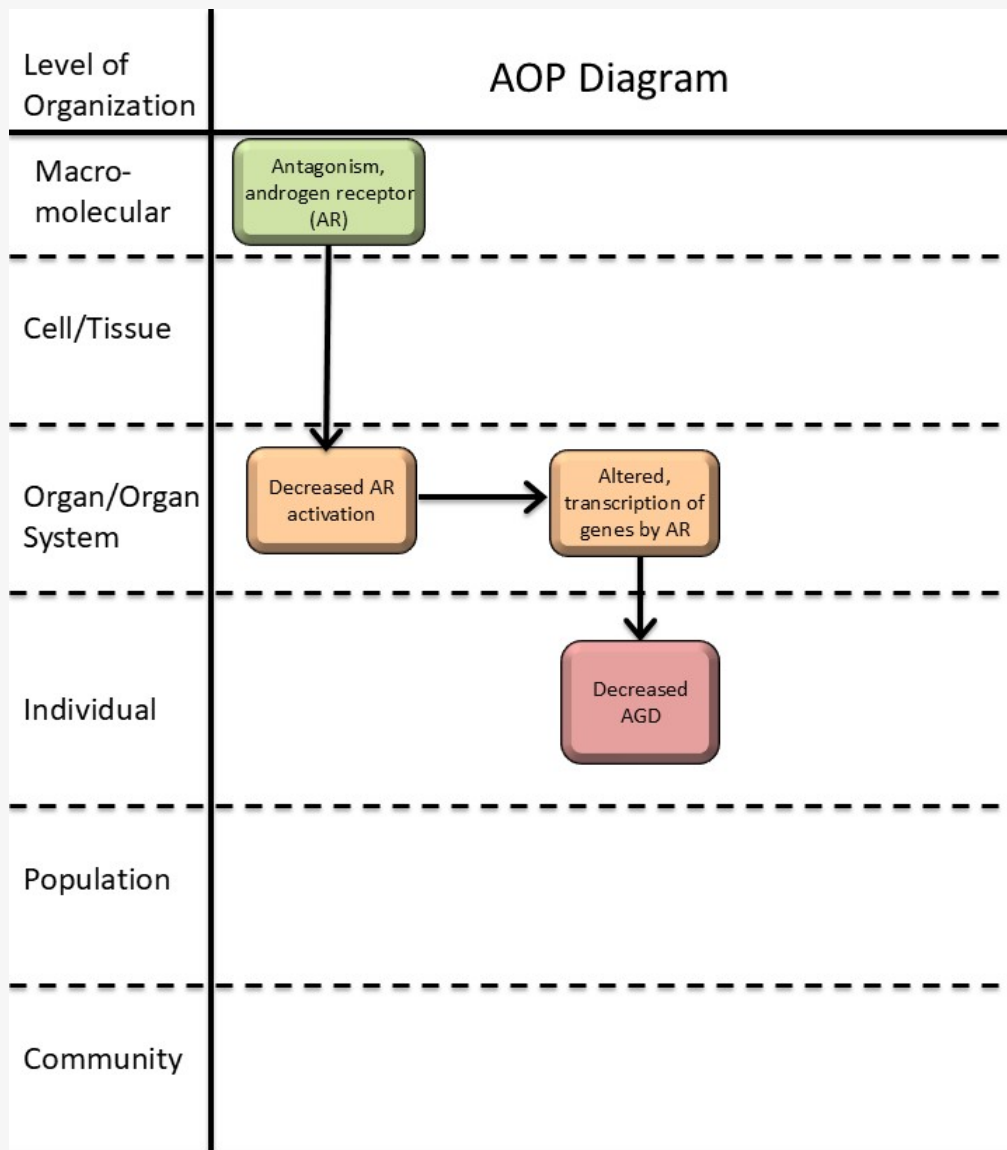


AOP ID and Title:

AOP 306: Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring

Short Title: AR antagonism leading to short AGD

Graphical Representation**Authors**

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

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

Terje Svingen; 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

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

Status

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 Do not cite	Under Review	1.90	Included in OECD Work Plan

Abstract

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

The AR is a nuclear receptor involved in the transcriptional regulation of various target genes during development and adulthood across species (Heemers & Tindall, 2007; Davey and Grossmann, 2016). Its main ligand is testosterone and dihydrotestosterone (DHT). Under normal physiological conditions, testosterone produced mainly by the testicles, is converted in peripheral tissues by 5 α -reductase into DHT, which in turn binds AR and activates downstream target genes (Davey and Grossmann, 2016). AR signaling is necessary for normal masculinization of the developing fetus, including differentiation of the levator ani/bulbocavernosus (LABC) muscle complex in male fetuses (Keller et al, 1996; Robitaille and Langlois, 2020). The LABC complex does not develop in the absence, or low levels of, androgen signaling, as in female fetuses.

A central step in this pathway is antagonism of the AR in target cells of the undifferentiated perineal region, which leads to inactivation of the AR and failure to properly masculinize the perineum/LABC complex. In this instance, the local levels of testosterone or DHT may be physiologically normal (or sufficient) levels, but prevented from binding the AR.

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 307 (Decreased testosterone synthesis leading to short AGD).

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

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	26	Antagonism, Androgen receptor	Antagonism, Androgen receptor
	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	286	Altered, Transcription of genes by the androgen receptor	Altered, Transcription of genes by the AR
	AO	1688	anogenital distance (AGD), decreased	AGD, decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Androgen receptor	adjacent	Decrease, androgen receptor activation	High	High
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	Moderate	

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

Stressors

Name Evidence

Flutamide

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

Essentiality of the Key Events

The essentiality of each key event (KE) was evaluated, meaning that if an upstream KE is blocked or does not occur, subsequent downstream KEs or the adverse outcome (AO) are prevented or altered. Both direct and indirect evidence of essentiality were assessed according to the OECD developer's handbook (see Supplementary Table S1, [4hsit0i1mm_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 306:

Event	Direct evidence	Indirect evidence	Contradictory evidence	Overall essentiality assessment
MIE-26	***	*		High
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 below Table:

ID	Assessment score	Rationale
Upstream KERs in AOP 306		
KER-2130	High	It is well established that antagonism of the AR leads to decreased AR activity.
Upstream KERs in AOP 305		
KER-1880	High	It is well established that 5 α -reductase converts testosterone to DHT and that decreased 5 α -reductase activity leads to decreased DHT levels.
KER-1935	High	It is well established that DHT activates the AR and that decreased DHT levels leads to decreased AR activation.
Upstream KERs in AOP 307		
KER-2131	High	It is well established that testosterone activates the AR and that decreased testosterone levels leads to decreased AR activation.
Downstream KERs shared by AOP 305-307		
KER-2124	High	It is well established that the AR regulates gene transcription, and that decreased AR activity leads to altered gene transcription.
KER-2820	High	It is well established that decreased AR activity leads to decreased AGD in male offspring.
KER-2127	Moderate	It is highly plausible that altered gene transcription in the perineum leads to decreased AGD in male offspring.

Quantitative Consideration

The quantitative understanding of this AOP remains low.

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* In Press (doi: 10.1002/etc.5972).
- Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, Guillette L (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* 7(3):248-64.
- Heemers HV, Tindall DJ (2007). Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. *Endocr Rev* 28(7):778-808.
- Holmer ML, Zilliacus J, Draskau MK, Hliseníková H, Beronius A, Svingen T (2024). Methodology for developing data-rich Key Event Relationships for Adverse Outcome Pathways exemplified by linking decreased androgen receptor activity with decreased anogenital distance. *Reprod Toxicol* 128:108662.
- Keller ET, Ershler WB, Chang C (1996). The androgen receptor: a mediator of diverse responses. *Front Biosci* 1:d59-71.
- Murashima A, Kishigami S, Thomson A, Yamada G (2015). Androgens and mammalian male reproductive tract development. *Biochim Biophys Acta* 1849(2):163-70.
- OECD (2008), Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment, OECD Series on Testing and Assessment, No. 43, OECD Publishing, Paris.

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

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

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

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

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

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

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

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

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

Appendix 1

List of MIEs in this AOP

[Event: 26: Antagonism, Androgen receptor](#)

Short Name: Antagonism, Androgen receptor

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	MolecularInitiatingEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	MolecularInitiatingEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male (mammalian) offspring	MolecularInitiatingEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	MolecularInitiatingEvent
Aop:595 - Nanoplastic effect	MolecularInitiatingEvent

Stressors

Name

Mercaptobenzole
 Triticonazole
 Flusilazole
 Epoxiconazole
 Prochloraz
 Propiconazole
 Tebuconazole
 Flutamide
 Cyproterone acetate
 Vinclozolin

Biological Context**Level of Biological Organization**

Molecular

Cell term**Cell term**

eukaryotic cell

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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 & Grossmann, 2016](#)). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutations studies from both humans and rodents showing strong correlation for AR-dependent development and function ([Walters et al., 2010](#)).

This KE is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across mammalian taxa. It is, however, acknowledged that this 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 DescriptionThe androgen receptor (AR) and its function

The AR is a ligand-activated transcription factor belonging to the steroid hormone nuclear receptor family ([Davey & Grossmann, 2016](#)). The AR has three domains: the N-terminal domain, the DNA-binding domain and the ligand-binding domain, with the latter being most evolutionary conserved. Testosterone (T) and the more biologically active dihydrotestosterone (DHT) are endogenous ligands for the AR ([MacLean et al., 1993](#); [MacLeod et al., 2010](#); [Schwartz et al., 2019](#)). In teleost fishes, 11-ketotestosterone is the second main ligand ([Schuppe et al., 2020](#)). Human AR mutations and mouse knock-out models have established a pivotal role for the AR in masculinization and spermatogenesis

(Walters et al, 2010). Apart from the essential role for AR in male reproductive development and function (Walters et al, 2010), the AR is also expressed in many other tissues and organs such as bone, muscles, ovaries, and the immune system (Rana et al, 2014).

AR antagonism as Key Event

The main function of the AR is to activate gene transcription in cells. Canonical signaling occurs by ligands (androgens) binding to AR in the cytoplasm which results in translocation to the cell nucleus, receptor dimerization and binding to specific regulatory DNA sequences (Heemers & Tindall, 2007). The gene targets regulated by AR activation depends on cell/tissue type and what stage of development activation occur, and is, for instance, dependent on available co-factors. Apart from the canonical signaling pathway, AR can also initiate cytoplasmic signaling pathways with other functions than the nuclear pathway, for instance rapid change in cell function by ion transport changes (Heinlein & Chang, 2002) and association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway (Leung & Sadar, 2017).

How it is Measured or Detected

AR antagonism can be measured in vitro by transient or stable transactivation assays to evaluate nuclear receptor activation. There is already a validated test guideline for AR (ant)agonism adopted by the OECD, Test No. 458: *Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals* (OECD, 2016). This test guideline contains three different methods. More information on limitations, advantages, protocols, and availability and description of cells are given in the test guideline.

Besides these validated methods, other transiently or stably transfected reporter cell lines are available as well as yeast based systems (Campana et al, 2015; Körner et al, 2004). AR nuclear translocation can be monitored by various assays (Campana et al 2015), for example by monitoring fluorescent rat AR movement in living cells (Tyagi et al 2020), with several human AR translocation assays being commercially available; e.g. Fluorescent AR Nuclear Translocation Assay (tGFP-hAR/HEK293) or Human Androgen NHR Cell Based Antagonist Translocation LeadHunter Assay.

Additional information on AR interaction can be obtained employing competitive AR binding assays (Freyberger et al 2010, Shaw et al 2018), which can also inform on relative potency of the compounds, though not on downstream effect of the AR binding.

The recently developed AR dimerization assay provides an assay with an improved ability to measure potential stressor-mediated disruption of dimerization/activation (Lee et al, 2021).

References

- Campana C, Pezzi V, Rainey WE (2015) Cell based assays for screening androgen receptor ligands. *Semin Reprod Med* 33: 225-234.
- Davey RA, Grossmann M (2016) Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin Biochem Rev* 37: 3-15
- Freyberger A, Weimer M, Tran HS, Ahr HJ. Assessment of a recombinant androgen receptor binding assay: initial steps towards validation. *Reprod Toxicol*. 2010 Aug;30(1):2-8. doi: 10.1016/j.reprotox.2009.10.001. Epub 2009 Oct 13. PMID: 19833195.
- Heemers HV, Tindall DJ (2007) Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. *Endocr Rev* 28: 778-808
- Heinlein CA, Chang C (2002) The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Mol Endocrinol* 16: 2181-2187
- Körner W, Vinggaard AM, Térouanne B, Ma R, Wieloch C, Schlumpf M, Sultan C, Soto AM (2004) Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals. *Environ Health Perspect* 112: 695-702
- Lee SH, Hong KY, Seo H, Lee HS, Park Y (2021) Mechanistic insight into human androgen receptor-mediated endocrine-disrupting potentials by a stable bioluminescence resonance energy transfer-based dimerization assay. *Chem Biol Interact* 349: 109655
- 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>
- MacLean HE, Chu S, Warne GL, Zajac JD (1993) Related individuals with different androgen receptor gene deletions. *J Clin Invest* 91: 1123-1128
- 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
- OECD. (2016) 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*, Paris.
- OECD (2022). Test No. 251: Rapid Androgen Disruption Activity Reporter (RADAR) assay. Paris: OECD Publishing

doi:10.1787/da264d82-en.

Rana K, Davey RA, Zajac JD (2014) Human androgen deficiency: insights gained from androgen receptor knockout mouse models. *Asian J Androl* **16**: 169-177

Satoh K, Ohyama K, Aoki N, Iida M, Nagai F (2004) Study on anti-androgenic effects of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. *Food Chem Toxicol* **42**: 983-993

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

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

Shaw J, Leveridge M, Norling C, Karén J, Molina DM, O'Neill D, Dowling JE, Davey P, Cowan S, Dabrowski M, Main M, Gianni D. Determining direct binders of the Androgen Receptor using a high-throughput Cellular Thermal Shift Assay. *Sci Rep.* 2018 Jan 9;8(1):163. doi: 10.1038/s41598-017-18650-x. PMID: 29317749; PMCID: PMC5760633.

Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK (2000) Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol Endocrinol* **14**: 1162-1174

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

List of Key Events in the AOP

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 ID and Name

Event Type

[Aop:576 - 5 \$\alpha\$ -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

Name

Flusilazole
 Prochloraz
 Propiconazole
 Stressor:286 Tebuconazole
 Triticonazole
 Vinclozalin

Biological Context**Level of Biological Organization**

Tissue

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

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

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

Key Event Description

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

The Androgen Receptor and its function

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

Altered transcription of genes by the AR as a Key Event

Upon activation by ligand-binding, the AR translocates from the cytoplasm to the cell nucleus, dimerizes, binds to androgen response elements in the DNA to modulate gene transcription (Davey and Grossmann 2016). The transcriptional targets vary between cells and tissues, as well as with developmental stages and is also dependent on available co-regulators (Bevan and Parker 1999; Heemers and Tindall 2007). It should also be mentioned that the AR can work in other 'non-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., Caillarec-Joly, A., Sallou, O., et al. (2018a). TOXsIgN: a cross-species repository for toxicogenomic signatures. *Bioinformatics* 34, 2116–2122. doi:10.1093/bioinformatics/bty040.
- Darde, T. A., Chalmel, F., and Svingen, T. (2018b). Exploiting advances in transcriptomics to improve on human-relevant toxicology. *Curr. Opin. Toxicol.* 11–12, 43–50. doi:10.1016/j.cotox.2019.02.001.
- Davey RA, Grossmann M (2016) Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin Biochem Rev* 37:3–15
- Estrada M, Espinosa A, Müller M, Jaimovich E (2003) Testosterone Stimulates Intracellular Calcium Release and Mitogen-Activated Protein Kinases Via a G Protein-Coupled Receptor in Skeletal Muscle Cells. *Endocrinology* 144:3586–3597. doi: 10.1210/en.2002-0164
- Heemers H V., Tindall DJ (2007) Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex. *Endocr. Rev.* 28:778–808
- Jin, Hong Jian, Jung Kim, and Jindan Yu. 2013. "Androgen Receptor Genomic Regulation." *Translational Andrology and Urology* 2(3):158–77. doi: 10.3978/j.issn.2223-4683.2013.09.01
- Keenan, A. B., Wojciechowicz, M. L., Wang, Z., Jagodnik, K. M., Jenkins, S. L., Lachmann, A., et al. (2019). Connectivity Mapping: Methods and Applications. *Annu. Rev. Biomed. Data Sci.* 2, 69–92. doi:10.1146/ANNUREV-BIODATASCI-072018-021211.
- Maclean HE, Chu S, Warne GL, Zajack JD Related Individuals with Different Androgen Receptor Gene Deletions
- MacLeod DJ, Sharpe RM, Welsh M, et al (2010) Androgen action in the masculinization programming window and development of male reproductive organs. In: *International Journal of Andrology*. Blackwell Publishing Ltd, pp 279–287
- Ngan S, Stronach EA, Photiou A, et al (2009) Microarray coupled to quantitative RT–PCR analysis of androgen-regulated genes in human LNCaP prostate cancer cells. *Oncogene* 28:2051–2063. doi: 10.1038/onc.2009.68
- Rana K, Davey RA, Zajack JD (2014) Human androgen deficiency: Insights gained from androgen receptor knockout mouse models. *Asian J. Androl.* 16:169–177
- Rooney, J. P., Chorley, B., Kleinstreuer, N., and Corton, J. C. (2018). Identification of Androgen Receptor Modulators in a Prostate Cancer Cell Line Microarray Compendium. *Toxicol. Sci.* 166, 146–162. doi:10.1093/TOXSCI/KFY187.
- Walters KA, Simanainen U, Handelsman DJ (2010) Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. *Hum Reprod Update* 16:543–558. doi: 10.1093/humupd/dmq003

List of Adverse Outcomes in this AOP

Event: 1688: anogenital distance (AGD), decreased**Short Name: AGD, decreased****Key Event Component**

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

AOPs Including This Key Event

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

Stressors

Name
Butylparaben
p,p'-DDE
Bis(2-ethylhexyl) phthalate
Dexamethasone
Fenitrothion
Finasteride
Flutamide
Ketoconazole
Linuron
Prochloraz
Procymidone
Triticonazole
Vinclozolin
di-n-hexyl phthalate
Dicyclohexyl phthalate
butyl benzyl phthalate
monobenzyl phthalate
di-n-heptyl phthalate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term

perineum

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

Foetal High

Sex Applicability

Sex Evidence

Male High

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

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

Key Event Description

The anogenital distance (AGD) refers to the distance between anus and the external genitalia. In rodents and humans, the male AGD is approximately twice the length as the female AGD ([Salazar-Martinez et al, 2004](#); [Schwartz et al, 2019](#)). This sexual dimorphism is a consequence of sex hormone-dependent development of secondary sexual characteristics ([Schwartz et al, 2019](#)). In males, it is believed that androgens (primarily DHT) activate AR-positive cells in non-myotonic cells in the fetal perineum region to initiate differentiation of the perineal *levator ani* and *bulbocavernosus* (LABC) muscle complex ([Ipulan et al, 2014](#)). This AR-dependent process occurs within a critical window of development, around gestational days 15-18 in rats ([MacLeod et al, 2010](#)). In females, the absence of DHT prevents this masculinization effect from occurring.

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

How it is Measured or Detected

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

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

Regulatory Significance of the AO

In regulatory toxicology, the AGD is mandatory inclusions in OECD test guidelines used to test for developmental and

reproductive toxicity of chemicals. Guidelines include 'TG 443 extended one-generation study', 'TG 421/422 reproductive toxicity screening studies' and 'TG 414 developmental toxicity study'.

References

- 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./ *Toxicol Sci***30 Spec No**: 79-96
- Hotchkiss AK, Parks-Salducci 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, Fiskens 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, Metzдорff 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, Lambricht C, Mann P, Price M, Cooper RL, Ostby J, Gray CLJ (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health***15**: 94-118
- Zhang L, Dong L, Ding S, Qiao P, Wang C, Zhang M, Zhang L, Du Q, Li Y, Tang N, Chang B (2014) Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E₂ levels in male rat offspring. *Environ Toxicol Pharmacol***37**: 705-717

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2130: Antagonism, Androgen receptor leads to Decrease, AR activation

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term Scientific Term Evidence Links

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 to mammals as AR expression and activity is highly conserved (Davey & Grossmann, 2016). AR activity is important for sexual development and reproduction in both males and females (Prizant et al., 2014; Walters et al., 2010). AR function is required during development, puberty, and adulthood. 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-activated steroid hormone nuclear receptor (Davey & Grossmann, 2016). In its inactive state, the AR locates to the cytoplasm (Roy et al., 2001). When activated, the AR translocates to the nucleus, dimerizes, and, together with co-regulators, binds to specific DNA regulatory sequences to regulate gene transcription (Davey & Grossmann, 2016) (Lamont and Tindall, 2010). This is considered the canonical AR signaling pathway. The AR can also activate non-genomic signalling (Jin et al., 2013). However, this KER focuses on the canonical pathway. The two main AR ligands are the androgens testosterone (T) and the more potent dihydrotestosterone (DHT). Androgens bind to the AR to mediate downstream androgenic responses, such as male development and masculinization (Rey, 2021; Walters et al., 2010). Antagonism of the AR would decrease AR activation and therefore the downstream AR-mediated effects.			
Evidence Supporting this KER			
Biological Plausibility			
The biological plausibility for this KER is considered high.			
The AR belongs to the steroid hormone nuclear receptor family. The AR has 3 main domains essential for its activity, the N-terminal domain, the ligand binding domain, and the DNA binding domain (Roy et al., 2001). Ligands, such as T and DHT, must bind to the ligand binding domain to activate AR allowing it to fulfill its role as a transcription factor. The binding of the ligand induces a change in AR conformation allowing it to translocate to the nucleus and congregate into a subnuclear compartment (Marcelli et al., 2006; Roy et al., 2001) homodimerize and bind to the DNA target sequences and regulate transcription of target genes. Regulation of AR target genes is greatly facilitated by numerous co-factors. Active AR signaling is essential for male reproduction and sexual development and is also crucial in several other tissues and organs such as ovaries, the immune system, bones, and muscles (Ogino et al., 2011; Prizant et al., 2014; Rey, 2021; William H. Walker, 2021) AR antagonists can compete with or prevent in different ways AR ligand binding, thereby preventing AR activation. Antagonism of the AR can prevent translocation to the nucleus, compartmentalization, dimerization and DNA binding. Consequently, AR cannot regulate transcription of target genes and androgen signalling is disrupted. This can be observed using different AR activation assays such as AR dimerization, translocation, DNA binding or transcriptional activity assays (Brown et al., 2023; OECD, 2020).			
Empirical Evidence			
The empirical evidence for this KER is considered high			
The effects of AR antagonism have been shown in many studies <i>in vivo</i> and <i>in vitro</i> .			
Several stressors can act as antagonists of the AR and lead to decreased AR activation. Some of these are detailed in an AOP key event relationship report by (Pedersen et al., 2022) and shown below, exhibiting evidence of dose-concordance:			
Stressors			
- Cyproterone acetate: Using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld, 2005) - Epoxiconazole: Using transiently AR-transfected CHO cells, epoxiconazole showed a LOEC of 1.6 µM and an IC50 of 10 µM (Kjærstad et al., 2010). - Flutamide: Using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 µM (Sonneveld, 2005).			

- Flusilazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.8 μM and an IC₅₀ of 2.8 (± 0.1) μM (Draskau et al., 2019).
- Prochloraz: Using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 μM and an IC₅₀ of 13 μM (Kjærstad et al., 2010).
- Propiconazole: Using transiently AR-transfected CHO cells, propiconazole showed a LOEC of 12.5 μM and an IC₅₀ of 18 μM (Kjærstad et al., 2010).
- Tebuconazole: Using transiently AR-transfected CHO cells, tebuconazole showed a LOEC of 3.1 μM and an IC₅₀ of 8.1 μM (Kjærstad et al., 2010).
- Triticonazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.2 μM and an IC₅₀ of 0.3 (± 0.01) μM (Draskau et al., 2019).
- Vinclozolin: Using the AR-CALUX reporter assay in antagonism mode, vinclozolin showed an IC₅₀ of 1.0 μM (Sonneveld, 2005). (Pedersen et al., 2022)

Other evidence:

Known AR antagonists are used for treatment of AR-sensitive cancers such as flutamide for prostate cancer (Mahler et al., 1998).

Uncertainties and Inconsistencies

Known antiandrogenic compounds like hydroxyflutamide have been shown to act as agonists when the AR carries certain mutations, therefore contributing to uncertainties (Yeh et al., 1997). Additionally, the levels of endogenous androgens (e.g., testosterone or dihydrotestosterone) and the variability in the presence and function of AR co-activators may modulate the effect of AR antagonism.

Quantitative Understanding of the Linkage

Response-response relationship

The quantitative relationship between AR antagonism and AR activation will depend on the type of antagonist.

Time-scale

Nuclear translocation in HeLa cells transfected with AR-GFP show a response within 2 hours after ligand exposure (Marcelli et al., 2006; Szafran et al., 2008). Another assay focusing on AR binding to promoters in LNCaP cells has shown that after ligand binding, AR is able to translocate and bind to the DNA sequences within 15min showing the speed of AR activation (Kang et al., 2002).

Known Feedforward/Feedback loops influencing this KER

AR antagonism can lead to increased AR transcript stability and levels as a compensatory mechanism in prostate cancer cells (Dart et al., 2020). In turn, in presence of increased AR levels, AR antagonists can exhibit agonistic activity (Chen et al., 2003).

References

- Brown, E. C., Hallinger, D. R., Simmons, S. O., Puig-Castellví, F., Eilebrecht, E., Arnold, L., & Bioscience, P. A. (2023). High-throughput AR dimerization assay identifies androgen disrupting chemicals and metabolites. *Front. Toxicol*, 5, 1134783. <https://doi.org/10.3389/ftox.2023.1134783>
- Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., & Sawyers, C. L. (2003). A R T I C L E S Molecular determinants of resistance to antiandrogen therapy. *NATURE MEDICINE*, 10(1). <https://doi.org/10.1038/nm972>
- Dart, D. A., Ashelford, K., & Jiang, W. G. (2020). *AR mRNA stability is increased with AR-antagonist resistance via 3'UTR variants*. <https://doi.org/10.1530/EC-19-0340>
- Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. In *Androgen Receptor Biology Clin Biochem Rev* (Vol. 37, Issue 1).
- Draskau, M. K., Boberg, J., Taxvig, C., Pedersen, M., Frandsen, H. L., Christiansen, S., & Svingen, T. (2019). In vitro and in vivo endocrine disrupting effects of theazole fungicides triticonazole and flusilazole. *Environmental Pollution*, 255, 113309. <https://doi.org/10.1016/j.envpol.2019.113309>
- Jin, H. J., Kim, J., & Yu, J. (2013). Androgen receptor genomic regulation. In *Translational Andrology and Urology* (Vol. 2, Issue 3, pp. 158–177). AME Publishing Company. <https://doi.org/10.3978/j.issn.2223-4683.2013.09.01>
- Kang, Z., Pirskanen, 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>
- Kjærstad, M. B., Taxvig, C., Nellemann, C., Vinggaard, A. M., & Andersen, H. R. (2010). Endocrine disrupting effects in vitro of

conazole antifungals used as pesticides and pharmaceuticals. *Reproductive Toxicology*, 30(4), 573–582. <https://doi.org/10.1016/j.reprotox.2010.07.009>

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.

Mahler, C., Verhelst, J., and Denis, L. (1998). Clinical pharmacokinetics of the antiandrogens and their efficacy in prostate cancer. *Clin. Pharmacokinet.* 34, 405–417. doi:10.2165/00003088-199834050-00005/METRICS.

Marcelli, M., Stenoien, D. L., Szafran, A. T., Simeoni, S., AgoulNIK, I. U., Weigel, N. L., Moran, T., Mikic, I., Price, J. H., & Mancini, M. A. (2006). Quantifying effects of ligands on androgen receptor nuclear translocation, intranuclear dynamics, and solubility. *Journal of Cellular Biochemistry*, 98(4), 770–788. <https://doi.org/10.1002/jcb.20593>

OECD (2020). Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals. OECD Guide. Paris: OECD Publishing doi:10.1787/9789264264366-en.

Ogino, Y., Miyagawa, S., Katoh, H., Prins, G. S., Iguchi, T., & Yamada, G. (2011). Essential functions of androgen signaling emerged through the developmental analysis of vertebrate sex characteristics. *Evolution & Development*, 13(3), 315–325. <https://doi.org/10.1111/j.1525-142X.2011.00482.x>

Pedersen, E. B., Christiansen, S., & Svingen, T. (2022). AOP key event relationship report: Linking androgen receptor antagonism with nipple retention. *Current Research in Toxicology*, 3, 100085. <https://doi.org/10.1016/j.crtox.2022.100085>

Prizant, H., Gleicher, N., & Sen, A. (2014). Androgen actions in the ovary: balance is key. *Journal of Endocrinology*, 222(3), R141–R151. <https://doi.org/10.1530/JOE-14-0296>

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

Roy, A. K., Tyagi, R. K., Song, C. S., Lavrovsky, Y., Ahn, S. C., Oh, T. S., & Chatterjee, B. (2001). Androgen receptor: Structural domains and functional dynamics after ligand-receptor interaction. *Annals of the New York Academy of Sciences*, 949, 44–57. <https://doi.org/10.1111/j.1749-6632.2001.tb04001.x>

Sonneveld, E. (2005). Development of Androgen- and Estrogen-Responsive Bioassays, Members of a Panel of Human Cell Line-Based Highly Selective Steroid-Responsive Bioassays. *Toxicological Sciences*, 83(1), 136–148. <https://doi.org/10.1093/toxsci/kfi005>

Szafran, A. T., Szwarc, M., Marcelli, M., & Mancini, M. A. (2008). Androgen Receptor Functional Analyses by High Throughput Imaging: Determination of Ligand, Cell Cycle, and Mutation-Specific Effects. *PLoS ONE*, 3(11), e3605. <https://doi.org/10.1371/journal.pone.0003605>

Walters, K. A., Simanainen, U., & Handelsman, D. J. (2010). Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. In *Human Reproduction Update* (Vol. 16, Issue 5, pp. 543–558). Hum Reprod Update. <https://doi.org/10.1093/humupd/dmq003>

William H. Walker. (2021). Androgen Actions in the Testis and the Regulation of Spermatogenesis. In *Advances in Experimental Medicine and Biology: Vol. volume 1381* (pp. 175–203).

Yeh, S., Miyamoto, H., & Chang, C. (1997). *ARA70 and androgenic activity of hydroxyflutamide Hydroxyflutamide may not always be a pure antiandrogen* (Vol. 349).

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

AOPs Referencing Relationship

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

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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 µg/L of the AR antagonist flutamide resulted in 674 differentially expressed genes at 96 h post fertilization whereas 500 µg/L flutamide resulted in 2871 differentially expressed genes (Ayobahan et al., 2023).

Uncertainties and Inconsistencies

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

Quantitative Understanding of the Linkage

Response-response relationship

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

Time-scale

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

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression in aging male rats	Tissue-specific alterations in AR activity with aging	(Supakar et al. 1993; Wu, Lin, and Gore 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Tut et al. 1997; Chamberlain et al. 1994)

Known Feedforward/Feedback loops influencing this KER

AR has been hypothesized to auto-regulate its mRNA and protein levels (Mora and Mahesh 1999).

References

- Ayobahan, S. U., Alvincz, J., Reinwald, H., Strompen, J., Salinas, G., Schäfers, C., et al. (2023). Comprehensive identification of gene expression fingerprints and biomarkers of sexual endocrine disruption in zebrafish embryo. *Ecotoxicol. Environ. Saf.* 250, 114514. doi:10.1016/j.ECOENV.2023.114514.
- Bennesch, Marcela A., and Didier Picard. 2015. "Minireview: Tipping the Balance: Ligand-Independent Activation of Steroid Receptors." *Molecular Endocrinology* 29(3):349–63.
- Chamberlain, Nancy L., Erika D. Driverand, and Roger L. Miesfeldi. 1994. *The Length and Location of CAG Trinucleotide Repeats in the Androgen Receptor N-Terminal Domain Affect Transactivation Function*. Vol. 22.
- Denolet, Evi, Karel De Gendt, Joke Allemeersch, Kristof Engelen, Kathleen Marchal, Paul Van Hummelen, Karen A. L. Tan, Richard M. Sharpe, Philippa T. K. Saunders, Johannes V. Swinnen, and Guido Verhoeven. 2006. "The Effect of a Sertoli Cell-Selective Knockout of the Androgen Receptor on Testicular Gene Expression in Prepubertal Mice." *Molecular Endocrinology* 20(2):321–34. doi: 10.1210/me.2005-0113.
- Fan, Wuqiang, Toshihiko Yanase, Masatoshi Nomura, Taijiro Okabe, Kiminobu Goto, Takashi Sato, Hirotaka Kawano, Shigeaki Kato, and Hajime Nawata. 2005. *Androgen Receptor Null Male Mice Develop Late-Onset Obesity Caused by Decreased Energy Expenditure and Lipolytic Activity but Show Normal Insulin Sensitivity With High Adiponectin Secretion*. Vol. 54.
- Holterhus, Paul-Martin, Olaf Hiort, Janos Demeter, Patrick O. Brown, and James D. Brooks. 2003. *Differential Gene-Expression Patterns in Genital Fibroblasts of Normal Males and 46,XY Females with Androgen Insensitivity Syndrome: Evidence for Early Programming Involving the Androgen Receptor*. Vol. 4.

- Ikeda, Yasumasa, Ken Ichi Aihara, Takashi Sato, Masashi Akaike, Masanori Yoshizumi, Yuki Suzaki, Yuki Izawa, Mitsunori Fujimura, Shunji Hashizume, Midori Kato, Shusuke Yagi, Toshiaki Tamaki, Hirotaka Kawano, Takahiro Matsumoto, Hirofumi Azuma, Shigeaki Kato, and Toshio Matsumoto. 2005. "Androgen Receptor Gene Knockout Male Mice Exhibit Impaired Cardiac Growth and Exacerbation of Angiotensin II-Induced Cardiac Fibrosis." *Journal of Biological Chemistry* 280(33):29661-66. doi: 10.1074/jbc.M411694200.
- Jin, Hong Jian, Jung Kim, and Jindan Yu. 2013. "Androgen Receptor Genomic Regulation." *Translational Andrology and Urology* 2(3):158-77.
- Kang, Zhigang, Asta Pirskanen, Olli A. Jänne, and Jorma J. Palvimo. 2002. "Involvement of Proteasome in the Dynamic Assembly of the Androgen Receptor Transcription Complex." *Journal of Biological Chemistry* 277(50):48366-71. doi: 10.1074/jbc.M209074200.
- Kanno, Yuichiro, Nao Saito, Ryota Saito, Tomohiro Kosuge, Ryota Shizu, Tomofumi Yatsu, Takuomi Hosaka, Kiyomitsu Nemoto, Keisuke Kato, and Kouichi Yoshinari. 2022. "Differential DNA-Binding and Cofactor Recruitment Are Possible Determinants of the Synthetic Steroid YK11-Dependent Gene Expression by Androgen Receptor in Breast Cancer MDA-MB 453 Cells." *Experimental Cell Research* 419(2). doi: 10.1016/j.yexcr.2022.113333.
- Karlsson, Sara A., Erik Studer, Petronella Kettunen, and Lars Westberg. 2016. "Neural Androgen Receptors Modulate Gene Expression and Social Recognition but Not Social Investigation." *Frontiers in Behavioral Neuroscience* 10(MAR). doi: 10.3389/fnbeh.2016.00041.
- Knapczyk-Stwora, Katarzyna, Anna Nynca, Renata E. Ciereszko, Lukasz Paukszt, Jan P. Jastrzebski, Elzbieta Czaja, Patrycja Witek, Marek Koziorowski, and Maria Slomczynska. 2019. "Flutamide-Induced Alterations in Transcriptional Profiling of Neonatal Porcine Ovaries." *Journal of Animal Science and Biotechnology* 10(1):1-15. doi: 10.1186/s40104-019-0340-y.
- Lamont, K. R., and Tindall, D. J. (2010). Androgen Regulation of Gene Expression. *Adv. Cancer Res.* 107, 137-162. doi:10.1016/S0065-230X(10)07005-3.
- MacLean, Helen E., W. S. Maria Chiu, Amanda J. Notini, Anna-Maree Axell, Rachel A. Davey, Julie F. McManus, Cathy Ma, David R. Plant, Gordon S. Lynch, and Jeffrey D. Zajac. 2008. "Impaired Skeletal Muscle Development and Function in Male, but Not Female, Genomic Androgen Receptor Knockout Mice." *The FASEB Journal* 22(8):2676-89. doi: 10.1096/fj.08-105726.
- Maiuri, Paolo, Anna Knezevich, Alex De Marco, Davide Mazza, Anna Kula, Jim G. McNally, and Alessandro Marcello. 2011. "Fast Transcription Rates of RNA Polymerase II in Human Cells." *EMBO Reports* 12(12):1280-85. doi: 10.1038/embor.2011.196.
- Mora, Gloria R., and Virendra B. Mahesh. 1999. *Autoregulation of the Androgen Receptor at the Translational Level: Testosterone Induces Accumulation of Androgen Receptor MRNA in the Rat Ventral Prostate Polyribosomes.*
- Peng, Yajie, Hui Zhu, Bing Han, Yue Xu, Xuemeng Liu, Huaidong Song, and Jie Qiao. 2021. "Identification of Potential Genes in Pathogenesis and Diagnostic Value Analysis of Partial Androgen Insensitivity Syndrome Using Bioinformatics Analysis." *Frontiers in Endocrinology* 12. doi: 10.3389/fendo.2021.731107.
- Rana, Kesha, Barbara C. Fam, Michele V Clarke, Tammy P. S. Pang, Jeffrey D. Zajac, and Helen E. Maclean. 2011. "Increased Adiposity in DNA Binding-Dependent Androgen Receptor Knockout Male Mice Associated with Decreased Voluntary Activity and Not Insulin Resistance." *Am J Physiol Endocrinol Me-Tab* 301:767-78. doi: 10.1152/ajpendo.00584.2010.-In.
- Roy, Arun K., Rakesh K. Tyagi, Chung S. Song, Yan Lavrovsky, Soon C. Ahn, Tae Sung Oh, and Bandana Chatterjee. 2001. "Androgen Receptor: Structural Domains and Functional Dynamics after Ligand-Receptor Interaction." Pp. 44-57 in *Annals of the New York Academy of Sciences* Vol. 949. New York Academy of Sciences.
- Russell, Patricia K., Michele V. Clarke, Jarrod P. Skinner, Tammy P. S. Pang, Jeffrey D. Zajac, and Rachel A. Davey. 2012. "Identification of Gene Pathways Altered by Deletion of the Androgen Receptor Specifically in Mineralizing Osteoblasts and Osteocytes in Mice." *Journal of Molecular Endocrinology* 49(1):1-10. doi: 10.1530/JME-12-0014.
- Shiina, Hiroko, Takahiro Matsumoto, Takashi Sato, Katsuhide Igarashi, Junko Miyamoto, Sayuri Takemasa, Matomo Sakari, Ichiro Takada, Takashi Nakamura, Daniel Metzger, Pierre Chambon, Jun Kanno, Hirofumi Yoshikawa, and Shigeaki Kato. 2006. *Premature Ovarian Failure in Androgen Receptor-Deficient Mice* Vol. 103.
- Supakar, P. C., C. S. Song, M. H. Jung, M. A. Slomczynska, J. M. Kim, R. L. Vellanoweth, B. Chatterjee, and A. K. Roy. 1993. "A Novel Regulatory Element Associated with Age-Dependent Expression of the Rat Androgen Receptor Gene." *Journal of Biological Chemistry* 268(35):26400-408. doi: 10.1016/s0021-9258(19)74328-2.
- Tut, Thein G., Farid J. Ghadessy, M. A. Trifiro, L. Pinsky, and E. L. Yong. 1997. *Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced Trans-Activation, Impaired Sperm Production, and Male Infertility**. Vol. 82.
- Wang, Ruey Sheng, Shuyuan Yeh, Lu Min Chen, Hung Yun Lin, Caixia Zhang, Jing Ni, Cheng Chia Wu, P. Anthony Di Sant'Agnese, Karen L. DeMesy-Bentley, Chii Ruey Tzeng, and Chawnschang Chang. 2006. "Androgen Receptor in Sertoli Cell Is Essential for Germ Cell Nursery and Junctional Complex Formation in Mouse Testes." *Endocrinology* 147(12):5624-33. doi: 10.1210/en.2006-0138.

Welsh, M., L. Moffat, K. Belling, L. R. de França, T. M. Segatelli, P. T. K. Saunders, R. M. Sharpe, and L. B. Smith. 2012. "Androgen Receptor Signalling in Peritubular Myoid Cells Is Essential for Normal Differentiation and Function of Adult Leydig Cells." *International Journal of Andrology* 35(1):25–40. doi: 10.1111/j.1365-2605.2011.01150.x.

Willems, Ariane, Sergio R. Batlouni, Arantza Esnal, Johannes V. Swinnen, Philippa T. K. Saunders, Richard M. Sharpe, Luiz R. França, Karel de Gendt, and Guido Verhoeven. 2010. "Selective Ablation of the Androgen Receptor in Mouse Sertoli Cells Affects Sertoli Cell Maturation, Barrier Formation and Cytoskeletal Development." *PLoS ONE* 5(11). doi: 10.1371/journal.pone.0014168.

Wu, D. I., Grace Lin, and Andrea C. Gore. 2009. "Age-Related Changes in Hypothalamic Androgen Receptor and Estrogen Receptor in Male Rats." *The Journal of Comparative Neurology* 512:688–701. doi: 10.1002/cne.21925.

Yu, I. Chen, Hung Yun Lin, Ning Chun Liu, Ruey Shen Wang, Janet D. Sparks, Shuyuan Yeh, and 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: 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
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., 2019). 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 5 α -Reductase Type 2 Deficiency." *Sexual Development* 16, no. 2-3 (2022): 171-83. <https://doi.org/10.1159/000525119>.
- Borch J, Ladefoged O, Hass U, and Vinggaard AM. "Steroidogenesis in Fetal Male Rats Is Reduced by DEHP and DINP, but

- Endocrine Effects of DEHP Are Not Modulated by DEHA in Fetal, Prepubertal and Adult Male Rats." *Reproductive Toxicology* (Elmsford, N.Y.) 18, no. 1 (2004): 53–61. <https://doi.org/10.1016/j.reprotox.2003.10.011>.
- Borch, Julie, Stine Broeng Metzдорff, Anne Marie Vinggaard, Leon Brokken, and Majken Dalgaard. "Mechanisms Underlying the Anti-Androgenic Effects of Diethylhexyl Phthalate in Fetal Rat Testis." *Toxicology* 223, no. 1–2 (June 2006): 144–55. <https://doi.org/10.1016/j.tox.2006.03.015>.
- Botelho, Giuliana G. K., Aedra C. Bufalo, Ana Claudia Boareto, Juliane C. Muller, Rosana N. Morais, Anderson J. Martino-Andrade, Karen R. Lemos, and Paulo R. Dalsenter. "Vitamin C and Resveratrol Supplementation to Rat Dams Treated with Di(2-Ethylhexyl)Phthalate: Impact on Reproductive and Oxidative Stress End Points in Male Offspring." *Archives of Environmental Contamination and Toxicology* 57, no. 4 (November 2009): 785–93. <https://doi.org/10.1007/s00244-009-9385-9>.
- Bowman, C. J., N. J. Barlow, K. J. Turner, D. G. Wallace, and P. M. D. Foster. "Effects of in Utero Exposure to Finasteride on Androgen-Dependent Reproductive Development in the Male Rat." *Toxicological Sciences* 74, no. 2 (August 1, 2003): 393–406. <https://doi.org/10.1093/toxsci/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 Metzдорff, and Ulla Hass. "Low-Dose Perinatal Exposure to Di(2-Ethylhexyl) Phthalate Induces Anti-Androgenic Effects in Male Rats." *Reproductive Toxicology* 30, no. 2 (September 2010): 313–21. <https://doi.org/10.1016/j.reprotox.2010.04.005>.
- Christiansen, Sofie, Martin Scholze, Majken Dalgaard, Anne Marie Vinggaard, Marta Axelstad, Andreas Kortenkamp, and Ulla Hass. "Synergistic Disruption of External Male Sex Organ Development by a Mixture of Four Antiandrogens." *Environmental Health Perspectives* 117, no. 12 (December 2009): 1839–46. <https://doi.org/10.1289/ehp.0900689>.
- Clark, R.L., C.A. Anderson, S. Prahalada, R.T. Robertson, E.A. Lochry, Y.M. Leonard, J.L. Stevens, and A.M. Hoberman. "Critical Developmental Periods for Effects on Male Rat Genitalia Induced by Finasteride, a 5 α -Reductase Inhibitor." *Toxicology and Applied Pharmacology* 119, no. 1 (March 1993): 34–40. <https://doi.org/10.1006/taap.1993.1041>.
- Colbert NK, Pelletier NC, Cote JM, Concannon JB, Jurdak NA, Minott SB, and Markowski VP. "Perinatal Exposure to Low Levels of the Environmental Antiandrogen Vinclozolin Alters Sex-Differentiated Social Play and Sexual Behaviors in the Rat." *Environmental Health Perspectives* 113, no. 6 (2005): 700–707. <https://doi.org/10.1289/ehp.7509>.
- Crouch, Ns, Lina Michala, Sm Creighton, and Gs Conway. "Androgen-Dependent Measurements of Female Genitalia in Women with Complete Androgen Insensitivity Syndrome: Measurements of Female Genitalia in Women with Complete Androgen Insensitivity Syndrome." *BJOG: An International Journal of Obstetrics & Gynaecology* 118, no. 1 (January 2011): 84–87. <https://doi.org/10.1111/j.1471-0528.2010.02778.x>.
- Culty, Martine, Raphael Thuillier, Wenping Li, Yan Wang, Daniel B. Martinez-Arguelles, Carolina Gesteira Benjamin, Kostantinos M. 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.crttox.2022.100069>.
- Eisenberg ML, Hsieh TC, Pastuszak AW, McIntyre MG, Walters RC, Lamb DJ, and Lipshultz LI. "The Relationship between Anogenital Distance and the Androgen Receptor CAG Repeat Length." *Asian Journal of Andrology* 15, no. 2 (2013): 286–89. <https://doi.org/10.1038/aja.2012.126>.
- Euling, S. Y. "Response-Surface Modeling of the Effect of 5 α -Dihydrotestosterone and Androgen Receptor Levels on the Response to the Androgen Antagonist Vinclozolin." *Toxicological Sciences* 69, no. 2 (October 1, 2002): 332–43. <https://doi.org/10.1093/toxsci/69.2.332>.
- Foster PM and Harris MW. "Changes in Androgen-Mediated Reproductive Development in Male Rat Offspring Following Exposure to a Single Oral Dose of Flutamide at Different Gestational Ages." *Toxicological Sciences: An Official Journal of the Society of Toxicology* 85, no. 2 (2005): 1024–32. <https://doi.org/10.1093/toxsci/kfi159>.
- Fussell, Karma C., Steffen Schneider, Roland Buesen, Sibylle Groeters, Volker Strauss, Stephanie Melching-Kollmuss, and Bennard Van Ravenzwaay. "Investigations of Putative Reproductive Toxicity of Low-Dose Exposures to Flutamide in Wistar Rats." *Archives of Toxicology* 89, no. 12 (December 2015): 2385–2402. <https://doi.org/10.1007/s00204-015-1622-6>.
- Goldspiel, Barry R., and David R. Kohler. "Flutamide: An Antiandrogen for Advanced Prostate Cancer." *DICP* 24, no. 6 (June

1990): 616–23. <https://doi.org/10.1177/106002809002400612>.

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

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

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

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

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

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

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

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

Hellwig, J., B. Van Ravenzwaay, M. Mayer, and C. Gembardt. "Pre- and Postnatal Oral Toxicity of Vinclozolin in Wistar and Long-Evans Rats." *Regulatory Toxicology and Pharmacology* 32, no. 1 (August 2000): 42–50. <https://doi.org/10.1006/rtph.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 Procyridone 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 Pastorski, Kiki Mastroyannopolou, and Jane MacDougall. "Androgen Insensitivity Syndrome." *Lancet* 2012 OCT, no. 20;380(9851) (June 13, 2012): 1419–28. [https://doi.org/doi:10.1016/S0140-6736\(12\)60071-3](https://doi.org/doi:10.1016/S0140-6736(12)60071-3).

Inawaka, Kunifumi, Noriyuki Kishimoto, Hashihiro Higuchi, and Satoshi Kawamura. "Maternal Exposure to Procyridone 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 Vivo Confirmation of an Androgen Receptor-Mediated Mechanism." *Toxicology and Applied Pharmacology* 142, no. 1 (January 1997): 192–200. <https://doi.org/10.1006/taap.1996.7966>.

Kita, Diogo H., Katlyn B. Meyer, Amanda C. Venturelli, Rafaella Adams, Daria L.B. Machado, Rosana N. Morais, Shanna H. Swan,

- Chris Gennings, and Anderson J. Martino-Andrade. "Manipulation of Pre and Postnatal Androgen Environments and Anogenital Distance in Rats." *Toxicology* 368-369 (August 2016): 152-61. <https://doi.org/10.1016/j.tox.2016.08.021>.
- Klinefelter, Gary R, John W Laskey, Witold M Winnik, Juan D Suarez, Naomi L Roberts, Lillian F Strader, Brandy W Riffle, and D N Rao Veeramachaneni. "Novel Molecular Targets Associated with Testicular Dysgenesis Induced by Gestational Exposure to Diethylhexyl Phthalate in the Rat: A Role for Estradiol." *REPRODUCTION* 144, no. 6 (December 2012): 747-61. <https://doi.org/10.1530/REP-12-0266>.
- Kojima, Hiroyuki, Eiji Katsura, Shinji Takeuchi, Kazuhito Niiyama, and Kunihiro Kobayashi. "Screening for Estrogen and Androgen Receptor Activities in 200 Pesticides by in Vitro Reporter Gene Assays Using Chinese Hamster Ovary Cells." *Environmental Health Perspectives* 112, no. 5 (April 2004): 524-31. <https://doi.org/10.1289/ehp.6649>.
- Labrie, F. "Mechanism of Action and Pure Antiandrogenic Properties of Flutamide." *Cancer* 72, no. S12 (December 15, 1993): 3816-27. [https://doi.org/10.1002/1097-0142\(19931215\)72:12+<3816::AID-CNCR2820721711>3.0.CO;2-3](https://doi.org/10.1002/1097-0142(19931215)72:12+<3816::AID-CNCR2820721711>3.0.CO;2-3).
- Lanzoni, Anna, Anna F Castoldi, George EN Kass, Andrea Terron, Guilhem De Seze, Anna Bal-Price, Frédéric Y Bois, et al. "Advancing Human Health Risk Assessment." *EFSA Journal* 17, no. Suppl 1 (July 8, 2019): e170712. <https://doi.org/10.2903/j.efsa.2019.e170712>.
- Lin, Han, Qing-Quan Lian, Guo-Xin Hu, Yuan Jin, Yunhui Zhang, Dianne O. Hardy, Guo-Rong Chen, et al. "In Utero and Lactational Exposures to Diethylhexyl-Phthalate Affect Two Populations of Leydig Cells in Male Long-Evans Rats1." *Biology of Reproduction* 80, no. 5 (May 1, 2009): 882-88. <https://doi.org/10.1095/biolreprod.108.072975>.
- Martínez, Ariadne Gutiérrez, Balia Pardo, Rafael Gámez, Rosa Mas, Miriam Noa, Gisela Marrero, Maikel Valle, et al. "Effects of In Utero Exposure to D-004, a Lipid Extract from *Roystonea Regia* Fruits, in the Male Rat: A Comparison with Finasteride." *Journal of Medicinal Food* 14, no. 12 (December 2011): 1663-69. <https://doi.org/10.1089/jmf.2010.0279>.
- Martino-Andrade AJ, Liu F, Sathyanarayana S, Barrett ES, Redmon JB, Nguyen RH, Levine H, and Swan SH. "Timing of Prenatal Phthalate Exposure in Relation to Genital Endpoints in Male Newborns." *Andrology* 4, no. 4 (2016): 585-93. <https://doi.org/10.1111/andr.12180>.
- Martino-Andrade, Anderson J., Rosana N. Morais, Giuliana G. K. Botelho, Graziela Muller, Simone W. Grande, Giovanna B. Carpentieri, Gabriel M. C. Leão, and Paulo R. Dalsenter. "Coadministration of Active Phthalates Results in Disruption of Foetal Testicular Function in Rats." *International Journal of Andrology* 32, no. 6 (December 2009): 704-12. <https://doi.org/10.1111/j.1365-2605.2008.00939.x>.
- Matsuura, Ikuo, Tetsuji Saitoh, Michiko Ashina, Yumi Wako, Hiroshi Iwata, Naoto Toyota, Yoshihito Ishizuka, Masato Namiki, Nobuhito Hoshino, and Minoru Tsuchitani. "EVALUATION OF A TWO-GENERATION REPRODUCTION TOXICITY STUDY ADDING ENDOPOINTS TO DETECT ENDOCRINE DISRUPTING ACTIVITY USING VINCLOZOLIN." *The Journal of Toxicological Sciences* 30, no. Special (2005): S163-188. <https://doi.org/10.2131/jts.30.S163>.
- McIntyre, B. S. "Androgen-Mediated Development in Male Rat Offspring Exposed to Flutamide in Utero: Permanence and Correlation of Early Postnatal Changes in Anogenital Distance and Nipple Retention with Malformations in Androgen-Dependent Tissues." *Toxicological Sciences* 62, no. 2 (August 1, 2001): 236-49. <https://doi.org/10.1093/toxsci/62.2.236>.
- Molina-Molina, J, A Hillenweck, I Jouanin, D Zalko, J Cravedi, M Fernandez, A Pillon, J Nicolas, N Olea, and P Balaguer. "Steroid Receptor Profiling of Vinclozolin and Its Primary Metabolites." *Toxicology and Applied Pharmacology* 216, no. 1 (October 1, 2006): 44-54. <https://doi.org/10.1016/j.taap.2006.04.005>.
- Moore, R W, T A Rudy, T M Lin, K Ko, and R E Peterson. "Abnormalities of Sexual Development in Male Rats with in Utero and Lactational Exposure to the Antiandrogenic Plasticizer Di(2-Ethylhexyl) Phthalate." *Environmental Health Perspectives* 109, no. 3 (March 2001): 229-37. <https://doi.org/10.1289/ehp.01109229>.
- Murashima, Aki, Satoshi Kishigami, Axel Thomson, and Gen Yamada. "Androgens and Mammalian Male Reproductive Tract Development." *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1849, no. 2 (February 2015): 163-70. <https://doi.org/10.1016/j.bbagr.2014.05.020>.
- Nightingale, Joanna, Khurram S. Chaudhary, Paul D. Abel, Andrew P. Stubbs, Hanna M. Romanska, Stephen E. Mitchell, Gordon W.H. Stamp, and El-Nasir Lalani. "Ligand Activation of the Androgen Receptor Downregulates E-Cadherin-Mediated Cell Adhesion and Promotes Apoptosis of Prostatic Cancer Cells." *Neoplasia* 5, no. 4 (July 2003): 347-61. [https://doi.org/10.1016/S1476-5586\(03\)80028-3](https://doi.org/10.1016/S1476-5586(03)80028-3).
- OECD. *Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals*. OECD Guidelines for the Testing of Chemicals, Section 4. OECD, 2023. <https://doi.org/10.1787/9789264264366-en>.
- Olier, Ivan, Yiqiang Zhan, Xiaoyu Liang, and Victor Volovici. "Causal Inference and Observational Data." *BMC Medical Research Methodology* 23, no. 1 (October 11, 2023): 227. <https://doi.org/10.1186/s12874-023-02058-5>.
- Orton, Frances, Erika Rosivatz, Martin Scholze, and Andreas Kortenkamp. "Widely Used Pesticides with Previously Unknown Endocrine Activity Revealed as in Vitro Antiandrogens." *Environmental Health Perspectives* 119, no. 6 (June 2011): 794-800. <https://doi.org/10.1289/ehp.1002895>.
- Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, and Gray LE Jr. "The Fungicide Procymidone Alters Sexual Differentiation in the Male Rat by Acting as an Androgen-Receptor Antagonist in Vivo and in Vitro." *Toxicology and Industrial Health* 15, no. 1

(1999): 80–93. <https://doi.org/10.1177/074823379901500108>.

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

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

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

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

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

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

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

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

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

Shimamura M, Kodaira K, Kenichi H, Ishimoto Y, Tamura H, and Iguchi T. "Comparison of Antiandrogenic Activities of Vinclozolin and D,L-Camphorquinone in Androgen Receptor Gene Transcription Assay in Vitro and Mouse in Utero Exposure Assay in Vivo." *Toxicology* 174, no. 2 (2002): 97–107. [https://doi.org/10.1016/s0300-483x\(02\)00044-6](https://doi.org/10.1016/s0300-483x(02)00044-6).

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

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

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

Sunman, Birce, Kadriye Yurdakok, Belma Kocer-Gumusel, Ozgur Ozyuncu, Filiz Akbiyik, Aylin Balci, Gizem Ozkemahli, Pinar Erkekoglu, and Murat Yurdakok. "Prenatal Bisphenol a and Phthalate Exposure Are Risk Factors for Male Reproductive System Development and Cord Blood Sex Hormone Levels." *REPRODUCTIVE TOXICOLOGY* 87 (2019): 146–55. <https://doi.org/10.1016/j.reprotox.2019.05.065>.

Swan, Shanna H. "Environmental Phthalate Exposure in Relation to Reproductive Outcomes and Other Health Endpoints in Humans." *ENVIRONMENTAL RESEARCH* 108, no. 2 (2008): 177–84. <https://doi.org/10.1016/j.envres.2008.08.007>.

Swan, Shanna H., Katharina M. Main, Fan Liu, Sara L. Stewart, Robin L. Kruse, Antonia M. Calafat, Catherine S. Mao, et al. "Decrease in Anogenital Distance among Male Infants with Prenatal Phthalate Exposure." *Environmental Health Perspectives* 113, no. 8 (August 2005): 1056–61. <https://doi.org/10.1289/ehp.8100>.

Thankamony, A., V. Pasterski, K. K. Ong, C. L. Acerini, and I. A. Hughes. "Anogenital Distance as a Marker of Androgen Exposure in Humans." *Andrology* 4, no. 4 (July 2016): 616–25. <https://doi.org/10.1111/andr.12156>.

Tut, Thein G., Farid J. Ghadessy, M. A. Trifiro, L. Pinsky, and E. L. Yong. "Long Polyglutamine Tracts in the Androgen Receptor Are Associated with Reduced *Trans*-Activation, Impaired Sperm Production, and Male Infertility¹." *The Journal of Clinical Endocrinology & Metabolism* 82, no. 11 (November 1997): 3777–82. <https://doi.org/10.1210/jcem.82.11.4385>.

Ungewitter, Erica, Emmi Rotgers, Tanika Bantukul, Yasuhiko Kawakami, Grace E. Kissling, and Humphrey Hung-Chang Yao. "Teratogenic Effects of *in Utero* Exposure to Di-(2-Ethylhexyl)-Phthalate (DEHP) in B6:129S4 Mice." *Toxicological Sciences*, January 25, 2017, kfx019. <https://doi.org/10.1093/toxsci/kfx019>.

Venturelli, Amanda Caroline, Katlyn Barp Meyer, Stefani Valéria Fischer, Diogo Henrique Kita, Rafaela Adams Philipsen, Rosana

- Nogueira Morais, and Anderson Joel Martino Andrade. "Effects of in Utero and Lactational Exposure to Phthalates on Reproductive Development and Glycemic Homeostasis in Rats." *Toxicology* 421 (June 2019): 30–40. <https://doi.org/10.1016/j.tox.2019.03.008>.
- Vo TT, Jung EM, Dang VH, Jung K, Baek J, Choi KC, and Jeung EB. "Differential Effects of Flutamide and Di-(2-Ethylhexyl) Phthalate on Male Reproductive Organs in a Rat Model." *The Journal of Reproduction and Development* 55, no. 4 (2009): 400–411. <https://doi.org/10.1262/jrd.20220>.
- Welsh, Michelle, Philippa T.K. Saunders, Mark Fiskien, Hayley M. Scott, Gary R. Hutchison, Lee B. Smith, and Richard M. Sharpe. "Identification in Rats of a Programming Window for Reproductive Tract Masculinization, Disruption of Which Leads to Hypospadias and Cryptorchidism." *Journal of Clinical Investigation* 118, no. 4 (April 1, 2008): 1479–90. <https://doi.org/10.1172/JCI34241>.
- Welsh, Michelle, Hiroko Suzuki, and Gen Yamada. "The Masculinization Programming Window." In *UNDERSTANDING DIFFERENCES AND DISORDERS OF SEX DEVELOPMENT (DSD)*, 27:17–27, 2014. <https://doi.org/10.1159/000363609>.
- Wenzel AG, Bloom MS, Butts CD, Wineland RJ, Brock JW, Cruze L, Unal ER, Kucklick JR, Somerville SE, and Newman RB. "Influence of Race on Prenatal Phthalate Exposure and Anogenital Measurements among Boys and Girls." *Environment International* 110 (2018): 61–70. <https://doi.org/10.1016/j.envint.2017.10.007>.
- Wilson, Vickie S., Kembra L. Howdeshell, Christy S. Lambright, Johnathan Furr, and L. Earl Gray. "Differential Expression of the Phthalate Syndrome in Male Sprague-Dawley and Wistar Rats after in Utero DEHP Exposure." *Toxicology Letters* 170, no. 3 (May 2007): 177–84. <https://doi.org/10.1016/j.toxlet.2007.03.004>.
- Wilson, Vickie S., Christy Lambright, Johnathan Furr, Joseph Ostby, Carmen Wood, Gary Held, and L. Earl Gray. "Phthalate Ester-Induced Gubernacular Lesions Are Associated with Reduced 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, Chlozoline, p,p'-DDE, and Ketoconazole) and Toxic Substances (Dibutyl- and Diethylhexyl Phthalate, PCB 169, and Ethane Dimethane Sulphonate) during Sexual Differentiation Produces Diverse Profiles of Reproductive Malformations in the Male Rat." *Toxicology and Industrial Health* 15, no. 1–2 (February 1999): 94–118. <https://doi.org/10.1177/074823379901500109>.
- Wong, Choi-iok, William R. Kelce, Madhabananda Sar, and Elizabeth M. Wilson. "Androgen Receptor Antagonist versus Agonist Activities of the Fungicide Vinclozolin Relative to Hydroxyflutamide." *Journal of Biological Chemistry* 270, no. 34 (August 1995): 19998–3. <https://doi.org/10.1074/jbc.270.34.19998>.
- Yamasaki Kanji, Noda Shuji, Muroi Takako, Mitoma Hideo, Takakura Saori, and Sakamoto Satoko. "Effects of in Utero and Lactational Exposure to Flutamide in SD Rats: Comparison of the Effects of Administration Periods." *Toxicology* 209, no. 1 (April 2005): 47–54. <https://doi.org/10.1016/j.tox.2004.12.004>.
- Yeh, Shuyuan, Meng-Yin Tsai, Qingquan Xu, Xiao-Min Mu, Henry Lardy, Ko-En Huang, Hank Lin, et al. "Generation and Characterization of Androgen Receptor Knockout (ARKO) Mice: An *in Vivo* Model for the Study of Androgen Functions in Selective Tissues." *Proceedings of the National Academy of Sciences* 99, no. 21 (October 15, 2002): 13498–503. <https://doi.org/10.1073/pnas.212474399>.
- Zhang, Jie, Yuanyuan Yao, Junlin Pan, Xiuxiu Guo, Xiaoying Han, Jun Zhou, and Xiaoqian Meng. "Maternal Exposure to Di-(2-Ethylhexyl) Phthalate (DEHP) Activates the PI3K/Akt/MTOR Signaling Pathway in F1 and F2 Generation Adult Mouse Testis." *Experimental Cell Research* 394, no. 2 (September 2020): 112151. <https://doi.org/10.1016/j.yexcr.2020.112151>.
- Zhang, Lian-Dong, Qian Deng, Zi-Ming Wang, Ming Gao, Lei Wang, Tie Chong, and He-Cheng Li. "Disruption of Reproductive Development in Male Rat Offspring Following Gestational and Lactational Exposure to Di-(2-Ethylhexyl) Phthalate and Genistein." *Biological Research* 46, no. 2 (2013): 139–46. <https://doi.org/10.4067/S0716-97602013000200004>.

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

AOPs Referencing Relationship

AOP Name

Adjacency

Weight of Evidence

Quantitative Understanding

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 as 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.bbagrm.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