International Journal of Andrology / Volume 33, Issue 2 / p. 279-287

Free Access

Androgen action in the masculinization programming window and development of male reproductive organs

D. J. MacLeod, R. M. Sharpe, M. Welsh, M. Fisken, H. M. Scott, G. R. Hutchison, A. J. Drake, S. Van Den Driesche

First published: 14 March 2010
https://doi.org/10.1111/j.1365-2605.2009.01005.x
Citations: 182

Richard M. Sharpe, MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK. E-mail: r.sharpe@hrsu.mrc.ac.uk

Summary

We have shown previously that deficient androgen action within a masculinization programming window (MPW; e15.5–e18.5 in rats) is important in the origin of male reproductive disorders and in programming male reproductive organ size, but that androgen action postnatally may be important to achieve this size. To further investigate importance of the MPW, we used two rat models, in which foetal androgen production or action was impaired during the MPW by exposing in utero to either di(*n*-butyl) phthalate (DBP) or to flutamide. Reduced anogenital distance (AGD) was used as a monitor of androgen production/action during the MPW. Offspring were evaluated in early puberty (Pnd25) to establish if reproductive organ size was altered. The testes, penis, ventral prostate (VP) and seminal vesicles (SV) were weighed and penis length measured. Both DBP and flutamide exposure in the MPW significantly reduced penis, VP and SV size along with AGD at Pnd25; AGD and organ size were highly correlated. In DBP-, but not flutamide-, exposed animals, testis weight was also reduced and correlated with AGD. Intratesticular testosterone was also measured in control and DBP-exposed males during (e17.5) or after (e21.5) the MPW and related to AGD at e21.5. To evaluate the importance of postnatal androgen action in reproductive organ growth, the effect of combinations of prenatal and postnatal maternal treatments on AGD and penis size at Pnd25 was evaluated. In prenatally DBP-exposed animals, further postnatal exposure to either DBP or flutamide significantly

reduced AGD and penis size in comparison with prenatal DBP exposure alone. In comparison, rats exposed postnatally to testosterone propionate after prenatal vehicle-exposure showed considerable increase in these parameters vs. controls. In conclusion, we show that the size of all male reproductive organs is programmed by androgen exposure in the MPW, but that growth towards this size is dependent on androgen action postnatally.

Introduction

Male reproductive health disorders such as cryptorchidism, hypospadias, testicular germ cell cancer or a low sperm count are believed to comprise a testicular dysgenesis syndrome (TDS; Skakkebæk *et al.*, 2001, 2007). It is hypothesized that abnormal testis development leads secondarily to hormonal or other malfunctions of the Leydig and/or Sertoli cells during testis development and masculinization, leading in turn to increased risk of the aforementioned disorders (Skakkebæk *et al.*, 2001, 2007). Human TDS disorders are common and may be associated with a decline in male fertility (Frederiksen *et al.*, 2007; Skakkebæk *et al.*, 2001, 2007). The rapid and geographically restricted increase in prevalence of some TDS disorders suggests that environmental and/or lifestyle factors e.g. exposure to environmental chemicals; may play a key role in the pathogenesis of TDS disorders (Sharpe & Skakkebæk, 2008; Skakkebæk *et al.*, 2001).

Support for the TDS concept has mainly come from two sets of studies using animal models. The first involves in utero exposure of rats to certain phthalate esters, such as di(*n*-butyl) phthalate (DBP), which induces a TDS-like spectrum of disorders in the male offspring (Fisher *et al.*, 2003; Foster, 2006; Mylchreest *et al.*, 2000). However, whilst DBP treatment induces a marked reduction in the production of the Leydig cell hormones testosterone and insulin-like factor 3 by the foetal testis, this effect is not uniform throughout gestation (Schultz *et al.*, 2001; Scott *et al.*, 2008) and is associated with only sporadic cases of hypospadias (Fisher *et al.*, 2003; Mahood *et al.*, 2007). An explanation for this 'discrepancy' comes from the second line of animal studies involving in utero exposure to the anti-androgen flutamide (Welsh *et al.*, 2008). This has shown the existence of a foetal 'masculinization programming window' (MPW; e15.5–e18.5 in rats) within which androgens must act to ensure correct later development of the male reproductive tract (Fig. 1; Welsh *et al.*, 2008). A corresponding MPW is thought also to occur in humans at ~8–14 weeks' gestation (Welsh *et al.*, 2008) and in non-human primates (Herman *et al.*, 2000; Prahalada *et al.*, 1997).

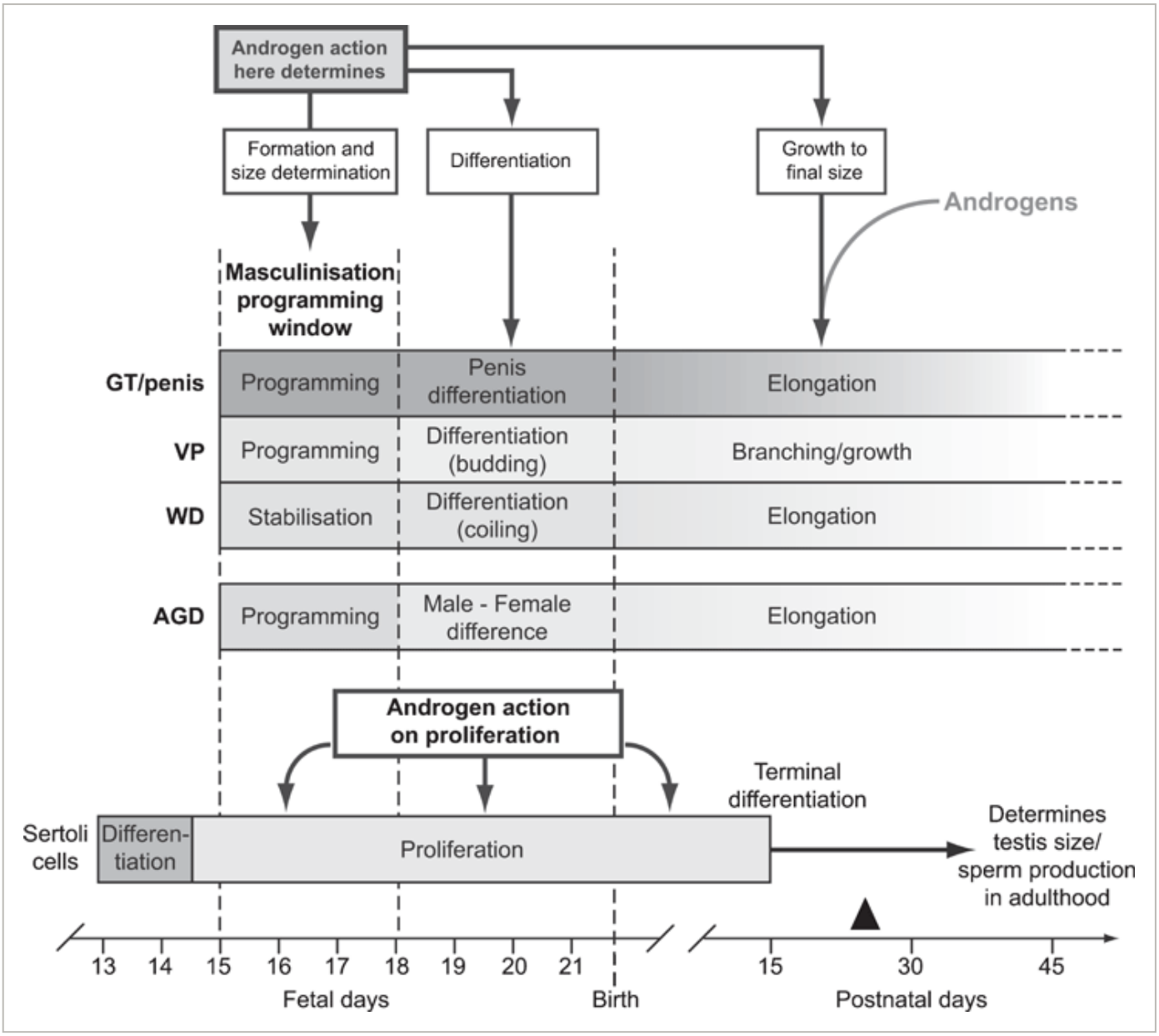

Figure 1

Open in figure viewer | PowerPoint

Schematic diagram summarizing our current hypothesis regarding the time-dependent roles that androgens play in determining formation, differentiation and growth of the component parts of the male reproductive tract. The masculinization programming window (MPW) in rats occurs between e15.5 and e18.5. Note that although androgen action in the MPW specifies the size to which the reproductive organs will grow, androgen action postnatally is still required to achieve this growth, as indicated in the upper right of the diagram. The dark triangle on the timing axis indicates Pnd25, when most of the sampling in the current studies was undertaken. GT, genital tubercle; VP, ventral prostate; WD, Wolffian duct; AGD, anogenital distance.

Only deficient androgen action within the MPW leads to cryptorchidism and hypospadias, which are both TDS disorders (Clark *et al.*, 1993; Spencer *et al.*, 1991; Welsh *et al.*, 2008). Moreover, the study by Welsh *et al.* (2008) showed that in rats anogenital distance (AGD), which is normally ~1.7 times longer in adult males than in females, is also determined by androgen action in the MPW and thus provides a lifelong 'read-out' of androgen action during the MPW. We have also shown in rats that testis size in adulthood, which reflects the capacity to produce sperm, is positively correlated to AGD in rats, suggesting that androgen action within the MPW may also be a determinant of sperm production/count in adulthood (Scott *et al.*, 2008). In humans, the penis undergoes androgen-dependent growth in late gestation, postnatally up to 4 years of age and at puberty (Boas *et al.*, 2006; Camurdan *et al.*, 2007; Husmann, 2002). In the rat, penis formation and its capacity to grow is determined foetally by androgen action during the MPW; based on studies using flutamide in rats, a similar scenario applies to the ventral prostate (VP) and to Wolffian duct-derived tissues such as the seminal vesicles (SV; Fig. 1; Welsh *et al.*, 2008, 2009).

These new developments in understanding provide additional support for the TDS hypothesis. However, the studies describing the inter-relationships between the MPW, AGD and reproductive development are largely based on studies using flutamide, which disrupts androgen action at the target organ rather than by causing testicular dysgenesis/malfunction, as suggested in the TDS hypothesis. The present studies therefore aimed to provide a more direct test of the TDS hypothesis by using DBP, which is known to disrupt foetal testis development (Foster, 2006), and to compare the effects observed with flutamide-exposed animals and to relate observed effects to the MPW as reflected by AGD. Furthermore, the effects of combined prenatal and postnatal treatments with DBP and/or flutamide on penis length and AGD were investigated to establish if the postnatal growth of these structures could be affected, as our current thinking predicted (Fig. 1). This study provides a detailed comparison of the effects of foetal exposure to flutamide or DBP on AGD and on the testis, penis, VP and SV.

Materials and methods

Animals and treatments

Wistar rats were maintained in our animal facility under standard conditions according to UK Home Office guidelines. Animals had free access to water and a soy-free breeding diet (SDS; Dundee, Scotland). Time-matings were established and presence of a vaginal plug was defined as embryonic day 0.5 (e0.5); for the present studies at least 3 litters were used per treatment group. All treatments are summarized in Table 1.

Table 1. Summary of the animal treatment periods and the age of sampling for the current studies

Treatment period	Prenatal window		Postnatal window		Termination
		e18.5			
	Flut (2 mg/kg)	e15.5–e18.5	–	–	Pnd25
Prenatal DBP	Corn oil	e13.5–e16.5	–	–	e17.5
	DBP (500 mg/kg)	e13.5–e16.5	–	–	e17.5
	Corn oil	e13.5–e20.5	–	–	e21.5
	DBP (500 mg/kg)	e13.5–e20.5	–	–	e21.5
	Corn oil	e13.5–e21.5	–	–	Pnd25
	DBP (100 mg/kg)	e13.5–e21.5	–	–	Pnd25
	DBP (500 mg/kg)	e13.5–e21.5	–	–	Pnd25
Combined prenatal and postnatal treatments	Oil	e13.5–e21.5	Oil	Pnd1–15	Pnd25

DBP, di(*n*-butyl) phthalate; TP, testosterone propionate; Flut, flutamide.

Prenatal flutamide exposure

Pregnant dams were dosed daily from e15.5 to e18.5 by oral gavage with either 0, 2, 5, 10 or 100 mg/kg flutamide (Sigma-Aldrich, Poole, UK) in 1 mL/kg corn oil/2.5% DMSO (Sigma-Aldrich); this treatment time window encompasses the MPW (Welsh *et al.*, 2008).

Prenatal DBP exposure

Pregnant dams were dosed daily with either 100 mg/kg DBP or 500 mg/kg DBP (Sigma-Aldrich) by oral gavage in 1 mL/kg corn oil. Depending on the age of termination, DBP was administered from e13.5 to e16.5 (termination e17.5), e13.5–e20.5 (termination e21.5) or e13.5–e21.5 (termination Pnd25).

Combined prenatal and postnatal treatments

To determine the effect of combined treatments, the following prenatal/postnatal treatment combinations were administered: oil/oil; oil/DBP; oil/flutamide; oil/testosterone propionate (TP; Sigma-Aldrich); DBP/oil; DBP/DBP; DBP/flutamide; flutamide/no treatment. For prenatal treatments, dams were treated daily with corn oil alone or DBP (500 mg/kg) from e13.5 to e21.5, whereas flutamide (100 mg/kg) was administered from e15.5 to e21.5 to coincide with the start of testosterone production (Habert & Picon, 1984). Postnatal treatment of dams with oil, DBP (500 mg/kg) or flutamide (100 mg/kg) was by daily oral gavage until Pnd15. For postnatal TP treatment, pups were injected subcutaneously with TP (20 mg/kg) in corn oil every third day from Pnd1 to 15.

Tissue recovery and measurements

To acquire foetal samples, dams were killed by inhalation of CO_2 followed by cervical dislocation at either e17.5 or e21.5. Foetuses were removed, decapitated and placed in ice cold phosphate buffered solution (PBS; Sigma-Aldrich). Testes were microdissected, snap frozen and stored at –80 °C for determination of intratesticular testosterone (ITT) as described previously (Fisher *et al.*, 2003). The limit of detection of the testosterone assay was 40 pg. For foetuses recovered at e21.5, AGD was measured using digital callipers (Faithfull Tools, Kent, UK) as a measure of androgen exposure during the MPW (Welsh *et al.*, 2008). Animals on Pnd25 were killed by inhalation of CO_2 followed by cervical dislocation. AGD was measured and the penis was recovered, weighed and its length measured using digital callipers (Welsh *et al.*, 2008). The testes, VP and SV were similarly recovered and weighed.

Statistical analysis

Data were analysed using GraphPad Prism version 5 (Graph Pad Software Inc., San Diego, CA, USA) and one-way Analysis of Variance followed by the Bonferroni post-test. Correlations were determined using linear regression. Data for foetal ITT levels were log transformed prior to analysis to normalize variances. As the present studies were focussed largely on exploring the relationship between androgen action in the MPW (as reflected by AGD) and the size of the various reproductive organs in individual animals, data have been analysed for individuals. However, comparable effects of treatment were found when litter means were used for statistical analysis (data not shown), although correlations were not undertaken using litter means.

Results

Effects of flutamide or DBP exposure during foetal life on AGD and male reproductive tract tissues at Pnd25

Foetal exposure to flutamide during the MPW (e15.5–e18.5) dose-dependently reduced AGD and the size of the penis, VP and SV at Pnd25 (Fig. 2), whereas testis weight was not significantly altered (Fig. 2), compared with the controls. Foetal exposure to 500 mg/kg DBP from e13.5 to e21.5 (i.e. including during the MPW) resulted at Pnd25 in significant reductions in AGD and in the size of testes, penis, VP and SV, although the magnitude of reduction varied between tissues (Fig. 2). Foetal exposure to 100 mg/kg DBP caused no significant effects, other than to reduce size of the VP.

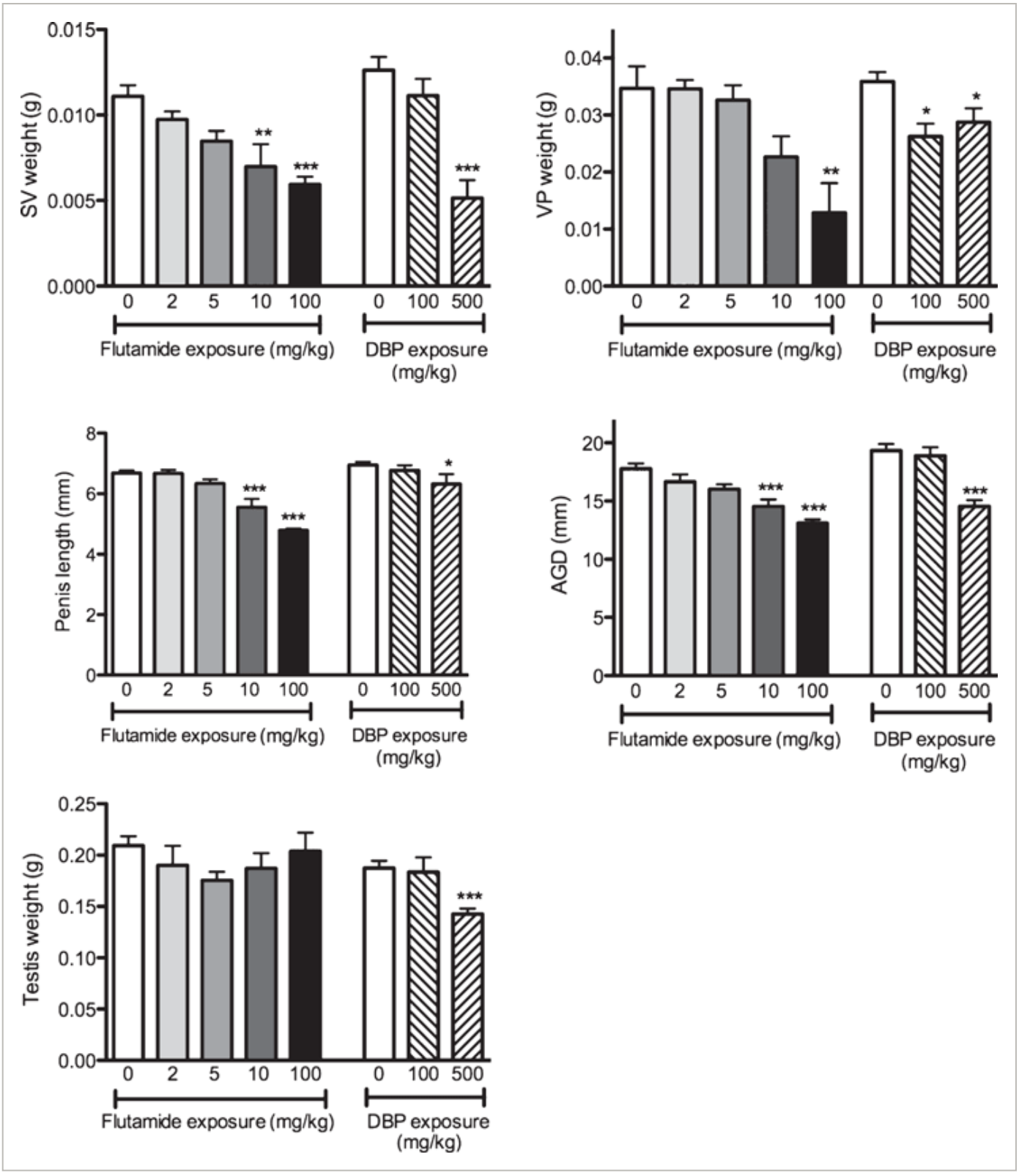

Figure 2

Open in figure viewer | PowerPoint

Effects of prenatal exposure to vehicle, different doses of flutamide (in mg/kg/day) during the MPW (e15.5–e18.5) or dibutyl phthalate (DBP: in mg/kg/day) from e13.5 to e21.5 on the

size of male reproductive tract organs and AGD at Pnd25. Values are mean ± SEM for 6–10 animals (flutamide) or 6–21 animals DBP per treatment group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ in comparison with respective control.

Effects of foetal exposure to DBP on foetal ITT and AGD and on male reproductive tract tissues and AGD at Pnd25

Foetal exposure to 500 mg/kg DBP significantly reduced ITT at both e17.5 (during the MPW) and e21.5 (after the MPW), although the mean reduction at e21.5 was greater than that at e17.5 (Fig. 3); effects of 100 mg/kg DBP were not studied. A much higher proportion of ITT values for DBP500 mg/kg-exposed foetuses at e21.5 (18 of 30 values; 60%) were below the range for control animals when compared with DBP-exposed animals at e17.5 (8 of 26 values; 31%; Fig. 3). This presumably explains the modest reduction (18%) in mean AGD in DBP500 mg/kg-exposed animals at e21.5 when compared with that induced by flutamide exposure (Fig. 3).

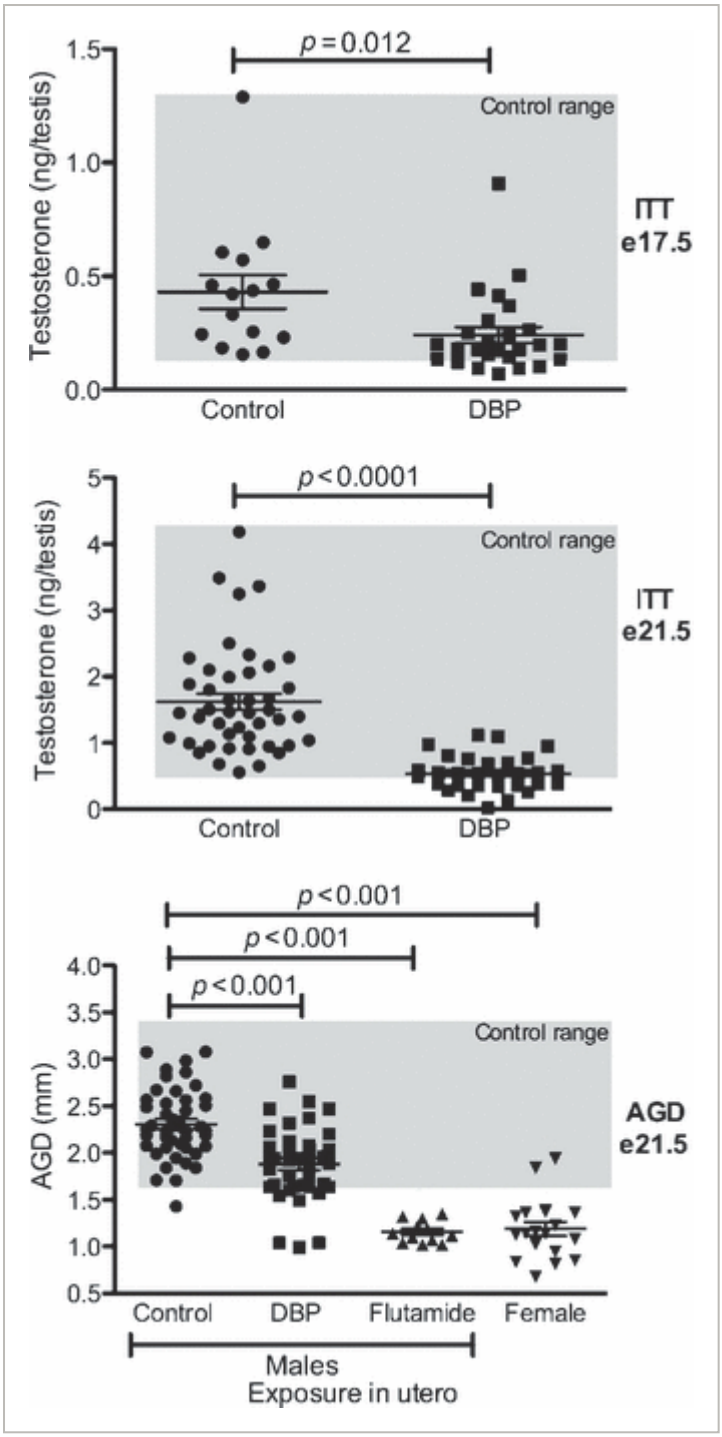

Figure 3

Open in figure viewer | PowerPoint

Effects of prenatal exposure to vehicle (control) or dibutyl phthalate (DBP: 500 mg/kg/day) on intratesticular testosterone (ITT) (e17.5 and e21.5) and on AGD (e21.5) in foetal life. Note that for AGD (bottom panel), data are also shown for normal females and for males exposed in utero to flutamide (100 mg/kg/day) for comparison. Small horizontal lines indicate the mean ± SEM for between 15 and 44 ITT or 40–46 AGD values from individual foetuses. Grey boxes indicate the range of control values.

Relationship between AGD and the size of reproductive organs at Pnd25 after foetal exposure to DBP or flutamide

Anogenital distance, reflecting androgen exposure during the MPW, was significantly correlated with the size of the penis, VP and SV in both DBP-exposed and flutamide-exposed animals at Pnd25 (Fig. 4). AGD was also significantly correlated with testis weight in DBP-exposed animals, but no such relationship was evident in flutamide-exposed animals (Fig. 4).

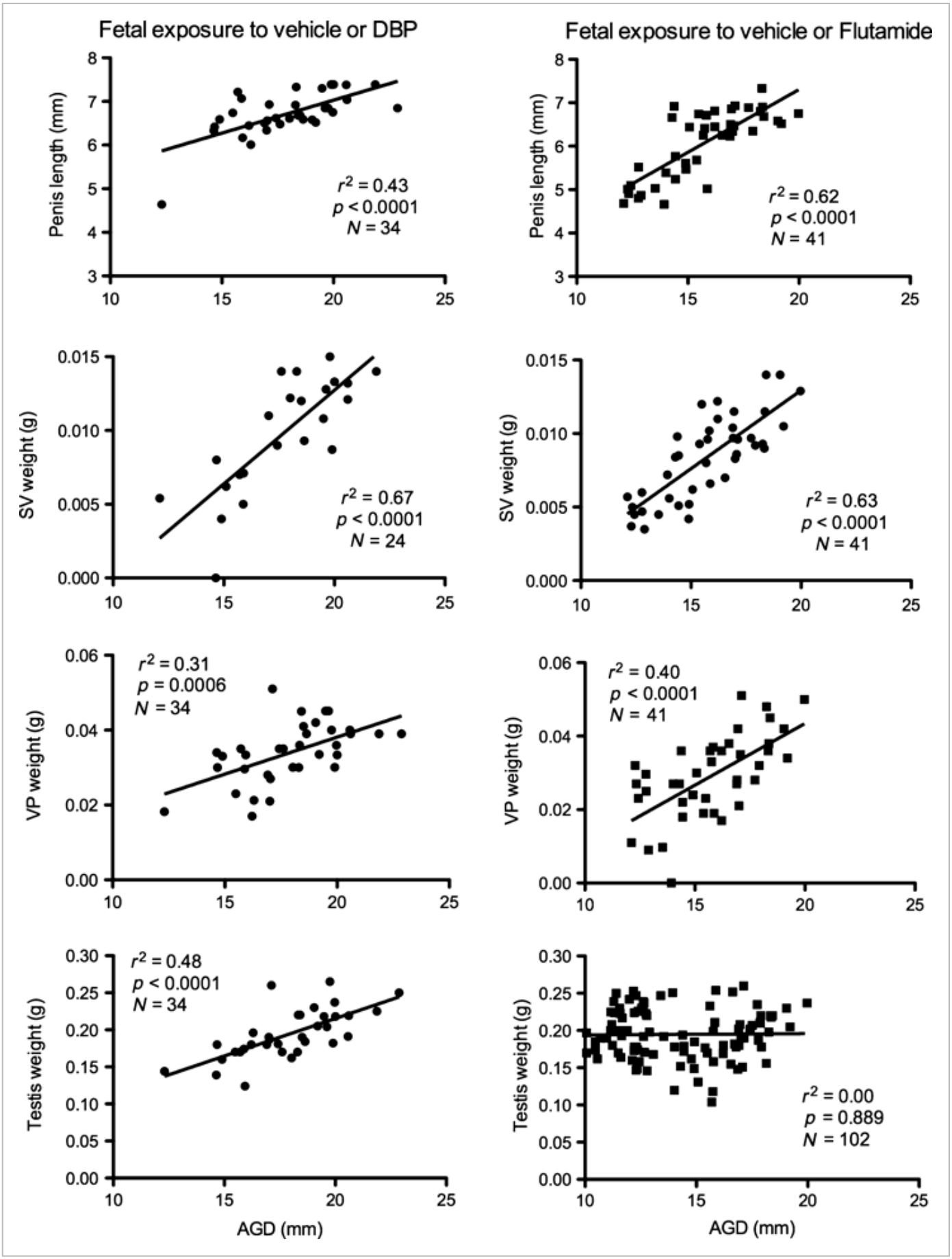
Fetal exposure to vehicle or DBP
Fetal exposure to vehicle or Flutamide
Penis length (mm)
$r^2 = 0.43$
$p < 0.0001$
$N = 34$
$r^2 = 0.62$
$p < 0.0001$
$N = 41$
SV weight (g)
$r^2 = 0.67$
$p < 0.0001$
$N = 24$
$r^2 = 0.63$
$p < 0.0001$
$N = 41$
VP weight (g)
$r^2 = 0.31$
$p = 0.0006$
$N = 34$
$r^2 = 0.40$
$p < 0.0001$
$N = 41$
Testis weight (g)
$r^2 = 0.48$
$p < 0.0001$
$N = 34$
$r^2 = 0.00$
$p = 0.889$
$N = 102$
AGD (mm)
AGD (mm)

Figure 4

Relationship between AGD and male reproductive organ weights at Pnd25 in animals exposed in utero to vehicle (control) or dibutyl phthalate (DBP: 500 mg/kg/day) (left hand panels) or animals exposed in utero to vehicle (control) or flutamide (2, 5, 10 or 100 mg/kg/day) (right hand panels).

Effect of combined prenatal and postnatal treatments on AGD and penis length at Pnd25

As outlined in Fig. 1, androgen action in the MPW predetermines ultimate reproductive organ size, but androgen action after birth is also thought to be required to ensure growth towards this predetermined level. We tested this using AGD and penis length as endpoints, after exposure prenatally and/or postnatally to various combinations of treatments (Table 1). Prenatal exposure to flutamide alone was used to identify the maximum reductions in AGD and penis length that could be achieved just by prenatal inhibition of androgen action (Fig. 5). Foetal DBP exposure alone caused a significant reduction in penis length and AGD, but combined foetal and postnatal exposure to DBP further reduced both AGD and penis length in comparison with controls or to foetal DBP exposure alone (Fig. 5). Postnatal exposure of rats to flutamide after the previous foetal exposure to DBP caused an even bigger decrease in penis length compared with foetal exposure to DBP followed by postnatal vehicle treatment, whereas postnatal exposure to flutamide alone had only a modest non-significant effect on either AGD or penis length (Fig. 5). Further evidence of the postnatal androgen-dependence of AGD and penile growth was provided by the marked increase (compared with controls) in both parameters induced by postnatal TP treatment (Fig. 5).

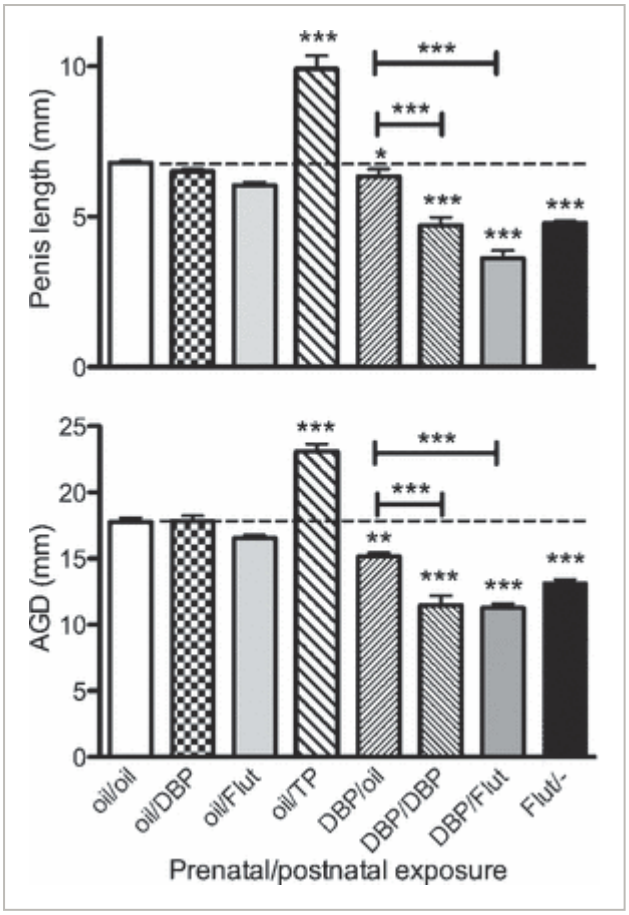

Figure 5

Open in figure viewer | PowerPoint

Effects of combined prenatal and postnatal exposure of rats on penis length and AGD at Pnd25. See Materials and methods and Table 1 for treatment details. Values are mean ± SEM for 5–15 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001 in comparison with respective control.

Discussion

The previous studies have highlighted the importance of the MPW in establishing the later development of the male reproductive tract based on experimental manipulation of peripheral androgen action (Fig. 1; Clark *et al.*, 1993; Spencer *et al.*, 1991; Welsh *et al.*, 2008), findings that are consistent with the original TDS hypothesis (Skakkebæk *et al.*, 2001). The primary aim of the present studies was to establish whether induction of foetal testis dysgenesis/dysfunction using DBP exposure would produce a similar spectrum of effects as occurs after blockage of peripheral androgen action via flutamide exposure; this would provide more direct support for the TDS hypothesis. The present results demonstrate that foetal exposure to DBP, including during the MPW, significantly reduces the size at puberty of the penis, testes, VP and SV in parallel with a significant reduction in AGD. This reduction in reproductive organs weight was

likely the result of reduced androgen exposure during the MPW as there was a strong and consistent correlation between organ size and AGD, the latter reflecting androgen action within the MPW (Welsh *et al.*, 2008). Moreover, we show presently that DBP exposure significantly suppresses ITT during the MPW (i.e. at e17.5) consistent with the previous literature (Schultz *et al.*, 2001; Scott *et al.*, 2008). However, our data also illustrate how variable this suppression is, especially in comparison with the degree of suppression observed later in gestation at e21.5. We believe that this variation provides a rational explanation for the variation in phenotype of DBP-exposed animals, especially the modest reduction in AGD and induction of only mild hypospadias in a minority of animals (Mahood *et al.*, 2007). It may also explain why the effects induced by foetal exposure to DBP are noticeably less severe than those induced by flutamide exposure as, at a dose of 100 mg/kg/day, the latter almost completely prevents masculinization of the reproductive tract in males by blocking peripheral androgen action (Welsh *et al.*, 2008, 2009). A notable exception was the absence of effect of foetal flutamide exposure on testis weight at Pnd25, whereas foetal exposure to DBP significantly reduced testis size. We believe this difference reflects the inability of flutamide to effectively antagonize androgen action within the foetal testis because of the high levels of ITT (Scott *et al.*, 2007); however, the DBP-induced reduction in perinatal germ cell numbers (Ferrara *et al.*, 2006), which reduces testis weight, could also be a factor. In summary, TDS induced by foetal DBP exposure can cause a range of male reproductive tract abnormalities that is similar to those induced by foetal flutamide treatment.

This study also investigated the importance of postnatal androgen action on growth of male reproductive tract tissues after altered foetal androgen action. The previous studies have demonstrated that postnatal androgen exposure is important for penis growth in humans/monkeys (Herman *et al.*, 2000; Prahalada *et al.*, 1997) and in rats (Husmann, 2002; Welsh *et al.*, 2009). Our present findings confirm this by showing that postnatal blockade of androgen production (by DBP treatment) or action (by flutamide treatment) in prepubertal rats significantly reduces penis size at Pnd25 whereas TP exposure during a similar, although shorter, period had the opposite effect and significantly increased penis size. Our studies show that these manipulations of pre and postnatal androgen action induce similar changes in AGD as they do in penis size. This implies that growth of the perineum to its final adult size, like the penis, is predetermined by androgen action within the MPW but also depends on sufficient androgen exposure after birth to grow to this size, consistent with the scheme in Fig. 1. This conclusion is also consistent with observations in mice with inactivating mutations of Kiss1/GPR54, which have normal AGD at birth, indicating normal androgen exposure during the MPW, but reduced AGD and penis size in adulthood as a result of absence of postnatal testosterone production (Kauffman *et al.*, 2007; Lapatto *et al.*, 2007), a phenotype that matches that observed in similarly affected humans (Chan *et al.*, 2009).

The present findings provide strong support for the TDS concept by demonstrating that a treatment that disrupts normal foetal testis development and somatic cell function 'DBP' leads to impaired development and/or growth of all of the reproductive tract organs, including the testis itself. Moreover, the demonstration of a strong correlation between AGD and size of each of the component organs at puberty (Pnd25), is consistent with a common causation, and strongly supports the view that androgen exposure within the MPW is of central importance in determining final size of male reproductive tract organs; it remains to be identified how this is mediated. Furthermore, low AGD at birth or during puberty may be predictive of TDS disorders that emerge in adulthood, such as a low sperm count or testicular cancer. This remains to be investigated, but recent studies in humans have shown that occurrence of hypospadias and cryptorchidism are both associated at birth with reduced AGD (Hsieh *et al.*, 2008; Swan *et al.*, 2005), and that these may coexist with reduced penis size (Acerini *et al.*, 2009; Swan *et al.*, 2005), all of which are consistent with the previous studies in rats (Welsh *et al.*, 2008, 2009).

Acknowledgements

This work was supported in part by the European Union (DEER; FP7-ENV-2007-1-212844) and the UK Medical Research Council (WBS U.1276.00.003.00003.01) and G0501904 (to AJD).

Acerini, C. L., Miles, H. L., Dunger, D. B., Ong, K. K. & Hughes, I. A. (2009) The descriptive epidemiology of congenital and acquired cryptorchidism in a UK infant cohort. *Archives of Diseases in Childhood* In press.

Boas, M., Boisen, K. A., Virtanen, H. E., Kaleva, M., Suomi, A.-M., Schmidt, I. M. *et al.* (2006) Postnatal penile length and growth rate correlate to serum testosterone levels: a longitudinal study of 1962 normal boys. *European Journal of Endocrinology* **154**, 125– 129.

Camurdan, A. D., Oz, M. O., Ihan, M. N., Camurdan, O. M., Sahin, F. & Beyazova, U. (2007) Current stretched penile length: cross-sectional study of 1040 healthy Turkish children aged 0 to 5 years. *Urology* **70**, 572– 575.

Chan, Y. M., Broder-Fingert, S. & Seminara, S. B. (2009) Reproductive functions of kisspeptin and gpr54 across the life cycle of mice and men. *Peptides* **30**, 42– 48.

Clark, R. L., Anderson, C. A., Prahalada, S., Robertson, R. T., Lochry, E. A., Leonard, Y. M., Stevens, J. L. & Hoberman, A. M. (1993) Critical developmental periods for effects on male rat genitalia by finasteride, a 5 alpha-reductase inhibitor. *Toxicology and Applied Pharmocology* **119**, 34– 40.

Ferrara, D., Hallmark, N., Scott, H., Brown, R., McKinnell, C., Mahood, I. K. & Sharpe, R. M. (2006) Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology* **147**, 5352– 5362.

Fisher, J. S., Macpherson, S., Marchetti, N. & Sharpe, R. M. (2003) Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Human Reproduction* **18**, 1383– 1394.

Foster, P. M. (2006) Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *International Journal of Andrology* **29**, 140– 147.

Frederiksen, H., Skakkebæk, N. E. & Andersson, A. M. (2007) Metabolism of phthalates in humans. *Molecular Nutrition and Food Research* **51**, 899– 911.

Habert, R. & Picon, R. (1984) Testosterone, dihydrotestosterone and estradiol-17 beta levels in maternal and fetal plasma and in fetal testes in the rat. *Journal of Steroid Biochemistry* **21**, 193– 198.

Herman, R. A., Jones, B., Mann, D. R. & Wallen, K. (2000) Timing of prenatal androgen exposure: anatomical and endocrine effects on juvenile male and female rhesus monkeys. *Hormones and Behaviour* **38**, 52– 66.

Hsieh, E. W., Breyer, B. N., Eisenberg, M. L. & Baskin, L. S. (2008) Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. *Current Urology Reports* **9**, 137– 142.

Husmann, D. A. (2002) Micropenis: an animal model and its human correlates. *Advances in Experimental Medicine and Biology* **511**, 41– 56.

Kauffman, A. S., Park, J. H., McPhie-Lalmansingh, A. A., Gottsch, M. L., Bodo, C., Hohmann, J. G. *et al.* (2007) The kisspeptin receptor gpr54 is required for sexual differentiation of the brain and behaviour. *The Journal of Neuroscience* **27**, 8826– 8835.

Lapatto, R., Pallain, J. C., Zhang, D., Chan, Y.-M., Mahan, A., Cerrato, F., Le, W. W., Hoffman, G. E. & Seminara, S. B. (2007) Kiss1–/– mice exhibit more variable hypogonadism than gpr54–/– mice. *Endocrinology* **148**, 4927– 4936.

Mahood, I. K., Scott, H. M., Brown, R., Hallmark, N., Walker, M. & Sharpe, R. M. (2007) In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. *Environmental Health Perspectives* **115**, 55– 61.

Mylchreest, E., Wallace, D. G., Cattley, R. C. & Foster, P. M. (2000) Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicological Sciences* **55**, 143– 151.

Prahalada, S., Tarantal, A. F., Harris, G. S., Ellsworth, K. P., Clarke, A. P., Skiles, G. L. *et al.* (1997) Effects of finasteride, a type 2 5-alpha reductase inhibitor, on fetal development in the rhesus monkey (*Macaca mulatta*). *Teratology* **55**, 119– 131.

Schultz, V. D., Phillips, S., Sar, M., Foster, P. M. D. & Gaido, K. W. (2001) Altered gene profiles in fetal rat testes after in *utero* exposure to di(*n*-butyl) phthalate. *Toxicological Sciences* **64**, 233– 242.

Scott, H. M., Hutchison, G. R., Mahood, I. K., Hallmark, N., Welsh, M., De Gendt, K., Verhoeven, G., O'Shaughnessy, P. & Sharpe, R. M. (2007) Role of androgens in fetal testis development and dysgenesis. *Endocrinology* **148**, 2027– 2036.

Scott, H. M., Hutchison, G. R., Jobling, M. S., McKinnell, C., Drake, A. J. & Sharpe, R. M. (2008) Relationship between androgen action in the "Male programming window," Fetal sertoli cell number, and adult testis size in the rat. *Endocrinology* **149**, 5280– 5287.

Sharpe, R. M. & Skakkebæk, N. E. (2008) Testicular disgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertility and Sterility* **89**, e33– e38.

Skakkebæk, N. E., Rajpert-De Meyts, E. & Main, K. M. (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Human Reproduction* **16**, 972– 978.

Skakkebæk, N. E., Rajpert-De Meyts, E., Jørgensen, N., Main, K. M., Leffers, H., Andersson, A. M., Juul, A., Jensen, T. K. & Toppari, J. (2007) Testicular cancer trends as 'whistle blowers' of testicular developmental problems in populations. *International Journal of Andrology* **30**, 198– 205.

Spencer, J. R., Torrado, T., Sanchez, R. S., Vaughan, E. D. J. & Imperato-McGinley, J. (1991) Effects of flutamide and finasteride on rat testicular descent. *Endocrinology* **129**, 741– 748.

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M. *et al.* (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environmental Health Perspectives* **113**, 1056– 1061.

Welsh, M., Saunders, P. T. K., Fisken, M., Scott, H. M., Hutchison, G. R., Smith, L. B. & Sharpe, R. M. (2008) Identification in rats of a programming window for reproductive tract masculinisation, disruption of which leads to hypospadias and cryptorchidism. *The Journal of Clinical Investigation* **118**, 1479– 1490.

Welsh, M., MacLeod, D. J., Walker, M., Smith, L. B. & Sharpe, R. M. (2009) Critical androgen-sensitive periods of rat penis and clitoris development. *International Journal of Andrology* **32**, 1– 9.

Panel discussion

René Habert (INSERM: CEA University of Paris 7, France)

We did not observe any constant effect of MEHP on testosterone production by mouse fetal testis in culture. In the three models of marmosets *in vivo*, and human and mouse fetal testis *in vitro*, phthalates act on germ cell development. This poses two important questions in regard to human health. Firstly, is the main effect of phthalates an antiandrogenic effect? Secondly, is the rat possibly not a good model to study the effects of phthalates?

Richard Sharpe

We will not know if the rat is a good model for humans until it can be shown for certain whether or not phthalates affect steroidogenesis in the human fetal testis. As you know, Dr Shanna Swan has shown that there is a correlation between maternal phthalate exposure and anogenital distance (AGD) in male offspring. All models are imperfect, and we are using rat exposure to dibutyl phthalate to look specifically at the development of TDS disorders. The main focus of our studies is not specifically to evaluate whether phthalates affect humans, nor the risks to humans. We are trying to discover the key pathways leading to TDS disorders and the phthalate-exposed rat model is extremely useful in this regard. This will improve our ability to identify what factors might target these pathways and thus pose a risk to the human male fetus. Based on present evidence, I consider the antiandrogenic effect of phthalates (in rats) to be the most important in relation to the development of TDS disorders. If phthalates do not, or cannot, inhibit testosterone production in the human and primate fetal testis then they may be largely irrelevant as a major risk for TDS disorders, but at present I do not know whether or not this is the case.

Earl Gray (US EPA, USA)

There are significant differences in the phthalate syndromes in different strains of rats. Your strain shows a high incidence of undescended testes whereas this occurs less frequently in our strain although we see more epididymal malformations.

Paul Foster

In our studies, we looked at malformation outcome including nipple retention in rats in addition to AGD as a result of exposure to DBP and flutamide. Did you include nipple retention in your studies?

Richard Sharpe

In our rat studies, we recorded nipple retention but this was not emphasized because it is not relevant to humans. Measurement of AGD is the first assessment that we undertake of the animals at death, and we have found this to be very accurate in predicting the rest of the animals reproductive phenotype such as the internal reproductive organs and (dissected) size of the penis. AGD is a very useful measurement in this regard and this may be relevant to humans.