



Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders

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Abstract

Male reproductive development is intricately dependent on fetal androgen action. Consequently, disrupted androgen action during fetal life can interfere with the development of the reproductive system resulting in adverse effects on reproductive function later in life. One biomarker used to evaluate fetal androgen action is the anogenital distance (AGD), the distance between the anus and the external genitalia. A short male AGD is strongly associated with genital malformations at birth and reproductive disorders in adulthood. AGD is therefore used as an effect readout in rodent toxicity studies aimed at testing compounds for endocrine activity and anti-androgenic properties, and in human epidemiological studies to correlate fetal exposure to endocrine disrupting chemicals to feminization of new-born boys. In this review, we have synthesized current data related to intrauterine exposure to xenobiotics and AGD measurements. We discuss the utility of AGD as a retrospective marker of in utero anti-androgenicity and as a predictive marker for male reproductive disorders, both with respect to human health and rodent toxicity studies. Finally, we highlight four areas that need addressing to fully evaluate AGD as a biomarker in both a regulatory and clinical setting.

Keywords Anogenital distance · Reproduction · Endocrine disruptors · Toxicology · Risks assessment

Introduction

Few things permeate our lives more than our sex and reproductive capacity, influencing not only our physical characteristics, but also behaviour and social perception. Ensuring proper reproductive development and life-long health therefore seems obvious, yet modern living is increasingly putting pressure on the processes of sexual differentiation and reproductive function. Exposure to endocrine disrupting chemicals (EDCs) during fetal life in both males and females

has been raised as particularly disconcerting, since this is the period when the sexual organs form and, in many respects, lay the foundation for adult reproductive health (Johansson et al. 2017; Skakkebaek et al. 2016). Much effort has thus been invested in understanding the relationships between fetal exposure to xenobiotics and reproductive disorders, as well as to devise testing strategies to screen chemicals for potential endocrine disrupting effects.

In males, reproductive disorders associated with impaired fetal testis development or function vary in both phenotype and time of manifestation. Often described by the ‘testicular dysgenesis syndrome’ hypothesis (Skakkebaek et al. 2016), these male disorders range from hypospadias and cryptorchidism in infants (Hsieh et al. 2008, 2012; Jain and Singal 2013; Thankamony et al. 2014), to low testosterone levels, impaired semen quality and fertility issues in adult men (Eisenberg et al. 2011, 2012; Mendiola et al. 2011). Because of this complexity, it is difficult to adopt a single biomarker to use in animal toxicity studies aimed at testing chemicals for potential adverse effects on male reproductive health. The anogenital distance (AGD), however, is considered a broad biomarker capable of both retrospectively determine

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early-life androgen disruption and predict late-life reproductive disorders in male offspring (Dean and Sharpe 2013; Thankamony et al. 2016).

The AGD refers to the distance between the anus and the external genitalia, and is approximately twice the length in male compared to female new-borns. This sexual dimorphism is apparent in rodents as well as humans (Salazar-Martinez et al. 2004; Thankamony et al. 2009) and is a consequence of the androgen-driven dimorphic differentiation of the two sexes (Fig. 1). This sexual bifurcation extends to the perineum, where a muscular complex develops in males, but not in females. A short male AGD is, therefore, considered a marker of disrupted androgen action. In rodents, a short male AGD largely predicts the same adverse effect outcomes as in humans. In fact, the idea of investigating AGD in human epidemiological studies came from rodent developmental and reproductive toxicity studies, where AGD had been used for decades as a marker of impaired fetal androgen action and regarded as an adverse outcome (Tables 1, 2).

In this review, we discuss current knowledge concerning AGD measurements, both as a clinical marker in humans and as a morphometric measure of fetal androgen disturbance in rodent toxicity studies. We have collated a growing body of toxicological studies that have reported on AGD measurements to gain a better overview of what evidence support the exclusive androgen-driven masculinization thesis, or to tease out other potential mechanisms that may lead to similar effects on AGD. However, first, to better appreciate why the AGD is a useful marker to assess early-life androgen disruption, we need to broadly outline how the two sexes develop.

Sexual differentiation and the importance of early androgen signaling

From gonadal sex determination to testosterone synthesis

At first, the male and female embryos are morphologically indistinguishable and only differentiate down two distinct trajectories after the appearance of either testes or ovaries. The specification of reproductive sex, or gonadal sex determination, is genetically controlled by the Y-linked sex determining gene *Sry* that is expressed in XY, but not in XX gonads, triggering testis differentiation in male fetuses (Koopman et al. 1991; Svingen and Koopman 2013). Subsequently, the testes differentiate into compartmentalized organs comprising testis cords and an interstitial space. Fetal Leydig cells differentiate within the interstitium and become the main site of androgen synthesis necessary for development of accessory male sex organs and general masculinization of the body (Svingen and Koopman 2013). The testes also produce the peptide hormone Insulin-like

factor 3 (INSL3) that is required for transabdominal testicular descent (Nef and Parada 1999), and anti-Müllerian hormone (AMH) which ensures regression of the Müllerian ducts; a paired structure that otherwise would develop into the female reproductive tract (Behringer 1994; Josso et al. 1993). In simple terms, the absence of these ‘male-centric’ factors allows for the female reproductive system to develop.

Testosterone drives masculinization

Simply put, testosterone regulates secondary sex differentiation. If testosterone is present, the body will develop male traits; if not present, the body will develop female traits (Fig. 1). However, the full picture is much more complex. First, many factors other than testosterone are involved, including AMH and INSL3 as mentioned above, but also others such as Hedgehog (Hh), Wingless-like (Wnt) and various growth factors. Second, androgen-dependent masculinization processes are likely influenced by actual hormone levels in target tissues, meaning that masculinization cannot be viewed as a simple ‘on–off’ switch, but rather a scenario, where more or less androgens can, to some degree, result in more or less masculine traits. From this viewpoint, it is more difficult to define when a morphometric change can be regarded as adverse or not. Nevertheless, as shown by Alfred Jost more than half a century ago (Jost 1947, 1953), androgens are the main drivers of male sex differentiation and are essential during a critical time window of development (MacLeod et al. 2010).

The fetal masculinization process involves numerous tissues and organs, including development of external genitalia and sex-specific differentiation of the perineum: the region between anus and genitalia (Fig. 1). In these tissues, the process seems largely governed by dihydro-testosterone (DHT) which is locally converted from testosterone by the enzyme 5 α -reductase. DHT acts by binding to and activating the Androgen receptor (AR), a nuclear hormone receptor responsible for regulating transcription of target genes (Fig. 2).

It has been suggested that the AGD is masculinized during development when AR activation stimulates the growth of the perineal muscles *levator ani* and *bulbocavernosus* (LABC) complex. In males, AR is expressed in non-myocytic cells of the LABC (Ipulan et al. 2014). Activation of AR in these cells prompts the growth of the LABC complex and the resulting size is thought to directly affect the AGD. Indeed, ablation of AR in rodents impairs development of the LABC complex and results in feminized male AGD (Ipulan et al. 2014; MacLean et al. 2008; Notini et al. 2005).

It is likely that more subtle changes in androgen levels, as for instance seen with fetal exposure to anti-androgenic compounds, can affect AGD by the same mechanism. This is supported by rodent toxicity studies, where a short AGD is

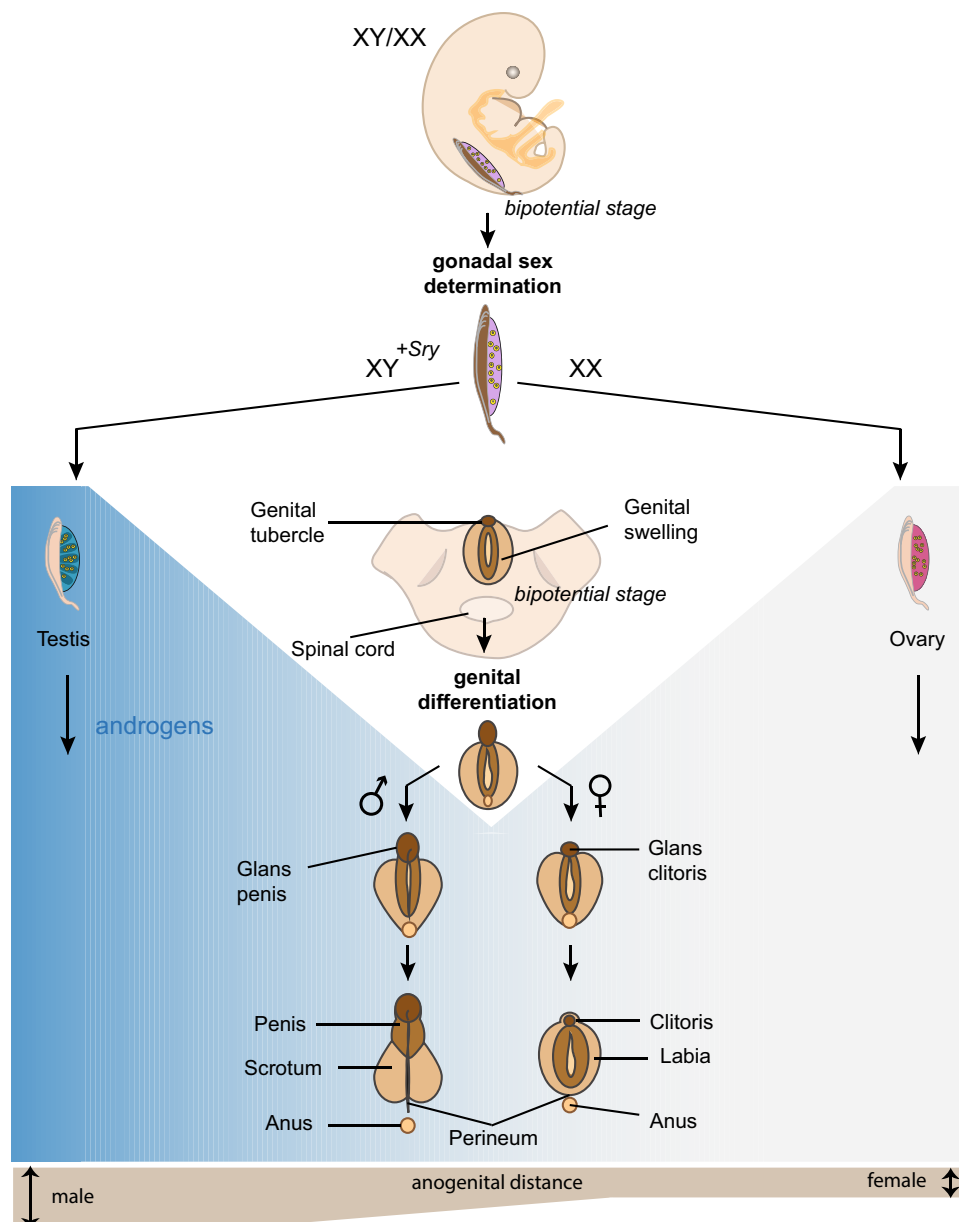


Fig. 1 Development of mammalian external genitalia. Initially, male (XY) and female (XX) fetuses are morphologically indistinguishable. During organogenesis, the bipotential gonadal ridges—located as paired structures running parallel on either side of the body midline—are instructed to differentiate into either testes or ovaries depending on the expression or not of the Y-chromosomal gene Sry in males and females, respectively. This takes place around gestational day 12 in rats and during gestational week 6 in humans. Following gonadal sex determination, the testes will quickly differentiate into compartmentalized structures comprising testis cords and an interstitial space. Steroidogenic Leydig cells appear in the interstitium and start synthesizing androgens—primarily testosterone—that are

secreted into the circulation, where they will prompt androgen-sensitive tissue to masculinize. The absence of Leydig cells and androgens in ovaries allows for female differentiation to occur. Locally, in the perineum, testosterone is converted to DHT, which in turn can activate the Androgen receptor and initiate a regulatory cascade that instructs the tissue to develop into male external genitalia, including differentiation of the LABC muscular complex, fusion of the genital swellings to form the scrotum, and growth of the genital tubercle into a penis. From this follows an elongation of the distance between genitalia and anus, referred to as the anogenital distance (AGD). On average, the male AGD is approximately twice that of female and it is a morphometric readout of fetal androgen exposure or activity

often associated with a reduced LABC weight (Christiansen et al. 2008). There are, however, indications that effects on AGD are more complicated than just androgens stimulating muscle growth and anti-androgens that depress this. First,

while AGD is thought to be relatively stable throughout life, the perineum is in fact responsive to postnatal changes in androgen levels (Kita et al. 2016; Mitchell et al. 2015). Second, certain EDCs have been found to induce both shorter

Table 1 Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to phthalates

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
<i>Increasing chain length (descending order)</i>					
DMP	750	n.e.	n.e.	n.e.	Gray et al. (2000)
DEP	750	n.e.	n.e.	n.e.	Gray et al. (2000)
DiBP	250	n.e.	5	x	Saillenfait et al. (2017)
	600	14	9	↑	Borch et al. (2006)
	625	22	x	n.e.	Saillenfait et al. (2008)
DBP	500	n.e.	x	x	Scott et al. (2007)
	500	9–14	12 ^(Martino-Andrade)	n.e. ^{Martino-Andrade}	Barlow et al. (2004), Howdeshell et al. (2007), Martino-Andrade et al. (2009) and Wolf et al. (1999)
	500	20–28	21 ^(de Mello Santos)	n.e. ^{Saillenfait}	Carruthers and Foster (2005), de Mello Santos et al. (2017), Mylchreest et al. (1999), Saillenfait et al. (2008), Scott et al. (2008) and Wolf et al. (1999)
	~640	11	10	x	Clewell et al. (2013)
	~650	43	26 ^(AGD/BW)	n.e.	Erma et al. (1998)
	~700	19 ^{Increases at other doses}	x	n.e.	Lee et al. (2004)
	750	x	9	x	Jiang et al. (2007)
	750	20–24	x	n.e. ^{Mylchreest}	Mylchreest et al. (1998) and van den Driesche et al. (2017)
	750	36	x	x	Van den Driesche et al. (2012)
	850	20	x	x	Jiang et al. (2011) and Liu et al. (2016)
	850	x	6	x	Jiang et al. (2015b)
	900	27	x	x	Li et al. (2015)
	1500	48	26 ^(AGD/BW)	n.e.	Erma et al. (2000)
MBuP	750	39	29	n.e.	Erma and Miyawaki (2001)

Table 1 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD	References
<i>Increasing chain length (descending order)</i>					
DEHP	30	x	n.e.	n.e.	Christiansen et al. (2009)
	150	n.e.	n.e.	n.e.	Martino-Andrade et al. (2009)
	300	x	5	n.e.	Nardelli et al. (2017)
	500	10	x	x	Howdeshell et al. (2007)
	500	18	18	x	Saillenfait et al. (2009b)
	750	17–18	17 ^(Kita)	x	Jarfelt et al. (2005), Kita et al. (2016) and Lin et al. (2009)
	750	30–34	x	n.e. ^{Gray}	Gray et al. (2000) and Wolf et al. (1999)
	900	14	x	x	Christiansen et al. (2010)
DHP	1000	30	11 ^(AGD/BW)	x	Li et al. (2013)
	1500	27	x	n.e.	Moore et al. (2001)
	500	20	23	x	Aydoğan Ahabab and Barlas (2015)
DCHP	~350	6	7	n.e.	Hoshino et al. (2005a)
	500	27	26	x	Aydoğan Ahabab and Barlas (2015)
BBP	750	17	13	n.e.	Saillenfait et al. (2009a)
	500	8–13	x	↑ ^{Nagao}	Hotchkiss et al. (2004) and Nagao et al. (2000)
	750	9	x	n.e.	Ty1 et al. (2004)
	750	30	x	n.e.	Gray et al. (2000)
	1000	38	29	n.e.	Ema and Miyawaki (2002)
	375	30	29	n.e.	Ema et al. (2003)
DnHP	500	18	18	x	Saillenfait et al. (2009b)
	750	35	31	↓	Saillenfait et al. (2009a)
DiHP	~500	15	x	n.e.	McKee et al. (2006)
DHPP	1000	11	10	n.e.	Saillenfait et al. (2011)
DnOP	1000	n.e.	n.e.	x	Saillenfait et al. (2011)
DOTP	750	n.e.	n.e.	n.e.	Gray et al. (2000)

Table 1 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD	References
DiNP	750	n.e.	n.e.	n.e. ^{Gray}	Clewell et al. (2013) and Gray et al. (2000)
	900	8	6	n.e.	Boberg et al. (2011)
	~1165	n.e.	x	n.e.	Masutomi et al. (2003)
DUDP	500	n.e.	4	n.e.	Saillenfait et al. (2013)
DTDP	1000	n.e.	n.e.	n.e.	Saillenfait et al. (2013)

AGD data after in utero exposure to various phthalates and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data were not available. A more complete compilation of data is found in Suppl. Table S1

x: not assessed; n.e. no effect, ↑ longer female AGD or AGDi, ↓ shorter female AGD or AGDi, DMP dimethyl phthalate, DEP diethyl phthalate, DBP dibutyl phthalate, MBuP monobutyl phthalate, DiBP di-isobutyl phthalate, DEHP diethylhexyl phthalate, DHP di-*n*-hexyl phthalate, DCHP dicyclohexyl phthalate, BBP benzyl butyl phthalate, MBeP monobenzyl phthalate, DiHP di-*n*-hexyl phthalate, DHPP di-*n*-heptyl phthalate, DiHP di-isoheptyl phthalate, DnOP di-*n*-octyl phthalate, DOTP dioctyl terephthalate, DiNP di-isononyl phthalate, DUDP diundecyl phthalate, DTDP dtridecyl phthalate

and longer female AGD or have multiple modes of action, as will be discussed later. Thus, more research is needed to fully understand how AGD is affected by fetal endocrine disruption.

Anogenital distance: a biomarker for fetal hormone action and late-life reproductive health

A large number of studies now support various aspects of the ‘testicular dysgenesis syndrome’ (TDS) hypothesis. However, it has also become increasingly clear that the relationship between hormone disruption and disease outcome is not always obvious. A simple reason for this is that male reproductive disorders can arise from various causes, not least genetic mutations or even genotypic predispositions. This can, in some instances, make it difficult to prove direct cause–effect relationships between chemical exposure and disease state in humans, especially if contributing genetics is present but not characterized. Another major complication is the considerable latency between exposure and disease manifestation, for instance, with regard to reduced sperm quality; a medical condition that can also be influenced by many other factors in the years between fetal life, birth, and adulthood. Hence, a single common biomarker that can predict a number of male reproductive disorders could prove valuable, both from a scientific and clinical point of view.

AGD measurements in human epidemiology and medicine

Fetal exposure to EDCs has been associated with a short AGD in new-born boys. Phthalates are the most frequently reported chemicals associated with a short AGD (Adibi et al. 2015; Bornehag et al. 2015; Bustamante-Montes et al. 2013; Marsee et al. 2006; Suzuki et al. 2012; Swan et al. 2005), but also other compounds including dioxins (Vafeiadi et al. 2013), bisphenol A (Mammadov et al. 2018; Miao et al. 2011) and mild analgesics (Fisher et al. 2016; Lind et al. 2017). Notably, several studies have not found significant correlations between exposure levels and short AGD in boys, including some phthalates (Jensen et al. 2016), dichlorodiphenyl-trichloroethane (DDT) (Bornman et al. 2016), triclosan (Lassen et al. 2016), and various pesticides (Dalsager et al. 2018). These discrepancies do not necessarily diminish the cause for concern, but rather highlight the challenges of obtaining evidence for causal relationships from human epidemiological studies.

With regard to reproductive disorders, many studies have reported significant correlations between short AGD in boys and for instance hypospadias (Cox et al. 2017; Gilboa et al. 2017; Hsieh et al. 2012; Singal et al. 2016),

Table 2 Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to compounds other than phthalates

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
<i>Drugs</i>					
Acetylsalicylic acid	400	38	x	n.e.	Gupta and Goldman (1986)
Aniline	93	x	20	x	Holm et al. (2015) (mouse study)
Paracetamol	150	9 ^a	10.5 ^a	x	Kristensen et al. (2011)
	150	x	15	x	Holm et al. (2015) (mouse study)
	350	8	9	x	van den Driesche et al. (2015)
	360	x	n.e.	n.e.	Axelstad et al. (2014)
Dexamethasone	0.1	10	x	x	Van den Driesche et al. (2012)
Finasteride	0.1	x	9	x	Christiansen et al. (2009)
	100	33	x	x	Bowman et al. (2003)
Flutamide	16–20	44 ^{Kita}	41–42	x	Hass et al. (2007); Kita et al. (2016)
	50	16–53	x	x	Foster and Harris (2005) and McIntyre et al. (2001)
	100	33–55	x	x	Mylchreest et al. (1999), Scott et al. (2007) and Welsh et al. (2007)
Ethinyl estradiol	(0.00–0.05)	n.e.	n.e.	(↑) ^{Mandrup}	Ferguson et al. (2011), Howdeshell et al. (2008) and Mandrup et al. (2013)
Ketoconazole	(50)	n.e.	x	x	Wolf et al. (1999)
	50	8	11	↓	Taxvig et al. (2008)
<i>Pesticides</i>					
Epoiconazole	3.75	5 ^a	5 ^a	↑	Hass et al. (2012)
	15	7 ^{(PND0)a}	10 ^{(GD21)a}	↑	Taxvig et al. (2007)
	50	n.e.	n.e.	n.e.	Taxvig et al. (2008)
Myclobutanil	145	12 ^{Increased}	x	x	Goetz et al. (2007)
Prochloraz	(0.01–35)	n.e.	x	(↑) ^{Melching, Hass}	Christiansen et al. (2009), Hass et al. (2012), Melching-Kollmuss et al. (2017) and Vinggaard et al. (2005)
	150	x	12	↑	Laier et al. (2006)
	250	6	x	↑	Noriega et al. (2005)
	50	n.e.	n.e.	n.e.	Taxvig et al. (2008)
Tebuconazole	~158	7 ^{Increased}	x	x	Goetz et al. (2007)
	12.5–50	n.e.	n.e.	(↑) ^{Hass}	Hass et al. (2012) and Taxvig et al. (2008)
Tebuconazole	100	n.e.	10 ^{Increased (only at GD21)}	↑	Taxvig et al. (2007)
	100	n.e.	n.e.	n.e.	Taxvig et al. (2008)
<i>Pesticides</i>					
Triadimefon	~114	3 ^{Increased}	x	x	Goetz et al. (2007)
Mancozeb	25	n.e.	n.e.	n.e.	Hass et al. (2012)
Vinclozolin	12	n.e.	n.e.	n.e.	Colbert et al. (2005)
	50–60	21 ^{Matsuura}	9–21	n.e. ^{Matsuura}	Christiansen et al. (2009) and Matsuura et al. (2005a)
	~100	28	22	x	Schneider et al. (2011)
	100	28	x	x	Ostby et al. (1999)
	160	x	35	x	Hass et al. (2007)
	200	46–56	x	(↓) ^{Gray}	Gray et al. (1994) and Wolf et al. (2004)
Procymidone	50	10	9	n.e.	Hass et al. (2012)
	100	24	n.e.	x	Wolf et al. (1999)
	150	x	37	x	Hass et al. (2007)

Table 2 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
Linuron	50	g ^{Not sig. in 2000}	x	n.e. ²⁰⁰²	McIntyre et al. (2002) and McIntyre et al. (2000)
	75–100	25–31	x	x	Hotchkiss et al. (2004) and Wolf et al. (1999)
<i>p,p'</i> -DDE	100	6–9	x	x	Wolf et al. (1999)
	50–200	x	11 (AGD/crown–rump length)	x	Loeffler and Peterson (1999)
Fenitrothion	25	16	x	n.e.	Turner et al. (2002)
Lindane	~16	n.e.	n.e.	(↓)	Matsuura et al. (2005b)
Methoxychlor	~82	n.e.	x	n.e.	Masutomi et al. (2003)
<i>UV filters</i>					
Benzophenone	(~130)	n.e.	n.e.	↓	Hoshino et al. (2005b)
HBM	(~3250)	x	n.e.	n.e.	Nakamura et al. (2015)
OMC	(1000)	n.e.	n.e.	n.e.	Axelstad et al. (2011)
<i>Preservative</i>					
Butylparaben	500	7	6	↓	Boberg et al. (2016)
	600	n.e.	n.e.	n.e.	Boberg et al. (2008)
	1000	16	x	x	Zhang et al. (2014)
<i>Plastic additive</i>					
Bisphenol A	0.25	7	x	↓	Christiansen et al. (2014)
	(0.0025–50)	n.e.	n.e. ^(Ferguson, Tinwell)	n.e. ^{Ferguson, Tinwell}	Ferguson et al. (2011), Howdeshell et al. (2008) and Tinwell et al. (2002)
	(5–385)	n.e.	x	n.e.	Takagi et al. (2004)
Nonylphenol	(~250)	n.e.	n.e.	↓	Takagi et al. (2004)
Genistein	~67	n.e.	x	n.e.	Masutomi et al. (2003)
<i>Other</i>					
TCDD	0.1	6–12 ^{Not sig. when BW or CR length taken into account}	x	x	Bjerke and Peterson (1994) and Gray et al. (1995)

AGD data after in utero exposure to various substances and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data were not available. A more complete compilation of data is found in Suppl. Table S1

x not assessed, n.e. no effect, ↑ longer female AGD or AGDi, ↓ shorter female AGD or AGDi, DDE DDT metabolite, dichloro-diphenyl-dichloro-ethylene, TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, HBM 2-hydroxy-4-methoxybenzene, OMC octyl methoxycinnamate

^aNon-monotonic (low-dose) effect

cryptorchidism (Jain and Singal 2013; Jiang et al. 2015a), penile length (Alaee et al. 2014; Thankamony et al. 2014), and sperm quality (Eisenberg et al. 2012; Mendiola et al. 2011). Together, these observations fit the model of a common ‘fetal origin of disease’, with androgen disruption at the root of the problem. However, they cannot provide definite proof for cause–effect relationships. There is also the complications of accounting for genetic variations of the fetus or maternal characteristics such as parity, factors that themselves can influence the AGD (Barrett et al. 2014; Eisenberg et al. 2013). This ultimately means that two individuals being exposed to the same chemicals could respond differently and display variable degree of changes to the AGD. Because of all these complexities with interpreting human data, the rodent models can be used to provide more robust causative evidence.

AGD measurements in rodent studies

In rats, a short AGD in male offspring after fetal exposure to anti-androgenic compounds often correlates with various reproductive disorders (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). There is evidence to suggest that the more pronounced the effect on the AGD, the more likely additional reproductive defects such as genital malformations are found (Christiansen et al. 2008). However, there is not always a clear correlation between the severity of AGD effects and severity or frequency of other reproductive malformations, such that AGD cannot always stand on its own in the prediction of perceived anti-androgenic effects.

High-exposure studies have reported on male pups with ‘female-like’ AGD, where male AGD in exposed offspring is

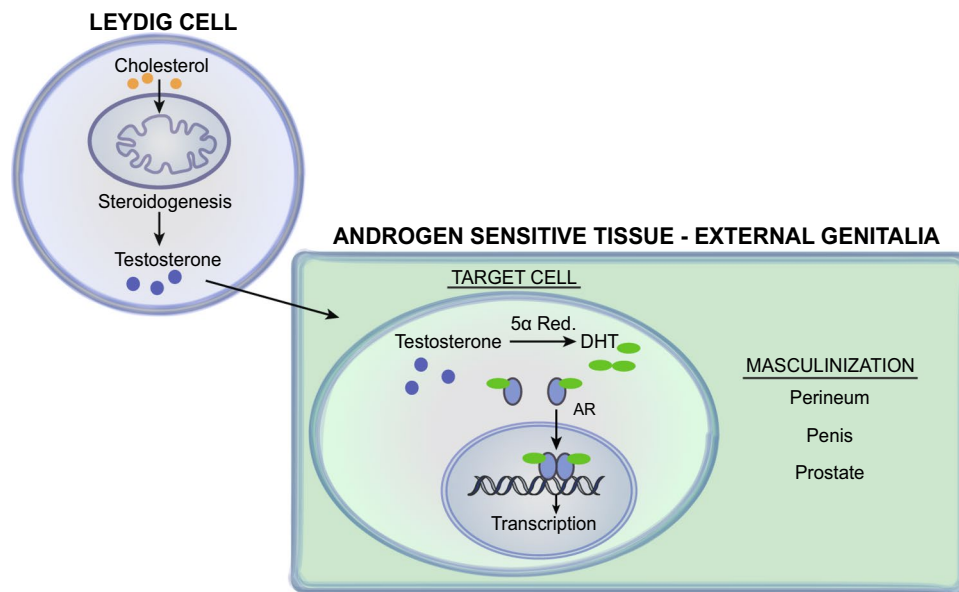


Fig. 2 Androgen signaling cascade required for fetal masculinization. Testosterone is synthesized in the testicular Leydig cells from precursor cholesterol. Testosterone is then secreted into the body circulation, where it acts on different androgen-sensitive tissues and organs. Differentiation of the internal male reproductive accessory organs (e.g., epididymis) is stimulated by testosterone, whereas external gen-

italia and perineum requires the local conversion of testosterone into DHT by the enzyme 5 α -Reductase. DHT can bind to the Androgen receptor (AR) which subsequently locates to the nucleus and regulate expression of target genes. Cell-dependent regulatory signaling pathways are activated and regulate the differentiation into specialized tissues and organs (e.g., LABC complex and external genitalia)

close to 50% that of control males. This does not only make the sex of the offspring difficult to determine (Christiansen et al. 2010; Hass et al. 2007; Ostby et al. 1999; Parks et al. 2000), but also result in additional phenotypes including nipple retention, genital malformations, and reduced reproductive organ weights (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). These reproductive phenotypes are all, at least to some degree, under androgen control during fetal development, making AGD a potentially robust marker for anti-androgenic effects.

AGD in animal toxicity studies: phthalates

The group of compounds most frequently reported to affect male AGD is the phthalate esters. As summarized in Table 1, many different phthalates have been tested in rats, with dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) being the most prevalent. From an early rat study on DEHP showing testicular toxicity (Gray et al. 1977) and structure–activity relationships suggesting linear side-chain esters of 4–6 carbons to be of specific concern (Foster et al. 1980), numerous toxicity studies on phthalates followed, providing increasing evidence for what later has been termed the ‘phthalate syndrome’ [(Foster 2006) and Table 1].

Fetal exposure to certain phthalates (chain length C4–C6) results in a short AGD in rat male offspring, without any significant effect on female AGD (Table 1). It is, for the most

part, a dose-dependent effect, where increasing dose levels result in progressively shorter AGD. Notably, the magnitude of AGD effects can differ greatly between studies, probably influenced by parameters such as rat strain, group size, or method used for AGD measurements. Body weights may also influence AGD measurements, but are unfortunately not always accounted for. In developmental toxicity studies, the offspring’s body weight is in fact frequently affected, particularly at higher exposure levels (Gallavan et al. 1999). Since the AGD correlates with the size of the fetus or newborn pup, body weight should ideally be accounted for by calculating the AGDi, or by including body weight as a covariate in the statistical analysis. Unfortunately, this is not always done, as indicated in Tables 1 and 2. This means that a significantly short AGD in many instances may not be *bona fide* feminization effect, but rather a readout of stunted growth. To remedy this problem for risk-assessment purposes, OECD test guidelines and guidance documents stipulate that bodyweight measurements must be included alongside AGD measurements (guidance documents 151 and 43), as discussed in “AGD measurements in regulatory toxicology”.

From the studies listed in Table 1, the most pronounced effect on AGD was seen for DBP at a dose of 1500 mg/kg/day. At this high perinatal exposure level, the average male AGD was 48% shorter than normal, but when accounting for pup body weight, the adjusted value (AGDi) was

26% shorter than normal (Ema et al. 2000). With this in mind, most of the remaining studies investigating the anti-androgenic phthalates have shown maximum shorter male AGD of between 15 and 30% at exposure doses between 100 and 500 mg/kg/day (Table 1). Thus, the active phthalates typically show marked effects on AGD, but rarely complete feminization (i.e., not comparable to female AGD), in a dose-dependent manner. These are high doses that also cause other reproductive disorders and in some cases show signs of systemic toxicity, reduced body weight, or increased liver weights. At lower exposure levels, these same phthalates typically cause less severe, yet statistically significant shorter male AGD, whereas other adverse effects on reproductive organs or systemic toxicity are less prevalent. Hence, a short AGD is often the most prevalent adverse effect observed at the lowest dose, supporting the use of AGD as a sensitive biomarker for these compounds.

Rat Leydig cells appear more sensitive to phthalate disruption than mouse and human Leydig cells (Svechnikov et al. 2016). For instance, the phthalate esters DEHP, MEHP, and DBP reportedly elevate testosterone levels in mice at early developmental stages, but suppress at late gestation, whereas no effects are observed in human fetal testis explants, with or without luteinizing hormone (LH) stimulation. Yet, in cultured mouse Leydig cells, MEHP can both inhibit and stimulate steroidogenesis depending on cell line (Svechnikov et al. 2016). These observations indicate that at least in some instances, disruption to testosterone output is not simply caused by direct disruption of steroidogenesis, or Leydig cells, but likely also includes endocrine signaling networks such as pituitary hormones. The implication of these discrepancies for using AGD as a biomarker for anti-androgenic effects remains uncertain. The best argument would be that since the rat seems to be the most sensitive model, it has the highest potential for safeguarding humans against detrimental effects in the wake of intrauterine exposures. However, more detailed knowledge about molecular events that are both similar and different between species is needed to devise the best test strategies for the future. The human relevance of rodent AGD data could then potentially be confirmed by testing chemicals in human-based cell and tissue systems.

AGD in animal toxicity studies: miscellaneous compounds

As summarized in Table 2, various substances from diverse chemical classes can affect the AGD in rat offspring. These include compounds with a clear anti-androgenic mode of action that cause even more severe effects on AGD than the potent anti-androgenic phthalates.

Prenatal exposure to high doses of certain AR antagonists completely feminizes the male pup AGD to 50% that

of control males. This is the case for the pesticide procymidone, vinclozolin, and the non-steroidal prostate cancer drug flutamide (Christiansen et al. 2008; Hass et al. 2007; Ostby et al. 1999; Parks et al. 2000). After exposure to these compounds, the male offspring also displays an increased rate of nipple retention, high incidence of genital malformations, and severely reduced male reproductive organ weights (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). As shown in Table 2, compounds such as the pesticide linuron, or the drugs finasteride and acetylsalicylic acid have also been shown to induce discernibly shorter male AGD (maximum response in the range of 31–38%). Yet, for the majority of the tested chemicals, the maximum effect on AGD in males ranges from 5 to 15% shorter than normal. Of note, for several of the listed compounds the relationship between chemical exposure and AGD does not always follow a continuous pattern, where reduction in the AGD index is proportional to exposure levels, or concomitant reproductive abnormalities.

Fetal exposure to both the antimicrobial preservative butyl paraben (Boberg et al. 2016; Zhang et al. 2014) and the industrial plasticizer bisphenol A (Christiansen et al. 2014) has been shown to shorten the male AGD around 7–16% in the male offspring, albeit there are studies reporting no effects on AGD for both butyl paraben (Boberg et al. 2008) and bisphenol A (Ferguson et al. 2011; Howdeshell et al. 2008; Takagi et al. 2004; Tinwell et al. 2002). In the cases where short AGD was observed with these compounds, clear effects on nipple retention or genital malformations were rarely seen. On the other hand, decreased sperm count or affected prostate development were observed, as were disrupted mammary glands in female offspring (Boberg et al. 2016; Hass et al. 2016; Mandrup et al. 2016). The intuitively appealing explanation for these discrepancies in phenotypic manifestations is that the latter compounds are considered mainly estrogenic. However, they also display weak anti-androgenic potential *in vitro* (Chen et al. 2007; Reif et al. 2010; Rosenmai et al. 2014; Satoh et al. 2005) and show effect in several *in vitro* toxicity assays for other mechanisms of action according to Toxicity Forecast [ToxCast; a program developed by the US Environmental Protection Agency to predict hazards and prioritize toxicity testing of environmental chemicals (Dix et al. 2007)]. This leaves the question of whether this small effect on AGD is the result of a weak anti-androgenic or an estrogenic effect, or yet another mechanism of action.

Fetal exposure to the estrogenic compound ethinyl estradiol does not seem to affect AGD in male offspring (Ferguson et al. 2012; Howdeshell et al. 2008; Mandrup et al. 2013), which then argue against the observed effect on male AGD following exposure to butyl paraben or BPA being estrogenic. It appears more likely that there are some weaker anti-androgenic effects exerted by these compounds

that do not increase significantly in efficacy at increasing doses. Another explanation could be lent from observations that estrogenic compounds can reduce the ability of Leydig cells to synthesize testosterone, as reviewed elsewhere (Svechnikov et al. 2010). A recent study where mice were exposed to diethylstilbestrol (DES) also resulted in a short AGD in male offspring, again hypothesized to be caused by reduced testosterone production (Stewart et al. 2018). This is in agreement with what has been observed in rats following exposure to high doses of estrogens, where AR expression is lost in all tissues that show ‘anti-androgenic’ effects, as well as a reduction in Leydig cell numbers (Williams et al. 2001). Nevertheless, more mechanistic insight is required to fully explain how estrogenic compounds give rise to seemingly anti-androgenic effects.

Another group of chemicals that can elicit effects on reproductive development, including AGD, is the azole fungicides. They are used by both the medical and agricultural industries for their anti-fungal properties. They can, however, provoke side effects in humans and are known to primarily interfere with CYP-family enzymes (Ashley et al. 2006), but also nuclear receptors (Dreisig et al. 2013). As shown in Table 2, there are six azoles that in some studies have been shown to affect the AGD in rats: the drug ketoconazole, and the pesticides epoxiconazole, myclobutanil, prochloraz, propiconazole, and tebuconazole. At tested doses, they do not cause large changes to AGD, but strikingly, many of them cause longer rather than shorter AGD in male offspring. This is not the case for ketoconazole, however, where fetal exposure to 50 mg/kg resulted in around 8–11% shorter male AGD (Taxvig et al. 2008). Notably, a contradictory study has reported no effect on AGD after exposure to ketoconazole at a similarly high dose (Wolf et al. 1999).

Prochloraz is an imidazole fungicide that can cause various adverse effects in rat fetuses at high doses. Regarding reproductive effects, prochloraz exposure can induce nipple retention in male offspring (Christiansen et al. 2009; Vinggaard et al. 2005), whereas effects on AGD are conflicting between studies. If taking birth-weight into account, there were no significant effects on male AGD at doses similar to those causing nipple retention (25–150 mg/kg) (Christiansen et al. 2009; Melching-Kollmuss et al. 2017; Noriega et al. 2005; Vinggaard et al. 2005). Another study reported around 10% shorter male AGD after fetal exposure to high doses of prochloraz concomitant with nipple retention at the same doses (Laier et al. 2006). Screening studies have shown that prochloraz can provoke multiple mechanisms of action in vitro, as it antagonizes the androgen and the estrogen receptor, agonizes the Ah receptor, and inhibits aromatase activity (Vinggaard et al. 2006). Whether or not all these mechanism are activated in vivo, and what effects this

would have on the developing fetus, remains to be properly clarified.

Exposure to myclobutanil, propiconazole, and tebuconazole can all seemingly induce longer AGD in male offspring, whereas with epoxiconazole, the picture is less clear with only weak indications that it may affect AGD (Goetz et al. 2007; Hass et al. 2012; Taxvig et al. 2007). By what mechanisms this occur remains unclear. Taken together, however, the azole fungicides seem to elicit different effects on the developing fetus, resulting in effect outcomes not readily explained due to our limited knowledge about mechanisms and modalities, which should be a focus area for future studies.

Note on mild analgesics and their endocrine disrupting properties

Non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol/acetaminophen represent a group of mild analgesics suggested to have endocrine disrupting properties. As recently reviewed (Kristensen et al. 2016), their use across the world has risen dramatically in recent years. Herein, we will not discuss in great detail the potential endocrine effects from the analgesics, but highlight studies where effects on AGD have been reported.

Fetal exposure to therapeutically relevant doses of paracetamol can result in 5–10% reduction in male rat AGDi (Kristensen et al. 2011). Another study using a similar high dose and exposure regimen failed to show a statistically significant change to male AGDi, but here, significant effects on nipple retention and reduction in LABC muscle complex weight were observed (Axelstad et al. 2014). Effects on AGD have also been observed in mice after in utero exposure to mild analgesics, and already in 1986, it was shown that male fetuses exposed to aspirin or indomethacin could present with shorter than normal AGD (Gupta and Goldman 1986). More recently, exposure to both aniline (which is metabolized to paracetamol in vivo) and paracetamol during pregnancy resulted in short AGD in male offspring (Holm et al. 2015). Although these studies strongly suggest that the mild analgesics act as anti-androgenic agents in the male fetuses, there are still not enough mechanistic studies available that convincingly support this conclusion.

Is AGD a useful biomarker for female reproduction?

In females, a longer AGD is considered a masculinization effect, something that would result from the presence of excess androgen levels or ectopic activation of AR. For example, longer than normal female AGD is associated with elevated testosterone levels (Mira-Escolano et al. 2014), exemplified by daughters born of women with polycystic ovarian syndrome (Barrett et al. 2018; Wu et al. 2017).

These clinical observations are further supported by rat studies (Hotchkiss et al. 2007; Ostby and Gray 2004; Ramezani Tehrani et al. 2014; Wolf et al. 2002), and are in line with what we know about how androgens masculinize fetal tissues. However, other mechanisms have also been proposed to underpin masculinization of female fetuses, for instance, ectopic activation of the progesterone receptor (PR).

The fungicide vinclozolin has been suggested to masculinize mouse female fetuses by activation of the PR, further supported by the fact that the synthetic progesterone medroxyprogesterone acetate also masculinizes the female offspring (Buckley et al. 2006). Notably, fetal exposure in rats had no significant effect on female AGD (Hass et al. 2007). Nevertheless, although synthetic progesterones can activate the PR, many synthetic progestins such as medroxyprogesterone also targets other nuclear receptors and can give rise to many off-target effects (Kuhl 2005). For instance, progestins were widely used to prevent miscarriage in the 1950s and 60s, but proved to masculinize the external genitalia of the female offspring (Money and Mathews 1982), and initially referred to as progestin-induced hermaphroditism (Wilkins et al. 1958). Even if these effects seem to be driven by PR-mediated signaling, it cannot be excluded that androgenic modes of action are involved. Not only are several progestins, including medroxyprogesterone, androgenic (Kuhl 2005), but androgen levels can seemingly also be elevated by high levels of progesterone, ultimately giving rise to androgenic effects (Auchus and Chang 2010).

Female rats exposed to the azole fungicide prochloraz present with longer AGD at birth, concomitant with elevated progesterone levels (Laier et al. 2006; Melching-Kollmuss et al. 2017). Prochloraz can induce progesterone synthesis *in vitro* by CYP17 inhibition at concentrations above 10 nM (Dreisig et al. 2013), a mechanism confirmed *in vivo*, where fetal plasma concentrations were measured at 24 nM following maternal exposure to 150 mg/kg bw/day of prochloraz (Laier et al. 2006). Although this does not prove that progesterone was acting via the PR to induce masculinization, androgen levels were not elevated in the same animals and hence suggest that this is an alternative mechanism, since azoles themselves do not seem to activate the AR. A definitive answer remains elusive, however, and much work remains to be done on azoles to characterize their sometimes perplexing modes of action.

Estrogens can seemingly also cause longer AGD in females. Although fetal exposure to ethinyl estradiol does not affect male AGD in rats, female offspring can present with longer AGD following exposure to supra-physiologically doses (Casanova et al. 1999; Delclos et al. 2009; Mandrup et al. 2013; NTP 2010; Ryan et al. 2010; Sawaki et al. 2003). Immediately, this effect seems counter-intuitive; however, very high doses of steroidal estrogens can agonize the AR (Vinggaard et al. 1999), which suggests that the effects are driven by androgen action rather than being estrogenic.

There are also examples, where female AGD is shorter than normal following *in utero* exposure, observations that evoke more questions than answers. The types of compounds capable of shortening female AGD in rodent are also variable and include bisphenol A (Christiansen et al. 2014), butyl paraben (Boberg et al. 2016), ketoconazole (Taxvig et al. 2008), paracetamol (Holm et al. 2016), as well as di-*n*-hexyl phthalate and dicyclohexyl phthalate (Aydoğan Ahabab and Barlas 2015). These studies are not covered in detail herein, and thus not presented with proper weight of evidence as yet any plausible mechanisms- or modes of action are lacking. It would, however, be of great interest to design studies specifically to answer such questions, as they may reveal insights of value to perineal development more broadly.

AGD measurements in regulatory toxicology

AGD in chemical risk assessment

Within a regulatory context, AGD measurements are mandatory to perform at either gestational days 20–21 or postnatal days 0–4 in several OECD test guidelines used to test for developmental and reproductive toxicity in chemical risk assessment. These guidelines include the extended one generation study (TG 443), the two reproductive toxicity screening studies (TG 421/422) and the newly updated TG 414 Developmental toxicity study (OECD 2012, 2016a, b, 2018). The OECD guidance documents (OECD GD 43 and GD 151), which guides the interpretation of these guidelines, states that “A statistically significant change in AGD that cannot be explained by the size of the animal indicates effects of the exposure and should be considered in setting the NOAEL (No Observed Adverse Effect Level)” (OECD 2008, 2013). This means that, when a statistically significant shorter AGD in male rat offspring is considered the critical effect, a NOAEL can be based on this information and used as the point of departure for setting safe exposure levels for humans. Since AGD measurements will be included as an endpoint when performing almost all future regulatory studies investigating developmental and reproductive toxicity, it will improve on the sensitivity for identifying endocrine disruptors and developmental toxicants in mammals. In addition, this inclusion will generate much data, which will contribute to more thorough evaluations of substances and information pertaining to their modes of action.

Other morphological biomarkers to support AGD

Nipples and mammary glands originate from bipotential structures that develop differentially between the sexes in response to specific molecular cues. In humans, both sexes are born with a pair of nipples and breast development in

girls is only initiated during puberty. In rats, the situation is somewhat different, as only the females retain their nipples; postnatal males only possess rudimentary structures (Kratochwil 1971).

In the developing male rodent, the presence of DHT causes regression, or apoptosis of the nipple anlagen (Imperato-McGinley et al. 1986). This process is blocked by fetal exposure to anti-androgens, and these male offspring subsequently display nipples similarly to their female littermates. Therefore, nipple retention in male pups is used alongside AGD as a morphometric marker of impaired androgen action. Although this morphological phenomenon does not occur in humans, it can, nevertheless, be used to predict anti-androgenic effects of chemicals. In other words, the fact that nipples are retained in exposed male rat offspring can be used to predict reproductive disorders caused by chemical exposures, even though nipples do not regress in human males. Whether AGD or nipple retention possess similar sensitivity, as was recently shown for an 18-chemical mixture of anti-androgens (Conley et al. 2018), or if one of the endpoints is more sensitive than the other, seems to depend on what chemical is being tested. Therefore, inclusion of both AGD and nipple retention in reproductive toxicity studies, in a weight-of-evidence manner, can significantly improve on the assessment of potential EDCs (OECD 2015). Nipple retention is also mandatory to assess in three separate OECD test guidelines (TG 443, TG 421/422).

Conclusions and perspectives

AGD has emerged a useful biomarker to detect fetal androgen insufficiency and is now applied in regulatory testing strategies for detecting endocrine disrupting effects. Its utility in a clinical setting is less defined, but it has received more traction in the last few years. It is our conviction that it will remain a standard retrospective biomarker in rodent toxicity studies and risk assessment. In humans, it will increasingly serve as a prospective biomarker, where a shorter than normal AGD in male offspring will be a warning flag for future reproductive complications, not least fertility issues. For this to fully eventuate, however, much more research focusing on answering many knowledge gaps is required. To this end, we would emphasize four areas that we believe should be focused on in the near future.

First, more efforts should be channelled into characterizing the morphoregulatory pathways of perineal development. Although the current dogma stipulates that the length of the AGD is controlled by the level of androgen action during the ‘masculinization programming window’, other regulatory pathways also seem to play a role, at the very least as effect-outcome modifiers.

Second, there is a need to better define the relationship between anti-androgenic effects and the length of the male AGD, both in rodents and humans. Should we rely purely on ‘statistically significant’ differences, or should a minimum percentage shorter than control mean be defined? And more importantly, can the magnitude of shortening be used to define magnitude of lost androgen action or future risk of contracting reproductive disease?

Third, to what extent can AGD measurements be used as a stand-alone biomarker to detect anti-androgenic effects in toxicity studies, and where do we need to supplement with additional effect measures? In a weight-of-evidence approach—which is currently recommended—what other measurements should be included, and when? For these evaluations, there should also be stronger emphasis on effect doses and to what extent supra-high doses reliably recapitulates what occurs at more human relevant doses. This latter point seems more important in view of chemicals that do not result in clear monotonic dose–response relationships.

Fourth, can AGD measurements be used as a biomarker in females? Certain perturbations can affect female AGD in either direction, but what does a long or short AGD really measure in female offspring? And is it linked to adverse health effects later in life? Concerning a longer than normal AGD, it would most often, if not always, be a sign of masculinization effects comparable to those observed when female fetuses receives too much androgens. With a shorter AGD, however, the jury is still out.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest pertaining to this work.

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