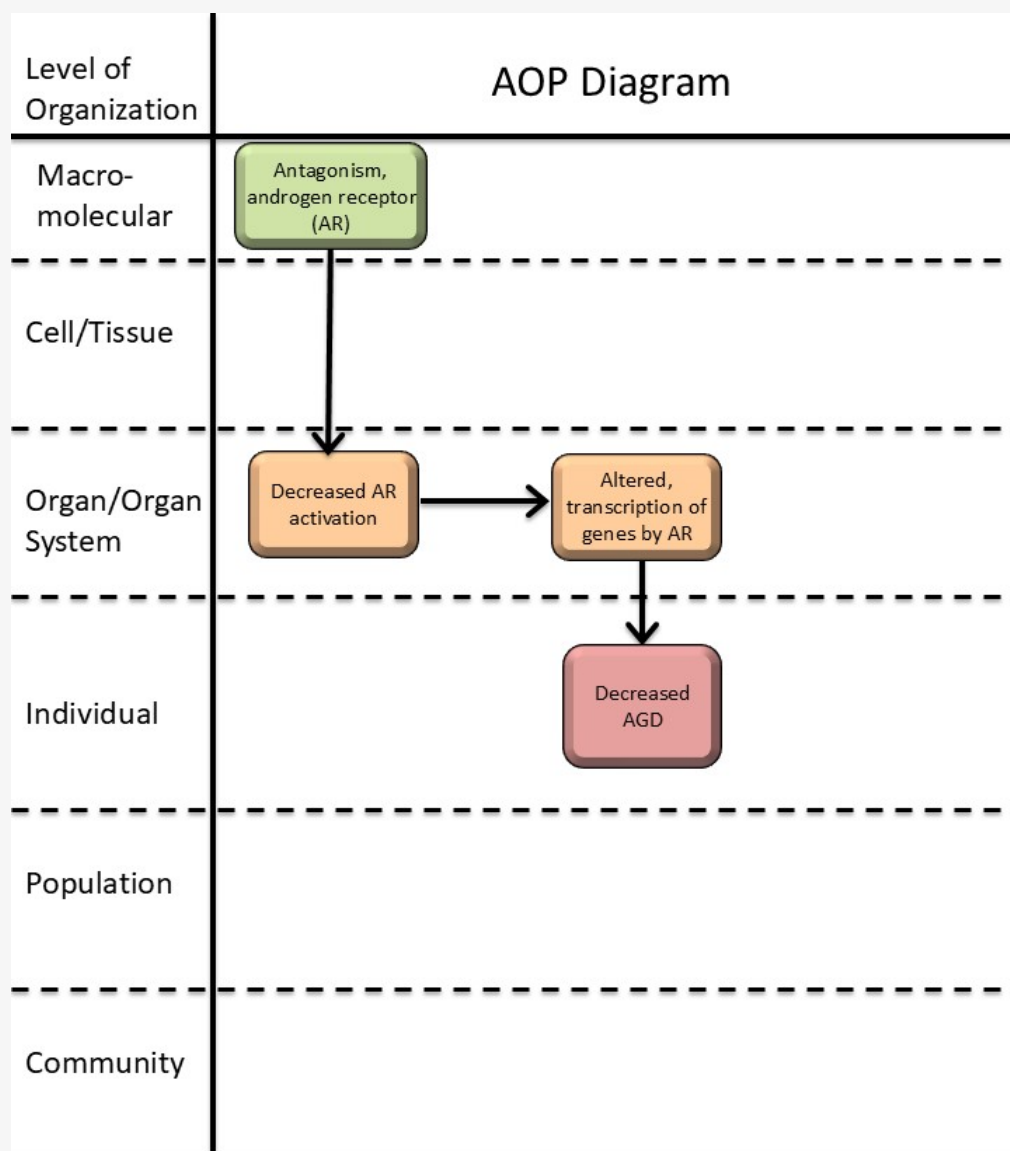


AOP ID and Title:

AOP 305: 5 α -reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring

Short Title: 5 α -reductase inhibition leading to short AGD

Graphical Representation**Authors**

Monica K. Draskau; National Food Institute, Technical University of Denmark, Lyngby, DK-2800, Denmark

Marie L. Holmer; National Food Institute, Technical University of Denmark, Lyngby, DK-2800, Denmark

Terje Svingen; National Food Institute, Technical University of Denmark, Lyngby, DK-2800, Denmark

Status

Author status

OECD status

OECD
project

SAAOP 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 Do not cite	Under Development	1.90	Included in OECD Work Plan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 5 α -reductase inhibition during fetal life 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 (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)

5 α -reductase is an enzyme responsible for the conversion of testosterone to DHT in target tissues (Azzouni et al 2012; Davey and Grossmann, 2016). DHT is more potent agonist of the Androgen receptor (AR) than testosterone, so that DHT is necessary for proper masculinization of e.g. male external genitalia. Under normal physiological conditions, testosterone produced mainly by the testes, is converted in peripheral tissues by 5 α -reductase into DHT, which in turn binds AR and activates downstream target genes (Davey and Grossmann, 2016). AR signaling is necessary for masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex in males (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 key step of this pathway is the inhibition of 5 α -reductase, which converts testosterone into the more potent dihydrotestosterone (DHT) in androgen-sensitive tissues. In the developing perineal region, low or absent DHT levels result in inactivation of the androgen receptor (AR), leading to failure in proper masculinization of the perineum and the levator ani-bulbocavernosus (LABC) complex.

Background

Androgen signaling is critical for male sex differentiation during fetal life and suboptimal action during critical life stages leads to under-masculinized offspring. Testosterone is a main androgen, but during fetal differentiation, particularly in tissues distant to the testes, the more potent androgen receptor ligand dihydro-testosterone (DHT) is critical. The formation of DHT from testosterone requires the enzyme 5 α -reductase, hence the role of both this enzyme and DHT must be considered when assessing overall effects of disrupted androgen signaling on sex differentiation.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1617	Inhibition, 5α-reductase	Inhibition, 5 α -reductase
2	KE	1613	Decrease, dihydrotestosterone (DHT) level	Decrease, DHT level
3	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
5	AO	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, 5α-reductase	adjacent	Decrease, dihydrotestosterone (DHT) level	High	High
Decrease, dihydrotestosterone (DHT) level	adjacent	Decrease, androgen receptor activation		
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	

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

Stressors

Name Evidence

Finasteride High

Finasteride

Finasteride is a type II 5alpha-reductase inhibitor that blocks conversion of testosterone to dihydrotestosterone (Clark et al 1990; Imperato-McGinley et al 1992). Intrauterine exposure in rats can result in shorter male AGD in male offspring (Bowman et al 2003; Christiansen et al 2009; Schwartz et al 2019)

References:

Bowman et al (2003), Toxicol Sci 74:393-406; doi: 10.1093/toxsci/kfg128

Christiansen et al (2009), Environ Health Perspect 117:1839-1846; doi: 10.1289/ehp.0900689

Clark et al (1990), Teratology 42:91-100; doi: 10.1002/tera.1420420111

Imperato-McGinley (1992), J Clin Endocrinol Metab 75:1022-1026; doi: 10.1210/jcem.75.4.1400866

Schwartz et al (2019), Toxicol Sci 169:303-311; doi: 10.1093/toxsci/kfz046

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage Evidence

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, 2019a). 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, 2019a).

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 305-307.

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
MIE-1617	*	*		Low
KE-1613		**		Intermediate
KE-1614	***	***		High
KE-286		***		High

Weight of Evidence Summary

Evidence for anti-androgenicity, by perturbing DHT signaling through 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 is summarized in the below Table:

ID	Assessment score	Rationale
KER-1880	High	It is well established that 5 α -reductase converts testosterone to DHT and that decreased 5 α -reductase activity leads to decreased DHT levels.
KER-1935	High	It is well established that DHT activates the AR and that decreased DHT 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 of the AOP is limited. A major challenge is that it is difficult to measure upstream and downstream events in the same study since MIE-26 and MIE-1617 are measured in vitro and KE-1614 focus on AR activation in vivo with no methods currently available to measure it.

References

Azzouni F, Godoy A, Li Y, Mohler J (2012). The 5 alpha-reductase isozyme family: a review of basic biology and their role in human diseases. *Adv Urol* 2012:530121.

Bhasin S, Cunningham GR, Hayes FJ, Matsumoto AM, Snyder PJ, Swerdloff RS, Montori VM; Task Force, Endocrine Society (2010). Testosterone therapy in men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 95(6):2536-59.

Chamberlain NL, Driver ED, Miesfeld RL (1994). The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* 22(15):3181-6.

Davey RA, Grossmann M (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin Biochem Rev* 37(1):3-15.

Draskau MK, Rosenmai AK, Bouftas N, Johansson HKL, Panagiotou EM, Holmer ML, Elmelund E, Zilliacus J, Beronius A, Damdimopolou P, van Duursen M, Svingen T (2024). AOP Report: An Upstream Network for Reduced Androgen Signaling Leading to Altered Gene Expression of Androgen Receptor-Responsive Genes in Target Tissues. *Environ Toxicol Chem* In Press (doi: 10.1002/etc.5972).

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, Guillelte L (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* 7(3):248-64.

Holmer ML, Zilliacus J, Draskau MK, Hliseníková H, Beronius A, Svingen T (2024). Methodology for developing data-rich Key Event Relationships for Adverse Outcome Pathways exemplified by linking decreased androgen receptor activity

with decreased anogenital distance. *Reprod Toxicol* 128:108662.

Keller ET, Ershler WB, Chang C (1996). The androgen receptor: a mediator of diverse responses. *Front Biosci* 1:d59-71.

Murashima A, Kishigami S, Thomson A, Yamada G (2015). Androgens and mammalian male reproductive tract development. *Biochim Biophys Acta* 1849(2):163-70.

OECD (2008), Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment, OECD Series on Testing and Assessment, No. 43, OECD Publishing, Paris.

OECD (2013) Guidance document in support of the test guideline on the extended one generation reproductive toxicity study no. 151.

Robitaille J, Langlois VS (2020). Consequences of steroid-5 α -reductase deficiency and inhibition in vertebrates. *Gen Comp Endocrinol* 290:113400.

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, Svingen T (2019). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch Toxicol* 93(2):253-272.

Supakar PC, Song CS, Jung MH, Slomczynska MA, Kim JM, Vellanoeweth RL, Chatterjee B, Roy AK (1993). A novel regulatory element associated with age-dependent expression of the rat androgen receptor gene. *J Biol Chem* 268(35):26400-8.

Svingen T, Villeneuve DL, Knapen D, Panagiotou EM, Draskau MK, Damdimopoulou P, O'Brien JM (2021). A Pragmatic Approach to Adverse Outcome Pathway Development and Evaluation. *Toxicol Sci* 184(2):183-190.

Thankamony A, Pasterski V, Ong KK, Acerini CL, Hughes IA (2016). Anogenital distance as a marker of androgen exposure in humans. *Andrology* 4(4):616-25.

Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL (1997). Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab* 82(11):3777-82.

Welsh M, Saunders PT, Fisker M, Scott HM, Hutchison GR, Smith LB, Sharpe RM (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* 118(4):1479-90.

Wu D, Lin G, Gore AC (2009). Age-related changes in hypothalamic androgen receptor and estrogen receptor alpha in male rats. *J Comp Neurol* 512(5):688-701.

Appendix 1

List of MIEs in this AOP

Event: 1617: Inhibition, 5 α -reductase

Short Name: Inhibition, 5 α -reductase

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	MolecularInitiatingEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

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

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

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

Key Event Description

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

There are three isozymes: 5 α -reductase type 1, 2, and 3. The substrates for 5 α -reductases are 3-oxo (3-keto), $\Delta^{4,5}$ C19/C21 steroids such as testosterone, progesterone, androstenedione, epi-testosterone, cortisol, aldosterone, and deoxycorticosterone. The enzymatic reaction leads to an irreversible breakage of the double bond between carbon 4 and 5 and subsequent insertion of a hydride anion at carbon 5 and insertion of a proton at carbon 4. The reaction is aided by the cofactor NADPH. The substrate affinity and reaction velocity differ depending on the combination of substrate and enzyme isoform, for instance 5 α -reductase type 2 has a higher substrate affinity for testosterone than the type 1 isoform of the enzyme, and the enzymatic reaction occurs at a higher velocity under optimal conditions. Likewise, inhibitors of 5 α -reductase may exhibit differential effects depending on isoforms (Azzouni et al., 2012).

How it is Measured or Detected

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

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

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

References

Andersson, S., & Russell, D. W. (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5 α -reductases. *Proc. Natl. Acad. Sci. USA*, 87, 3640–3644. <https://www.pnas.org>

Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. <https://doi.org/10.1155/2012/530121>

Peng, H. M., Valentin-Goyco, J., Im, S. C., Han, B., Liu, J., Qiao, J., & Auchus, R. J. (2020). Expression in escherichia coli, purification, and functional reconstitution of human steroid 5 α -reductases. *Endocrinology (United States)*, 161(8), 1-11. <https://doi.org/10.1210/ENDOCR/BQAA117>

Robitaille, J., & Langlois, V. S. (2020). Consequences of steroid-5 α -reductase deficiency and inhibition in vertebrates. In *General and Comparative Endocrinology* (Vol. 290). Academic Press Inc. <https://doi.org/10.1016/j.ygcen.2020.113400>

Thigpens, A. E., Cala, K. M., & Russell, D. W. (1993). Characterization of Chinese Hamster Ovary Cell Lines Expressing Human Steroid 5 α -Reductase Isozymes. *The Journal of Biological Chemistry*, 268(23), 17404-17412.

Yamana, K., Fernand, L., Luu-The, V., & Luu-The, V. (2010). Human type 3 5 α -reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride. *Hormone Molecular Biology and Clinical Investigation*, 2(3), 293-299. <https://doi.org/10.1515/HMBCI.2010.035>

List of Key Events in the AOP

[Event: 1613: Decrease, dihydrotestosterone \(DHT\) level](#)

Short Name: Decrease, DHT level

Key Event Component

Process	Object	Action
hormone biosynthetic process	17beta-Hydroxy-2-oxa-5alpha-androstan-3-one	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:527 - Decreased, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) leads to Hypospadias, increased	KeyEvent

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 (Swerdlhoff 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 5 α -Reductases (5 α -REDs) (Swerdlhoff 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 5 α -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 (Swerdlhoff et al.).

The initial conversion of DHT into inactive steroids is primarily through 3 α -hydroxysteroid dehydrogenase (3 α -HSD) and 3 β -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 (Swerdlhoff 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 (Swerdlhoff 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.

References

- Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. <https://doi.org/10.1155/2012/530121>
- Gerald, T., & Raj, G. (2022). Testosterone and the Androgen Receptor. In *Urologic Clinics of North America* (Vol. 49, Issue 4, pp. 603–614). W.B. Saunders. <https://doi.org/10.1016/j.ucl.2022.07.004>
- Hammes, S. R., & Levin, E. R. (2019). Impact of estrogens in males and androgens in females. In *Journal of Clinical Investigation* (Vol. 129, Issue 5, pp. 1818–1826). American Society for Clinical Investigation. <https://doi.org/10.1172/JCI125755>
- Hsing, A. W., Stanczyk, F. Z., Bélanger, A., Schroeder, P., Chang, L., Falk, R. T., & Fears, T. R. (2007). Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. *Cancer Epidemiology Biomarkers and Prevention*, 16(5), 1004–1008.

<https://doi.org/10.1158/1055-9965.EPI-06-0792>

Miller, W. L., & Auchus, R. J. (2019). The “backdoor pathway” of androgen synthesis in human male sexual development. *PLoS Biology*, 17(4). <https://doi.org/10.1371/journal.pbio.3000198>

Naamneh Elzenaty, R., du Toit, T., & Flück, C. E. (2022). Basics of androgen synthesis and action. In *Best Practice and Research: Clinical Endocrinology and Metabolism* (Vol. 36, Issue 4). Bailliere Tindall Ltd. <https://doi.org/10.1016/j.beem.2022.101665>

OECD (2023), Test No. 456: H295R Steroidogenesis Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264122642-en>.

Renfree, M. B., and Shaw, G. (2023). The alternate pathway of androgen metabolism and window of sensitivity. *J. Endocrinol.*, JOE-22-0296. doi:10.1530/JOE-22-0296.

Shiraishi, S., Lee, P. W. N., Leung, A., Goh, V. H. H., Swerdloff, R. S., & Wang, C. (2008). Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography-tandem mass spectrometry. *Clinical Chemistry*, 54(11), 1855–1863. <https://doi.org/10.1373/clinchem.2008.103846>

Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C., & Salameh, W. A. (2017). Dihydrotestosterone: Biochemistry, physiology, and clinical implications of elevated blood levels. In *Endocrine Reviews* (Vol. 38, Issue 3, pp. 220–254). Endocrine Society. <https://doi.org/10.1210/er.2016-1067>

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I. M., Edlund, K., Lundberg, E., Navani, S., Szgyarto, C. A. K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., ... Pontén, F. (2015). Tissue-based map of the human proteome. *Science*, 347(6220). <https://doi.org/10.1126/science.1260419>

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 <i>in vivo</i>. 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 <i>in vivo</i> activation, with most methods that can be used to measure AR activity carried out <i>in vitro</i>. 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.	
References	
Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. <i>The Clinical Biochemist. Reviews</i>, 37(1), 3–15. Gao, W., Bohl, C. E., & Dalton, J. T. (2005). Chemistry and structural biology of androgen receptor. <i>Chemical Reviews</i>, 105(9), 3352–3370. https://doi.org/10.1021/cr020456u Heinlein, C. A., & Chang, C. (2002). Androgen Receptor (AR) Coregulators: An Overview. https://academic.oup.com/edrv/article/23/2/175/2424160 Leung, J. K., & Sadar, M. D. (2017). Non-Genomic Actions of the Androgen Receptor in Prostate Cancer. <i>Frontiers in Endocrinology</i>, 8. https://doi.org/10.3389/fendo.2017.00002 OECD (2022). Test No. 251: Rapid Androgen Disruption Activity Reporter (RADAR) assay. Paris: OECD Publishing doi:10.1787/da264d82-en.	

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 fetus (mammals)	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:495 - Androgen receptor activation leading to prostate cancer	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent

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 <i>in vivo</i>. 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.	
<u>The Androgen Receptor and its function</u>	
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).	
<u>Altered transcription of genes by the AR as a Key Event</u>	
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-canonical' 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).	
References	
Bevan C, Parker M (1999) The role of coactivators in steroid hormone action. Exp. Cell Res. 253:349–356	

- Bolton EC, So AY, Chaivorapol C, et al (2007) Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes Dev* 21:2005–2017. doi: 10.1101/gad.1564207
- Darde, T. A., Gaudriault, P., Beranger, R., Lancien, C., Caillairec-Joly, A., Sallou, O., et al. (2018a). TOXsigN: a cross-species repository for toxicogenomic signatures. *Bioinformatics* 34, 2116–2122. doi:10.1093/bioinformatics/bty040.
- Darde, T. A., Chalmel, F., and Svingen, T. (2018b). Exploiting advances in transcriptomics to improve on human-relevant toxicology. *Curr. Opin. Toxicol.* 11–12, 43–50. doi:10.1016/j.cotox.2019.02.001.
- Davey RA, Grossmann M (2016) Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin Biochem Rev* 37:3–15
- Estrada M, Espinosa A, Müller M, Jaimovich E (2003) Testosterone Stimulates Intracellular Calcium Release and Mitogen-Activated Protein Kinases Via a G Protein-Coupled Receptor in Skeletal Muscle Cells. *Endocrinology* 144:3586–3597. doi: 10.1210/en.2002-0164
- Heemers H V., Tindall DJ (2007) Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex. *Endocr. Rev.* 28:778–808
- Jin, Hong Jian, Jung Kim, and Jindan Yu. 2013. “Androgen Receptor Genomic Regulation.” *Translational Andrology and Urology* 2(3):158–77. doi: 10.3978/j.issn.2223-4683.2013.09.01
- Keenan, A. B., Wojciechowicz, M. L., Wang, Z., Jagodnik, K. M., Jenkins, S. L., Lachmann, A., et al. (2019). Connectivity Mapping: Methods and Applications. *Annu. Rev. Biomed. Data Sci.* 2, 69–92. doi:10.1146/ANNUREV-BIODATASCI-072018-021211.
- Maclean HE, Chu S, Warne GL, Zajack JD Related Individuals with Different Androgen Receptor Gene Deletions
- MacLeod DJ, Sharpe RM, Welsh M, et al (2010) Androgen action in the masculinization programming window and development of male reproductive organs. In: *International Journal of Andrology*. Blackwell Publishing Ltd, pp 279–287
- Ngan S, Stronach EA, Photiou A, et al (2009) Microarray coupled to quantitative RT–PCR analysis of androgen-regulated genes in human LNCaP prostate cancer cells. *Oncogene* 28:2051–2063. doi: 10.1038/onc.2009.68
- Rana K, Davey RA, Zajack JD (2014) Human androgen deficiency: Insights gained from androgen receptor knockout mouse models. *Asian J. Androl.* 16:169–177
- Rooney, J. P., Chorley, B., Kleinstreuer, N., and Corton, J. C. (2018). Identification of Androgen Receptor Modulators in a Prostate Cancer Cell Line Microarray Compendium. *Toxicol. Sci.* 166, 146–162. doi:10.1093/TOXSCI/KFY187.
- Walters KA, Simanainen U, Handelsman DJ (2010) Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. *Hum Reprod Update* 16:543–558. doi: 10.1093/humupd/dmq003

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
 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 dimorphism 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-myotonic 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'.

References

- Aydoğar Ahabab M, Barlas N (2015) Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats. *Toxicol Lett* **233**: 125-137
- Boberg J, Axelstad M, Svingen T, Mandrup K, Christiansen S, Vinggaard AM, Hass U (2016) Multiple endocrine disrupting effects in rats perinatally exposed to butylparaben. *Toxicol Sci* **152**: 244-256
- Boberg J, Metzдорff S, Wortziger R, Axelstad M, Brokken L, Vinggaard AM, Dalgaard M, Nellemann C (2008) Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. *Toxicology* **250**: 75-81
- Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PM (2003) Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat. *Toxicol Sci* **74**: 393-406
- Christiansen S, Boberg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzдорff SB, Hass U (2010) Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod Toxicol* **30**: 313-321
- Christiansen S, Scholze M, Dalgaard M, Vinggaard AM, Axelstad M, Kortenkamp A, Hass U (2009) Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. *Environ Health Perspect* **117**: 1839-1846
- Draskau MK, Boberg J, Taxvig C, Pedersen M, Frandsen HL, Christiansen S, Svingen T (2019) In vitro and in vivo endocrine disrupting effects of the azole fungicides triticonazole and flusilazole. *Environ Pollut* **255**: 113309
- Ema M, Miyawaki E (2002) Effects on development of the reproductive system in male offspring of rats given butyl benzyl phthalate during late pregnancy. *Reprod Toxicol* **16**: 71-76

- Ema M, Miyawaki E, Hirose A, Kamata E (2003) Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate. *Reprod Toxicol* **17**: 407-412
- Foster PM, Harris MW (2005) Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci* **85**: 1024-1032
- Gray LE, Jr., Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L (2000) Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* **58**: 350-365
- Gray LEJ, Ostby JS, Kelce WR (1994) Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol* **129**: 46-52
- Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A, Clemmensen LH, Axelstad M (2012) Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reprod Toxicol* **34**: 261-274
- Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Metzдорff SB, Kortenkamp A (2007) Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environ Health Perspect* **115 Suppl. 1**: 122-128
- Hoshino N, Iwai M, Okazaki Y (2005) A two-generation reproductive toxicity study of dicyclohexyl phthalate in rats. *Toxicol Sci* **30 Spec No**: 79-96
- Hotchkiss AK, Parks-Saldutti LG, Ostby JS, Lambright C, Furr J, Vandenberg JG, Gray LEJ (2004) A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biol Reprod* **71**: 1852-1861
- Howdeshell KL, Furr J, Lambright CR, Rider CV, Wilson VS, Gray LE, Jr. (2007) Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. *Toxicol Sci* **99**: 190-202
- Ipulan LA, Suzuki K, Sakamoto Y, Murashima A, Imai Y, Omori A, Nakagata N, Nishinakamura R, Valasek P, Yamada G (2014) Nonmyocytic androgen receptor regulates the sexually dimorphic development of the embryonic bulbocavernosus muscle. *Endocrinology* **155**: 2467-2479
- Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H, Ladefoged O (2005) Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. *Reprod Toxicol* **19**: 505-515
- Jost A (1953) Problems of fetal endocrinology: The gonadal and hypophyseal hormones. *Recent Prog Horm Res* **8**: 379-418
- Juul A, Almstrup K, Andersson AM, Jensen TK, Jorgensen N, Main KM, Rajpert-De Meyts E, Toppari J, Skakkebaek NE (2014) Possible fetal determinants of male infertility. *Nat Rev Endocrinol* **10**: 553-562
- Kita DH, Meyer KB, Venturelli AC, Adams R, Machado DL, Morais RN, Swan SH, Gennings C, Martino-Andrade AJ (2016) Manipulation of pre and postnatal androgen environments and anogenital distance in rats. *Toxicology* **368-369**: 152-161
- Laier P, Metzдорff SB, Borch J, Hagen ML, Hass U, Christiansen S, Axelstad M, Kledal T, Dalgaard M, McKinnell C, Brokken LJ, Vinggaard AM (2006) Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol Appl Pharmacol* **213**: 2
- Li M, Qiu L, Zhang Y, Hua Y, Tu S, He Y, Wen S, Wang Q, Wei G (2013) Dose-related effect by maternal exposure to di-(2-ethylhexyl) phthalate plasticizer on inducing hypospadiac male rats. *Environ Toxicol Pharmacol* **35**: 55-60
- Lin H, Lian QQ, Hu GX, Jin Y, Zhang Y, Hardy DO, Chen GR, Lu ZQ, Sottas CM, Hardy MP, Ge RS (2009) In utero and lactational exposures to diethylhexyl-phthalate affect two populations of Leydig cells in male Long-Evans rats. *Biol Reprod* **80**: 882-888
- Loeffler IK, Peterson RE (1999) Interactive effects of TCDD and p,p'-DDE on male reproductive tract development in in utero and lactationally exposed rats. *Toxicol Appl Pharmacol* **154**: 28-39
- MacLeod DJ, Sharpe RM, Welsh M, Fiskin M, Scott HM, Hutchison GR, Drake AJ, van den Driesche S (2010) Androgen action in the masculinization programming window and development of male reproductive organs. *Int J Androl* **33**: 279-287
- Matsuura I, Saitoh T, Ashina M, Wako Y, Iwata H, Toyota N, Ishizuka Y, Namiki M, Hoshino N, Tsuchitani M (2005) Evaluation of a two-generation reproduction toxicity study adding endpoints to detect endocrine disrupting activity using vinclozolin. *J Toxicol Sci* **30 Spec No**: 163-168
- McIntyre BS, Barlow NJ, Foster PM (2001) Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* **62**: 236-249
- McIntyre BS, Barlow NJ, Sar M, Wallace DG, Foster PM (2002) Effects of in utero linuron exposure on rat Wolffian duct

development. *Reprod Toxicol***16**: 131-139

Melching-Kollmuss S, Fussell KC, Schneider S, Buesen R, Groeters S, Strauss V, van Ravenzwaay B (2017) Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: example prochloraz. *Arch Toxicol***91**: 143-162

Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE (2001) Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. *Environ Health Perspect***109**: 229-237

Mylchreest E, Sar M, Cattley RC, Foster PM (1999) Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol***156**: 81-95

Nagao T, Ohta R, Marumo H, Shindo T, Yoshimura S, Ono H (2000) Effect of butyl benzyl phthalate in Sprague-Dawley rats after gavage administration: a two-generation reproductive study. *Reprod Toxicol***14**: 513-532

Nardelli TC, Albert O, Lalancette C, Culty M, Hales BF, Robaire B (2017) In utero and lactational exposure study in rats to identify replacements for di(2-ethylhexyl) phthalate. *Sci Rep***7**: 3862

Noriega NC, Ostby J, Lambright C, Wilson VS, Gray LE, Jr. (2005) Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring. *Biol Reprod***72**: 1324-1335

OECD. (2013) Guidance document in support of the test guideline on the extended one generation reproductive toxicity study No. 151.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, Gray CLJ (1999) The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicol Ind Health***15**: 80-93

Saillenfait AM, Gallissot F, Sabaté JP (2009a) Differential developmental toxicities of di-n-hexyl phthalate and dicyclohexyl phthalate administered orally to rats. *J Appl Toxicol***29**: 510-521

Saillenfait AM, Roudot AC, Gallissot F, Sabaté JP (2011) Prenatal developmental toxicity studies on di-n-heptyl and di-n-octyl phthalates in Sprague-Dawley rats. *Reprod Toxicol***32**: 268-276

Saillenfait AM, Sabaté JP, Gallissot F (2009b) Effects of in utero exposure to di-n-hexyl phthalate on the reproductive development of the male rat. *Reprod Toxicol***28**: 468-476

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M (2004) Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. *Environ Health***3**: 8

Schneider S, Kaufmann W, Strauss V, van Ravenzwaay B (2011) Vinclozolin: a feasibility and sensitivity study of the ILSI-HESI F1-extended one-generation rat reproduction protocol. *Regulatory Toxicology and Pharmacology***59**: 91-100

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, 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

Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, Verhoeven H, O'Shaughnessy P, Sharpe RM (2007) Role of androgens in fetal testis development and dysgenesis. *Endocrinology***148**: 2027-2036

Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod***16**: 972-978

Taxvig C, Vinggaard AM, Hass U, Axelstad M, Metzdrorf S, Nellemann C (2008) Endocrine-disrupting properties in vivo of widely used azole fungicides. *Int J Androl***31**: 170-177

Turner KJ, Barlow NJ, Struve MF, Wallace DG, Gaido KW, Dorman DC, Foster PM (2002) Effects of in utero exposure to the organophosphate insecticide fenitrothion on androgen-dependent reproductive development in the Crl:CD(SD)BR rat. *Toxicol Sci***68**: 174-183

Tyl RW, Myers CB, Marr MC, Fail PA, Seely JC, Brine DR, Barter RA, Butala JH (2004) Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. *Reprod Toxicol***18**: 241-264

Van den Driesche S, Kolovos P, Platts S, Drake AJ, Sharpe RM (2012) Inter-relationship between testicular dysgenesis and Leydig cell function in the masculinization programming window in the rat. *PLoS one***7**: e30111

Welsh M, Saunders PT, Fiskens M, Scott HM, Hutchison GR, Smith LB, Sharpe RM (2008) Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest***118**: 1479-1490

Welsh M, Saunders PT, Sharpe RM (2007) The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology***148**: 3185-3195

Wolf CJ, LeBlanc GA, Gray LE, Jr. (2004) Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. *Toxicol Sci***78**: 135-143

Wolf CJJ, Lambricht C, Mann P, Price M, Cooper RL, Ostby J, Gray CLJ (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* **15**: 94-118

Zhang L, Dong L, Ding S, Qiao P, Wang C, Zhang M, Zhang L, Du Q, Li Y, Tang N, Chang B (2014) Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E₂ levels in male rat offspring. *Environ Toxicol Pharmacol* **37**: 705-717

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Key Event Relationship Description

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

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

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

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is considered high.

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

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

Empirical Evidence

The empirical evidence for this KER is considered high

Dose concordance

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

Table 1: Dose concordance from selected *in vitro* test systems

Test system	Model description	Stressor	Effect	Reference
HEK-293 cells	Cells stably transfected human 5 α -reductase type 1 and 2 used to measure conversion of [¹⁴ C]labeled steroids	Finasteride	Type 1: IC ₅₀ = 106.9 μ M Type 2: IC ₅₀ = 14.3 μ M	(Yamana et al., 2010)
		Dutasteride	Type 1: IC ₅₀ = 8.7 μ M Type 2: IC ₅₀ = 57 μ M	
COS cells	Cell homogenates from transfected cells with human and rat 5 α -reductase (unknown isoform) used to measure conversion of radiolabeled testosterone	Finasteride	Human: IC ₅₀ $\approx$ 1 μ M K _i = 340-620 nM Rat: IC ₅₀ $\approx$ 0.1 μ M K _i = 3-5 nM	(Andersson & Russell, 1990)
		4-MA	Human: IC ₅₀ $\approx$ 0.1 μ M K _i = 7-8 nM Rat: IC ₅₀ $\approx$ 0.1 μ M K _i = 5-7 nM	
CHO cells	Stably transfected with human 5 α -reductase type 1 and 2	Finasteride	Type 1: K _i = 325 nM Type 2: K _i = 12 nM	(Thigpens et al., 1993)

		4-MA	Type 1: $K_i = 8 \text{ nM}$ Type 2: $K_i = 4 \text{ nM}$	
Isolated enzyme	Human 5 α -reductase type 1 and 2 used to measure conversion of radiolabeled substrate of both isoforms	Finasteride	Type 1: $K_i = > 200 \text{ nM}$ Type 2: $K_i = 0.45 \text{ nM}$	(Peng et al., 2020)
		Dutasteride	Type 1: $K_i = 39 \text{ nM}$ Type 2: $K_i = 1.1 \text{ nM}$	

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

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

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

Other evidence

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

Quantitative Understanding of the Linkage

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

These differences in isoform specificity reflect in the effects on DHT serum levels, hence the broader specificity of dutasteride leads to > 90% decrease in patients with benign prostatic hyperplasia, in comparison to 70% with finasteride administration (Nikolaou et al., 2021).

Response-response relationship

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

Time-scale

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

Known Feedforward/Feedback loops influencing this KER

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

References

- Aleman, M. (2022). The Roles of Androgens in Humans: Biology, Metabolic Regulation and Health. In *International Journal of Molecular Sciences* (Vol. 23, Issue 19). MDPI. <https://doi.org/10.3390/ijms231911952>
- Andersson, S., Bishop, R. W., & Russell, D. W. (1989). *THE JOURNAL OF BIOLOGICAL CHEMISTRY Expression Cloning and Regulation of Steroid 5 α -Reductase, an Enzyme Essential for Male Sexual Differentiation** (Vol. 264, Issue 27).
- Andersson, S., & Russell, D. W. (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5 α -reductases. *Proc. Natl. Acad. Sci. USA*, 87, 3640–3644. <https://www.pnas.org>

- Azzouni, F., Godoy, A., Li, Y., & Mohler, J. (2012). The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. In *Advances in Urology*. <https://doi.org/10.1155/2012/530121>
- Berman, D. M., & Russell, D. W. (1993). Cell-type-specific expression of rat steroid 5a-reductase isozymes (sexual development/androgens/prostate/stroma/epithelium). In *Proc. Natl. Acad. Sci. USA* (Vol. 90). <https://www.pnas.org>
- Clark, R. V., Hermann, D. J., Cunningham, G. R., Wilson, T. H., Morrill, B. B., & Hobbs, S. (2004). Marked Suppression of Dihydrotestosterone in Men with Benign Prostatic Hyperplasia by Dutasteride, a Dual 5 α -Reductase Inhibitor. *Journal of Clinical Endocrinology and Metabolism*, 89(5), 2179–2184. <https://doi.org/10.1210/jc.2003-030330>
- Drake, L., Hordinsky, M., Fiedler, V., Swinehart, J., Unger, W. P., Cotterill, P. C., Thiboutot, D. M., Lowe, N., Jacobson, C., Whiting, D., Stieglitz, S., Kraus, S. J., Griffin, E. I., Weiss, D., Carrington, P., Gencheff, C., Cole, G. W., Pariser, D. M., Epstein, E. S., ... City, O. (1999). *The effects of finasteride on scalp skin and serum androgen levels in men with androgenetic alopecia*
- Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, 32(1), 81–151. <https://doi.org/10.1210/er.2010-0013>
- Miller, W. L., & Auchus, R. J. (2019). The “backdoor pathway” of androgen synthesis in human male sexual development. *PLoS Biology*, 17(4). <https://doi.org/10.1371/journal.pbio.3000198>
- Nikolaou, N., Hodson, L., & Tomlinson, J. W. (2021). The role of 5-reduction in physiology and metabolic disease: evidence from cellular, pre-clinical and human studies. In *Journal of Steroid Biochemistry and Molecular Biology* (Vol. 207). Elsevier Ltd. <https://doi.org/10.1016/j.jsbmb.2021.105808>
- Peng, H. M., Valentin-Goyco, J., Im, S. C., Han, B., Liu, J., Qiao, J., & Auchus, R. J. (2020). Expression in escherichia coli, purification, and functional reconstitution of human steroid 5 α -reductases. *Endocrinology (United States)*, 161(8), 1–11. <https://doi.org/10.1210/ENDOCR/BQAA117>
- Robitaille, J., & Langlois, V. S. (2020). Consequences of steroid-5 α -reductase deficiency and inhibition in vertebrates. In *General and Comparative Endocrinology* (Vol. 290). Academic Press Inc. <https://doi.org/10.1016/j.ygcen.2020.113400>
- Russell, D. W., & Wilson, J. D. (1994). *STEROID 5a-REDUCTASE: TWO GENES/TWO ENZYMES*. www.annualreviews.org
- Thigpens, A. E., Cala, K. M., & Russell, D. W. (1993). Characterization of Chinese Hamster Ovary Cell Lines Expressing Human Steroid 5a-Reductase Isozymes. *The Journal of Biological Chemistry*, 268(23), 17404–17412.
- Yamana, K., Fernand, L., Luu-The, V., & Luu-The, V. (2010). Human type 3 5 α -reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride. *Hormone Molecular Biology and Clinical Investigation*, 2(3), 293–299. <https://doi.org/10.1515/HMBCI.2010.035>

Relationship: 1935: Decrease, DHT level 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
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 5 α -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 5 α -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 5 α -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 5 α -reductase inhibitor, dose-dependently decreases AR-mediated prostate cancer cell line proliferation (Bologna et al., 1995). 0.001 μ M finasteride decreased the growth rate with 44%, 0.1 μ M 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 5 α -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 5 α -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 5 α -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)
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, precursor of DHT	(Bhasin et al., 2010)
Castration	Removal of testicles	Reduced levels of circulating testosterone, precursor 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 5 α -reductase expression, but for 5 α -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.

References

- Aggarwal, S., Thareja, S., Verma, A., Bhardwaj, T.R., Kumar, M., 2010. An overview on 5 α -reductase inhibitors. *Steroids* 75, 109-153. <https://doi.org/10.1016/j.steroids.2009.10.005>
- Bennesch, M.A., Picard, D., 2015. Minireview: Tipping the Balance: Ligand-Independent Activation of Steroid Receptors. *Mol. Endocrinol.* 29, 349-363. <https://doi.org/10.1210/ME.2014-1315>
- Bhasin, S., Cunningham, G.R., Hayes, F.J., Matsumoto, A.M., Snyder, P.J., Swerdloff, R.S., Montori, V.M., 2010. Testosterone Therapy in Men with Androgen Deficiency Syndromes: An Endocrine Society Clinical Practice Guideline. *J. Clin. Endocrinol. Metab.* 95, 2536-2559. <https://doi.org/10.1210/JC.2009-2354>
- Bologna, M., Muzi, P., Biordi, L., Festuccia, C., Vicentini, C., 1995. Finasteride dose-dependently reduces the proliferation rate of the LnCap human prostatic cancer cell line in vitro. *Urology* 45, 282-290. [https://doi.org/10.1016/0090-4295\(95\)80019-0](https://doi.org/10.1016/0090-4295(95)80019-0)

- Bowman, C.J., Barlow, N.J., Turner, K.J., Wallace, D.G., Foster, P.M.D., 2003. Effects of in Utero Exposure to Finasteride on Androgen-Dependent Reproductive Development in the Male Rat. *Toxicol. Sci.* 74, 393–406. <https://doi.org/10.1093/TOXSCI/KFG128>
- Chamberlain, N.L., Driver, E.D., Miesfeld, R.L., 1994. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res.* 22, 3181. <https://doi.org/10.1093/NAR/22.15.3181>
- Costa, E.F., Domenice, S., Sircili, M., Inacio, M., Mendonca, B., 2012. DSD due to 5 α -reductase 2 deficiency - From diagnosis to long term outcome. *Semin. Reprod. Med.* 30, 427–431. <https://doi.org/10.1055/S-0032-1324727/ID/JR00766-20/BIB>
- Dalton, J.T., Mukherjee, A., Zhu, Z., Kirkovsky, L., Miller, D.D., 1998. Discovery of nonsteroidal androgens. *Biochem. Biophys. Res. Commun.* 244, 1–4. <https://doi.org/10.1006/bbrc.1998.8209>
- Davey, R.A., Grossmann, M., 2016. Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin. Biochem. Rev.* 37, 3–15.
- Eder, I.E., Culig, Z., Putz, T., Nessler-Menardi, C., Bartsch, G., Klocker, H., 2001. Molecular Biology of the Androgen Receptor: From Molecular Understanding to the Clinic. *Eur. Urol.* 40, 241–251. <https://doi.org/10.1159/000049782>
- Grino, P.B., Griffin, J.E., Wilson, J.D., 1990. Testosterone at High Concentrations Interacts with the Human Androgen Receptor Similarly to Dihydrotestosterone. *Endocrinology* 126, 1165–1172. <https://doi.org/10.1210/endo-126-2-1165>
- Heinlein, C.A., Chang, C., 2002. Androgen Receptor (AR) Coregulators: An Overview. *Endocr. Rev.* 23, 175–200. <https://doi.org/10.1210/EDRV.23.2.0460>
- Huggins, C., Hodges, C. V., 1941. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res.* 1, 293–297.
- Kang, Z., Pirskanen, A., Jänne, O.A., Palvimo, J.J., 2002. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. *J. Biol. Chem.* 277, 48366–48371. <https://doi.org/10.1074/jbc.M209074200>
- Keller, E.T., Ershler, W.B., Chang, C., 1996. The androgen receptor: a mediator of diverse responses. *Front. Biosci. (Landmark Ed)* 1, 59–71. <https://doi.org/10.2741/A116>
- Krotkiewski, M., Kral, J.G., Karlsson, J., 1980. Effects of castration and testosterone substitution on body composition and muscle metabolism in rats. *Acta Physiol. Scand.* 109, 233–237. <https://doi.org/10.1111/J.1748-1716.1980.TB06592.X>
- Lee, D.K., Chang, C., 2003. Expression and Degradation of Androgen Receptor: Mechanism and Clinical Implication. *J. Clin. Endocrinol. Metab.* 88, 4043–4054. <https://doi.org/10.1210/JC.2003-030261>
- Marks, L.S., 2004. 5Alpha-Reductase: History and Clinical Importance. *Rev. Urol.* 6 Suppl 9, S11–21.
- Nightingale, J., Chaudhary, K.S., Abel, P.D., Stubbs, A.P., Romanska, H.M., Mitchell, S.E., Stamp, G.W.H., Lalani, E.N., 2003. Ligand Activation of the Androgen Receptor Downregulates E-Cadherin-Mediated Cell Adhesion and Promotes Apoptosis of Prostatic Cancer Cells. *Neoplasia* 5, 347. [https://doi.org/10.1016/S1476-5586\(03\)80028-3](https://doi.org/10.1016/S1476-5586(03)80028-3)
- OECD, 2020. Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. <https://doi.org/10.1787/9789264264366-en>
- Robitaille, J., Langlois, V.S., 2020. Consequences of steroid-5 α -reductase deficiency and inhibition in vertebrates. *Gen. Comp. Endocrinol.* 290. <https://doi.org/10.1016/j.ygcen.2020.113400>
- Schaufele, F., Carbonell, X., Guerbodot, M., Borngraeber, S., Chapman, M.S., Ma, A.A.K., Miner, J.N., Diamond, M.I., 2005. The structural basis of androgen receptor activation: Intramolecular and intermolecular amino-carboxy interactions. *Proc. Natl. Acad. Sci. U. S. A.* 102, 9802–9807. <https://doi.org/10.1073/pnas.0408819102>
- Schuppe, E.R., Miles, M.C., Fuxjager, M.J., 2020. Evolution of the androgen receptor: Perspectives from human health to dancing birds. *Mol. Cell. Endocrinol.* 499, 110577. <https://doi.org/10.1016/j.mce.2019.110577>
- Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., 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. <https://doi.org/10.1007/s00204-018-2350-5>
- Supakar, P.C., Song, C.S., Jung, M.H., Slomczynska, M.A., Kim, J.M., Vellanoweth, R.L., Chatterjee, B., Roy, A.K., 1993. A novel regulatory element associated with age-dependent expression of the rat androgen receptor gene. *J. Biol. Chem.* 268, 26400–26408. [https://doi.org/10.1016/S0021-9258\(19\)74328-2](https://doi.org/10.1016/S0021-9258(19)74328-2)
- Tut, T.G., Ghadessy, F.J., Trifiro, M.A., Pinsky, L., Yong, E.L., 1997. Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced Trans-Activation, Impaired Sperm Production, and Male Infertility. *J. Clin. Endocrinol. Metab.* 82, 3777–3782. <https://doi.org/10.1210/JCEM.82.11.4385>
- Williams, A.J., Grulke, C.M., Edwards, J., McEachran, A.D., Mansouri, K., Baker, N.C., Patlewicz, G., Shah, I., Wambaugh, J.F., Judson, R.S., Richard, A.M., 2017. The CompTox Chemistry Dashboard: a community data resource for environmental

chemistry. J. Cheminform. 9, 61. <https://doi.org/10.1186/s13321-017-0247-6>

Wu, D., Lin, G., Gore, A.C., 2009. Age-related Changes in Hypothalamic Androgen Receptor and Estrogen Receptor α in Male Rats. J. Comp. Neurol. 512, 688. <https://doi.org/10.1002/CNE.21925>

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).

References

- Ayobahan, S. U., Alvincz, J., Reinwald, H., Strompen, J., Salinas, G., Schäfers, C., et al. (2023). Comprehensive identification of gene expression fingerprints and biomarkers of sexual endocrine disruption in zebrafish embryo. *Ecotoxicol. Environ. Saf.* 250, 114514. doi:10.1016/j.ECOENV.2023.114514.
- Bennesch, Marcela A., and Didier Picard. 2015. "Minireview: Tipping the Balance: Ligand-Independent Activation of Steroid Receptors." *Molecular Endocrinology* 29(3):349-63.
- Chamberlain, Nancy L., Erika D. Driverand, and Roger L. Miesfeldi. 1994. *The Length and Location of CAG Trinucleotide Repeats in the Androgen Receptor N-Terminal Domain Affect Transactivation Function*. Vol. 22.
- Denolet, Evi, Karel De Gendt, Joke Allemeersch, Kristof Engelen, Kathleen Marchal, Paul Van Hummelen, Karen A. L.

- Tan, Richard M. Sharpe, Philippa T. K. Saunders, Johannes V. Swinnen, and Guido Verhoeven. 2006. "The Effect of a Sertoli Cell-Selective Knockout of the Androgen Receptor on Testicular Gene Expression in Prepubertal Mice." *Molecular Endocrinology* 20(2):321-34. doi: 10.1210/me.2005-0113.
- Fan, Wuqiang, Toshihiko Yanase, Masatoshi Nomura, Taijiro Okabe, Kiminobu Goto, Takashi Sato, Hirotaka Kawano, Shigeaki Kato, and Hajime Nawata. 2005. *Androgen Receptor Null Male Mice Develop Late-Onset Obesity Caused by Decreased Energy Expenditure and Lipolytic Activity but Show Normal Insulin Sensitivity With High Adiponectin Secretion*. Vol. 54.
- Holterhus, Paul-Martin, Olaf Hiort, Janos Demeter, Patrick O. Brown, and James D. Brooks. 2003. *Differential Gene-Expression Patterns in Genital Fibroblasts of Normal Males and 46,XY Females with Androgen Insensitivity Syndrome: Evidence for Early Programming Involving the Androgen Receptor*. Vol. 4.
- Ikeda, Yasumasa, Ken Ichi Aihara, Takashi Sato, Masashi Akaike, Masanori Yoshizumi, Yuki Suzaki, Yuki Izawa, Mitsunori Fujimura, Shunji Hashizume, Midori Kato, Shusuke Yagi, Toshiaki Tamaki, Hirotaka Kawano, Takahiro Matsumoto, Hiroyuki Azuma, Shigeaki Kato, and Toshio Matsumoto. 2005. "Androgen Receptor Gene Knockout Male Mice Exhibit Impaired Cardiac Growth and Exacerbation of Angiotensin II-Induced Cardiac Fibrosis." *Journal of Biological Chemistry* 280(33):29661-66. doi: 10.1074/jbc.M411694200.
- Jin, Hong Jian, Jung Kim, and Jindan Yu. 2013. "Androgen Receptor Genomic Regulation." *Translational Andrology and Urology* 2(3):158-77.
- Kang, Zhigang, Asta Pirskanen, Olli A. Jänne, and Jorma J. Palvimo. 2002. "Involvement of Proteasome in the Dynamic Assembly of the Androgen Receptor Transcription Complex." *Journal of Biological Chemistry* 277(50):48366-71. doi: 10.1074/jbc.M209074200.
- Kanno, Yuichiro, Nao Saito, Ryota Saito, Tomohiro Kosuge, Ryota Shizu, Tomofumi Yatsu, Takuomi Hosaka, Kiyomitsu Nemoto, Keisuke Kato, and Kouichi Yoshinari. 2022. "Differential DNA-Binding and Cofactor Recruitment Are Possible Determinants of the Synthetic Steroid YK11-Dependent Gene Expression by Androgen Receptor in Breast Cancer MDA-MB 453 Cells." *Experimental Cell Research* 419(2). doi: 10.1016/j.yexcr.2022.113333.
- Karlsson, Sara A., Erik Studer, Petronella Kettunen, and Lars Westberg. 2016. "Neural Androgen Receptors Modulate Gene Expression and Social Recognition but Not Social Investigation." *Frontiers in Behavioral Neuroscience* 10(MAR). doi: 10.3389/fnbeh.2016.00041.
- Knapczyk-Stwora, Katarzyna, Anna Nynca, Renata E. Ciereszko, Lukasz Paukszt, Jan P. Jastrzebski, Elzbieta Czaja, Patrycja Witek, Marek Koziorowski, and Maria Slomczynska. 2019. "Flutamide-Induced Alterations in Transcriptional Profiling of Neonatal Porcine Ovaries." *Journal of Animal Science and Biotechnology* 10(1):1-15. doi: 10.1186/s40104-019-0340-y.
- Lamont, K. R., and Tindall, D. J. (2010). Androgen Regulation of Gene Expression. *Adv. Cancer Res.* 107, 137-162. doi:10.1016/S0065-230X(10)07005-3.
- MacLean, Helen E., W. S. Maria Chiu, Amanda J. Notini, Anna-Maree Axell, Rachel A. Davey, Julie F. McManus, Cathy Ma, David R. Plant, Gordon S. Lynch, and Jeffrey D. Zajac. 2008. "Impaired Skeletal Muscle Development and Function in Male, but Not Female, Genomic Androgen Receptor Knockout Mice." *The FASEB Journal* 22(8):2676-89. doi: 10.1096/fj.08-105726.
- Maiuri, Paolo, Anna Knezevich, Alex De Marco, Davide Mazza, Anna Kula, Jim G. McNally, and Alessandro Marcello. 2011. "Fast Transcription Rates of RNA Polymerase II in Human Cells." *EMBO Reports* 12(12):1280-85. doi: 10.1038/embor.2011.196.
- Mora, Gloria R., and Virendra B. Mahesh. 1999. *Autoregulation of the Androgen Receptor at the Translational Level: Testosterone Induces Accumulation of Androgen Receptor mRNA in the Rat Ventral Prostate Polyribosomes*.
- Peng, Yajie, Hui Zhu, Bing Han, Yue Xu, Xuemeng Liu, Huaidong Song, and Jie Qiao. 2021. "Identification of Potential Genes in Pathogenesis and Diagnostic Value Analysis of Partial Androgen Insensitivity Syndrome Using Bioinformatics Analysis." *Frontiers in Endocrinology* 12. doi: 10.3389/fendo.2021.731107.
- Rana, Kesha, Barbara C. Fam, Michele V. Clarke, Tammy P. S. Pang, Jeffrey D. Zajac, and Helen E. Maclean. 2011. "Increased Adiposity in DNA Binding-Dependent Androgen Receptor Knockout Male Mice Associated with Decreased Voluntary Activity and Not Insulin Resistance." *Am J Physiol Endocrinol Me-Tab* 301:767-78. doi: 10.1152/ajpendo.00584.2010.-In.
- Roy, Arun K., Rakesh K. Tyagi, Chung S. Song, Yan Lavrovsky, Soon C. Ahn, Tae Sung Oh, and Bandana Chatterjee. 2001. "Androgen Receptor: Structural Domains and Functional Dynamics after Ligand-Receptor Interaction." Pp. 44-57 in *Annals of the New York Academy of Sciences* Vol. 949. New York Academy of Sciences.
- Russell, Patricia K., Michele V. Clarke, Jarrod P. Skinner, Tammy P. S. Pang, Jeffrey D. Zajac, and Rachel A. Davey. 2012. "Identification of Gene Pathways Altered by Deletion of the Androgen Receptor Specifically in Mineralizing Osteoblasts and Osteocytes in Mice." *Journal of Molecular Endocrinology* 49(1):1-10. doi: 10.1530/JME-12-0014.
- Shiina, Hiroko, Takahiro Matsumoto, Takashi Sato, Katsuhide Igarashi, Junko Miyamoto, Sayuri Takemasa, Matomo Sakari, Ichiro Takada, Takashi Nakamura, Daniel Metzger, Pierre Chambon, Jun Kanno, Hiroyuki Yoshikawa, and Shigeaki Kato. 2006. *Premature Ovarian Failure in Androgen Receptor-Deficient Mice*. Vol. 103.

- Supakar, P. C., C. S. Song, M. H. Jung, M. A. Slomczynska, J. M. Kim, R. L. Vellanoweth, B. Chatterjee, and A. K. Roy. 1993. "A Novel Regulatory Element Associated with Age-Dependent Expression of the Rat Androgen Receptor Gene." *Journal of Biological Chemistry* 268(35):26400-408. doi: 10.1016/s0021-9258(19)74328-2.
- Tut, Thein G., Farid J. Ghadessy, M. A. Trifiro, L. Pinsky, and E. L. Yong. 1997. *Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced Trans-Activation, Impaired Sperm Production, and Male Infertility**. Vol. 82.
- Wang, Ruey Sheng, Shuyuan Yeh, Lu Min Chen, Hung Yun Lin, Caixia Zhang, Jing Ni, Cheng Chia Wu, P. Anthony Di Sant'Agnese, Karen L. DeMesy-Bentley, Chii Ruey Tzeng, and Chawnschang Chang. 2006. "Androgen Receptor in Sertoli Cell Is Essential for Germ Cell Nursery and Junctional Complex Formation in Mouse Testes." *Endocrinology* 147(12):5624-33. doi: 10.1210/en.2006-0138.
- Welsh, M., L. Moffat, K. Belling, L. R. de França, T. M. Segatelli, P. T. K. Saunders, R. M. Sharpe, and L. B. Smith. 2012. "Androgen Receptor Signalling in Peritubular Myoid Cells Is Essential for Normal Differentiation and Function of Adult Leydig Cells." *International Journal of Andrology* 35(1):25-40. doi: 10.1111/j.1365-2605.2011.01150.x.
- Willems, Ariane, Sergio R. Batlouni, Arantza Esnal, Johannes V. Swinnen, Philippa T. K. Saunders, Richard M. Sharpe, Luiz R. França, Karel de Gendt, and Guido Verhoeven. 2010. "Selective Ablation of the Androgen Receptor in Mouse Sertoli Cells Affects Sertoli Cell Maturation, Barrier Formation and Cytoskeletal Development." *PLoS ONE* 5(11). doi: 10.1371/journal.pone.0014168.
- Wu, D. I., Grace Lin, and Andrea C. Gore. 2009. "Age-Related Changes in Hypothalamic Androgen Receptor and Estrogen Receptor in Male Rats." *The Journal of Comparative Neurology* 512:688-701. doi: 10.1002/cne.21925.
- Yu, I. Chen, Hung Yun Lin, Ning Chun Liu, Ruey Shen Wang, Janet D. Sparks, Shuyuan Yeh, and Chawnschang Chang. 2008. "Hyperleptinemia without Obesity in Male Mice Lacking Androgen Receptor in Adipose Tissue." *Endocrinology* 149(5):2361-68. doi: 10.1210/en.2007-0516.
- Yu, Shengqiang, Chuan Ren Yeh, Yuanjie Niu, Hong Chiang Chang, Yu Chieh Tsai, Harold L. Moses, Chih Rong Shyr, Chawnschang Chang, and Shuyuan Yeh. 2012. "Altered Prostate Epithelial Development in Mice Lacking the Androgen Receptor in Stromal Fibroblasts." *Prostate* 72(4):437-49. doi: 10.1002/pros.21445.
- Zhang, Caixia, Shuyuan Yeh, Yen-Ta Chen, Cheng-Chia Wu, Kuang-Hsiang Chuang, Hung-Yun Lin, Ruey-Sheng Wang, Yu-Jia Chang, Chamindrani Mendis-Handagama, Liquan Hu, Henry Lardy, Chawnschang Chang, and † † George. 2006. *Oligozoospermia with Normal Fertility in Male Mice Lacking the Androgen Receptor in Testis Peritubular Myoid Cells*
- Zhou, Wei, Gensheng Wang, Christopher L. Small, Zhilin Liu, Connie C. Weng, Lizhong Yang, Michael D. Griswold, and Marvin L. Meistrich. 2011. "Erratum: Gene Expression Alterations by Conditional Knockout of Androgen Receptor in Adult Sertoli Cells of Utp14bjsd/Jsd (Jsd) Mice (Biology of Reproduction (2010) 83, (759-766) DOI: 10.1095/Biolreprod.110.085472)." *Biology of Reproduction* 84(2):400-408.

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
------------	----------

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 <i>in vitro</i> assay receptor binding and transactivation assays	Decreased male AGD after prenatal exposure in studies in rat
Procymidone	AR antagonism in <i>in vitro</i> assay receptor binding and transactivation assays	Decreased male AGD after prenatal exposure in studies in rat
Vinclozolin	AR antagonism in <i>in vitro</i> 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 <i>in vitro</i> 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 deduced that fetal exposure to substances known to decrease androgen receptor activation through antagonism of the AR (vinclozolin, procymidone, flutamide), inhibition of testosterone synthesis (DEHP) 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.

References

- Amato, Ciro M., Humphrey H-C. Yao, and Fei Zhao. "One Tool for Many Jobs: Divergent and Conserved Actions of Androgen Signaling in Male Internal Reproductive Tract and External Genitalia." *Frontiers in Endocrinology* 13 (2022). <https://www.frontiersin.org/articles/10.3389/fendo.2022.910964>.
- Andersson, S, and D W Russell. "Structural and Biochemical Properties of Cloned and Expressed Human and Rat Steroid 5 Alpha-Reductases." *Proceedings of the National Academy of Sciences* 87, no. 10 (May 1990): 3640-44. <https://doi.org/10.1073/pnas.87.10.3640>.
- Andrade AJ, Grande SW, Talsness CE, Grote K, Golombiewski A, Sterner-Kock A, and Chahoud I. "A Dose-Response Study Following in Utero and Lactational Exposure to Di-(2-Ethylhexyl) Phthalate (DEHP): Effects on Androgenic Status, Developmental Landmarks and Testicular Histology in Male Offspring Rats." *Toxicology* 225, no. 1 (2006): 64-74. <https://doi.org/10.1016/j.tox.2006.05.007>.
- Arbuckle TE, Agarwal A, MacPherson SH, Fraser WD, Sathyanarayana S, Ramsay T, Dodds L, et al. "Prenatal Exposure to Phthalates and Phenols and Infant Endocrine-Sensitive Outcomes: The MIREC Study." *Environment International* 120 (2018): 572-83. <https://doi.org/10.1016/j.envint.2018.08.034>.
- Barrett, E. S., L. E. Parlett, J. B. Redmon, and S. H. Swan. "Evidence for Sexually Dimorphic Associations Between Maternal Characteristics and Anogenital Distance, a Marker of Reproductive Development." *American Journal of Epidemiology* 179, no. 1 (January 1, 2014): 57-66. <https://doi.org/10.1093/aje/kwt220>.
- Batista, Rafael L., and Berenice B. Mendonca. "The Molecular Basis of 5 α -Reductase Type 2 Deficiency." *Sexual Development* 16, no. 2-3 (2022): 171-83. <https://doi.org/10.1159/000525119>.
- Borch J, Ladefoged O, Hass U, and Vinggaard AM. "Steroidogenesis in Fetal Male Rats Is Reduced by DEHP and DINP, but Endocrine Effects of DEHP Are Not Modulated by DEHA in Fetal, Prepubertal and Adult Male Rats." *Reproductive Toxicology (Elmsford, N.Y.)* 18, no. 1 (2004): 53-61. <https://doi.org/10.1016/j.reprotox.2003.10.011>.
- Borch, Julie, Stine Broeng Metzдорff, Anne Marie Vinggaard, Leon Brokken, and Majken Dalgaard. "Mechanisms Underlying the Anti-Androgenic Effects of Diethylhexyl Phthalate in Fetal Rat Testis." *Toxicology* 223, no. 1-2 (June 2006): 144-55. <https://doi.org/10.1016/j.tox.2006.03.015>.
- Botelho, Giuliana G. K., Aedra C. Bufalo, Ana Claudia Boareto, Juliane C. Muller, Rosana N. Morais, Anderson J. Martino-Andrade, Karen R. Lemos, and Paulo R. Dalsenter. "Vitamin C and Resveratrol Supplementation to Rat Dams Treated with Di(2-Ethylhexyl)Phthalate: Impact on Reproductive and Oxidative Stress End Points in Male Offspring." *Archives of Environmental Contamination and Toxicology* 57, no. 4 (November 2009): 785-93. <https://doi.org/10.1007/s00244-009-9385-9>.
- Bowman, C. J., N. J. Barlow, K. J. Turner, D. G. Wallace, and P. M. D. Foster. "Effects of in Utero Exposure to Finasteride on Androgen-Dependent Reproductive Development in the Male Rat." *Toxicological Sciences* 74, no. 2 (August 1, 2003): 393-406. <https://doi.org/10.1093/toxsci/kfq128>.
- Casto, J, O Ward, and A Bartke. "Play, Copulation, Anatomy, and Testosterone in Gonadally Intact Male Rats Prenatally Exposed to Flutamide." *Physiology & Behavior* 79, no. 4-5 (September 2003): 633-41. [https://doi.org/10.1016/S0031-9384\(03\)00120-3](https://doi.org/10.1016/S0031-9384(03)00120-3).
- Chamberlain, Nancy L., Erika D. Driver, and Roger L. Miesfeld. "The Length and Location of CAG Trinucleotide Repeats in the Androgen Receptor N-Terminal Domain Affect Transactivation Function." *Nucleic Acids Research* 22, no. 15 (1994): 3181-86. <https://doi.org/10.1093/nar/22.15.3181>.
- Christiansen, Sofie, Julie Boberg, Marta Axelstad, Majken Dalgaard, Anne Marie Vinggaard, Stine Broeng Metzдорff, and Ulla Hass. "Low-Dose Perinatal Exposure to Di(2-Ethylhexyl) Phthalate Induces Anti-Androgenic Effects in Male Rats." *Reproductive Toxicology* 30, no. 2 (September 2010): 313-21. <https://doi.org/10.1016/j.reprotox.2010.04.005>.
- Christiansen, Sofie, Martin Scholze, Majken Dalgaard, Anne Marie Vinggaard, Marta Axelstad, Andreas Kortenkamp, and Ulla Hass. "Synergistic Disruption of External Male Sex Organ Development by a Mixture of Four Antiandrogens." *Environmental Health Perspectives* 117, no. 12 (December 2009): 1839-46. <https://doi.org/10.1289/ehp.0900689>.
- Clark, R.L., C.A. Anderson, S. Prahalada, R.T. Robertson, E.A. Lochry, Y.M. Leonard, J.L. Stevens, and A.M. Hoberman. "Critical Developmental Periods for Effects on Male Rat Genitalia Induced by Finasteride, a 5 α -Reductase Inhibitor." *Toxicology and Applied Pharmacology* 119, no. 1 (March 1993): 34-40. <https://doi.org/10.1006/taap.1993.1041>.
- Colbert NK, Pelletier NC, Cote JM, Concannon JB, Jurdak NA, Minott SB, and Markowski VP. "Perinatal Exposure to Low Levels of the Environmental Antiandrogen Vinclozolin Alters Sex-Differentiated Social Play and Sexual Behaviors in the Rat." *Environmental Health Perspectives* 113, no. 6 (2005): 700-707. <https://doi.org/10.1289/ehp.7509>.
- Crouch, Ns, Lina Michala, Sm Creighton, and Gs Conway. "Androgen-Dependent Measurements of Female Genitalia in Women with Complete Androgen Insensitivity Syndrome: Measurements of Female Genitalia in Women with Complete Androgen Insensitivity Syndrome." *BJOG: An International Journal of Obstetrics & Gynaecology* 118, no. 1 (January 2011): 84-87. <https://doi.org/10.1111/j.1471-0528.2010.02778.x>.
- Culty, Martine, Raphael Thuillier, Wenping Li, Yan Wang, Daniel B. Martinez-Arguelles, Carolina Gesteira Benjamin, Kostantinos M. Triantafyllou, Barry R. Zirkin, and Vassilios Papadopoulos. "In Utero Exposure to Di-(2-Ethylhexyl) Phthalate Exerts Both Short-Term and Long-Lasting Suppressive Effects on Testosterone Production in the Rat1." *Biology of Reproduction* 78, no. 6 (June 1, 2008): 1018-28. <https://doi.org/10.1095/biolreprod.107.065649>.
- Do, Rylee Phuong, Richard W. Stahlhut, Davide Ponzi, Frederick S. Vom Saal, and Julia A. Taylor. "Non-Monotonic Dose Effects

of in Utero Exposure to Di(2-Ethylhexyl) Phthalate (DEHP) on Testicular and Serum Testosterone and Anogenital Distance in Male Mouse Fetuses." *Reproductive Toxicology* 34, no. 4 (December 2012): 614–21.

<https://doi.org/10.1016/j.reprotox.2012.09.006>.

Draskau, Monica Kam, Anne-Sofie Ravn Ballegaard, Louise Ramhøj, Josephine Bowles, Terje Svingen, and Cassy M. Spiller. "AOP Key Event Relationship Report: Linking Decreased Retinoic Acid Levels with Disrupted Meiosis in Developing Oocytes." *Current Research in Toxicology* 3 (2022): 100069. <https://doi.org/10.1016/j.crttox.2022.100069>.

Eisenberg ML, Hsieh TC, Pastuszak AW, McIntyre MG, Walters RC, Lamb DJ, and Lipshultz LI. "The Relationship between Anogenital Distance and the Androgen Receptor CAG Repeat Length." *Asian Journal of Andrology* 15, no. 2 (2013): 286–89. <https://doi.org/10.1038/aja.2012.126>.

Euling, S. Y. "Response-Surface Modeling of the Effect of 5alpha-Dihydrotestosterone and Androgen Receptor Levels on the Response to the Androgen Antagonist Vinclozolin." *Toxicological Sciences* 69, no. 2 (October 1, 2002): 332–43. <https://doi.org/10.1093/toxsci/69.2.332>.

Foster PM and Harris MW. "Changes in Androgen-Mediated Reproductive Development in Male Rat Offspring Following Exposure to a Single Oral Dose of Flutamide at Different Gestational Ages." *Toxicological Sciences: An Official Journal of the Society of Toxicology* 85, no. 2 (2005): 1024–32. <https://doi.org/10.1093/toxsci/kfi159>.

Fussell, Karma C., Steffen Schneider, Roland Buesen, Sibylle Groeters, Volker Strauss, Stephanie Melching-Kollmuss, and Bennard Van Ravenzwaay. "Investigations of Putative Reproductive Toxicity of Low-Dose Exposures to Flutamide in Wistar Rats." *Archives of Toxicology* 89, no. 12 (December 2015): 2385–2402. <https://doi.org/10.1007/s00204-015-1622-6>.

Goldspiel, Barry R., and David R. Kohler. "Flutamide: An Antiandrogen for Advanced Prostate Cancer." *DICP* 24, no. 6 (June 1990): 616–23. <https://doi.org/10.1177/106002809002400612>.

Goto, Kazunori, Keiji Koizumi, Hitoshi Takaori, Yoshinobu Fujii, Yuko Furuyama, Osamu Saika, Hiroetsu Suzuki, Kenichi Saito, and Katsushi Suzuki. "EFFECTS OF FLUTAMIDE ON SEX MATURATION AND BEHAVIOR OF OFFSPRING BORN TO FEMALE RATS TREATED DURING LATE PREGNANCY." *The Journal of Toxicological Sciences* 29, no. 5 (2004): 517–34. <https://doi.org/10.2131/jts.29.517>.

Gray, L. E., J Ostby, J Furr, M Price, D N Rao Veeramachaneni, and L Parks. "Perinatal Exposure to the Phthalates DEHP, BBP, and DINP, but Not DEP, DMP, or DOTP, Alters Sexual Differentiation of the Male Rat." *Toxicological Sciences* 58, no. 2 (December 1, 2000): 350–65. <https://doi.org/10.1093/toxsci/58.2.350>.

Gray, L.E., J.S. Ostby, and W.R. Kelce. "Developmental Effects of an Environmental Antiandrogen: The Fungicide Vinclozolin Alters Sex Differentiation of the Male Rat." *Toxicology and Applied Pharmacology* 129, no. 1 (November 1994): 46–52. <https://doi.org/10.1006/taap.1994.1227>.

Gray, Leon Earl, Norman J. Barlow, Kembra L. Howdeshell, Joseph S. Ostby, Johnathan R. Furr, and Clark L. Gray. "Transgenerational Effects of Di (2-Ethylhexyl) Phthalate in the Male CRL:CD(SD) Rat: Added Value of Assessing Multiple Offspring per Litter." *Toxicological Sciences* 110, no. 2 (August 2009): 411–25. <https://doi.org/10.1093/toxsci/kfp109>.

Hannas, Bethany R., Christy S. Lambricht, Johnathan Furr, Nicola Evans, Paul M. D. Foster, Earl L. Gray, and Vickie S. Wilson. "Genomic Biomarkers of Phthalate-Induced Male Reproductive Developmental Toxicity: A Targeted RT-PCR Array Approach for Defining Relative Potency." *Toxicological Sciences* 125, no. 2 (February 2012): 544–57. <https://doi.org/10.1093/toxsci/kfr315>.

Hannas, Bethany R., Christy S. Lambricht, Johnathan Furr, Kembra L. Howdeshell, Vickie S. Wilson, and Leon E. Gray. "Dose-Response Assessment of Fetal Testosterone Production and Gene Expression Levels in Rat Testes Following InUtero Exposure to Diethylhexyl Phthalate, Diisobutyl Phthalate, Diisooheptyl Phthalate, and Diisononyl Phthalate." *Toxicological Sciences* 123, no. 1 (September 2011): 206–16. <https://doi.org/10.1093/toxsci/kfr146>.

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Metzдорff SB, and Kortenkamp A. "Combined Exposure to Anti-Androgens Exacerbates Disruption of Sexual Differentiation in the Rat." *Environmental Health Perspectives* 115 (2007): 122–28. <https://doi.org/10.1289/ehp.9360>.

Hass, Ulla, Julie Boberg, Sofie Christiansen, Pernille Rosenskjoeld Jacobsen, Anne Marie Vinggaard, Camilla Taxvig, Mette Erecius Poulsen, et al. "Adverse Effects on Sexual Development in Rat Offspring after Low Dose Exposure to a Mixture of Endocrine Disrupting Pesticides." *REPRODUCTIVE TOXICOLOGY* 34, no. 2 (2012): 261–74. <https://doi.org/10.1016/j.reprotox.2012.05.090>.

Hellwig, J., B. Van Ravenzwaay, M. Mayer, and C. Gembardt. "Pre- and Postnatal Oral Toxicity of Vinclozolin in Wistar and Long-Evans Rats." *Regulatory Toxicology and Pharmacology* 32, no. 1 (August 2000): 42–50. <https://doi.org/10.1006/rtp.2000.1400>.

Henriksen LS, Frederiksen H, Jørgensen N, Juul A, Skakkebaek NE, Toppari J, Petersen JH, and Main KM. "Maternal Phthalate Exposure during Pregnancy and Testis Function of Young Adult Sons." *The Science of the Total Environment*, 2023, 161914. <https://doi.org/10.1016/j.scitotenv.2023.161914>.

Hosokawa, Shunji, Masakazu Murakami, Mariko Ineyama, Tomoya Yamada, Akira Yoshitake, Hirohiko Yamada, and Junshi Miyamoto. "The Affinity of Procymidone to Androgen Receptor in Rats and Mice." *The Journal of Toxicological Sciences* 18, no. 2 (1993): 83–93. <https://doi.org/10.2131/jts.18.83>.

Hughes, Ieuan A, John D Davies, Trevor I Bunch, Vickie Pasterski, Kiki Mastroyannopoulou, and Jane MacDougall. "Androgen Insensitivity Syndrome." *Lancet* 2012 OCT, no. 20;380(9851) (June 13, 2012): 1419–28. [https://doi.org/doi:10.1016/S0140-6736\(12\)60071-3](https://doi.org/doi:10.1016/S0140-6736(12)60071-3).

- Inawaka, Kunifumi, Noriyuki Kishimoto, Hashihiro Higuchi, and Satoshi Kawamura. "Maternal Exposure to Procymidone Has No Effects on Fetal External Genitalia Development in Male Rabbit Fetuses in a Modified Developmental Toxicity Study." *The Journal of Toxicological Sciences* 35, no. 3 (2010): 299-307. <https://doi.org/10.2131/jts.35.299>.
- Ipulan LA, Raga D, Suzuki K, Murashima A, Matsumaru D, Cunha G, and Yamada G. "Investigation of Sexual Dimorphisms through Mouse Models and Hormone/Hormone-Disruptor Treatments." *Differentiation; Research in Biological Diversity* 91, no. 4 (2016): 78-89. <https://doi.org/10.1016/j.diff.2015.11.001>.
- Jarfelt, K, M Dalgaard, U Hass, J Borch, H Jacobsen, and O Ladefoged. "Antiandrogenic Effects in Male Rats Perinatally Exposed to a Mixture of Di(2-Ethylhexyl) Phthalate and Di(2-Ethylhexyl) Adipate." *Reproductive Toxicology* 19, no. 4 (April 2005): 505-15. <https://doi.org/10.1016/j.reprotox.2004.11.005>.
- Jensen TK, Frederiksen H, Kyhl HB, Lassen TH, Swan SH, Bornehag CG, Skakkebaek NE, et al. "Prenatal Exposure to Phthalates and Anogenital Distance in Male Infants from a Low-Exposed Danish Cohort (2010-2012)." *Environmental Health Perspectives* 124, no. 7 (2016): 1107-13. <https://doi.org/10.1289/ehp.1509870>.
- Kang, Hong-Yo, Ko-En Huang, Shihuh Young Chang, Wen-Lung Ma, Wen-Jye Lin, and Chawnshang Chang. "Differential Modulation of Androgen Receptor-Mediated Transactivation by Smad3 and Tumor Suppressor Smad4." *Journal of Biological Chemistry* 277, no. 46 (November 2002): 43749-56. <https://doi.org/10.1074/jbc.M205603200>.
- Kelce, William R., Christy R. Lambright, L.Earl Gray, and Kenneth P. Roberts. "Vinclozolin Andp,P'-DDE Alter Androgen-Dependent Gene Expression:In VivoConfirmation of an Androgen Receptor-Mediated Mechanism." *Toxicology and Applied Pharmacology* 142, no. 1 (January 1997): 192-200. <https://doi.org/10.1006/taap.1996.7966>.
- Kita, Diogo H., Katlyn B. Meyer, Amanda C. Venturelli, Rafaella Adams, Daria L.B. Machado, Rosana N. Morais, Shanna H. Swan, Chris Gennings, and Anderson J. Martino-Andrade. "Manipulation of Pre and Postnatal Androgen Environments and Anogenital Distance in Rats." *Toxicology* 368-369 (August 2016): 152-61. <https://doi.org/10.1016/j.tox.2016.08.021>.
- Klinefelter, Gary R, John W Laskey, Witold M Winnik, Juan D Suarez, Naomi L Roberts, Lillian F Strader, Brandy W Riffle, and D N Rao Veeramachaneni. "Novel Molecular Targets Associated with Testicular Dysgenesis Induced by Gestational Exposure to Diethylhexyl Phthalate in the Rat: A Role for Estradiol." *REPRODUCTION* 144, no. 6 (December 2012): 747-61. <https://doi.org/10.1530/REP-12-0266>.
- Kojima, Hiroyuki, Eiji Katsura, Shinji Takeuchi, Kazuhito Niiyama, and Kunihiro Kobayashi. "Screening for Estrogen and Androgen Receptor Activities in 200 Pesticides by in Vitro Reporter Gene Assays Using Chinese Hamster Ovary Cells." *Environmental Health Perspectives* 112, no. 5 (April 2004): 524-31. <https://doi.org/10.1289/ehp.6649>.
- Labrie, F. "Mechanism of Action and Pure Antiandrogenic Properties of Flutamide." *Cancer* 72, no. S12 (December 15, 1993): 3816-27. [https://doi.org/10.1002/1097-0142\(19931215\)72:12+<3816::AID-CNCR2820721711>3.0.CO;2-3](https://doi.org/10.1002/1097-0142(19931215)72:12+<3816::AID-CNCR2820721711>3.0.CO;2-3).
- Lanzoni, Anna, Anna F Castoldi, George EN Kass, Andrea Terron, Guilhem De Seze, Anna Bal-Price, Frédéric Y Bois, et al. "Advancing Human Health Risk Assessment." *EFSA Journal* 17, no. Suppl 1 (July 8, 2019): e170712. <https://doi.org/10.2903/j.efsa.2019.e170712>.
- Lin, Han, Qing-Quan Lian, Guo-Xin Hu, Yuan Jin, Yunhui Zhang, Dianne O. Hardy, Guo-Rong Chen, et al. "In Utero and Lactational Exposures to Diethylhexyl-Phthalate Affect Two Populations of Leydig Cells in Male Long-Evans Rats1." *Biology of Reproduction* 80, no. 5 (May 1, 2009): 882-88. <https://doi.org/10.1095/biolreprod.108.072975>.
- Martínez, Ariadne Gutiérrez, Balía Pardo, Rafael Gámez, Rosa Mas, Miriam Noa, Gisela Marrero, Maikel Valle, et al. "Effects of In Utero Exposure to D-004, a Lipid Extract from *Roystonea Regia* Fruits, in the Male Rat: A Comparison with Finasteride." *Journal of Medicinal Food* 14, no. 12 (December 2011): 1663-69. <https://doi.org/10.1089/jmf.2010.0279>.
- Martino-Andrade AJ, Liu F, Sathyanarayana S, Barrett ES, Redmon JB, Nguyen RH, Levine H, and Swan SH. "Timing of Prenatal Phthalate Exposure in Relation to Genital Endpoints in Male Newborns." *Andrology* 4, no. 4 (2016): 585-93. <https://doi.org/10.1111/andr.12180>.
- Martino-Andrade, Anderson J., Rosana N. Morais, Giuliana G. K. Botelho, Graziela Muller, Simone W. Grande, Giovanna B. Carpentieri, Gabriel M. C. Leão, and Paulo R. Dalsenter. "Coadministration of Active Phthalates Results in Disruption of Foetal Testicular Function in Rats." *International Journal of Andrology* 32, no. 6 (December 2009): 704-12. <https://doi.org/10.1111/j.1365-2605.2008.00939.x>.
- Matsuura, Ikuo, Tetsuji Saitoh, Michiko Ashina, Yumi Wako, Hiroshi Iwata, Naoto Toyota, Yoshihito Ishizuka, Masato Namiki, Nobuhito Hoshino, and Minoru Tsuchitani. "EVALUATION OF A TWO-GENERATION REPRODUCTION TOXICITY STUDY ADDING ENDOPOINTS TO DETECT ENDOCRINE DISRUPTING ACTIVITY USING VINCLOZOLIN." *The Journal of Toxicological Sciences* 30, no. Special (2005): S163-188. <https://doi.org/10.2131/jts.30.S163>.
- McIntyre, B. S. "Androgen-Mediated Development in Male Rat Offspring Exposed to Flutamide in Utero: Permanence and Correlation of Early Postnatal Changes in Anogenital Distance and Nipple Retention with Malformations in Androgen-Dependent Tissues." *Toxicological Sciences* 62, no. 2 (August 1, 2001): 236-49. <https://doi.org/10.1093/toxsci/62.2.236>.
- Molina-Molina, J, A Hillenweck, I Jouanin, D Zalko, J Cravedi, M Fernandez, A Pillon, J Nicolas, N Olea, and P Balaguer. "Steroid Receptor Profiling of Vinclozolin and Its Primary Metabolites." *Toxicology and Applied Pharmacology* 216, no. 1 (October 1, 2006): 44-54. <https://doi.org/10.1016/j.taap.2006.04.005>.
- Moore, R W, T A Rudy, T M Lin, K Ko, and R E Peterson. "Abnormalities of Sexual Development in Male Rats with in Utero and

Lactational Exposure to the Antiandrogenic Plasticizer Di(2-Ethylhexyl) Phthalate." *Environmental Health Perspectives* 109, no. 3 (March 2001): 229–37. <https://doi.org/10.1289/ehp.01109229>.

Murashima, Aki, Satoshi Kishigami, Axel Thomson, and Gen Yamada. "Androgens and Mammalian Male Reproductive Tract Development." *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1849, no. 2 (February 2015): 163–70. <https://doi.org/10.1016/j.bbagr.2014.05.020>.

Nightingale, Joanna, Khurram S. Chaudhary, Paul D. Abel, Andrew P. Stubbs, Hanna M. Romanska, Stephen E. Mitchell, Gordon W.H. Stamp, and El-Nasir Lalani. "Ligand Activation of the Androgen Receptor Downregulates E-Cadherin-Mediated Cell Adhesion and Promotes Apoptosis of Prostatic Cancer Cells." *Neoplasia* 5, no. 4 (July 2003): 347–61. [https://doi.org/10.1016/S1476-5586\(03\)80028-3](https://doi.org/10.1016/S1476-5586(03)80028-3).

OECD. *Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals*. OECD Guidelines for the Testing of Chemicals, Section 4. OECD, 2023. <https://doi.org/10.1787/9789264264366-en>.

Olier, Ivan, Yiqiang Zhan, Xiaoyu Liang, and Victor Volovici. "Causal Inference and Observational Data." *BMC Medical Research Methodology* 23, no. 1 (October 11, 2023): 227. <https://doi.org/10.1186/s12874-023-02058-5>.

Orton, Frances, Erika Rosivatz, Martin Scholze, and Andreas Kortenkamp. "Widely Used Pesticides with Previously Unknown Endocrine Activity Revealed as *in Vitro* Antiandrogens." *Environmental Health Perspectives* 119, no. 6 (June 2011): 794–800. <https://doi.org/10.1289/ehp.1002895>.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, and Gray LE Jr. "The Fungicide Procymidone Alters Sexual Differentiation in the Male Rat by Acting as an Androgen-Receptor Antagonist in Vivo and in Vitro." *Toxicology and Industrial Health* 15, no. 1 (1999): 80–93. <https://doi.org/10.1177/074823379901500108>.

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, and Gray LE Jr. "The Plasticizer Diethylhexyl Phthalate Induces Malformations by Decreasing Fetal Testosterone Synthesis during Sexual Differentiation in the Male Rat." *Toxicological Sciences: An Official Journal of the Society of Toxicology* 58, no. 2 (2000): 339–49. <https://doi.org/10.1093/toxsci/58.2.339>.

Rittmaster, Roger S., and Alastair J.J. Wood. "Finasteride." *New England Journal of Medicine* 330, no. 2 (January 13, 1994): 120–25. <https://doi.org/10.1056/NEJM199401133300208>.

Saillenfait, Anne-Marie, Jean-Philippe Sabaté, and Frédéric Gallissot. "Diisobutyl Phthalate Impairs the Androgen-Dependent Reproductive Development of the Male Rat." *Reproductive Toxicology* 26, no. 2 (October 2008): 107–15. <https://doi.org/10.1016/j.reprotox.2008.07.006>.

Sato, Takashi, Takahiro Matsumoto, Hirotaka Kawano, Tomoyuki Watanabe, Yoshikatsu Uematsu, Keisuke Sekine, Toru Fukuda, et al. "Brain Masculinization Requires Androgen Receptor Function." *Proceedings of the National Academy of Sciences* 101, no. 6 (February 10, 2004): 1673–78. <https://doi.org/10.1073/pnas.0305303101>.

Schaufele, Fred, Xavier Carbonell, Martin Guerbodot, Sabine Borngraeber, Mark S. Chapman, Aye Aye K. Ma, Jeffrey N. Miner, and Marc I. Diamond. "The Structural Basis of Androgen Receptor Activation: Intramolecular and Intermolecular Amino-Carboxy Interactions." *Proceedings of the National Academy of Sciences* 102, no. 28 (July 12, 2005): 9802–7. <https://doi.org/10.1073/pnas.0408819102>.

Schneider, Steffen, Wolfgang Kaufmann, Volker Strauss, and Bennard Van Ravenzwaay. "Vinclozolin: A Feasibility and Sensitivity Study of the ILSI-HESI F1-Extended One-Generation Rat Reproduction Protocol." *Regulatory Toxicology and Pharmacology* 59, no. 1 (February 2011): 91–100. <https://doi.org/10.1016/j.yrtph.2010.09.010>.

Scholze M, Taxvig C, Kortenkamp A, Boberg J, Christiansen S, Svingen T, Lauschke K, et al. "Quantitative in Vitro to in Vivo Extrapolation (QIVIVE) for Predicting Reduced Anogenital Distance Produced by Anti-Androgenic Pesticides in a Rodent Model for Male Reproductive Disorders." *Environmental Health Perspectives* 128, no. 11 (2020): 117005. <https://doi.org/10.1289/EHP6774>.

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, and Svingen T. "Anogenital Distance as a Toxicological or Clinical Marker for Fetal Androgen Action and Risk for Reproductive Disorders." *Archives of Toxicology* 93, no. 2 (2019): 253–72. <https://doi.org/10.1007/s00204-018-2350-5>.

Sharpe, Richard M. "Androgens and the Masculinization Programming Window: Human–Rodent Differences." *Biochemical Society Transactions* 48, no. 4 (August 28, 2020): 1725–35. <https://doi.org/10.1042/BST20200200>.

Shimamura M, Kodaira K, Kenichi H, Ishimoto Y, Tamura H, and Iguchi T. "Comparison of Antiandrogenic Activities of Vinclozolin and D,L-Camphorquinone in Androgen Receptor Gene Transcription Assay in Vitro and Mouse in Utero Exposure Assay in Vivo." *Toxicology* 174, no. 2 (2002): 97–107. [https://doi.org/10.1016/S0300-483X\(02\)00044-6](https://doi.org/10.1016/S0300-483X(02)00044-6).

Simard, J., I. Luthy, J. Guay, A. Bélanger, and F. Labrie. "Characteristics of Interaction of the Antiandrogen Flutamide with the Androgen Receptor in Various Target Tissues." *Molecular and Cellular Endocrinology* 44, no. 3 (March 1986): 261–70. [https://doi.org/10.1016/0303-7207\(86\)90132-2](https://doi.org/10.1016/0303-7207(86)90132-2).

Song, Jae W., and Kevin C. Chung. "Observational Studies: Cohort and Case-Control Studies." *Plastic and Reconstructive Surgery* 126, no. 6 (December 2010): 2234–42. <https://doi.org/10.1097/PRS.0b013e3181f44abc>.

Stoner, Elizabeth. "The Clinical Development of a 5 α -Reductase Inhibitor, Finasteride." *The Journal of Steroid Biochemistry and Molecular Biology* 37, no. 3 (November 1990): 375–78. [https://doi.org/10.1016/0960-0760\(90\)90487-6](https://doi.org/10.1016/0960-0760(90)90487-6).

- Sunman, Birce, Kadriye Yurdakok, Belma Kocer-Gumusel, Ozgur Ozyuncu, Filiz Akbiyik, Aylin Balci, Gizem Ozkemahli, Pinar Erkekoglu, and Murat Yurdakok. "Prenatal Bisphenol a and Phthalate Exposure Are Risk Factors for Male Reproductive System Development and Cord Blood Sex Hormone Levels." *REPRODUCTIVE TOXICOLOGY* 87 (2019): 146-55. <https://doi.org/10.1016/j.reprotox.2019.05.065>.
- Swan, Shanna H. "Environmental Phthalate Exposure in Relation to Reproductive Outcomes and Other Health Endpoints in Humans." *ENVIRONMENTAL RESEARCH* 108, no. 2 (2008): 177-84. <https://doi.org/10.1016/j.envres.2008.08.007>.
- Swan, Shanna H., Katharina M. Main, Fan Liu, Sara L. Stewart, Robin L. Kruse, Antonia M. Calafat, Catherine S. Mao, et al. "Decrease in Anogenital Distance among Male Infants with Prenatal Phthalate Exposure." *Environmental Health Perspectives* 113, no. 8 (August 2005): 1056-61. <https://doi.org/10.1289/ehp.8100>.
- Thankamony, A., V. Pasterski, K. K. Ong, C. L. Acerini, and I. A. Hughes. "Anogenital Distance as a Marker of Androgen Exposure in Humans." *Andrology* 4, no. 4 (July 2016): 616-25. <https://doi.org/10.1111/andr.12156>.
- Tut, Thein G., Farid J. Ghadessy, M. A. Trifiro, L. Pinsky, and E. L. Yong. "Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced *Trans*-Activation, Impaired Sperm Production, and Male Infertility ¹." *The Journal of Clinical Endocrinology & Metabolism* 82, no. 11 (November 1997): 3777-82. <https://doi.org/10.1210/jcem.82.11.4385>.
- Ungewitter, Erica, Emmi Rotgers, Tanika Bantukul, Yasuhiko Kawakami, Grace E. Kissling, and Humphrey Hung-Chang Yao. "Teratogenic Effects of *in Utero* Exposure to Di-(2-Ethylhexyl)-Phthalate (DEHP) in B6:129S4 Mice." *Toxicological Sciences*, January 25, 2017, kfx019. <https://doi.org/10.1093/toxsci/kfx019>.
- Venturelli, Amanda Caroline, Katlyn Barp Meyer, Stefani Valéria Fischer, Diogo Henrique Kita, Rafaela Adams Philipsen, Rosana Nogueira Morais, and Anderson Joel Martino Andrade. "Effects of *in Utero* and Lactational Exposure to Phthalates on Reproductive Development and Glycemic Homeostasis in Rats." *Toxicology* 421 (June 2019): 30-40. <https://doi.org/10.1016/j.tox.2019.03.008>.
- Vo TT, Jung EM, Dang VH, Jung K, Baek J, Choi KC, and Jeung EB. "Differential Effects of Flutamide and Di-(2-Ethylhexyl) Phthalate on Male Reproductive Organs in a Rat Model." *The Journal of Reproduction and Development* 55, no. 4 (2009): 400-411. <https://doi.org/10.1262/jrd.20220>.
- Welsh, Michelle, Philippa T.K. Saunders, Mark Fiskien, Hayley M. Scott, Gary R. Hutchison, Lee B. Smith, and Richard M. Sharpe. "Identification in Rats of a Programming Window for Reproductive Tract Masculinization, Disruption of Which Leads to Hypospadias and Cryptorchidism." *Journal of Clinical Investigation* 118, no. 4 (April 1, 2008): 1479-90. <https://doi.org/10.1172/JCI34241>.
- Welsh, Michelle, Hiroko Suzuki, and Gen Yamada. "The Masculinization Programming Window." In *UNDERSTANDING DIFFERENCES AND DISORDERS OF SEX DEVELOPMENT (DSD)*, 27:17-27, 2014. <https://doi.org/10.1159/000363609>.
- Wenzel AG, Bloom MS, Butts CD, Wineland RJ, Brock JW, Cruze L, Unal ER, Kucklick JR, Somerville SE, and Newman RB. "Influence of Race on Prenatal Phthalate Exposure and Anogenital Measurements among Boys and Girls." *Environment International* 110 (2018): 61-70. <https://doi.org/10.1016/j.envint.2017.10.007>.
- Wilson, Vickie S., Kembra L. Howdeshell, Christy S. Lambright, Johnathan Furr, and L. Earl Gray. "Differential Expression of the Phthalate Syndrome in Male Sprague-Dawley and Wistar Rats after *in Utero* DEHP Exposure." *Toxicology Letters* 170, no. 3 (May 2007): 177-84. <https://doi.org/10.1016/j.toxlet.2007.03.004>.
- Wilson, Vickie S., Christy Lambright, Johnathan Furr, Joseph Ostby, Carmen Wood, Gary Held, and L. Earl Gray. "Phthalate Ester-Induced Gubernacular Lesions Are Associated with Reduced *Ins3* Gene Expression in the Fetal Rat Testis." *Toxicology Letters* 146, no. 3 (February 2004): 207-15. <https://doi.org/10.1016/j.toxlet.2003.09.012>.
- Wolf, C. J., LeBlanc, G.A., and Gray LE Jr. "Interactive Effects of Vinclozolin and Testosterone Propionate on Pregnancy and Sexual Differentiation of the Male and Female SD Rat." *Toxicological Sciences* 78, no. 1 (January 21, 2004): 135-43. <https://doi.org/10.1093/toxsci/kfh018>.
- Wolf, C. J., LeBlanc, G.A., J.S. Ostby, and Gray LE Jr. "Characterization of the Period of Sensitivity of Fetal Male Sexual Development to Vinclozolin." *Toxicological Sciences* 55, no. 1 (May 1, 2000): 152-61. <https://doi.org/10.1093/toxsci/55.1.152>.
- Wolf, Cynthia, Christy Lambright, Peter Mann, Matthew Price, Ralph L. Cooper, Joseph Ostby, and L. Earl Gray. "Administration of Potentially Antiandrogenic Pesticides (Procymidone, Linuron, Iprodione, Chlozolate, p,p'-DDE, and Ketoconazole) and Toxic Substances (Dibutyl- and Diethylhexyl Phthalate, PCB 169, and Ethane Dimethane Sulphonate) during Sexual Differentiation Produces Diverse Profiles of Reproductive Malformations in the Male Rat." *Toxicology and Industrial Health* 15, no. 1-2 (February 1999): 94-118. <https://doi.org/10.1177/074823379901500109>.
- Wong, Choi-iok, William R. Kelce, Madhabananda Sar, and Elizabeth M. Wilson. "Androgen Receptor Antagonist versus Agonist Activities of the Fungicide Vinclozolin Relative to Hydroxyflutamide." *Journal of Biological Chemistry* 270, no. 34 (August 1995): 19998-3. <https://doi.org/10.1074/jbc.270.34.19998>.
- Yamasaki Kanji, Noda Shuji, Muroi Takako, Mitoma Hideo, Takakura Saori, and Sakamoto Satoko. "Effects of *in Utero* and Lactational Exposure to Flutamide in SD Rats: Comparison of the Effects of Administration Periods." *Toxicology* 209, no. 1 (April 2005): 47-54. <https://doi.org/10.1016/j.tox.2004.12.004>.
- Yeh, Shuyuan, Meng-Yin Tsai, Qingquan Xu, Xiao-Min Mu, Henry Lardy, Ko-En Huang, Hank Lin, et al. "Generation and Characterization of Androgen Receptor Knockout (ARKO) Mice: An *in Vivo* Model for the Study of Androgen Functions in

Selective Tissues." *Proceedings of the National Academy of Sciences* 99, no. 21 (October 15, 2002): 13498–503. <https://doi.org/10.1073/pnas.212474399>.

Zhang, Jie, Yuanyuan Yao, Junlin Pan, Xiuxiu Guo, Xiaoying Han, Jun Zhou, and Xiaoqian Meng. "Maternal Exposure to Di-(2-Ethylhexyl) Phthalate (DEHP) Activates the PI3K/Akt/MTOR Signaling Pathway in F1 and F2 Generation Adult Mouse Testis." *Experimental Cell Research* 394, no. 2 (September 2020): 112151. <https://doi.org/10.1016/j.yexcr.2020.112151>.

Zhang, Lian-Dong, Qian Deng, Zi-Ming Wang, Ming Gao, Lei Wang, Tie Chong, and He-Cheng Li. "Disruption of Reproductive Development in Male Rat Offspring Following Gestational and Lactational Exposure to Di-(2-Ethylhexyl) Phthalate and Genistein." *Biological Research* 46, no. 2 (2013): 139–46. <https://doi.org/10.4067/S0716-97602013000200004>.

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