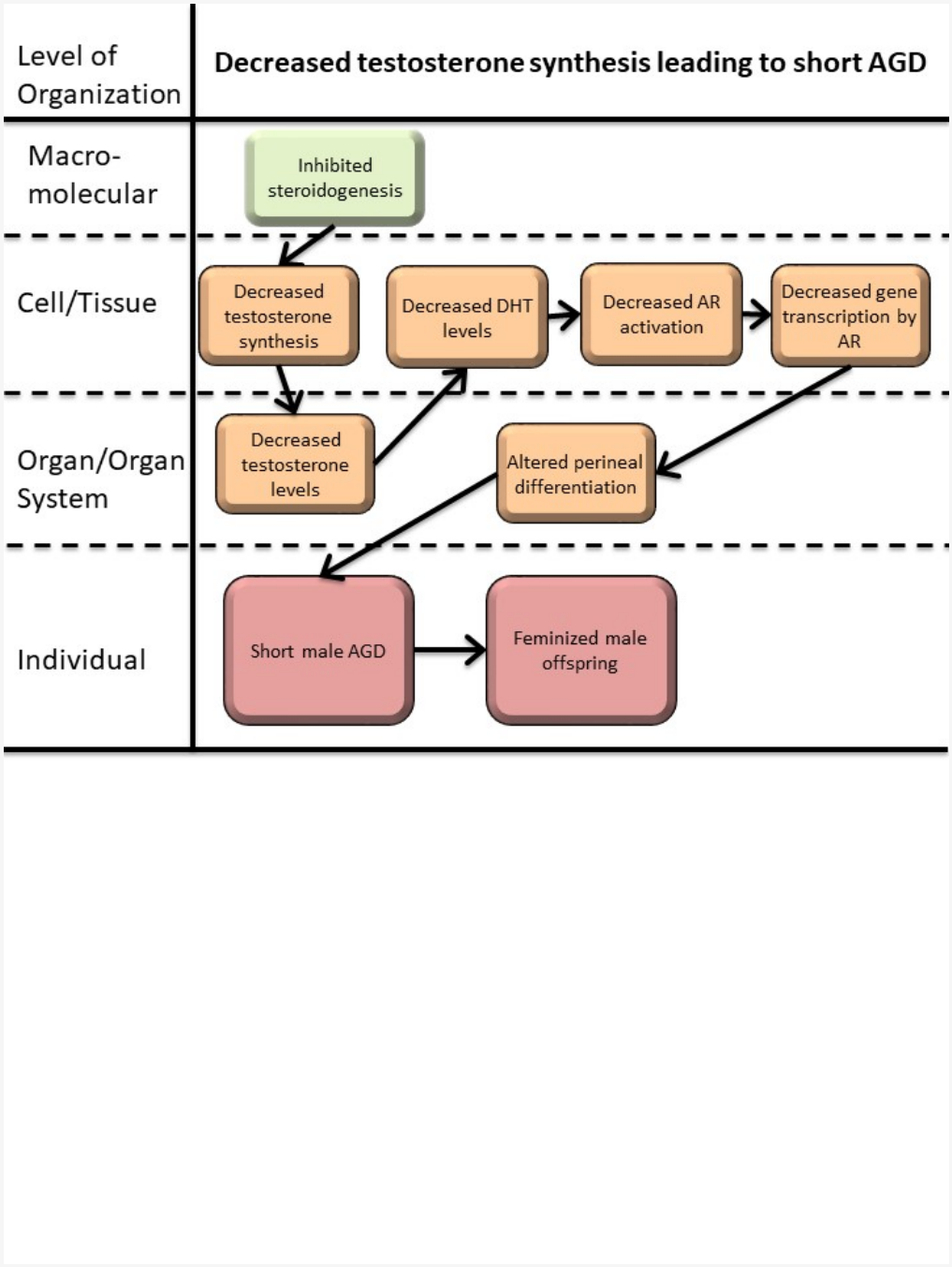
AOP ID and Title:

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

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.90	Included in OECD Work Plan

Abstract

This AOP links decreased testosterone synthesis by fetal Leydig cells with short anogenital distance (AGD) in male offspring. 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. 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).

Testosterone is primarily synthesized by fetal Leydig cells of the fetal testes by the process of steroidogenesis. The precursor molecule cholesterol is converted to testosterone via several enzymatic steps and includes for instance key 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. Under normal physiological conditions, testosterone produced by the testicles, is converted in peripheral tissues by 5α-reductase into DHT, which in turn binds AR and activates downstream target genes. AR signaling is necessary for masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex in males. The LABC complex does not develop in the absence, or low levels of, androgen signaling, as in female fetuses.

The key events in this pathway is inhibition of testosterone synthesis in the fetal Leydig cells. 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.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	KE	413	Reduction, Testosterone synthesis in Leydig cells	Reduction, Testosterone synthesis in Leydig cells
	KE	1690	Decrease, testosterone levels	Decrease, testosterone levels
	KE	1613	Decrease, dihydrotestosterone (DHT) level	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	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Reduction, Testosterone synthesis in Leydig cells	adjacent	Decrease, testosterone levels	High	Moderate
Decrease, testosterone levels	adjacent	Decrease, dihydrotestosterone (DHT) level	Moderate	Low
Decrease, dihydrotestosterone (DHT) level	adjacent	Decrease, androgen receptor activation	High	Moderate
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	Moderate
Altered, Transcription of genes by the androgen receptor	adjacent	anogenital distance (AGD), decreased	Moderate	Moderate
Decrease, testosterone levels	non-adjacent	Decrease, androgen receptor activation	Moderate	Moderate
Decrease, androgen receptor activation	non-adjacent	anogenital distance (AGD), decreased		

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

References

1. Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U and**Svingen T** (2019), Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch Toxicol* 93: 253-272.

Appendix 1

List of Key Events in the AOP

Event: 413: Reduction, Testosterone synthesis in Leydig cells

Short Name: Reduction, Testosterone synthesis in Leydig cells

Key Event Component

Process	Object	Action
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males	KeyEvent
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent

Biological Context

Level of Biological Organization

Level of Biological Organization

Cell term

Cell term
testosterone secreting cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
mice	Mus sp.	Low	NCBI

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

Biological state

Testosterone is a steroid hormone from the androgen group and is found in humans and other vertebrates.

Biological compartments

In humans and other mammals, testosterone is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females and other steroidogenic tissues (e.g., brain, adipose). It either acts locally /or is transported to other tissues via blood circulation. Testosterone synthesis takes place within the mitochondria of Leydig cells, the testosterone-producing cells of the testis. It is produced upon stimulation of these cells by Luteinizing hormone (LH) that is secreted in pulses into the peripheral circulation by the pituitary gland in response to Gonadotropin-releasing hormone (GnRH) from the hypothalamus. Testosterone and its aromatized product, estradiol, feed back to the hypothalamus and pituitary gland to suppress transiently LH and thus testosterone production. In response to reduced testosterone levels, GnRH and LH are produced. This negative feedback cycle results in pulsatile secretion of LH followed by pulsatile production of testosterone (Ellis, Desjardins, and Fraser 1983), (Chandrashekar and Bartke 1998).

General role in biology

Testosterone is the principal male sex hormone and an anabolic steroid. Male sexual differentiation depends on testosterone (T), dihydrotestosterone (DHT), and the expression of androgen receptors by target cells (Manson and Carr 2003). During the development secretion of androgens by Leydig cells is essential for masculinization of the foetus (Nef 2000). The foetal Leydig cells develop in utero. These cells become competent to produce testosterone in rat by gestational day (GD) 15.5, with increasing production thereafter. Peak steroidogenic activity is reached just prior to birth, on GD19 (Chen, Ge, and Zirkin 2009). Testosterone secreted by foetal Leydig cells is required for the differentiation of the male urogenital system late in gestation (Huhtaniemi and Pelliniemi 1992). Foetal Leydig cells also play a role in the scrotal descent of the testis through their synthesis of insulin-like growth factor 3 (Insl3), for review see (Nef 2000).

In humans, the first morphological sign of testicular differentiation is the formation of testicular cords, which can be seen between 6 and 7 weeks of gestation. Steroid-secreting Leydig cells can be seen in the testis at 8 weeks of gestation. At this period, the concentration of androgens in the testicular tissue and blood starts to rise, peaking at 14-16 weeks of gestation. This increase comes with an increase in the number of Leydig cells for review see (Rouiller-Fabre et al. 2009).

Adult Leydig cells, which are distinct from the foetal Leydig cells, form during puberty and supply the testosterone required for the onset of spermatogenesis, among other functions. Distinct stages of adult Leydig cell development have been identified and characterized. The stem Leydig cells are undifferentiated cells that are capable of indefinite self-renewal but also of differentiation to steroidogenic cells. These cells give rise to progenitor Leydig cells, which proliferate, continue to differentiate, and give rise to the immature Leydig cells. Immature Leydig cells synthesize high levels of testosterone metabolites and develop into terminally differentiated adult Leydig cells, which produce high levels of testosterone. With aging, both serum and testicular testosterone concentrations progressively decline, for review see (Nef 2000).

Androgens play a crucial role in the development and maintenance of male reproductive and sexual functions. Low levels of circulating androgens can cause disturbances in male sexual development, resulting in congenital abnormalities of the male reproductive tract. Later in life, this may cause reduced fertility, sexual dysfunction,

decreased muscle formation and bone mineralisation, disturbances of fat metabolism, and cognitive dysfunction. Testosterone levels decrease as a process of ageing: signs and symptoms caused by this decline can be considered a normal part of ageing.

How it is Measured or Detected

OECD TG 456 [1] is the validated test guideline for an in vitro screen for chemical effects on steroidogenesis, specifically the production of 17ß-estradiol (E2) and testosterone (T). The testosterone syntheis can be measured in vitro cultured Leydig cells. The methods for culturing Leydig cells can be found in the Database Service on Alternative Methods to animal experimentation (DB-ALM): Leydig Cell-enriched Cultures [2], Testicular Organ and Tissue Culture Systems [3].

Testosterone synthesis in vitro cultured cells can be measured indirectly by testosterone radioimmunoassay or analytical methods such as LC-MS.

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Event: 1690: Decrease, testosterone levels

Short Name: Decrease, testosterone levels

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) stem Leydig cells leads to Impaired, Spermatogenesis	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

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. 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

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.

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.

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Event: 1613: Decrease, dihydrotestosterone (DHT) level

Short Name: Decrease, DHT level

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: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:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:527 - Decreased Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) stem Leydig cells leads to Hypospadias, increased	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

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

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: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

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

Name
Bis(2-ethylhexyl) phthalate
Dexamethasone
Fenitrothion
Finasteride
Flutamide
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: 2125: Reduction, Testosterone synthesis in Leydig cells leads to Decrease, testosterone levels

AOPs Referencing Relationship

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

Relationship: 2126: Decrease, testosterone levels leads to Decrease, DHT level

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

T and DHT are androgens present in all vertebrates. They play a role in development and fertility in both males and females (Ogino et al., 2011; Prizant et al., 2014; Rey, 2021; Swerdloff et al., 2017). All tissues expressing 5α-reductase are applicable to this KER (Azzouni et al., 2012).

Key Event Relationship Description

Testosterone (T) and dihydrotestosterone (DHT) are androgens that are involved in numerous developmental and functional processes across animal taxa. In vertebrates, testosterone can be aromatized into estrogens catalyzed by the enzyme aromatase (CYP19) or be metabolized to DHT by the enzyme 5α-reductase (Azzouni et al., 2012; Naamneh Elzenaty et al., 2022; Swerdloff et al., 2017). Both T and DHT binds to the androgen receptor (AR), but with different affinities. DHT has a higher affinity for the AR than T. DHT also has a longer half-life and slower dissociation rate than T and cannot be aromatized into estrogens (Gerald & Raj, 2022; Naamneh Elzenaty et al., 2022; Swerdloff et al., 2017).

During mammalian development, T is primarily produced by the fetal testes and is needed for differentiation of the Wolffian ducts, the epididymis, and the ejaculatory duct. In pubertal and adult mammals, T is produced by the testes, the ovaries (although at a much lower level), and the adrenal glands (Ogino et al., 2011; Rey, 2021). In peripheral tissues (i.e. relative to the testes), DHT is converted from T by 5α-reductase to induce the differentiation of the urogenital sinus and genital tubercle to form the prostate, penis, scrotum and urethra (Swerdloff et al., 2017). Both

androgens are essential for masculinization, sexual development, and fertility.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

It is well established that DHT is synthesized from circulating T. 5α-reductase is the enzyme responsible for the conversion of T into DHT. Multiple isoforms of this enzyme are expressed in different tissues. Expression of 5α-reductase in peripheral tissues dictates where DHT will be formed from circulating T (Azzouni et al., 2012; Swerdloff et al., 2017).

Since T can be converted to DHT, it stands to reason that a lack of T can lead to a lack of DHT. Therefore, if there is a marked reduction in the availability of T, it can be surmised that DHT levels are consequently affected. However, to what extent T needs to be diminished in tissues before there is a functionally relevant decrease in DHT is largely unknown. In addition, the quantitative relationship between substrate (T) availability and levels of synthesized DHT is not well characterized in tissues *in vivo*. Notably, DHT can be produced via other steroid intermediates through the 'backdoor pathway' in mammals such as marsupials and humans (Renfree & Shaw 2023).

Empirical Evidence

The empirical evidence for this KER is considered moderate

As per Table 1, empirical data exists for effects on both T and DHT following chemical exposures, but it is not always possible to deduce if the reduction in DHT is a direct consequence of reduced T or because of other mechanisms such as e.g. interference with 5α-reductase. However, some studies do include 5α-reductase mRNA expression or measure the ratio of T/DHT which if unchanged, indicates that the decrease would most likely be due to decrease in T availability.

Table 1

Compound	Species	Effect level	KE: testosterone, decrease	KE: DHT, decrease	Details	References
DEHP	rat	LOEL = 117 mg/kg/day	Significant decrease day 1, 2, 3: from 4 to 2 ng/testis	Significant decrease day 1 from 2.5 to 1 ng/testis	In utero exposure, fetal testes ex vivo from GD20 rats.	(Culty et al., 2008)
Ibuprofen	human	One concentration tested: 10^{-5}M	48h: significant decrease -36.8%	48h: significant decrease -70.2%	Human fetal testes explants, measurements were done using the exposure media. No effect on SRD5A3 mRNA levels (5α-R3)	(Ben Maamar et al., 2017)
Rosiglitazone	human	One concentration tested: 8mg/day	significant decrease of production rates 318±±62 µg/h to 272±7±72 µg/h	significant decrease of production rates 21±±6 µg/h to 17±±5 µg/h	Serum levels after 7 days of treatment in healthy men: "Calculated from the product of the known infusion rate (Rt) and the ratio of tracer infusate enrichment (Et) to tracer dilution in the plasma" Ratio T/DHT remained unchanged.	(Vierhapper et al., 2003)

Dose concordance:

All the exposure data shown above indicates dose-concordance, since the same concentration tested affects both the upstream and downstream key event.

Other evidence

One study focused on the condition Leydig cell hypoplasia (LCH) in one patient. This patient had mutations in the LHCGR, and when measuring the levels of testosterone and DHT before and after hCG stimulation a decrease in both levels under the normal range were observed, even with hCG stimulation (Xu et al., 2018).

PTU	rats	One concentration tested: 240 mg/kg/day	significant decrease ˜2ng/ml to 0.15ng/ml	significant decrease ˜0.5ng/ml to 0.17ng/ml	Oral exposure of 14day old rats treated until day 51. Serum testosterone and DHT measured	(Marty et al., 2001)(
Dibutyltin	Carp fish	One concentration tested: 100µM	significant decrease -16%	significant decrease -24%	Gonad microsomes. Dibultyltin **inhibited 5α-reductase**, whichdecreases possibility that this is solely due to decrease of testosterone	(Thibaut & Porte, 2004)
TCDD	rats	Effects observed at 15µg/kg	significant decrease -90%	significant decrease -75%	Oral exposure of 66-68 day old rats. Serum or plasma measurements. Dose dependent decrease of both was observed. Ratio T/DHT indicates effect is due to reduced testosterone.	(Moore et al., 1985)

Uncertainties and Inconsistencies

The levels of T do not always reflect the levels of DHT. T is also converted to estradiol (Naamneh Elzenaty et al., 2022). Therefore, the decrease in T may lead to a decrease in estradiol while DHT levels remain unchanged.

Several studies have shown the existence of an alternative ('backdoor') pathway for DHT synthesis that is independent of T in marsupials and humans, but not in rodents (Marilyn B. Renfree et al., 1995). Instead of proceeding through the canonical pathway, progesterone or 17-OH progesterone, can be converted into allopregnanolone and 17OH-allopregnanolone. 17-OH allopregnanolone is then converted into androsterone leading to androstanediol that can finally be oxidized to produce DHT. Therefore, through this pathway, DHT can be synthesized without the presence of T (Auchus, 2004; Miller & Auchus, 2019).

Quantitative Understanding of the Linkage

Response-response relationship

The response-response relationship is not clearly established.

Time-scale

Different time scales have been observed in the studies above, the shortest one found being 48h. With Ibuprofen treatment, a decrease in both testosterone and DHT was observed after 48h in human fetal explant's exposure media (Ben Maamar et al., 2017). However, it is not evident that this effect is direct and only due to a decrease in T.

Known Feedforward/Feedback loops influencing this KER

Activity of 5α-reductase type 1 and 2: The activity of this enzyme determines how much T is converted into DHT. There are two isomers, with type 2 being the primary isomer expressed in DHT target organs. In deficiencies of this enzyme, there are studies that observe maintained DHT levels. This indicates that the type 1 enzyme can take over if needed (Azzouni et al., 2012).

Conversion of T to estradiol (E2): Aromatase can convert T into estrogens. The activity of this enzyme may push towards a decrease of T levels and an increase in estrogen levels without necessarily affecting DHT levels (Naamneh Elzenaty et al., 2022).

Hypothalamus-pituitary-gonadal (HPG) axis: Like most sex steroids, T production is controlled by the HPG axis during puberty and adulthood, but also during certain periods of development. For humans, the HPG axis is active following birth between 1-3 months in both males and females. Increase of LH and FSH are observed in infants up to 4-6months old. This stage is also known as the minipuberty (Lanciotti et al., 2018; Renault et al., 2020). Once GnRH is released from the hypothalamus, the pituitary gland secretes LH in pulses, which then stimulates the cells in the testes to produce T. A negative feedback loop can then occur, where testosterone then inhibits the release of GnRH and LH, in turn slowing down T production (Gerald & Raj, 2022; Naamneh Elzenaty et al., 2022; Nef & Parada, 2000).

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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
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent		

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)
Age	AR expression changes with aging	Tissue-specific alterations in AR activity with aging	(Supakar et al., 1993; Wu et al., 2009)

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
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
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

This KER is applicable 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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Relationship: 2127: Altered, Transcription of genes by the AR leads to AGD, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	Moderate

List of Non 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
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	Moderate	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: 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		