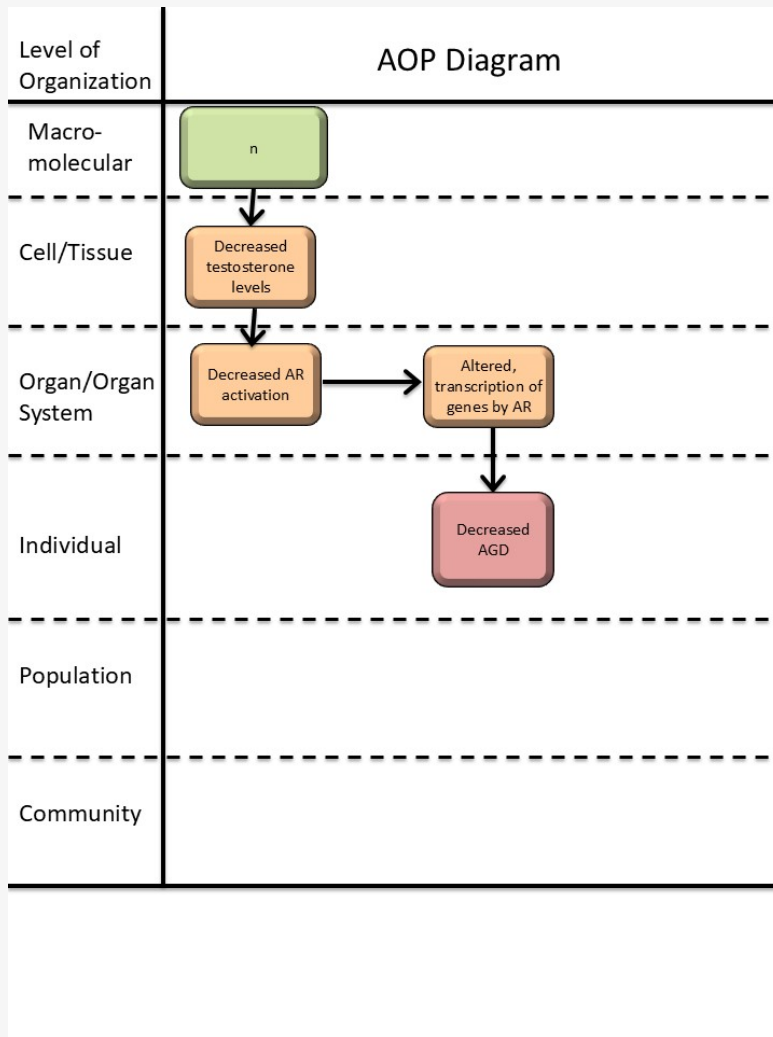


AOP ID and Title:

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

Short Title: Decreased testosterone synthesis leading to short AGD**Graphical Representation****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

Emilie Elmelund; National Food Institute, Technical University of Denmark, Lyngby, DK-2800, Denmark

Anna O. Bindel; National Food Institute, Technical University of Denmark, Lyngby, DK-2800, Denmark

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

Henrik Holbech; University of Southern Denmark, Odense M, DK-5230, Denmark

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Abstract

This AOP links decreased intratesticular testosterone levels with short anogenital distance (AGD) in male offspring. It does not yet contain an MIE, as several upstream mechanisms can lead to 'reduced testosterone' synthesis in fetal testis, such as inhibiting key steroidogenic enzymes. Testosterone is synthesized from cholesterol through several enzymatic steps, including those catalyzed by CYP enzymes such as CYP11 and CYP17. Once synthesized, testosterone is released into circulation and transported to target tissues where it initiates masculinization by binding to and activating the androgen receptor (AR) in target cells. Notably, testosterone can be converted to DHT by 5 α -reductase, with DHT being a more potent AR agonist than testosterone; this testosterone-to-DHT conversion is critical during development for differentiation of male traits, including masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex (Davey and Grossmann, 2016; Keller et al, 1996; Robitaille and Langlois, 2020). The LABC complex fails to develop in the absence or insufficiency of androgen signaling, as for instance observed in female fetuses.

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

A central event in this pathway is the inhibition of testosterone synthesis in the fetal testes, leading to reduced circulating testosterone levels and decreased DHT conversion by 5 α -reductase. Insufficient DHT fails to effectively activate AR in target tissues, including the developing perineal region, which leads to failure to properly masculinize the perineum/LABC complex and ultimately a short AGD.

Background

This AOP was developed as part of an AOP network for developmental androgen signalling-inhibition leading to short AGD in male offspring. The other AOPs in this network are AOP 305 (5 α -reductase inhibition leading to short AGD) and 306 (AR antagonism leading to short AGD).

Androgen signaling is critical for male sex differentiation during fetal life, and suboptimal signaling during critical life stages leads to under-masculinized offspring. Androgens, primarily testosterone and DHT, exert their effects by binding to and activating the AR in target cells. Blocking the AR basically blocks androgen signaling and masculinization of tissues that otherwise should masculinize in male fetuses. One morphometric marker for reduced fetal androgen action is shorter AGD compared to control males.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	KE	1690	Decrease, circulating testosterone levels	Decrease, circulating testosterone levels
	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	286	Altered, Transcription of genes by the androgen receptor	Altered, Transcription of genes by the AR
	KE	2298	Decrease, intratesticular testosterone levels	Decrease, intratesticular testosterone
	AO	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

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

Stressors

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

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage Evidence

Foetal High

Taxonomic Applicability

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

Sex Applicability

Sex Evidence

Male High

The upstream part of the AOP, converging on 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, but only males due to the specification of intratesticular testosterone in KE-2298. It could be extended to cover non-mammalian vertebrates by adding additional relevant knowledge, as previously discussed (Draskau et al, 2024). The overall applicability domain is limited by AO-1688 (decreased AGD). The AGD is strongly influenced by androgen action during critical fetal stages in mammals, with evidence from humans (Murashima et al, 2015; Thankamony et al, 2016), and from numerous gestational exposure studies in rats and mice to anti-androgenic chemicals (Gray et al, 2001; Schwartz et al, 2019). The male

masculinization programming window occurs at a developmental stage included in the applicability domain of these AOPs and corresponds to around gestational day 16-20 in rats and gestation weeks 8-14 in humans (Welsh et al, 2008). Only males are included in the applicability domain since the male AGD, but not the female AGD, is shortened by decreased androgen action (Schwartz et al, 2019).

Essentiality of the Key Events

The essentiality of each key event (KE) was evaluated, meaning that if an upstream KE is blocked or does not occur, subsequent downstream KEs or the adverse outcome (AO) are prevented or altered. Both direct and indirect evidence of essentiality were assessed according to the OECD developer's handbook (see Supplementary Table S1, [Syrainxq_Supplementary_Table_S1_Essentiality_table_AOPs_305_307_forwiki_R2.pdf](#)), with a summary provided in Table 1.

Table 1: Essentiality assessment of KEs of AOP 307.

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
KE-2298		***		Moderate
KE-1690		***		Moderate
KE-1614	***	***		High
KE-286		***		Moderate

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

Weight of Evidence Summary

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

ID	Assessment score	Rationale
KER-3448	High	It is considered canonical knowledge that testis is primary site of testosterone synthesis, and that circulating T will be directly impacted by testis production.
KER-2131	High	It is well established that testosterone activates the AR and that decreased testosterone levels leads to decreased AR activation.
KER-2124	High	It is well established that the AR regulates gene transcription, and that decreased AR activity leads to altered gene transcription.
KER-3449	High	It is well established that testis is the main site of testosterone synthesis and impacts circulating T levels, which again impacts masculinization, including AGD.
KER-3449	High	It is well established that decreased serum testosterone levels impact masculinization of the male fetus, including a feminized AGD.
KER-2820	High	It is well established that decreased AR activity leads to decreased AGD in male offspring.
KER-2127	Moderate	It is highly plausible that altered gene transcription in the perineum leads to decreased AGD in male offspring.

Quantitative Consideration

The quantitative understanding between in vitro test data and in vivo is low. There is some quantitative understanding about the magnitude of reduction in explanted fetal testis testosterone production and effect on AGD (and other masculinization parameters) in rats, related to phthalate exposures. The dose-response relationship appears non-linear, with a low incidence rate of male under-virilization effects when testosterone production is reduced by less than 60% but with a steep increase in rate of malformations, including a decreasing length of the perineum, when testosterone is reduced by more than 60% (Earl Gray et al, 2024). This relationship has not been systematically evaluated for other chemicals.

References

- 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* 43(11):2329-2337.
- Earl Gray L Jr (2023). Biologically relevant reductions in fetal testosterone and InsI3 induced by in utero exposure to high levels of di-isononyl phthalate (DINP) in male rats. *Toxicol Appl Pharmacol* 465:116454.
- Earl Gray L Jr, Lambright CS, Evans N, Ford J, Conley JM (2024). Using targeted fetal rat testis genomic and endocrine alterations to predict the effects of a phthalate mixture on the male reproductive tract. *Curr Res Toxicol*. 7:100180. doi: 10.1016/j.crttox.2024.100180
- Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, Guillette 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, Hlisníková 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, Vellanoweth 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, Pasternski 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, 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(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 Key Events in the AOP

Event: 1690: Decrease, circulating testosterone levels

Short Name: Decrease, circulating testosterone levels

Key Event Component

Process	Object	Action
hormone biosynthetic process	testosterone	decreased
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:526 - Decreased, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) leads to Impaired, Spermatogenesis	KeyEvent
Aop:124 - HMG-CoA reductase inhibition leading to decreased fertility	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males	KeyEvent
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents	KeyEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction [in zebrafish]	KeyEvent
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	KeyEvent
Aop:120 - Inhibition of 5α-reductase leading to Leydig cell tumors (in rat)	KeyEvent
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:595 - Nanoplastic effect	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

blood

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

This key event (KE) is applicable to all mammals, as the synthesis and role of testosterone are evolutionarily conserved (Vitousek et al., 2018). Both sexes produce and require testosterone, which plays critical roles throughout life, from development to adulthood; albeit there are differences in life stages when testosterone exerts specific effects and function (Luetjens & Weinbauer, 2012; Naamneh Elzenaty et al., 2022). Accordingly, this KE applies to both males and females across all life stages, but life stage should be considered when embedding in AOPs.

Notably, the key enzymes involved in testosterone production first appeared in the common ancestor of amphioxus and vertebrates (Baker, 2011). This suggests that the KE has a broader domain of applicability, encompassing non-mammalian vertebrates. AOP developers are encouraged to integrate additional knowledge to expand its relevance beyond mammals to other vertebrates.

Key Event Description

Testosterone is an endogenous steroid hormone that acts by binding the androgen receptor (AR) in androgen-responsive tissues (Murashima et al., 2015). As with all steroid hormones, testosterone is produced through steroidogenesis, an enzymatic pathway converting cholesterol into all the downstream steroid hormones. Briefly, androstenedione or androstenediol is converted to testosterone by the enzymes 17 β -hydroxysteroid dehydrogenase (HSD) or 3 β -HSD, respectively. Testosterone can then be converted to the more potent androgen, dihydrotestosterone (DHT) by 5 α -reductase, or aromatized by CYP19A1 (Aromatase) into estrogens. Testosterone secreted in blood circulation can be found free or bound to SHBG or albumin (Trost & Mulhall, 2016).

Testosterone is produced mainly by the testes (in males), ovaries (in females) and to a lesser degree in the adrenal glands. The output of testosterone from different tissues varies with life stages. During fetal development testosterone is crucial for the differentiation of male reproductive tissues and the overall male phenotype. In adulthood, testosterone synthesis is controlled by the Hypothalamus-Pituitary-Gonadal (HPG) axis. GnRH is released from the hypothalamus inducing LH pulses secreted by the anterior pituitary. This LH surge leads to increased testosterone production, both in testes (males) and ovaries (females). If testosterone reaches low levels, this axis is once again stimulated to increase testosterone synthesis. This feedback loop is essential for maintenance of appropriate testosterone levels (Chandrashekar & Bartke, 1998; Ellis et al., 1983; Rey, 2021).

By disrupting e.g. steroidogenesis or the HPG-axis, testosterone synthesis or homeostasis may be disrupted and can lead to less testosterone being synthesized and released into circulation.

General role in biology

Androgens are essential hormones responsible for the development of the male phenotype during fetal life and for sexual maturation at puberty. In adulthood, androgens remain essential for the maintenance of male reproductive function and behavior but is also essential for female fertility. Apart from their effects on reproduction, androgens affect a wide variety of non-reproductive tissues such as skin, bone, muscle, and brain (Heemers et al 2006). Androgens, principally testosterone and DHT, exert most of their effects by interacting with the AR (Murashima et al 2015).

How it is Measured or Detected

Testosterone levels can be quantified in serum (in vivo), cell culture medium (in vitro), or tissue (ex vivo, in vitro). Methods include traditional immunoassays such as ELISA and RIA, advanced techniques like LC-MS/MS, and liquid scintillation spectrometry following radiolabeling (Shiraishi et al., 2008).

The H295R Steroidogenesis Assay (OECD TG 456) is (currently; anno 2025) primarily used to measure estradiol and testosterone production. This validated OECD test guideline uses adrenal H295R cells, with hormone levels measured in the cell culture medium (OECD, 2011). H295R adrenocortical carcinoma cells express the key enzymes and hormones of the steroidogenic pathway, enabling broad analysis of steroidogenesis disruption by quantifying hormones in the medium using LC-MS/MS. Initially designed to assess testosterone and estradiol levels, the assay now extends to additional steroid hormones, such as progesterone and pregnenolone. The U.S. EPA's ToxCast program further advanced this method, enabling high-throughput measurement of 11 steroidogenesis-related hormones (Haggard et al., 2018). While the H295R assay indirectly reflects disruptions in overall steroidogenesis (e.g., changes in testosterone levels), it does not provide mechanistic insights.

Testosterone can be measured by immunoassays and by isotope-dilution gas chromatography-mass spectrometry in serum (Taieb et al., 2003; Paduch et al., 2014). Testosterone levels may also be measured by: Fish Lifecycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500), Male pubertal assay (PP Male Assay) (US EPA OPPTS 890.1500), OECD TG 441: Hersherberger Bioassay in Rats (H Assay).

References

- Baker, M.E. (2011). Insights from the structure of estrogen receptor into the evolution of estrogens: implications for endocrine disruption. *Biochem Pharmacol*, 82(1), 1-8. <https://doi.org/10.1016/j.bcp.2011.03.008>
- Chandrashekar, V., & Bartke, A. (1998). The Role of Growth Hormone in the Control of Gonadotropin Secretion in Adult Male Rats*. *Endocrinology*, 139(3), 1067-1074. <https://doi.org/10.1210/endo.139.3.5816>
- Ellis, G. B., Desjardins, C., & Fraser, H. M. (1983). Control of Pulsatile LH Release in Male Rats. *Neuroendocrinology*, 37(3), 177-183. <https://doi.org/10.1159/000123540>
- Haggard, D. E., Karmaus, A. L., Martin, M. T., Judson, R. S., Setzer, R. W., & Paul Friedman, K. (2018). High-Throughput H295R Steroidogenesis Assay: Utility as an Alternative and a Statistical Approach to Characterize Effects on Steroidogenesis. *Toxicological Sciences*, 162(2), 509-534. <https://doi.org/10.1093/toxsci/kfx274>
- Heemers, H. V., Verhoeven, G., & Swinnen, J. V. (2006). Androgen activation of the sterol regulatory element-binding protein pathway: Current insights. *Molecular Endocrinology* (Baltimore, Md.), 20(10), 2265-77. doi:10.1210/me.2005-0479
- Luetjens, C. M., & Weinbauer, G. F. (2012). Testosterone: biosynthesis, transport, metabolism and (non-genomic) actions. In *Testosterone* (pp. 15-32). Cambridge University Press. <https://doi.org/10.1017/CBO9781139003353.003>
- Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015). Androgens and mammalian male reproductive tract development. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1849(2), 163-170. <https://doi.org/10.1016/j.bbagrm.2014.05.020>
- Naamneh Elzenaty, R., du Toit, T., & Flück, C. E. (2022). Basics of androgen synthesis and action. *Best Practice & Research Clinical Endocrinology & Metabolism*, 36(4), 101665. <https://doi.org/10.1016/j.beem.2022.101665>
- Paduch, D. A., Brannigan, R. E., Fuchs, E. F., Kim, E. D., Marmar, J. L., & Sandlow, J. I. (2014). The laboratory diagnosis of testosterone deficiency. *Urology*, 83(5), 980-

8. <https://doi.org/10.1016/j.urology.2013.12.024>

Rey, R. A. (2021). The Role of Androgen Signaling in Male Sexual Development at Puberty. *Endocrinology*, 162(2). <https://doi.org/10.1210/endo/bqaa215>

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>

Taieb, J., Mathian, B., Millot, F., Patricot, M.-C., Mathieu, E., Queyrel, N., ... Boudou, P. (2003). Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. *Clinical Chemistry*, 49(8), 1381–95.

Trost, L. W., & Mulhall, J. P. (2016). Challenges in Testosterone Measurement, Data Interpretation, and Methodological Appraisal of Interventional Trials. *The Journal of Sexual Medicine*, 13(7), 1029–1046. <https://doi.org/10.1016/j.jsxm.2016.04.068>

Vitousek, M. N., Johnson, M. A., Donald, J. W., Francis, C. D., Fuxjager, M. J., Goymann, W., Hau, M., Husak, J. F., Kircher, B. K., Knapp, R., Martin, L. B., Miller, E. T., Schoenle, L. A., Uehling, J. J., & Williams, T. D. (2018). HormoneBase, a population-level database of steroid hormone levels across vertebrates. *Scientific Data*, 5(1), 180097. <https://doi.org/10.1038/sdata.2018.97>

Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:288 - Inhibition of 17α-hydrolase/C 10.20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:111 - Decrease in androgen receptor activity leading to Leydig cell tumors (in rat)	MolecularInitiatingEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Key Event Description

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

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

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

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

How it is Measured or Detected

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

References

Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical Biochemist. Reviews*, 37(1), 3–15.

Gao, W., Bohl, C. E., & Dalton, J. T. (2005). Chemistry and structural biology of androgen receptor. *Chemical Reviews*, 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. *Frontiers in Endocrinology*, 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 foetus (mammals)	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:495 - Androgen receptor activation leading to prostate cancer	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction [in zebrafish]	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:576 - 5α-reductase inhibition leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	KeyEvent

Stressors

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

Name

Vinclozalin

Biological Context**Level of Biological Organization**

Tissue

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

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

Key Event Description

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

The Androgen Receptor and its function

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

Altered transcription of genes by the AR as a Key Event

Upon activation by ligand-binding, the AR translocates from the cytoplasm to the cell nucleus, dimerizes, binds to androgen response elements in the DNA to modulate gene transcription (Davey and Grossmann 2016). The transcriptional targets vary between cells and tissues, as well as with developmental stages and is also dependent on available co-regulators (Bevan and Parker 1999; Heemers and Tindall 2007). It should also be mentioned that the AR can work in other 'non-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., Caillaire-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

Event: 2298: Decrease, intratesticular testosterone levels

Short Name: Decrease, intratesticular testosterone

Key Event Component

Process	Object	Action
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:575 - Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

testis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex Evidence

Male High

This key event (KE) is applicable to all male vertebrates with testis that produce testosterone.

Key Event Description

This KE refers to decreased testosterone biosynthesis in the testis (male); i.e. intratesticular testosterone levels. It is therefore considered distinct from KEs describing circulating testosterone levels, or levels in any other tissue or organ of vertebrate animals. It is also distinct from indirect cell-based assays measuring effects on testosterone synthesis, including in vitro Leydig cells.

In males, the testis is the primary site of testosterone biosynthesis via the steroidogenesis pathway – an enzymatic pathway converting cholesterol into all the downstream steroid hormones (Miller and Auchus 2010). In mammals, the Leydig cells are considered the primary site of steroidogenesis in the testis. Although generally correct, there is evidence to suggest the involvement of Sertoli cells during fetal stages in e.g. mouse and human testis, but with Leydig cells being sufficient in adult life (O'Donnell et al 2022).

Testicular testosterone synthesis is primarily regulated by the hypothalamic-pituitary-gonadal (HPG) axis, with Gonadotropin-releasing hormone (GnRH) from the hypothalamus controlling the secretion of Luteinizing hormone (LH) from the pituitary that ultimately binds to the LH receptors on Leydig cells to stimulate steroidogenesis. Notably, the timing of HPG axis activation during development varies between species. In humans, human chorionic gonadotropin (hCG) act similarly to LH and appear to be critical in stimulating testosterone synthesis in the fetal testis (Huhtaniemi 2025), whereas in the mouse testosterone synthesis in the fetal testis appears to be independent of pituitary gonadotropins even though LH is detectable during late gestation O'Shaughnessy et al 1998). Irrespective of testosterone being stimulated by gonadotropins or occurring de novo, however, it is essential for masculinization of the developing fetus, initiation of puberty, and maintain reproductive, and other, functions in adulthood.

Notably, intratesticular testosterone concentration is significantly higher than serum testosterone levels, typically ranging from 30- to 200-fold greater in mammals, including humans (Turner et al 1984; McLachlan et al 2002; Coviello et al 2004).

How it is Measured or Detected

Testosterone levels can be quantified in testis tissue (ex vivo, in vivo). Methods include traditional immunoassays such as ELISA and RIA, advanced techniques like LC-MS/MS, and liquid scintillation spectrometry following radiolabeling (Shiraishi et al., 2008).

References

- Coviello, A.D., Bremner, W.J., Matsumoto, A.M., Herbst, K.L., Amory, J.K., Anawalt, B.D., Yan, X., Brown, T.R., Wright, W.W., Zirkin, B.R. and Jarow, J.P. (2004). Intratesticular Testosterone Concentrations Comparable With Serum Levels Are Not Sufficient to Maintain Normal Sperm Production in Men Receiving a Hormonal Contraceptive Regimen. *J Androl*, 25:931-938. <https://doi.org/10.1002/j.1939-4640.2004.tb03164.x>
- Huhtaniemi, I.T. (2025). Luteinizing hormone receptor knockout mouse: What has it taught us? *Andrology*, In Press. <https://doi.org/10.1111/andr.70000>
- McLachlan, R.I., O'Donnell, L., Stanton, P.G., Balourdos, G., Frydenberg, M., de Kretser, D.M. and Robertson, D.M. (2002). Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. *J Clin Endocrinol Metab*, 87:546-556. <https://doi.org/10.1210/jcem.87.2.8231>
- Miller, W.L. and Auchus, R.J. (2010). The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders. *Endocr Rev*, 32(1):81-151. <https://doi.org/10.1210/er.2010-0013>
- O'Donnell, L., Whiley, P.A.F., and Loveland, K.L. (2022). Activin A and Sertoli Cells: Key to Fetal Testis Steroidogenesis. *Front Endocrinol*, 13:898876. <https://doi.org/10.3389/fendo.2022.898876>
- O'Shaughnessy, P.J., Baker, P., Sohnius, U., Haavisto, A.M., Charlton, H.M. and Huhtaniemi, I. (1998). Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology*, 139:1141-1146. <https://doi.org/10.1210/endo.139.3.5788>
- 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>
- Turner, T.T., Jones, C.E., Howards, S.S., Ewing, L.L., Zegeye, B. and Gonsalus, G.L. (1984). On the androgen microenvironment of maturing spermatozoa. *Endocrinology*, 115:1925-1932. <https://doi.org/10.1210/endo-115-5-1925>

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

Name

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

- Aydoğan 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. *J 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, Metzdorff 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 CJ, Lambright 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: 3448: Decrease, intratesticular testosterone leads to Decrease, circulating testosterone levels

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
Life Stage Applicability			
Life Stage	Evidence		
All life stages	High		
Sex Applicability			
Sex	Evidence		
Male	High		
Taxonomic applicability			
The KER is assessed applicable to mammals, as testicular testosterone synthesis is common for all mammals. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates.			
Sex applicability			
This KER is only applicable to males, as testes are only found in males.			
Life stage applicability			
This KER is applicable to all life stages. Once formed, the testes produce and secrete testosterone during fetal development and throughout postnatal life, although testosterone levels do vary between life stages (Vesper et al., 2015).			
Key Event Relationship Description			
This KE describes a decrease in intratesticular testosterone production leading to a decrease in circulating levels of testosterone. Intratesticular testosterone can be measured in whole testicular tissue samples by testing <i>ex vivo</i> testicular testosterone production, and circulating testosterone is measured in plasma or serum. In males, the testes produce and secrete the majority of the circulating testosterone, with only a small contribution from the adrenal gland (Naamneh Elzenaty et al., 2022). In mammals, intratesticular testosterone levels are 30- to 100-fold higher than serum testosterone levels (Coviello et al., 2004; McLachlan et al., 2002; Turner et al., 1984). Reducing testicular testosterone will consequently lead to a reduction in circulating levels as well.			
Evidence Supporting this KER			
Biological Plausibility			
The biological plausibility for this KER is considered high. The testes are the primary testosterone-producing organs in male mammals and the main contributors to the circulating testosterone levels in males (Naamneh Elzenaty et al., 2022). A decrease in intratesticular testosterone will therefore lead to a decrease in secretion of testosterone and consequently lower circulating levels of testosterone.			
Empirical Evidence			
The empirical evidence for this KER is overall judged as high .			
In vivo toxicity studies in rats and mice have shown that exposure to substances that lowers intratesticular testosterone also lowers circulating testosterone levels. This includes <i>in utero</i> exposure and measurements in fetal males (Borch J et al., 2004; Vinggaard AM et al., 2005) as well as exposure and measurements postnatally in male rodents (Hou X et al., 2020; Ji et al., 2010; Jiang XP et al., 2017)			
Supporting this evidence are castration studies in male rats and monkeys, showing a marked reduction in circulating testosterone levels when removing the testes (Gomes & Jain, 1976; Perachio et al., 1977).			
Lastly, in humans, males with hypogonadism or gonadal dysgenesis present with lower circulating testosterone levels (Hirose Y et al., 2007; Jones LW et al., 1970).			
Dose concordance			
In vivo toxicity studies support dose concordance for this KER, as exemplified below.			
In pre-pubertal/pubertal male rats, chlorocholine chloride exposure (postnatal day (PND) 23-60) in three doses reduced both intratesticular and serum testosterone levels at PND60 at all doses tested (Hou X et al., 2020).			
Perinatal exposure (gestational day (GD) 10-birth) of male mice to diethylhexyl phthalate (DEHP) in three doses (100, 500, and 1000 mg/kg bw/day) reduced intratesticular testosterone at 500 and 1000 mg/kg bw/day at PND1, while only 1000 mg/kg bw/day reduced serum levels of testosterone, although this was measured later, at PND56 (Xie Q et al., 2024)			
In utero exposure (GD7-21) of male rats to DEHP in doses of 300 or 750 mg/kg bw/day reduced intratesticular testosterone levels at GD21, while only the high dose also reduced plasma testosterone levels (Borch J et al., 2004).			
Temporal concordance			
In vivo toxicity studies moderately support temporal concordance for this KER, as exemplified below.			
Several studies show that a decrease in intratesticular and circulating testosterone can be measured at the same time point (Borch J et al., 2004; Hou X et al., 2020; Jiang XP et al., 2017; Vinggaard AM et al., 2005).			
In utero exposure of male mice to DEHP from GD10 to birth reduced intratesticular testosterone levels at PND1 with LOAEL 500 mg/kg bw/day, and when measured at PND56, circulating testosterone levels were decreased, but with LOAEL 1000 mg/kg bw/day (Xie Q et al., 2024).			
In Fisher JS et al., 2003, exposure of male rats from GD13-21 to 500 mg/kg bw/day dibutyl phthalate reduced intratesticular testosterone by ~90% (measured at GD19). When analyzing circulating testosterone levels at PND4, 10, 15, 25, and 90, only the testosterone levels on PND25 were decreased.			
One study report conflicting results on the temporal concordance of this KER (Caceres et al., 2023). Here, male rats were exposed for 20 weeks from PND60 to a mixture of the phytoestrogens genistein and daidzein (combined dose of either 29 or 290 mg/kg bw/day). Intratesticular testosterone was measured every 4 weeks, while serum levels of testosterone were measured every second week. While the mixture caused a reduction of serum testosterone after 2 weeks of exposure, a reduction in intratesticular testosterone was not measured until after 8 weeks. The discrepancy might be explained by the multiple mechanisms of action of the phytoestrogens, as they, besides affecting testicular testosterone synthesis, may also influence peripheral aromatization of testosterone to estrogens (van Duursen et al., 2011).			

Incidence concordance

Incidence concordance can not be evaluated for this KER.

Uncertainties and Inconsistencies

There are examples of *in vivo* studies, in which stressors exposure have caused a reduction in intratesticular testosterone levels without a reduction in circulating testosterone levels.

Quantitative Understanding of the Linkage**Time-scale**

The time-scale for this KER is likely minutes or hours, as testosterone is secreted into the blood from the testes after synthesis. *In vivo*, a decrease in intratesticular and circulating testosterone can be measured at the same time, both in fetal and postnatal studies (Borch J et al., 2004; Hou X et al., 2020; Jiang XP et al., 2017; Vinggaard AM et al., 2005). *Ex vivo*, chemically-induced reduction in testicular production of testosterone can be measured in culture media after 3 hours incubation (earlier time points were not measured) (Wilson et al., 2009).

Known Feedforward/Feedback loops influencing this KER

Testosterone is a part of the hypothalamic-pituitary-gonadal (HPG) axis, which controls testosterone synthesis in puberty and adulthood. In this axis, gonatropin-releasing hormone (GnRH) is released from the hypothalamus and stimulates release of luteinizing hormone (LH) from the pituitary. LH acts on the testes to produce and secrete testosterone. Elevated circulating testosterone levels exerts negative feedback on the HPG axis (decreasing GnRH secretion) to keep testosterone levels in balance (Tilbrook & Clarke, 2001).

Importantly, there are species-specific differences in when the HPG axis is functional during development. In the mouse, fetal testosterone synthesis is independent of pituitary LH (O'Shaughnessy et al., 1998), whereas in humans, human chorionic gonadotropin (hCG) act similarly to LH and appear to be critical in stimulating testosterone synthesis in the fetal testis (Huhtaniemi, 2025).

References

- Borch J, Ladefoged O, Hass U, & Vinggaard AM. (2004). 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(1), 53–61. <https://doi.org/10.1016/j.reprotox.2003.10.011>
- Caceres, S., Crespo, B., Alonso-Diez, A., De Andrés, P. J., Millan, P., Silván, G., Illera, M. J., & Illera, J. C. (2023). Long-Term Exposure to Isoflavones Alters the Hormonal Steroid Homeostasis-Impairing Reproductive Function in Adult Male Wistar Rats. *Nutrients*, 15(5), 1261. <https://doi.org/10.3390/nu15051261>
- Coviello, A. D., Bremner, W. J., Matsumoto, A. M., Herbst, K. L., Amory, J. K., Anawalt, B. D., Yan, X., Brown, T. R., Wright, W. W., Zirkin, B. R., & Jarow, J. P. (2004). Intratesticular Testosterone Concentrations Comparable With Serum Levels Are Not Sufficient to Maintain Normal Sperm Production in Men Receiving a Hormonal Contraceptive Regimen. *Journal of Andrology*, 25(6), 931–938. <https://doi.org/10.1002/j.1939-4640.2004.tb03164.x>
- Fisher JS, Macpherson S, Marchetti N, & Sharpe RM. (2003). Human “testicular dysgenesis syndrome”: A possible model using in-utero exposure of the rat to dibutyl phthalate. *Human Reproduction (Oxford, England)*, 18(7), 1383–1394. <https://doi.org/10.1093/humrep/deg273>
- Gomes, W. R., & Jain, S. K. (1976). Effect of unilateral and bilateral castration and cryptorchidism on serum gonadotrophins in the rat. *The Journal of Endocrinology*, 68(02), 191–196. <https://doi.org/10.1677/joe.0.0680191>
- Hirose Y, Sasa M, Bando Y, Hirose T, Morimoto T, Kurokawa Y, Nagao T, & Tangoku A. (2007). Bilateral male breast cancer with male potential hypogonadism. *World Journal of Surgical Oncology*, 5, 60. <https://doi.org/10.1186/1477-7819-5-60>
- Hou X, Hu H, Xiagedeer B, Wang P, Kang C, Zhang Q, Meng Q, & Hao W. (2020). Effects of chlorocholine chloride on pubertal development and reproductive functions in male rats. *Toxicology Letters*, 319, 1–10. <https://doi.org/10.1016/j.toxlet.2019.10.024>
- Huhtaniemi, I. T. (2025). Luteinizing hormone receptor knockout mouse: What has it taught us? *Andrology*, andr.70000. <https://doi.org/10.1111/andr.70000>
- Ji, Y.-L., Wang, H., Liu, P., Wang, Q., Zhao, X.-F., Meng, X.-H., Yu, T., Zhang, H., Zhang, C., Zhang, Y., & Xu, D.-X. (2010). Pubertal cadmium exposure impairs testicular development and spermatogenesis via disrupting testicular testosterone synthesis in adult mice. *Reproductive Toxicology*, 29(2), 176–183. <https://doi.org/10.1016/j.reprotox.2009.10.014>
- Jiang XP, Tang JY, Xu Z, Han P, Qin ZQ, Yang CD, Wang SQ, Tang M, Wang W, Qin C, Xu Y, Shen BX, Zhou WM, & Zhang W. (2017). Sulforaphane attenuates di-N-butylphthalate-induced reproductive damage in pubertal mice: Involvement of the Nrf2-antioxidant system. *Environmental Toxicology*, 32(7), 1908–1917. <https://doi.org/10.1002/tox.22413>
- Jones LW, Isaacs H Jr, Edelbrock H, & Donnell GN. (1970). Reifenstein's syndrome: Hereditary familial hypogonadism with hypospadias and gynecomastia. *The Journal of Urology*, 104(4), 608–611. [https://doi.org/10.1016/s0022-5347\(17\)61793-2](https://doi.org/10.1016/s0022-5347(17)61793-2)
- McLachlan, R. I., O'Donnell, L., Stanton, P. G., Balourdos, G., Frydenberg, M., de Kretser, D. M., & Robertson, D. M. (2002). Effects of Testosterone Plus Medroxyprogesterone Acetate on Semen Quality, Reproductive Hormones, and Germ Cell Populations in Normal Young Men. *The Journal of Clinical Endocrinology & Metabolism*, 87(2), 546–556. <https://doi.org/10.1210/jcem.87.2.8231>
- Naamneh Elzenaty, R., Du Toit, T., & Flück, C. E. (2022). Basics of androgen synthesis and action. *Best Practice & Research Clinical Endocrinology & Metabolism*, 36(4), 101665. <https://doi.org/10.1016/j.beem.2022.101665>
- O'Shaughnessy, P. J., Baker, P., Sohnius, U., Haavisto, A.-M., Charlton, H. M., & Huhtaniemi, I. (1998). Fetal Development of Leydig Cell Activity in the Mouse Is Independent of Pituitary Gonadotroph Function*. *Endocrinology*, 139(3), 1141–1146. <https://doi.org/10.1210/endo.139.3.5788>
- Perachio, A. A., Alexander, M., Marr, L. D., & Collins, D. C. (1977). Diurnal variations of serum testosterone levels in intact and gonadectomized male and female rhesus monkeys. *Steroids*, 29(1), 21–33. [https://doi.org/10.1016/0039-128X\(77\)90106-4](https://doi.org/10.1016/0039-128X(77)90106-4)
- Tilbrook, A. J., & Clarke, I. J. (2001). Negative Feedback Regulation of the Secretion and Actions of Gonadotropin-Releasing Hormone in Males. *Biology of Reproduction*, 64(3), 735–742. <https://doi.org/10.1095/biolreprod64.3.735>
- Turner, T. T., Jones, C. E., Howards, S. S., Ewing, L. L., Zegeye, B., & Gunsalus, G. L. (1984). On the androgen microenvironment of maturing spermatozoa. *Endocrinology*, 115(5), 1925–1932. <https://doi.org/10.1210/endo-115-5-1925>
- van Duursen, M. B. M., Nijmeijer, S. M., de Morree, E. S., de Jong, P. Chr., & van den Berg, M. (2011). Genistein induces breast cancer-associated aromatase and stimulates estrogen-dependent tumor cell growth in in vitro breast cancer model. *Toxicology*, 289(2), 67–73. <https://doi.org/10.1016/j.tox.2011.07.005>
- Vesper, H. W., Wang, Y., Vidal, M., Botelho, J. C., & Caudill, S. P. (2015). Serum Total Testosterone Concentrations in the US Household Population from the NHANES 2011–2012 Study Population. *Clinical Chemistry*, 61(12), 1495–1504. <https://doi.org/10.1373/clinchem.2015.245969>
- Vinggaard AM, Christiansen S, Laier P, Poulsen ME, Breinholt V, Jarfelt K, Jacobsen H, Dalgaard M, Nellemann C, & Hass U. (2005). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 85(2), 886–897. <https://doi.org/doi.org/10.1093/toxsci/kfi150>
- Wilson, V. S., Lambright, C. R., Furr, J. R., Howdeshell, K. L., & Gray, L. E., Jr. (2009). The herbicide linuron reduces testosterone production from the fetal rat testis during both in utero and in vitro exposures. *TOXICOLOGY LETTERS*, 186(2), 73–77. <https://doi.org/10.1016/j.toxlet.2008.12.017>

Xie Q, Cao H, Liu H, Xia K, Gao Y, & Deng C. (2024). Prenatal DEHP exposure induces lifelong testicular toxicity by continuously interfering with steroidogenic gene expression. *Translational Andrology and Urology*, 13(3), 369–382. <https://doi.org/10.21037/tau-23-503>

Relationship: 2131: Decrease, circulating testosterone levels leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of 17α-hydrolase/C 10.20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	adjacent	High	High
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	Moderate
Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	adjacent	High	
Decreased testosterone synthesis leading to increased nipple retention (NR) in male (rodent) offspring	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Taxonomic applicability

KER2131 is assessed applicable to mammals, as T and AR activation are known to be related in mammals. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Sex applicability

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

Life-stage applicability

KER2131 is considered applicable to developmental and adult life stages, as T-mediated AR activation is relevant from the AR is expressed.

Key Event Relationship Description

This key event relationship links decreased testosterone (T) levels to decreased androgen receptor (AR) activation. T is an endogenous steroid hormone important for, amongst other things, reproductive organ development and growth as well as muscle mass and spermatogenesis (Marks, 2004). T is, together with dihydrotestosterone (DHT), a primary ligand for the AR in mammals (Schuppe et al., 2020). Besides its genomic actions, the AR can also mediate rapid, non-genomic second messenger signaling (Davey & Grossmann, 2016). When T levels are reduced, less substrate is available for the AR, and hence, AR activation is decreased (Gao et al., 2005).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

AR activation is dependent on ligand binding (though a few cases of ligand-independent AR activation has been shown, see *uncertainties and inconsistencies*). T is a primary ligand for the AR, and when T levels are decreased there is less substrate for the AR, and hence, AR activation is decreased. In the male, T is primarily synthesized by the testes, and in some target tissues T is irreversibly metabolized to the more potent metabolite DHT. T and DHT both bind to the AR, but DHT has a higher binding affinity (Gao et al., 2005). The lower binding affinity of T compared to DHT is due to the faster dissociation rate of T from the full-length AR, as T has less effective FXXLF motif binding to AF2 (Askew et al., 2007). Binding of T or DHT has different effects in different tissues. E.g. in the developing male, T is required for development of the internal sex organs (epididymis, vas deferens and the seminal vesicles), whereas DHT is crucial for development of the external sex organs (Keller et al., 1996). In the adult male, androgen action in the reproductive tissues is DHT dependent, whereas action in muscle and bone is DHT independent (Gao et al., 2005). In patients with male androgen deficiency syndrome (AIS), clinically low levels of T leads to reduced AR activation (either due to low T or DHT in target tissue), which manifests as both androgenic related symptoms (such as incomplete or delayed sexual development, loss of body hair, small or shrinking testes, low or zero sperm count) as well as anabolic related symptoms (such as height loss, low trauma fracture, low bone mineral density, reduced muscle bulk and strength, increased body fat). All symptoms can be counteracted by treatment with T, which acts directly on the AR receptor in anabolic tissue (Bhasin et al., 2010). Similarly, removal of the testicles in weanling rats results in a feminized body composition and muscle metabolism, which is reversed by administration of T (Krotkiewski et al., 1980). As this demonstrates, the consequences of low T regarding AR activation will depend on tissue, life stage, species etc.

Empirical Evidence

The empirical evidence for this KER is considered high

Dose concordance

There is a positive dose-response relationship between increasing concentrations of T and AR activation (U.S. EPA., 2023).

Other evidence

- In male patients with androgen deficiency, treatment with T counteracts anabolic (DHT independent) related symptoms such as height loss, low

- trauma fracture, low bone mineral density, reduced muscle bulk and strength, increased body fat (Bhasin et al., 2010; Katznelson et al., 1996)
- Removal of the testicles in weanling rats result in a feminized body composition and muscle metabolism, which is reversed by administration of T (Krotkiewski et al., 1980).

Uncertainties and Inconsistencies

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

Quantitative Understanding of the Linkage

Response-response relationship

There is a positive dose-response relationship between increasing concentrations of T and AR activation (U.S. EPA., 2023). However, there is not enough data, or overview of the data, to define a quantitative linkage *in vivo*, and such a relationship will differ between biological systems (species, tissue, cell type).

Time-scale

AR and promoter interactions occur within 15 minutes of ligand binding, and RNA polymerase II and coactivator recruitment are then proposed to occur transiently with cycles of approximately 90 minutes (Kang et al., 2002).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression changes with aging	Tissue-specific alterations in AR activity with aging	(Supakar et al., 1993; Wu et al., 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Chamberlain et al., 1994; Tut et al., 1997)
Male androgen deficiency syndrome	Low circulating testosterone levels due to primary (testicular) or secondary (pituitary-hypothalamic) hypogonadism	Reduced levels of circulating testosterone	(Bhasin et al., 2010)
Castration	Removal of testicles	Reduced levels of circulating testosterone	(Krotkiewski et al., 1980)

Known Feedforward/Feedback loops influencing this KER

Androgens can upregulate and downregulate AR expression (Lee & Chang, 2003).

References

- Askew, E. B., Gampe, R. T., Stanley, T. B., Faggart, J. L., & Wilson, E. M. (2007). Modulation of Androgen Receptor Activation Function 2 by Testosterone and Dihydrotestosterone. *Journal of Biological Chemistry*, 282(35), 25801–25816. <https://doi.org/10.1074/jbc.M703268200>
- Bennesch, M. A., & Picard, D. (2015). Minireview: Tipping the Balance: Ligand-Independent Activation of Steroid Receptors. *Molecular Endocrinology*, 29(3), 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. *The Journal of Clinical Endocrinology & Metabolism*, 95(6), 2536–2559. <https://doi.org/10.1210/jc.2009-2354>
- Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical Biochemist. Reviews*, 37(1), 3–15. <http://www.ncbi.nlm.nih.gov/pubmed/27057074>
- Gao, W., Bohl, C. E., & Dalton, J. T. (2005). Chemistry and Structural Biology of Androgen Receptor. *Chemical Reviews*, 105(9), 3352–3370. <https://doi.org/10.1021/cr020456u>
- Kang, Z., Pirsanen, A., Jänne, O. A., & Palvimo, J. J. (2002). Involvement of Proteasome in the Dynamic Assembly of the Androgen Receptor Transcription Complex. *Journal of Biological Chemistry*, 277(50), 48366–48371. <https://doi.org/10.1074/jbc.M209074200>
- Katznelson, L., Finkelstein, J. S., Schoenfeld, D. A., Rosenthal, D. I., Anderson, E. J., & Klibanski, A. (1996). Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. *The Journal of Clinical Endocrinology & Metabolism*, 81(12), 4358–4365. <https://doi.org/10.1210/jcem.81.12.8954042>
- Keller, E. T., Ershler, W. B., & Chang, Chawnshang. (1996). The androgen receptor: A mediator of diverse responses. *Frontiers in Bioscience*, 1(4), 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 Physiologica Scandinavica*, 109(3), 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. *The Journal of Clinical Endocrinology & Metabolism*, 88(9), 4043–4054. <https://doi.org/10.1210/jc.2003-030261>
- Marks, L. S. (2004). 5alpha-reductase: history and clinical importance. *Reviews in Urology*, 6 Suppl 9(Suppl 9), S11–21. <http://www.ncbi.nlm.nih.gov/pubmed/16985920>
- Schuppe, E. R., Miles, M. C., and Fuxjager, M. J. (2020). Evolution of the androgen receptor: Perspectives from human health to dancing birds. *Mol. Cell. Endocrinol.* 499, 110577. doi:10.1016/j.mce.2019.110577.
- U.S. EPA. (2023). *ToxCast & Tox21 AR agonism of testosterone*. Retrieved from <https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data> June 23, 2023. Data Released October 2018.

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

AOPs Referencing Relationship

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

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

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

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

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

Empirical Evidence

The empirical evidence for this KER is considered high

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

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

Dose concordance has also been observed for instance in zebrafish embryos; a dose of 50 μ g/L of the AR antagonist flutamide resulted in 674 differentially expressed genes at 96 h post fertilization whereas 500 μ g/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 (Bennessch 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.
- Ikedo, Yasumasa, Ken Ichi Aihara, Takashi Sato, Masashi Akaike, Masanori Yoshizumi, Yuki Suzuki, 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 Metab* 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 Chawnshang 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 Chawnshang 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, Chiu Ren Yeh, Yuanjie Niu, Hong Chiang Chang, Yu Chieh Tsai, Harold L. Moses, Chih Rong Shyr, Chawnshang 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, Chawnshang 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: 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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Fetal to Parturition	High

Sex Applicability

Sex	Evidence
Male	High

Species

This KER applies to humans, mice, and rats based on biological plausibility. Current empirical evidence is from rat studies only.

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., 2019a). 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

The length of the AGD is programmed during fetal life during the masculinization programming window. 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., 2019a).

Sex

A decrease in the male AGD is a consequence of disrupted androgen action (Welsh et al 2008). 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 2019a).

Key Event Relationship Description

During male reproductive development, the androgen receptor (AR) regulates gene transcription in target tissues to induce masculinization. Target tissues include the perineum, the tissue located between the anus and the genitals. This tissue is sexually dimorphic, with males developing the levator ani-bulbocavernosus (LABC)

muscle complex in response to androgen signaling. The anogenital distance (AGD) is about twice as long in newborn males than in females in many mammals such mice, rats and humans.

A consequence of reduced androgen action during the masculinization programming window in utero, the male AGD will end up being shorter, approaching female AGD when AR signaling is almost blocked. Measuring of the AGD thus serves as a morphometric biomarker for compromised androgen action during fetal life and is used in OECD test guidelines for assessing endocrine disruption.

This KER refers to a tissue-specific, in this case the perineum, alteration in AR-mediated gene transcription during fetal development leading to a decreased AGD in male offspring.

Evidence Supporting this KER

Biological Plausibility

Sexual differentiation initiates during fetal life when a surge in testosterone induces masculinization of a range of tissues and organs (Welsh et al). Testosterone and the more potent metabolite DHT mediate masculinization via activation of the AR; a nuclear transcription factor. Androgens thus induce masculinization via altered AR gene transcription in target tissues. This includes the perineum (Niel et al 2008; Ipulan et al 2014) which can be measured as the AGD and is approximately twice as long in newborn male rodents and humans compared to female (Schwartz et al 2019a). This is also evident in male AR knockout mice which present with an AGD that is indistinguishable from wildtype female littermates (MacLean et al 2008; Notini et al 2005).

Empirical Evidence

Current evidence for direct transcriptional changes mediated by AR disruption in the perineum leading to shorter male AGD is limited. Two studies were identified investigating the transcriptional footprint in the perineum after anti-androgen exposure:

Gestational exposure of rats to the 5 α -reductase inhibitor finasteride (leading to decreased DHT levels) decreased fetal male AGD with 37% at gestational day (GD) 21. Microarray was used to compare transcriptional profiles between control males, finasteride-exposed males, and control females, revealing a sexually dimorphic transcriptional profile of the perineum, with the profile of finasteride-exposed males being intermediary to the male and female control groups (Schwartz et al 2019b).

Gestational exposure of rats to the AR antagonist triticonazole induced decreased fetal male AGD at GD21 and a differentially expressed set of genes investigated by whole transcriptome sequencing in the perineum at both GD17 and GD21 (Draskau et al 2022).

References

- Draskau MK, Schwartz CL, Evrard B, Lardenois A, Pask A, Chalmel F and Svingen T (2022). The anti-androgenic fungicide triticonazole induces region-specific transcriptional changes in the developing rat perineum and phallus. *Chemosphere* 308(Pt 2):136346. doi: 10.1016/j.chemosphere.2022.136346
- Ipulan LA, Suzuki K, Sakamoto Y, Murashima A, Imai Y, Omori A, Nakagata N, Nishinakamura R, Valasek P and Yamada G (2014). Nonmyocytic androgen receptor regulates the sexually dimorphic development of the embryonic bulbocavernosus muscle. *Endocrinology* 155(7):2467-79. doi: 10.1210/en.2014-1008
- MacLean HE, Chiu WS, Notini AJ, Axell AM, Davey RA, McManus JF, Ma C, Plant DR, Lynch GS and Zajac JD (2008). Impaired skeletal muscle development and function in male, but not female, genomic androgen receptor knockout mice. *FASEB J* 22(8):2676-89. doi: 10.1096/fj.08-105726
- 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>.
- Niel L, Willemsen KR, Volante SN and Monks DA (2008). Sexual dimorphism and androgen regulation of satellite cell population in differentiating rat levator ani muscle. *Dev Neurobiol* 68(1):115-22. doi: 10.1002/dneu.20580
- Notini AJ, Davey RA, McManus JF, Bate KL and Zajac JD (2005). Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model. *J Mol Endocrinol* 35(3):547-55. doi: 10.1677/jme.1.0188
- Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U and Svingen T (2019a). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch Toxicol* 93(2):253-272. doi: 10.1007/s00204-018-2350-5
- Schwartz CL, Vinggaard AM, Christiansen S, Darde TA, Chalmel F and Svingen T (2019b). Distinct Transcriptional Profiles of the Female, Male, and Finasteride-Induced Feminized Male Anogenital Region in Rat Fetuses. *Toxicol Sci* 169(1):303-311. doi: 10.1093/toxsci/kfz046
- 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>.
- Welsh M, Saunders PT, Fiskin M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. 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. doi: 10.1172/JCI34241

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	High	
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	High	
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Fetal to Parturition	High

Sex Applicability

Sex	Evidence
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Sex Evidence

Male High

Taxonomic

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

Life stage

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

Sex

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

Key Event Relationship Description

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

Evidence Supporting this KER**Biological Plausibility**

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

- Sexual differentiation happens in fetal life. The testes are developed and start to produce testosterone that is converted in other tissues by the enzyme 5-alpha-reductase to the more potent androgen dihydrotestosterone (DHT). Both hormones bind and activate the nuclear receptor and transcription factor AR that in turn drives masculinization of the male fetus (Welsh et al., 2014; Schwartz et al., 2019).
- Fetal masculinization depends on activation of androgen signaling during a critical time window, the masculinization programming window (MPW), from gestational day (GD) 15.5-18.5 in rats, 14.5-16.5 in mice and presumably gestation weeks (GWs) 8-14 in humans (Welsh et al., 2008; Amato et al., 2022). The onset of AR expression in the tissues of the reproductive tract follows the timing of the MPW (Welsh et al., 2008).
- The fetal masculinization process involves a range of tissues and organs, including the perineum. Perineum length can be measured as the AGD, which is the distance between the anus and the genitalia. The AGD is approximately twice as long in male as in female newborn rodents and humans (Schwartz et al., 2019).
- Male AR knockout mice present shorter AGD than wildtype males, so short that it is indistinguishable from wildtype female littermates (Yeh et al., 2002; Sato et al., 2004).
- In human males, mutations decreasing AR activity also lead to feminization. One example is the androgen insensitivity syndrome (AIS), where mutations in the AR lead to an impaired or abolished response to androgens, and thereby some degree of feminization of XY individuals and even XY sex reversal in individuals with complete AIS (CAIS) (Thankamony et al., 2016; Hughes et al., 2012; Crouch et al., 2011). XY individuals with CAIS present as women with internally placed testes. A study showed that the clitoral to urethral distance in these individuals was similar to a control group of women, but it is not clear whether this measurement can work as a proxy for measuring the AGD (Thankamony et al 2016, Crouch 2011). Unfortunately, it seems the AGD has not at present been measured in CAIS individuals. Another example is human males lacking 5-alpha-reductase, also presenting female-like genitalia (Batista & Mendonca, 2022).
- The detailed mechanism by which androgens regulate the AGD is not known but it is hypothesized that the AGD is influenced by the size of the levator-ani and bulbocavernosus (LABC) muscle complex in the perineum. The growth of this complex is stimulated by AR activation, it is sexually dimorphic and larger in males than in females and (Schwartz et al., 2019). AR is required for the development of the LABC complex as demonstrated by AR general and muscle specific knockout mice. AR is expressed in non-myocytic cells in the LABC complex, starting at E15.5 in mice, and knockout of AR in these cells results in defects in the muscle formation (Ipulan et al., 2016;). Differential gene expression profiles in the perineum of male and female rats as well as in antiandrogen-exposed male rats have been identified providing further mechanistic understanding (Schwartz et al, 2019; Draskau et al, 2022).

Empirical Evidence**Animal *in vivo* data**

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

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

The empirical evidence for this KER from animal studies in vivo is based on studies using five different substances that result in decreased AR activation by different mechanisms. Flutamide, procymidone and vinclozolin bind to the AR and inhibit the receptor activity and thereby act as AR antagonists, see MIE26. Finasteride inhibits the 5-alpha-reductase enzyme that converts testosterone to DHT, see MIE1617. DEHP exposure during prenatal development in rats results in reduced fetal testosterone levels, see KE1690. (MIE26, MIE1617 and KE1690 can be found in AOP Wiki).

The evidence for the upstream KE is mainly based on data from in vitro assays (AR antagonism or 5-alpha-reductase inhibition in vitro) whereas the evidence for the downstream KE is based on in vivo studies, and there is generally not evidence for both KEs from the same study. However, decreased testosterone levels can be measured in vivo, and Borch et al., 2004 measured the effect of developmental DEHP exposure on both testosterone levels and AGD (see section about "Dose concordance").

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

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

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

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

From table 3, it can be 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: [8dh20j155i_FINAL_Appendix_KER2820_For_Wiki.pdf](#).

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 5α-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 Metzdrorff, 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/kfg128>.
- 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 Metzdrorff, 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 5α-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. Triantafilou, 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.crtox.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, Diisooctyl 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, Metzdrorf 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 Rosenskold 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 Pasternski, Kiki Mastroyannopoulou, and Jane MacDougall. "Androgen Insensitivity Syndrome." *Lancet* 2012 OCT, no. 20:380(9851) (June 13, 2012): 1419–28. [https://doi.org/10.1016/S0140-6736\(12\)60071-3](https://doi.org/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, Shih Young Chang, Wen-Lung Ma, Wen-Jye Lin, and Chawnsiang 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. Lambricht, 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 Kunihiko 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-CNCR282072711>3.0.CO;2-3](https://doi.org/10.1002/1097-0142(19931215)72:12+<3816::AID-CNCR282072711>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.bbagrm.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, Hirota 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 Guerbadot, 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 Philipson, 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 *Ins13* 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, 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." *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: 3449: Decrease, intratesticular testosterone leads to AGD, decreased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI
rat	<i>Rattus norvegicus</i>	High	NCBI

Life Stage Applicability

Life Stage Evidence

Foetal	High
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Sex Applicability

Sex Evidence

Male	High
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Taxonomic applicability

Testosterone, synthesized in the testis, is essential for growth (masculinization) of the male perineum in all mammals. It is therefore biologically plausible that this KER is applicable to all mammals (Murashima et al., 2015a). The empirical evidence in this KER strongly supports the applicability to rats in particular. Given the knowledge of normal reproductive development, the KER is also considered applicable to humans.

Sex applicability

Testes are the primary male sex organs; hence, this KER is only applicable to males. The empirical evidence in this KER supports the applicability in males.

Life stage applicability

The perineum is programmed by androgens during the masculinization programming window, a fetal period during which the testes produce high levels of testosterone. The masculinization programming window is ~gestational day (GD) 16-20 in rats and suggested to be gestational weeks (GW) 8-14 in humans (Sharpe RM, 2020; Welsh M et al., 2014). Once programmed in fetal life, the AGD is believed to be relatively stable, but the perineum can in some cases be responsive to postnatal changes in androgen levels (Schwartz CL et al., 2019; Sharpe RM, 2020; Thankamony A et al., 2016). The empirical evidence in this KER supports the fetal life stage applicability.

Key Event Relationship Description

This non-adjacent KER describes a fetal decrease in testicular testosterone leading to short AGD in male offspring. In this KER, intratesticular testosterone levels can both be measured in whole testes extracts or by measuring *ex vivo* testosterone production from exposed testes.

In male mammals, the testes first differentiate in early fetal life and start synthesizing testosterone through the steroidogenesis pathway. Although the adrenal glands may also produce testosterone, the testes are the main site of testosterone production (Naamneh Elzenaty et al., 2022). Testosterone is secreted to initiate male reproductive differentiation in the peripheral tissues, either directly acting on the androgen receptor (AR) or through conversion to the more potent androgen dihydrotestosterone (DHT). The androgen hormones initiate masculinization, including elongation of the perineum, which is suggested to involve the perineal muscle complex *levator ani bulbocavernosus* (LABC). LABC expresses AR and increases in size by androgen programming (Schwartz CL et al., 2019). The perineum is programmed in the masculinization programming window (GD 16-20 in rats, GW 8-14 in humans), when testicular testosterone production is high (Sharpe RM, 2020; Welsh M et al., 2014). Thus, a decrease in testicular testosterone levels in this window may limit the AR signaling in the LABC, leading to less elongation of the perineum and a short AGD.

Evidence Supporting this KER**Biological Plausibility**

The biological plausibility for this KER is judged to be **high** given the canonical biological knowledge on normal reproductive development.

Sexual differentiation in males, including elongation (masculinization) of the perineum, is initiated and programmed in fetal life. Around GW 8 in humans and GD16 in rats, the testes have formed and start synthesizing testosterone through the steroidogenesis pathway. Testicular testosterone is secreted to either act directly on the AR or be converted to the more potent androgen hormone (DHT). AR activation in the perineum of males programs it to elongate, resulting in a longer AGD in males than in females (~twice the length in rats and humans) (Murashima et al., 2015b; Trost & Mulhall, 2016; Welsh M et al., 2014).

The programming of the reproductive tissues, including masculinization of the perineum happens in the masculinization programming window (GD 16-20 in rats, GW 8-14 in humans) (Welsh M et al., 2014).

Given the dependency of testosterone for elongation of the perineum, either through direct AR activation or conversion to DHT, it is highly plausible that a decrease in testicular levels of testosterone will lead to a shorter AGD in males.

Empirical Evidence

The overall empirical evidence for this KER is judged as **strong**.

A total of 19 data sets were collected as evidence for this KER (table 3 and appendix 2: [Microsoft Word - KER 3449_appendix 2.docx](#)). Of these, 14 studies, all in rats, show that exposure to a stressor that decreased fetal intratesticular testosterone also caused a short AGD.

Table 3 Empirical evidence for KER 3449 LOAEL: Lowest observed adverse effect level; NOAEL: No observed adverse effect level; ITT: Intratesticular testosterone measured in whole testes; *Ex vivo*: Testosterone production measured by incubation of testes and collection of media. See appendix 2 for specifications.

Species	Stressors(s)	Effect on upstream event (intratesticular or <i>ex vivo</i> testosterone)	Effect on downstream event (AGD) ¹	Reference
Rat	α -cypermethrin	LOAEL 5 mg/kg	No effect NOAEL 10 mg/kg	(Saillenfait AM et al., 2017)
Rat	Butyl benzyl phthalate	LOAEL 500 mg/kg	LOAEL 500 mg/kg at PND2, but not adult	(Hotchkiss AK et al., 2004)
Rat	Butyl benzyl phthalate + Linuron	LOAEL 500 + 75 mg/kg	LOAEL 500 + 75 mg/kg	(Hotchkiss AK et al., 2004)
Rat	Dibutyl phthalate	LOAEL 500 mg/kg	LOAEL 500 mg/kg	(Lourenço AC et al., 2014)
Rat	Dibutyl phthalate	LOAEL 500 mg/kg	LOAEL 100 mg/kg for AGDi, LOAEL 500 mg/kg for AGD	(Martino-Andrade AJ et al., 2009)
Rat	Dibutyl phthalate	LOAEL 500 mg/kg	LOAEL 500 mg/kg	(Pike et al., 2014)
Rat	Dibutyl phthalate	LOAEL 100 mg/kg	LOAEL 500 mg/kg	(Struve MF et al., 2009)
Rat	Dibutyl phthalate	LOAEL 750 mg/kg	LOAEL 750 mg/kg	(van den Driesche et al., 2020)

Rat	Dibutyl phthalate + Diethylhexyl phthalate	LOAEL 100 + 150 mg/kg	No effect NOAEL 100 + 150 mg/kg	(Martino-Andrade AJ et al., 2009)
Rat	Diethylhexyl phthalate	LOAEL 750 mg/kg	LOAEL 750 mg/kg	(Borch J et al., 2004)
Rat	Diethylhexyl phthalate + Diethylhydroxylamine	LOAEL 750 + 400 mg/kg	LOAEL 750 + 400 mg/kg	(Borch J et al., 2004)
Rat	Diisonyl phthalate	Decreased ITT at 600 mg/kg but not at 750 and 900 mg/kg No effect <i>ex vivo</i>	LOAEL 900 mg/kg	(Boberg J et al., 2011)
Rat	Diisobutyl phthalate	LOAEL 250 mg/kg	LOAEL 250 mg/kg for AGD index, no effect on AGD	(Saillenfait AM et al., 2017)
Rat	Diisonyl phthalate	LOAEL 250 mg/kg at GD19 No effect at GD20 NOAEL 750 mg/kg	No effect NOAEL 750 mg/kg	(Clewett et al., 2013)
Rat	Ketoconazole	LOAEL 50 mg/kg No effect <i>ex vivo</i>	LOAEL 50 mg/kg	(Taxvig et al 2008)
Rat	Linuron	LOAEL 75 mg/kg	LOAEL 75 mg/kg	(Hotchkiss AK et al., 2004)
Rat	Prochloraz	LOAEL 50 mg/kg	LOAEL 50 mg/kg	(Laier P et al., 2006)
Rat	Prochloraz	ITT LOAEL 30 mg/kg No effect <i>ex vivo</i>	No effect NOAEL 30 mg/kg	(Vinggaard AM et al., 2005)
Rat	Mixture (prochloraz, deltamethrin, methiocarb, simazine, tribenuron)	LOAEL 20 mg/kg	No effect NOAEL 20 mg/kg	(Vinggaard AM et al., 2005)
Rat	Mixture (BBP, DBP, DCHP, DEHP, DHEP, DHP, DIBP, DIHEP, DPEP, LIN, DDE, PCZ, PCD, PFQ, VIN)	LOAEL 6.25% of full dose	LOAEL 12.5% of full dose for AGD index, LOAEL 25% of full dose for AGD	(Conley JM et al., 2021)

¹NOAEL and LOAEL were, when available, based on AGDi data. For some datasets, only AGD or AGD/bw were available, see appendix 1 for details on each dataset.

Dose concordance

Overall, the empirical evidence supports dose concordance, although with some inconsistencies.

Five different studies show that *in utero* dibutyl phthalate exposure reduces intratesticular testosterone and AGD. Three of these studies report the same LOAEL for reduced intratesticular testosterone and short AGD, respectively (Lourenço AC et al., 2014; Pike et al., 2014; van den Driesche S et al., 2020). In one study, the LOAEL for reduced intratesticular testosterone was 500 mg/kg/day, while the LOAEL for short AGD was 100 mg/kg/day, thus not showing dose concordance (Martino-Andrade AJ et al., 2009). In contrast, another study reports 100 mg/kg/day as LOAEL for reduced intratesticular testosterone and 500 mg/kg/day as LOAEL for short AGD (Struve MF et al., 2009).

Two studies used prochloraz as the stressor. One study showed a reduction in testosterone in the testes at a dose of 30 mg/kg bw/day, but no effect on *ex vivo* testosterone production or on AGD (Vinggaard AM et al., 2005). The other study tested 50 and 150 mg/kg bw/day prochloraz and found an effect on both intratesticular testosterone (both in testes and *ex vivo*) and on AGD in the male offspring (Laier P et al., 2006).

Of the remaining empirical evidence, most studies report the same LOAEL for reduced intratesticular testosterone and short AGD, but for many of these cases only one chemical dose was tested.

Temporal concordance

Overall, the empirical evidence supports temporal concordance.

In several of the studies, AGD was measured at a later timepoint, often postnatally, than intratesticular testosterone. For example, exposure of rats from GD14-18 to a mixture of butyl benzyl phthalate (500 mg/kg bw/day) and linuron (75 mg/kg bw/day) reduced intratesticular testosterone levels at GD18 and caused short AGD in the males, which could be measured at both PND2 and in adult rats (Hotchkiss AK et al., 2004). Exposure to only 500 mg/kg bw/day butyl benzyl phthalate (500 mg/kg bw/day) from GD14-18 also reduced intratesticular testosterone levels at GD18 and caused short AGD at PND2, but in adult males, the effect on AGD was no longer significant. Exposure to linuron alone (75 mg/kg bw/day) in the same study caused both reduced intratesticular testosterone and short AGD at PND2 and in adult males (Hotchkiss AK et al., 2004). This may indicate that the fetal effect on AGD is best detected in early postnatal life.

In ten studies, AGD was measured prenatally, either at the same time or a day after the intratesticular testosterone measurements. Three of these studies did not find an effect on short AGD when intratesticular testosterone was measured. For example, exposure to α -cypermethrin from GD13-19 in four different doses reduced *ex vivo* testosterone production in testes at GD19 with LOAEL of 5 mg/kg bw/day, but AGD at GD19 was not affected at this dose or at 10 mg/kg bw/day (Saillenfait AM et al., 2017). In the same study, however, exposure to diisobutyl phthalate (250 mg/kg bw/day) caused both a reduction in *ex vivo* testosterone and AGD on GD19, although the effect on AGD was small (Saillenfait AM et al., 2017).

Incidence concordance

Because the data mainly includes chemicals at different doses and exposure windows, and all data are continuous, they do not firmly establish incidence concordance of this KER. However, a few studies have used the same stressors at the same doses and provide some information on incidence concordance.

Five studies used the stressor dibutyl phthalate, three of them testing the same two doses, 100 and 500 mg/kg bw/day, although with slightly different exposure windows and timepoints of AGD measurement. Of these three studies, two found the LOAEL for reduced intratesticular testosterone to be 500 mg/kg bw/day (Martino-Andrade AJ et al., 2009; Pike et al., 2014), while one detected a reduction in testosterone at 100 mg/kg bw/day (Struve MF et al., 2009). Regarding AGD, one study reported 100 mg/kg bw/day as the LOAEL, i.e. lower than the LOAEL for intratesticular testosterone (Martino-Andrade AJ et al., 2009), while the others reported 500 mg/kg bw/day as the LOAEL (Pike et al., 2014; Struve MF et al., 2009). Thus, these studies are conflicting regarding incidence concordance.

There are also three studies using diisobutyl phthalate as stressor. These vary more in terms of doses, but overall they see subtle and more uncertain effects on both intratesticular testosterone and AGD with LOAELs ranging from 50 to 250 mg/kg bw/day for both measurements (Clewell et al., 2013; Saillenfait AM et al., 2017; Taxvig C et al., 2008).

Uncertainties and Inconsistencies

One uncertainty in empirical data for this KER is the studies where intratesticular testosterone was measured in an *ex vivo* testes incubation experiment. With this method, there is an uncertainty of the direct relationship between the *ex vivo* secretion, as testosterone was measured in media, and the exact intratesticular testosterone levels. However, in most of the studies using this *ex vivo* method, intratesticular testosterone was also measured in testes homogenates (see appendix 2) with similar outcomes using both methods, suggesting that *ex vivo* testosterone production after incubation can be used as a proxy for intratesticular testosterone, exemplified by very identical measurements in (Borch J et al., 2004). In the three studies, only measure testosterone production *ex vivo* (Conley JM et al., 2021; Saillenfait AM et al., 2017), the uncertainty in this measurements should be kept in mind.

Five data sets did not measure any effect of the stressors on AGD. In two cases, this could be due to the AGD measurements either being measured too early to measure detectable differences between groups (Saillenfait AM et al., 2017) or having too high variance to gain statistical significance (Martino-Andrade AJ et al., 2009). In the three other cases, the lack of effect on AGD was likely due to only testing one dose of the stressor (Vinggaard AM et al., 2005) (dose concordance) or the doses tested were too low (Clewell et al., 2013).

Another uncertainty is the inconsistencies between studies for the stressor dibutyl phthalate. One study report the LOAEL for reduced intratesticular testosterone as 100 mg/kg/day (Struve MF et al., 2009), while others report 500 mg/kg/day (one of these only use on dose) (Lourenço AC et al., 2014; Martino-Andrade AJ et al., 2009; Pike et al., 2014). Similarly, the LOAEL for short AGD is inconsistent, with 500 mg/kg/day being reported in three studies (Lourenço AC et al., 2014; Pike et al., 2014; Struve MF et al., 2009), and 100 mg/kg/day being reported in one (Martino-Andrade AJ et al., 2009).

Finally, one study containing uncertainties is a study on diisonyl phthalate (Boberg et al., 2011). In this study, exposure from GD7-21 to 600 mg/kg/day, but not 750 or 900 mg/kg bw/day reduced intratesticular testosterone, while 900 mg/kg/day caused short AGD. However, both 750 and 900 mg/kg bw/day diisonyl phthalate tended to decrease intratesticular testosterone levels, and the lack of statistical significance may therefore be explained by a low sample size for these measurements (n=3-4 litters, 1-2 testes per litter).

Quantitative Understanding of the Linkage

Response-response relationship

There are no direct models for reductions in intratesticular testosterone levels and AGD. A model for phthalates has been developed, aiming to predict reductions in AGD based on the reduction in *ex vivo* testosterone production. In this model, a 5-parameter logistic regression model, around 60% testosterone reduction can cause a decreased AGD, with a steep declining curve as testosterone production decreases. It must be emphasized that this model, however, was only developed for the phthalates and does therefore not directly evidence the same relationship for other stressors reducing testosterone levels (Earl Gray L Jr et al., 2024).

Time-scale

The exact timescale of this KER depends on the species, but it may take days or weeks for growth changes in the perineum to be measurable. In humans, testosterone production in the testes begin around GW8, and sexual dimorphism of the perineum between males and females can be measured by GW11-13, reaching the full 2:1 male:female ratio in length at GW17-20 (Thankamony A et al., 2016)

Known modulating factors

There are no known modulating factors for this KER.

Known Feedforward/Feedback loops influencing this KER

There are no known feedback/feedforward loops for this KER

References

- Boberg, J., Christiansen, S., Axelstad, M., Kledal, T. S., Vinggaard, A. M., Dalgaard, M., Nellemann, C., & Hass, U. (2011). Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats. *REPRODUCTIVE TOXICOLOGY*, 31(2), 200–209. <https://doi.org/10.1016/j.reprotox.2010.11.001>
- Borch J, Ladefoged O, Hass U, & Vinggaard AM. (2004). 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(1), 53–61. <https://doi.org/10.1016/j.reprotox.2003.10.011>
- Clewell, R. A., Sochaski, M., Edwards, K., Creasy, D. M., Willson, G., & Andersen, M. E. (2013). Disposition of diisononyl phthalate and its effects on sexual development of the male fetus following repeated dosing in pregnant rats. *REPRODUCTIVE TOXICOLOGY*, 35, 56–69. <https://doi.org/10.1016/j.reprotox.2012.07.001>
- Conley JM, Lambricht CS, Evans N, Cardon M, Medlock-Kakaley E, Wilson VS, & Gray LE Jr. (2021). A mixture of 15 phthalates and pesticides below individual chemical no observed adverse effect levels (NOAELs) produces reproductive tract malformations in the male rat. *Environment International*, 156, 106615. <https://doi.org/10.1016/j.envint.2021.106615>
- Earl Gray L Jr, Lambricht CS, Evans N, Ford J, & Conley JM. (2024). Using targeted fetal rat testis genomic and endocrine alterations to predict the effects of a phthalate mixture on the male reproductive tract. *Current Research in Toxicology*, 7, 100180. <https://doi.org/10.1016/j.crtox.2024.100180>
- Holmer, M. L., Zilliacus, J., Draskau, M. K., Hlšíšková, 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. *Reproductive Toxicology*, 128, 108662. <https://doi.org/10.1016/j.reprotox.2024.108662>
- Hotchkiss AK, Parks-Saldutti LG, Ostby JS, Lambricht C, Furr J, Vandenbergh JG, & Gray LE Jr. (2004). A mixture of the “antiandrogens” linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biology of Reproduction*, 71(6), 1852–1861. <https://doi.org/10.1095/biolreprod.104.031674>
- 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. *Toxicology and Applied Pharmacology*, 213(2), 160–171. <https://doi.org/10.1016/j.taap.2005.10.013>

- Lourenço AC, Gomes C, Boareto AC, Mueller RP, Nihi F, Andrade LF, Trindade ES, Coelho I, Naliwaiko K, Morais RN, & Martino-Andrade AJ. (2014). Influence of oily vehicles on fetal testis and lipid profile of rats exposed to di-butyl phthalate. *Human & Experimental Toxicology*, 33(1), 54–63. <https://doi.org/10.1177/0960327112474847>
- Martino-Andrade AJ, Morais RN, Botelho GG, Muller G, Grande SW, Carpentieri GB, Leão GM, & Dalsenter PR. (2009). Coadministration of active phthalates results in disruption of foetal testicular function in rats. *International Journal of Andrology*, 32(6), 704–712. <https://doi.org/10.1111/j.1365-2605.2008.00939.x>
- Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015a). Androgens and mammalian male reproductive tract development. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1849(2), 163–170. <https://doi.org/10.1016/j.bbagrm.2014.05.020>
- Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015b). Androgens and mammalian male reproductive tract development. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1849(2), 163–170. <https://doi.org/10.1016/j.bbagrm.2014.05.020>
- Naamneh Elzenaty, R., Du Toit, T., & Flück, C. E. (2022). Basics of androgen synthesis and action. *Best Practice & Research Clinical Endocrinology & Metabolism*, 36(4), 101665. <https://doi.org/10.1016/j.beem.2022.101665>
- Pike, J., McDowell, E., McCahan, S., & Johnson, K. (2014). Identification of gene expression changes in postnatal rat foreskin after in utero anti-androgen exposure. *REPRODUCTIVE TOXICOLOGY*, 47, 42–50. <https://doi.org/10.1016/j.reprotox.2014.05.011>
- Saillenfait AM, Sabaté JP, Denis F, Antoine G, Robert A, Roudot AC, Ndiaye D, & Eljarrat E. (2017). Evaluation of the effects of α -cypermethrin on fetal rat testicular steroidogenesis. *Reproductive Toxicology (Elmsford, N.Y.)*, 72, 106–114. <https://doi.org/10.1016/j.reprotox.2017.06.133>
- 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. *Archives of Toxicology*, 93(2), 253–272. <https://doi.org/10.1007/s00204-018-2350-5>
- Sharpe RM. (2020). Androgens and the masculinization programming window: Human-rodent differences. *Biochemical Society Transactions*, 48(4), 1725–1735. <https://doi.org/10.1042/BST20200200>
- Struve MF, Gaido KW, Hensley JB, Lehmann KP, Ross SM, Sochaski MA, Willson GA, & Dorman DC. (2009). Reproductive toxicity and pharmacokinetics of di-n-butyl phthalate (DBP) following dietary exposure of pregnant rats. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, 86(4), 345–354. <https://doi.org/10.1002/bdrb.20199>
- Taxvig C, Vinggaard AM, Hass U, Axelstad M, Metzдорff S, & Nellemann C. (2008). Endocrine-disrupting properties in vivo of widely used azole fungicides. *International Journal of Andrology*, 31(2), 170–177. <https://doi.org/10.1111/j.1365-2605.2007.00838.x>
- Thankamony A, Pasterski V, Ong KK, Acerini CL, & Hughes IA. (2016). Anogenital distance as a marker of androgen exposure in humans. *Andrology*, 4(4), 616–625. <https://doi.org/10.1111/andr.12156>
- Trost, L. W., & Mulhall, J. P. (2016). Challenges in Testosterone Measurement, Data Interpretation, and Methodological Appraisal of Interventional Trials. *The Journal of Sexual Medicine*, 13(7), 1029–1046. <https://doi.org/10.1016/j.jsxm.2016.04.068>
- van den Driesche S, Shoker S, Inglis F, Palermo C, Langsch A, & Otter R. (2020). Systematic comparison of the male reproductive tract in fetal and adult Wistar rats exposed to DBP and DINP in utero during the masculinisation programming window. *Toxicology Letters*, 335, 37–50. <https://doi.org/10.1016/j.toxlet.2020.10.006>
- Vinggaard AM, Christiansen S, Laier P, Poulsen ME, Breinholt V, Jarfelt K, Jacobsen H, Dalgaard M, Nellemann C, & Hass U. (2005). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 85(2), 886–897. <https://doi.org/10.1093/toxsci/kfi150>
- Welsh M, Suzuki H, & Yamada G. (2014). The masculinization programming window. *Endocrine Development*, 27, 17–27. <https://doi.org/10.1159/000363609>

Relationship: 3349: Decrease, circulating testosterone levels leads to AGD, decreased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage Evidence

Foetal	High
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Sex Applicability

Sex Evidence

Male	High
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Taxonomic applicability

Male-specific development of the fetal perineum in male mammals is strongly influenced by androgen signaling. It is therefore biologically plausible that this KER is applicable to all mammals (Murashima et al., 2015). The empirical evidence in this KER provides strong support that reduced circulating testosterone levels in fetal life can cause short AGD in rats. The empirical evidence for this KER in humans is sparse and conflicting; however, given the known role of androgens in human male reproductive development, the KER is considered applicable to humans.

Sex applicability

The empirical evidence in this KER supports that reduced circulating testosterone in fetal life can cause reduced AGD in males. Females do have circulating testosterone, but in much lower concentrations than males (Vesper et al., 2015), and it is unlikely that further reduction can cause a short AGD in females (Schwartz CL et al., 2019). Of note is that ‘reduced AGD’ in males is not a reduction per se, but a failure to elongate in response to androgen action.

Life stage applicability

This KER is applicable to fetal life, as this is when the perineum is programmed by androgen hormones in males. The masculinization programming window is around gestational days (GD) 16-20 in rats, and suggested to be gestational weeks (GW) 8-14 in humans (Sharpe RM, 2020; Welsh M et al., 2014). [Once programmed in fetal life, the AGD is believed to be relatively stable, but the perineum can in some cases be responsive to postnatal changes in androgen levels](#) (Schwartz CL et al., 2019; Sharpe RM, 2020; Thankamony A et al., 2016). The empirical evidence in this KER supports the fetal life stage applicability.

Key Event Relationship Description

This non-adjacent KER describes a fetal decrease in circulating testosterone (often measured in serum or plasma) leading to short anogenital distance (AGD) in male offspring.

In male mammals, testosterone is one of the primary hormonal drivers of male reproductive differentiation. Produced by the fetal testes, testosterone is transported through blood to the peripheral reproductive tissues to bind the androgen receptor (AR) or be converted to the higher potency androgen hormone dihydrotestosterone (Murashima et al., 2015). The androgen hormones signal through AR to program the reproductive tissue to differentiate along the male pathway. This includes elongation of the perineum, which is suggested to involve the perineal muscle complex *levator ani bulbocavernosus* (LABC). LABC expresses AR and increases in size by androgen programming (Schwartz CL et al., 2019). The male programming of the tissue happens during fetal life in the masculinization programming window (GD 16-20 in rats, GW 8-14 in humans), when circulating testosterone levels are high (Sharpe RM, 2020; Welsh M et al., 2014). Thus, a decrease in circulating testosterone levels in this window may limit the AR signaling in the LABC, leading to less elongation of the perineum and a short AGD.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is judged to be **high** given the canonical biological knowledge on normal reproductive development.

Sexual differentiation in males, including elongation (masculinization) of the perineum, is initiated and programmed in fetal life. Once the testes have formed, they start producing testosterone through the steroidogenesis pathway and secrete testosterone into circulation. Testosterone is transported in the blood either as free testosterone or bound to albumin or sex-hormone binding globulin. In peripheral tissues, testosterone can be converted to the more potent androgen hormone dihydrotestosterone (DHT) by the enzyme 5 α -reductase. Both DHT and testosterone bind and activate the androgen receptor (AR) to program fetal tissues to differentiate along the male pathway, including elongation of the perineum, resulting in a longer AGD in males than in females (~twice the length in rats and humans) (Murashima et al., 2015; Trost & Mulhall, 2016; Welsh M et al., 2014).

Testosterone is produced from around GD15 in fetal rats and GW8 in humans, which is also the onset of when testosterone levels can be measured in circulation. The programming of the reproductive tissues, including masculinization of the perineum happens in the masculinization programming window (GD16-20 in rats, GW8-14 in humans) (Welsh M et al., 2014).

Given the dependency of testosterone for elongation of the perineum, either through direct AR activation or conversion to DHT, it is highly plausible that a decrease in circulating levels of testosterone will lead to a shorter AGD in males

Empirical Evidence

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

From the data collection, ten data sets were extracted. The data sets included different stressors causing reduced fetal levels of testosterone, all in rats (table 3 and appendix 2: [99hykht8a4_KER_3349_Appendix_2.pdf](#)). Of these ten data sets, eight showed concurrent short AGD.

Table 3 Empirical evidence for KER 3349 LOAEL: Lowest observed adverse effect level; NOAEL: No observed adverse effect level. See appendix 2 for specifications.

Species	Stressors(s)	Effect on upstream event (circulating testosterone)	Effect on downstream event (AGD) ¹	Reference
Rat	2,3,7,8-Tetrachlorodibenzo-p-dioxin	LOAEL 1 μ g/kg	LOAEL 1 μ g/kg	(Mably TA et al., 1992)
Rat	Dicyclohexyl phthalate	LOAEL 100 mg/kg	LOAEL 20 mg/kg	(Aydoğan Ahbab M & Barlas N, 2015)
Rat	Diethylhexyl phthalate	LOAEL 750 mg/kg	LOAEL 750 mg/kg	(Borch J et al., 2004)
Rat	Diethylhexyl phthalate + Diethylhydroxylamine	LOAEL 750 + 400 mg/kg	LOAEL 750 + 400 mg/kg	(Borch J et al., 2004)
Rat	Di-n-hexyl phthalate	LOAEL 20 mg/kg	Short AGD at 20 and 500 mg/kg, but not 100 mg/kg	(Aydoğan Ahbab M & Barlas N, 2015)
Rat	Perfluorotridecanoic acid	LOAEL 1 mg/kg	LOAEL 10 mg/kg	(Li C et al., 2021)
Rat	Prochloraz	LOAEL 30 mg/kg	No effect NOAEL 30 mg/kg	(Vinggaard AM et al., 2005)
Rat	Prochloraz	LOAEL 50 mg/kg ¹	LOAEL 50 mg/kg	(Laier P et al., 2006)
Rat	Zearalenone	LOAEL 10 mg/kg	LOAEL 5 mg/kg	(Pan P et al., 2020)
Rat	Mixture (prochloraz, deltamethrin, methiocarb, simazine, tribenuron)	LOAEL 20 mg/kg	No effect NOAEL 20 mg/kg	(Vinggaard AM et al., 2005)

¹NOAEL and LOAEL were, when available, based on AGDi data. For some datasets, only AGD or AGD/bw were available, see appendix 2 for details on each dataset.

²No statistics available as samples were pooled for measurement of testosterone.

Supporting epidemiological evidence

No studies have shown a direct association between fetal circulating testosterone levels and AGD. A few epidemiologic studies can inform indirectly on the human evidence for this KER, and the current studies on this are conflicting.

As some phthalates are known to reduce testosterone production, an association between phthalate exposure and short AGD could support the KER in humans. A meta-analysis found an association between maternal urinary concentrations of some phthalate metabolites and short AGD (Zarean M et al., 2019). Moreover, in a Taiwan Maternal and Infant Cohort study, maternal urinary concentrations of some phthalate metabolites were also associated with a shorter AGD in male infants, although there was no association between cord blood testosterone levels and AGD, and the metabolites were not associated with lower cord blood testosterone levels, either (Lu et al., 2024). Another longitudinal mother-child cohort study did not find an association between AGD in adult men with maternal serum concentrations of phthalate metabolites during pregnancy (Henriksen LS et al., 2023).

One study related cord blood testosterone levels to AGD in infants boys and did not find associations between the two (Liu C et al., 2016).

In adult men, anogenital distance was significantly associated with serum testosterone levels (Eisenberg ML et al., 2012).

Dose concordance

The *in vivo* rat toxicity studies for this KER moderately supports dose concordance.

One study with the stressor perfluorotridecanoic acid showed dose concordance with the LOAEL for reduced serum testosterone being 1 mg/kg bw/day, while the LOAEL for short AGD was 10 mg/kg (Li C et al., 2021).

In another study with two doses of prochloraz, the LOAEL was the same (50 mg/kg) for decreased testosterone and short AGD (Laier P et al., 2006).

Finally, in two studies with dicyclohexyl phthalate and zearalenone, respectively, AGD was shortened at lower doses than testosterone was reduced (Aydoğan Ahabab M & Barlas N, 2015; Pan P et al., 2020), and these do therefore not support dose concordance. However, in both cases the testosterone levels tended to be lower in the non-significant doses as well, and the lack of effect could therefore be due to high variation in the testosterone measurements.

Temporal concordance

Overall, the empirical evidence supports temporal concordance between the events.

Mably TA et al., 1992 followed plasma testosterone levels in male rats after *in utero* exposure to 1 µg/kg 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin on GD15. From GD17-21, testosterone levels steadily decreased in both control and exposed males, but the overall levels in the exposed males were lower than in control males. After birth, the testosterone levels in exposed male increased to match values in control males. At PND1, 3, and 5, plasma levels were normal in exposed male rats, while their AGD were reduced at all days.

Five of the data sets also measure circulating testosterone prenatally (GD20 or GD21) and AGD postnatally (between PND0 and PND3), and three of these observe short AGD when testosterone is reduced during fetal life (Borch J et al., 2004; Laier P et al., 2006). In one of these studies, where diethylhexyl phthalate alone or diethylhexyl phthalate in combination with diethylhydroxylamine was administered from GD7-PND17, serum testosterone levels were decreased at GD21, but not at PND22 or PD90 (Borch J et al., 2004). Similarly, PND16 serum testosterone was not altered by perinatal (GD7-PND16) prochloraz exposure which reduced serum testosterone at GD21 and AGD at PND1 (Laier P et al., 2006).

Incidence concordance

The data does not inform incidence concordance.

Uncertainties and Inconsistencies

Two data sets, both from the same study (Vinggaard AM et al., 2005), showed no effect of decreased circulating testosterone levels on AGD, which may be due to too low doses of the stressors (prochloraz and a mixture). For two studies (Aydoğan Ahabab M & Barlas N, 2015; Pan P et al., 2020), the LOAEL was lower for the downstream event, short AGD, than the upstream event, reduced circulating testosterone. In both cases, lower doses of stressors tended to lower testosterone levels as well, and the inconsistency could therefore be due to high variance in testosterone measurements.

Another uncertainty is the AGD results in the study investigating di-n-hexyl phthalate exposure from GD6-19 in rats (Aydoğan Ahabab M & Barlas N, 2015). Three doses of the phthalate (20, 100, and 500 mg/kg bw/day) all reduced plasma testosterone levels, but only 20 and 500 mg/kg bw/day caused short AGD, when calculating the anogenital distance index (AGDi, $AGD/bw^{1/3}$). When analyzing the direct AGD, all doses of di-n-hexyl phthalate decreased AGD. In contrast, when analyzing the relative AGD (AGD/bw), only the highest dose (500 mg/kg bw/day) decreased the AGD. This study thus identified different LOAELs for AGD, depending on if and how body weight was considered, posing an uncertainty on the results.

Quantitative Understanding of the Linkage

Time-scale

Testosterone is secreted from around GW8 in humans (GD16 in rats), marking the beginning of the masculinization programming window and programming of the perineal tissue. Depending on the species, the time scale for observing effects on tissue growth is days or weeks. In humans, sexual dimorphism of the AGD can be measured by GW11-13, reaching the full 2:1 male:female ratio in length at GW17-20 (Thankamony A et al., 2016).

Known modulating factors

There are no known modulating factors for this KER.

Known Feedforward/Feedback loops influencing this KER

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

References

Aydoğan Ahabab M & Barlas N. (2015). Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats. *Toxicology Letters*, 233(2), 125-137. <https://doi.org/10.1016/j.toxlet.2015.01.015>

- Borch J, Ladefoged O, Hass U, & Vinggaard AM. (2004). 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(1), 53–61. <https://doi.org/10.1016/j.reprotox.2003.10.011>
- Eisenberg ML, Jensen TK, Walters RC, Skakkebaek NE, & Lipshultz LI. (2012). The relationship between anogenital distance and reproductive hormone levels in adult men. *The Journal of Urology*, 187(2), 594–598. <https://doi.org/10.1016/j.juro.2011.10.041>
- Henriksen LS, Frederiksen H, Jørgensen N, Juul A, Skakkebaek NE, Toppari J, Petersen JH, & Main KM. (2023). Maternal phthalate exposure during pregnancy and testis function of young adult sons. *The Science of the Total Environment*, 871, 161914. <https://doi.org/10.1016/j.scitotenv.2023.161914>
- Holmer, M. L., Zilliacus, J., Draskau, M. K., Hlisková, 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. *Reproductive Toxicology*, 128, 108662. <https://doi.org/10.1016/j.reprotox.2024.108662>
- 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. *Toxicology and Applied Pharmacology*, 213(2), 160–171. <https://doi.org/10.1016/j.taap.2005.10.013>
- Li C, Zou C, Yan H, Li Z, Li Y, Pan P, Ma F, Yu Y, Wang Y, Wen Z, & Ge RS. (2021). Perfluorotridecanoic acid inhibits fetal Leydig cell differentiation after in utero exposure in rats via increasing oxidative stress and autophagy. *Environmental Toxicology*, 36(6), 1206–1216. <https://doi.org/10.1002/tox.23119>
- Liu C, Xu X, Zhang Y, Li W, & Huo X. (2016). Associations between maternal phenolic exposure and cord sex hormones in male newborns. *Human Reproduction (Oxford, England)*, 31(3), 648–656. <https://doi.org/10.1093/humrep/dev327>
- Lu, C.-L., Wen, H.-J., Chen, M.-L., Sun, C.-W., Hsieh, C.-J., Wu, M.-T., Wang, S.-L., & TMICS study grp. (2024). Prenatal phthalate exposure and sex steroid hormones in newborns: Taiwan Maternal and Infant Cohort Study. *PLOS ONE*, 19(3). <https://doi.org/10.1371/journal.pone.0297631>
- Mably TA, Moore RW, & Peterson RE. (1992). In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 1. Effects on androgenic status. *Toxicology and Applied Pharmacology*, 114(1), 97–107. [https://doi.org/10.1016/0041-008x\(92\)90101-w](https://doi.org/10.1016/0041-008x(92)90101-w)
- Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015). Androgens and mammalian male reproductive tract development. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1849(2), 163–170. <https://doi.org/10.1016/j.bbaggm.2014.05.020>
- Pan P, Ma F, Wu K, Yu Y, Li Y, Li Z, Chen X, Huang T, Wang Y, & Ge RS. (2020). Maternal exposure to zearalenone in masculinization window affects the fetal Leydig cell development in rat male fetus. *Environmental Pollution (Barking, Essex : 1987)*, 263, 114357. <https://doi.org/10.1016/j.envpol.2020.114357>
- 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. *Archives of Toxicology*, 93(2), 253–272. <https://doi.org/10.1007/s00204-018-2350-5>
- Sharpe RM. (2020). Androgens and the masculinization programming window: Human-rodent differences. *Biochemical Society Transactions*, 48(4), 1725–1735. <https://doi.org/10.1042/BST20200200>
- Thankamony A, Pasterski V, Ong KK, Acerini CL, & Hughes IA. (2016). Anogenital distance as a marker of androgen exposure in humans. *Andrology*, 4(4), 616–625. <https://doi.org/10.1111/andr.12156>
- Trost, L. W., & Mulhall, J. P. (2016). Challenges in Testosterone Measurement, Data Interpretation, and Methodological Appraisal of Interventional Trials. *The Journal of Sexual Medicine*, 13(7), 1029–1046. <https://doi.org/10.1016/j.jsxm.2016.04.068>
- Vesper, H. W., Wang, Y., Vidal, M., Botelho, J. C., & Caudill, S. P. (2015). Serum Total Testosterone Concentrations in the US Household Population from the NHANES 2011–2012 Study Population. *Clinical Chemistry*, 61(12), 1495–1504. <https://doi.org/10.1373/clinchem.2015.245969>
- Vinggaard AM, Christiansen S, Laier P, Poulsen ME, Breinholt V, Jarfelt K, Jacobsen H, Dalgaard M, Nellemann C, & Hass U. (2005). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 85(2), 886–897. <https://doi.org/10.1093/toxsci/kfi150>
- Welsh M, Suzuki H, & Yamada G. (2014). The masculinization programming window. *Endocrine Development*, 27, 17–27. <https://doi.org/10.1159/000363609>
- Zarean M, Keikha M, Feizi A, Kazemitabae M, & Kelishadi R. (2019). The role of exposure to phthalates in variations of anogenital distance: A systematic review and meta-analysis. *Environmental Pollution (Barking, Essex : 1987)*, 247, 172–179. <https://doi.org/10.1016/j.envpol.2019.01.026>