay which can measure a total of 11 hormones from the steroidogenesis pathway (Haggard et al., 2018). The H295R can be considered an indirect measurement as it provides information on a disruption of overall steroidogenesis that would result in a change of testosterone levels but not the underlying mechanism.

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 are measured i.a. in: 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

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 offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	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). In this way, this KE is a placeholder for tissue-specific responses to AR activation or inactivation that will depend on the adverse outcome (AO) for which it is included.

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

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: 1688: anogenital distance (AGD), decreased

Short Name: AGD, decreased

Key Event Component

Process	Object	Action
androgen receptor signaling pathway	Musculature of male perineum	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	AdverseOutcome
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	AdverseOutcome

Stressors

Name
Butylparaben
p,p'-DDE
Bis(2-ethylhexyl) phthalate
Dexamethasone
Fenitrothion
Finasteride
Flutamide

Name
Ketoconazole
Linuron
Prochloraz
Procymidone
Triticonazole
Vinclozolin
di-n-hexyl phthalate
Dicyclohexyl phthalate
butyl benzyl phthalate
monobenzyl phthalate
di-n-heptyl phthalate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
perineum

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Foetal	High

Sex Applicability

Sex	Evidence
Male	High

A short AGD in male offspring is a marker of insufficient androgen action during critical fetal developmental stages (Schwartz et al, 2019; Welsh et al, 2008). A short AGD is thus a sign of undervirilization, which is also associated with a series of male reproductive disorders, including genital malformations and infertility in humans (Juul et al, 2014; Skakkebaek et al, 2001).

There are numerous human epidemiological studies showing associations with intrauterine exposure to anti-androgenic chemicals and short AGD in newborn boys alongside other reproductive disorders (Schwartz et al, 2019). This underscores the human relevance of this AO. However, in reproductive toxicity studies and chemical risk assessment, rodents (rats and mice) are what is tested on. The list of chemicals inducing short male AGD in male rat offspring is extensive, as evidenced by the ‘stressor’ list and reviewed by (Schwartz et al, 2019).

Key Event Description

The anogenital distance (AGD) refers to the distance between anus and the external genitalia. In rodents and humans, the male AGD is approximately twice the length as the female AGD (Salazar-Martinez et al, 2004; Schwartz et al, 2019). This sexual dimorphisms is a consequence of sex hormone-dependent development of secondary sexual characteristics (Schwartz et al, 2019). In males, it is believed that androgens (primarily DHT) activate AR-positive cells

in non-myotic cells in the fetal perineum region to initiate differentiation of the perineal *levator ani* and *bulbocavernosus* (LABC) muscle complex (Ipulan et al, 2014). This AR-dependent process occurs within a critical window of development, around gestational days 15-18 in rats (MacLeod et al, 2010). In females, the absence of DHT prevents this masculinization effect from occurring.

The involvement of androgens in masculinization of the male fetus, including the perineum, has been known for a very long time (Jost, 1953), and AGD has historically been used to, for instance, sex newborn kittens. It is now well established that the AGD in newborns is a proxy readout for the intrauterine sex hormone milieu the fetus was developing. Too low androgen levels in XY fetuses makes the male AGD shorter, whereas excess (ectopic) androgen levels in XX fetuses makes the female AGD longer, in humans and rodents (Schwartz et al, 2019).

How it is Measured or Detected

The AGD is a morphometric measurement carried out by trained technicians (rodents) or medical staff (humans).

In rodent studies AGD is assessed as the distance between the genital papilla and the anus, and measured using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) is often calculated by dividing AGD by the cube root of the body weight. It is important in statistical analysis to use litter as the statistical unit. This is done when more than one pup from each litter is examined. Statistical analyses is adjusted using litter as an independent, random and nested factor. AGD are analysed using body weight as covariate as recommended in Guidance Document 151 (OECD, 2013).

Regulatory Significance of the AO

In regulatory toxicology, the AGD is mandatory inclusions in OECD test guidelines used to test for developmental and reproductive toxicity of chemicals. Guidelines include ‘TG 443 extended one-generation study’, ‘TG 421/422 reproductive toxicity screening studies’ and ‘TG 414 developmental toxicity study’.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2131: Decrease, 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

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

KER2131 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

KER2131 is assessed applicable to both sexes, as T activates AR in both males and females.

Life-stage applicability

KER2131 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 (AIS), clinically low levels of T leads to reduced AR activation (either due to low T or DHT in target tissue), which manifests as both androgenic 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

Ligand-independent actions of the AR have 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	Moderate	Moderate
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent		
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

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: 2820: Decrease, AR activation leads to AGD, decreased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human, mouse, rat	human, mouse, rat	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Fetal to Parturition	High

Sex Applicability

Sex	Evidence
Male	High

Taxonomic

Fetal masculinization including the AGD is regulated by androgens interacting with the AR in all mammals, including humans (Murashima et al., 2015; Thankamony et al., 2016), although, the size of the AGD and difference between the sexes vary between species. A large number of studies exist showing that fetal exposure to anti-androgens causes shortened AGD in male rats and mice (Schwartz et al., 2019, see also Table 2). Some epidemiological studies find associations between exposure to anti-androgenic compounds and shorter AGD in boys (Thankamony et al., 2016). However, the associations are not very clear and confidence in the data is limited by conflicting results, possibly due to differences in study design and methods for exposure measurements and analyses. Nevertheless, the KER is considered applicable to humans, based on current understanding of the role of AR activation in fetal masculinization.

Life stage

Programming of the AGD occurs during the masculinization programming window in fetal life. This takes place in rats around embryonic days 15.5-19.5 (GD16-20) and likely gestation weeks 8-14 in humans (Welsh et al., 2008). It should be mentioned that though AGD is believed to be relatively stable throughout life, it can be responsive to postnatal changes in androgen levels (Schwartz et al., 2019).

Sex

Data presented in this KER support that disruption of androgen action during fetal life can lead to a short AGD in male offspring. While exposure to chemicals during fetal life can also shorten female AGD, the biological significance and the mechanism driving the effect is unknown (Schwartz et al., 2019).

Key Event Relationship Description

This KER refers to a decrease in androgen receptor (AR) activation during fetal development leading to decreased anogenital distance (AGD) in male 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 androgen receptor in vivo, while most methods that can be used to measure AR activity are carried out in vitro. Indirect information about this KE may for example be provided from assays showing in vitro AR antagonism, decreased in vitro or in vivo testosterone production/levels or decreased in vitro or in vivo dihydrotestosterone (DHT) production/levels.

Evidence Supporting this KER

Biological Plausibility

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

- 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 that in turn drives masculinization of the male fetus (Welsh et al., 2014; Schwartz et. al, 2019).

- Fetal masculinization depends on activation of androgen signaling during a critical time window, the masculinization programming window (MPW), from gestational day (GD) 15.5-18.5 in rats, 14.5-16.5 in mice and presumably gestation weeks (GWs) 8-14 in humans (Welsh et al., 2008; Amato et al., 2022). The onset of AR expression in the tissues of the reproductive tract follows the timing of the MPW (Welsh et al., 2008).

- The fetal masculinization process involves a range of tissues and organs, including the perineum. Perineum length can be measured as the AGD, which is the distance between the anus and the genitalia. The AGD is approximately twice as long in male as in female newborn rodents and humans (Schwartz et al., 2019).

- Male AR knockout mice present shorter AGD than wildtype males, so short that it is indistinguishable from wildtype female littermates (Yeh et al., 2002, Sato et al., 2004).

- In human males, mutations decreasing AR activity also lead to feminization. One example is the androgen insensitivity syndrome (AIS), where mutations in the AR lead to an impaired or abolished response to androgens, and thereby some degree of feminization of XY individuals and even XY sex reversal in individuals with complete AIS (CAIS) (Thankamony et al., 2016; Hughes et al., 2012; Crouch et al., 2011). XY individuals with CAIS present as women with internally placed testes. A study showed that the clitoral to urethral distance in these individuals was similar to a control group of women, but it is not clear whether this measurement can work as a proxy for measuring the AGD (Thankamony et al 2016, Crouch 2011). Unfortunately, it seems the AGD has not at present been measured in CAIS individuals. Another example is human males lacking 5-alpha-reductase, also presenting female-like genitalia (Batista & Mendonca, 2022).

- The detailed mechanism by which androgens regulate the AGD is not known but it is hypothesized that the AGD is influenced by the size of the levator-ani and bulbocavernosus (LABC) muscle complex in the perineum. The growth of this complex is stimulated by AR activation, it is sexually dimorphic and larger in males than in females and (Schwartz et al., 2019). AR is required for the development of the LABC complex as demonstrated by AR general and muscle specific knockout mice. AR is expressed in non-myocytic cells in the LABC complex, starting at E15.5 in mice, and knockout of AR in these cells results in defects in the muscle formation (Ipulan et al., 2016;). Differential gene expression profiles in the perineum of male and female rats as well as in antiandrogen-exposed male rats have been identified providing further mechanistic understanding (Schwartz et al, 2019; Draskau et al, 2022).

Empirical Evidence

Animal *in vivo* data

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

It should be noted that the KE decreased androgen receptor activation (KE-1614 in AOP Wiki) specifically focuses on decreased activation of the androgen receptor 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 five 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 MIE26. Finasteride inhibits the 5-alpha-reductase enzyme that converts testosterone to DHT, see MIE1617. DEHP exposure during prenatal development in rats results in reduced fetal testosterone levels, see KE1690. (MIE26, MIE1617 and KE1690 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 not evidence for both KEs from the same study. However, decreased testosterone levels can be measured in vivo, and Borch et al., 2004 measured the effect of developmental DEHP exposure on both testosterone levels and AGD (see section about “Dose concordance”).

The empirical animal evidence for the five substances is summarized in table 3.

Table 3. Summary of empirical evidence for decreased androgen receptor activation, leading to decreased male AGD. References for the studies supporting the empirical evidence are found in section “Evidence for decreased AR activation (KE1614) by flutamide, procymidone, and vinclozolin, finasteride and DEHP” and in table 2.

Stressor(s)	Upstream effect (decreased AR activation)	Downstream effect (decreased male AGD)

Flutamide	AR antagonism in in vitro assay receptor binding and transactivation assays	Decreased male AGD after prenatal exposure in studies in rat
Procymidone	AR antagonism in in vitro assay receptor binding and transactivation assays	Decreased male AGD after prenatal exposure in studies in rat
Vinclozolin	AR antagonism in in vitro assay receptor binding and transactivation assays	Decreased male AGD after prenatal exposure in studies in rat and mouse
Finasteride	Inhibition of 5-alpha-reductase enzyme in in vitro assays	Decreased male AGD 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	Decreased male AGD after prenatal exposure in studies in rat

From table 3, it can be deducted that fetal exposure to substances known to decrease androgen receptor activation through antagonism of the AR (vinclozolin, procymidone, flutamide), inhibition of testosterone synthesis (DEHP) or inhibition of conversion of testosterone to DHT (finasteride), results in decreased AGD in rat and mouse male offspring.

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

Flutamide, a pharmaceutical, binds the AR and inhibits the receptor activity, thereby acting as an AR antagonist. It has been used as an antiandrogen for 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 COS 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 et al., 1997) or AR transfected COS-1 cells (Wong et al., 1995).
Vinclozolin also inhibits agonist activated transcription in AR reporter assays (Euling et al, 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 & Russel, 1990; Rittmaster & Wood, 1994; Stoner, 1990).

Prenatal exposure to DEHP in rats results in 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 (Borch et al., 2004; Borch et al., 2006; Culty et al., 2008; Hannas et al., 2011; Hannas et al., 2012; Klinefelter et al., 2012; Parks et al., 2000; Wilson et al., 2004; Wilson et al., 2007; Vo et al., 2009). Two studies don't show an effect on testosterone levels in testis or fetal plasma testosterone levels, respectively (Andrade et al., 2006; Borch et al., 2006). The precise underlying mechanism is presently unknown.

Evidence for decreased AGD in males (KE1688) by prenatal exposure to flutamide, procymidone, vinclozolin, finasteride and DEHP

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 a decreased male AGD. The conclusion was that the level of confidence was strong for all five substances. The studies are summarized in table 4.

Empirical evidence for the included substances

Table 4. Empirical evidence for decreased AGD in males (KE1688) by prenatal exposure to flutamide, procymidone, vinclozolin,

finasteride and DEHP. *One dose only.

>>>>>TABLE 4<<<<<

Species	Exposure window	Measurement timepoint	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Reference
Flutamide					
rat	GD12-21	PND1 and PND100	No	6.25	McIntyre et al., 2001
rat	GD16, 17, 18 or 19	PND1 and PND100	--*	50	Foster & Harris, 2005
rat	GD7-21	PND1	No	0.5	Hass et al., 2007
rat	GD6-17 + GD16-21	GD21	No	3	Goto et al., 2004
rat	GD6-PND4	PND4	0.4	2	Yamasaki et al., 2005
rat	GD6-PND1	PND1	0.25	2.5	Fussell et al., 2015
rat	GD13-20	PND4 and PND23	--*	20	Kita et al., 2016
rat	GD11-21	PND 14, 21 and 120	--*	5 mg per rat	Casto et al., 2003
Procymidone					
rat	GD7-PND16	at birth, GD22-24	No	12.5	Hass et al., 2012
rat	GD7-PND16	at birth, GD22-24	10	25	Hass et al., 2007
rabbit	GD6-28	GD29	125	No effect	Inawaka et al., 2010
rat	GD14-PND3	PND2	No	25	Ostby et al., 1999
Vinclozolin					
Rat	GD16-17 + GD18-19	PND1	--*	400	Wolf et al., 2000
Rat	GD14-19	PND1	No	200	Wolf et al., 2000
Rat	GD7-21	PND1	5	10	Hass et al., 2007
Mouse	GD10-18	PND1 and 7	--*	100	Shimamura et al., 2002
Rat	GD4-PND3	PND2	No	3.125	Gray et al., 1994
Finasteride					
rat	GD12-21	PND1 and PND90	No	0.01	Bowman et al., 2003
rat	GD7-21	PND0	0.01	0.1	Christiansen et al., 2009
rat	GD15-21	PND1	0.0003	0.03	Clark et al., 1993
rat	GD15-21	PND22 and PND114-117	0.03	3	Clark et al., 1993
rat	GD12-21	PND1 and PND90	--*	10	Martinez et al., 2011

Epidemiological data on DEHP

The biggest relevant epidemiological dataset was identified on associations between DEHP and AGD.

Six prospective cohort studies and one cross-sectional study on the association between maternal DEHP metabolites and length of AGD (anopenile distance (APD) and anoscrotal distance (ASD)) in boys were assessed as reliable without or with restriction. Decreased AGD (anopenile distance (APD) and/or anoscrotal distance (ASD)) was observed in three prospective cohort studies (Martino-Adrade et al., 2016; Swan et al., 2005 reviewed and updated in Swan 2008; Wenzel et al., 2018). In contrast, no significant association was observed in three other prospective cohort studies (Arbuckle et al., 2018; Henriksen et al., 2023; Jensen et al., 2016) and the cross-sectional study (Sunman et al., 2019). This inconsistency introduces a level of uncertainty regarding the overall association. Therefore, the level of confidence was judged as weak.

Dose concordance

Dose concordance is challenging to assess for this KER since in vivo AR activity is currently not possible to measure, but only

can be informed indirectly by measures of upstream events.

However, some studies provide useful information that support dose concordance between the KEs.

In a publication by Borch et al., rats were exposed in utero to DEHP at GD7-21. Fetal testosterone levels in testes and serum and testosterone production in fetal testes ex vivo were investigated at GD21, whereas AGD was investigated at PND3. The LOAELs for reduced testosterone production in ex vivo fetal testes and reduced testosterone levels in fetal testes were 300 mg/kg/d, whereas the LOAEL for decreased AGD in male offspring was 750 mg/kg/d (Borch et al., 2004).

In a publication by Scholze et al, AR antagonism and decreased testosterone synthesis was quantitatively assessed (IC50) in vitro for a list of substances. In addition, internal concentrations in male fetuses and effects on AGD were measured after fetal exposure to the same substances. In utero exposure to all the substances lead to reduced AGDIndex (AGDI) in the exposed male offspring. Further, for all substances except Cyprodinil, the internal exposure levels in the fetuses leading to reduced AGD exceeded the IC50 levels observed in one or both of the in vitro assays.
Three different doses of linuron exposure were included. The medium exposure dose led to a higher level of internal exposure and a higher degree of AGDI reduction than the low dose. AGDI could not be determined in the highest dose due to maternal toxicity (Scholze et al., 2020).

Temporal concordance

Temporal concordance can only be considered from a theoretical perspective since the downstream event, decreased AGD, is usually measured at GD21, PND0 or PND1 in rats, and due to the size of the fetuses is not feasible to measure at earlier timepoints.

Considering the biology, the upstream event – decreased AR activation *in vivo* – is foreseen to happen minutes to hours after exposure. 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, though it is uncertain exactly when the change will be big enough to be measurable. On the other hand, the downstream event – decreased AGD - is a measurement of relative growth of the perineal tissue, which is expected to take days in the developing fetus.

Uncertainties and Inconsistencies

For the model substances, 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.

Species differences in effects of phthalates (including DEHP and DBP) on fetal testes testosterone production have been observed between humans, mice and rats. In human fetal testes exposed to DEHP or DBP in vitro or ex vivo, no suppression of testosterone production is observed, which contrasts observations in rat fetal testes under similar conditions. Also in mice, testosterone production in the fetal testes is unaffected by treatment with DEHP or DBP in vitro or in utero (Sharpe, 2020).

The species differences described above are specific for some phthalates and their interference with fetal testicular testosterone production. This uncertainty should not be reflected on other antiandrogenic substances, especially not those acting through other mechanisms of action.
The association between exposure to DEHP and reduced AGD in humans is judged to be weak, which may further support a species difference between rodents and humans, but it may also reflect the large uncertainties inherent in the epidemiological studies.

Observational epidemiological studies face challenges in proving cause-effect relationships as they cannot control conditions like experimental animal and in vitro studies. Human studies can identify associations between variables but cannot offer conclusive proof of causation (Lanzoni et al., 2019). Various study designs and statistical methods are employed to strengthen evidence within the inherent limitations of observational research (Song & Chung, 2010; Olier et al., 2023). Inconsistencies in epidemiological data arise from various factors, such as different methodologies used in exposure and outcome measurement and also in statistical analyses.

These differences collectively contribute to the complexity of interpreting and weighing the evidence in epidemiological research.

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 event in the same study.

Response-response relationship

In one study, a quantitative model was developed to predict the decrease in AGD from in vitro AR antagonism or in vitro decreased testosterone synthesis. The authors conclude that predicting the effect on AGD in vivo based on the in vitro results is only possible on a qualitative level, but the model cannot predict AGD reductions quantitatively (Scholze et al., 2020).

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 growth of a tissue (in this case the perineum) is closer to days and weeks depending on species. For instance, in humans, the masculinization programming window is presumed to start around GW 8, while a sexual dimorphism of the AGD can first be observed from around GWs 11-13 (Thankamony et al., 2016) and reaches its maximum 2-fold difference around GWs 17-20 (Sharpe, 2020).

It has been demonstrated that exposure to flutamide for one day (Foster & Harris, 2005) or vinclozolin for two days (Wolf et al., 2000) during the sensitive window of exposure can elicit a detectable decrease in the AGD in male rat offspring.

Known modulating factors

A well established modulating factor 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 (Tut et al 1997, Chamberlain et al 1994) and a shorter AGD in adult men (Eisenberg et al., 2013). Other modulating factors being discussed in the literature is maternal age and parity (Barrett et al., 2014), but these associations are only suggestive with more studies needed to confirm the associations (Barrett et al., 2014).

Known Feedforward/Feedback loops influencing this KER

Not relevant for this KER.

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Relationship: 2127: Altered, Transcription of genes by the AR leads to AGD, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	Moderate	
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	Moderate	
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	Moderate	Low