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CLINICAL STUDIES ON TESTICULAR GROWTH AND DESCENT

Jaakko Koskenniemi

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To Krista and Joonatan

ABSTRACT

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Clinical studies on testicular growth and descent

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Recent population-based cohort studies indicate that semen quality is declining and simultaneously the incidence of testicular germ-cell cancer is increasing globally. A failure of testicular descent i.e. cryptorchidism and reduced testicular volume are risk factors both for testicular germ-cell cancer and reduced semen quality. Epidemiological and experimental studies suggest that these disorders may originate from testicular dysgenesis and reduced intratesticular androgen action during fetal period, which may be caused by genetic factors or exposure to antiandrogen endocrine disrupters.

In this study, we explored postnatal testicular descent and the physiological significance of the so-called 'minipuberty', the transient activation of the hypothalamic-pituitary-gonadal axis after birth for postnatal testicular position. In addition, we assessed whether the levels of persistent organic pollutants such as polychlorinated biphenyl (PCBs), polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs, or 'dioxins') and polybrominated diphenyl ethers (PBDEs) are associated with congenital cryptorchidism. Finally, we assessed the testicular development during puberty among boys with a history of congenital cryptorchidism.

We observed that testicular descent continued until the age of three months, and was followed by a 'physiological' testicular ascent, which coincided with the decline in circulating reproductive hormones. We also discovered that the circulating concentration of insulin-like growth factor 1 and hormonal indices reflecting Sertoli and Leydig cell function correlated with testicular position. Our results also suggest that the exposure to dioxins may increase the risk of congenital cryptorchidism. Furthermore, we showed that boys who had a history of congenital cryptorchidism display poor testicular growth during puberty in comparison to controls, which may predispose them to reduced semen quality and subfertility.

Keywords: testis, cryptorchidism, endocrine disruptors, dioxins, PCB, PCDD/F, PBDE, reproductive health

TIIVISTELMÄ

Jaakko Koskenniemi
Kliinisiä tutkimuksia kivesten kasvusta ja laskeutumisesta

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia, Turun kliininen tohtoriohjelma, Integratiivisen fysiologian ja farmakologian tutkimusyksikkö, Turku

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Viimeaikaisten tutkimusten mukaan siemennesteen laatu on heikentynyt ja kivessyövän esiintyvyys lisääntynyt ympäri maailmaa. Laskeutumaton kives eli piilokives ja kivesten pieni koko altistavat sekä kivessyövälle että heikentyneelle siemennesteen laadulle. Epidemiologisten tutkimusten sekä eläinmallien tulosten perusteella nämä häiriöt saattavat johtua kiveksen kehityshäiriöstä ja vähentyneestä sikiökautisesta kiveksensisäisestä androgeenivaikutuksesta. Tämä puolestaan saattaa johtua perintötekijöiden lisäksi altistuksesta ympäristön antiandrogeenisille hormonaalisille haitta-aineille.

Tässä väitöskirjassa selvitimme syntymän jälkeisen ohimenevän hypotalamus-aivolisäke-kives –akselin aktivoitumisen eli niin sanotun minipuberteetin merkitystä kivesten laskeutumiselle syntymän jälkeen. Väitöskirjassani myös selvitettiin altistavatko ympäristön pysyvät hormonaaliset haitta-aineet kuten polyklooratut bifenyylit (PCB), dioksiinit (PCDD/F) tai polybromatut difenyylieetterit (PBDE) piilokiveksisyydelle. Lisäksi tutkimme piilokiveksisten ja verrokkien kiveskasvua murrosiässä.

Tutkimustulostemme mukaan kivekset laskeutuvat kolmen kuukauden ikään asti, minkä jälkeen kivekset nousevat samaan aikaan kun sukupuolihormonien pitoisuus verenkierrossa vähenee. Väitöskirjani mukaan insuliinin kaltaisen kasvutekijä 1:n pitoisuus sekä Leydigin ja Sertolin solujen toimintaa kuvaavat sukupuolihormoni-indeksit olivat yhteydessä kiveksen laskeutumiseen syntymän jälkeen. Lisäksi altistuminen pysyville hormonaalisille haitta-aineille, erityisesti dioksiineille, näyttää olevan yhteydessä synnynnäiseen piilokiveksisyyteen. Lisäksi synnynnäinen piilokives kasvaa verrokkien kiveksiä heikommin murrosiän aikana, mikä ennustaa heikompaa siemennesteen laatua ja hedelmällisyyttä.

Avainsanat: kives, piilokives, hormonaaliset haitta-aineet, dioksiinit, PCB, PCDD/F, PBDE, lisääntymisterveys

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ABBREVIATIONS

2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
5α-R1	5α-reductase1
5α-R2	5α-reductase2
95% CI	95% confidence interval
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
ALSC	Adult Leydig stem cell
AMH	Anti-Müllerian hormone
ARNT	AhR nuclear translocator protein
AR	Androgen receptor
BDE	Brominated diphenyl ether
CDD/F	Chlorinated dibenzo-*p*-dioxin and/or furan
CDGP	Constitutional delay in growth and puberty
CGRP	Calcitonin gene-related peptide
CV	Coefficient of variation
DHT	Dihydrotestosterone
E	Embryonic day
EU	European Union
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GA	Gestational age
GNRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HPT-axis	Hypothalamic-pituitary-testicular axis
IGF-I	Insulin-like growth factor 1
INSL3	Insulin-like 3
IPCS	International Panel on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
LH	Luteinising hormone
LHR	Luteinising hormone receptor
LHCGR	Luteinising hormone / choriogonadotropin receptor
LOD	Limit of detection
LOQ	Limit of quantification
MKRN3	Makorin ring finger protein 3
MPW	Masculinisation programming window
NA	Not applicable
OR	Odds ratio

PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorinated dibenzo-*p*-dioxins and/or furan
POP	Persistent organic pollutant
RXFP2	Relaxin/insulin-like family peptide receptor 2
SD	Standard deviation
SE	Standard error
SHBG	Sex-hormone binding globulin
SRY	Sex-determining region Y
StAR	Steroidogenic acute regulatory protein
TDI	Tolerable daily intake

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following publications, which are referred in the text by Roman numerals (I.-IV.). The original publications have been reproduced with the permission of the copyright holders.

I. Koskenniemi JJ, Virtanen HE, Wohlfahrt-Veje C, Löyttyniemi E, Skakkebaek NE, Juul A, Andersson AM, Main KM, Toppari J. Postnatal changes in testicular position are associated with IGF-I and function of Sertoli and Leydig cells. Submitted.

II. Virtanen HE, Koskenniemi JJ, Sundqvist E, Main KM, Kiviranta H, Tuomisto JT, Tuomisto J, Viluksela M, Vartiainen T, Skakkebaek NE, Toppari J. Associations between congenital cryptorchidism in newborn boys and levels of dioxins and PCBs in placenta. Int. J. Androl. 2012;35(3):283–93.

III. Koskenniemi JJ, Virtanen HE, Kiviranta H, Damgaard IN, Matomäki J, Thorup JM, Hurme T, Skakkebaek NE, Main KM, Toppari J. Association between levels of persistent organic pollutants in adipose tissue and cryptorchidism in early childhood: a case-control study. Environ. Health 2015;14(1):78.

IV. Sadov S*, Koskenniemi JJ*, Virtanen HE, Perheentupa A, Petersen JH, Skakkebaek NE, Main KM, Toppari J. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. J. Clin. Endocrinol. Metab. 2016;101(6):2570–7. *Both authors contributed equally to this publication

1 INTRODUCTION

In addition to infertility[1–3], testicular dysfunction is associated with decreased quality of life[4], cardiovascular and psychiatric morbidity[5,6] as well as the overall risk of cancer and all-cause mortality[7].

There is ample evidence of a global reduction in semen quality[8] and an increase in incidence of testicular germ-cell cancer[9,10]. In Finland, 42% of the men born in 1987 have sperm concentration below 40 million/ml[11], which is associated with delayed time to pregnancy[1,3]. In parallel, the incidence of testicular germ-cell cancer in the Turku area is now six times higher compared to 1960s[11], and it is still projected to increase by 70% by 2025 in Finland and by 25% in the European Union as a whole[12].

Adult testicular function and the risk of testicular germ-cell cancer seem to be largely programmed by events that occur early during fetal development[13,14]. Furthermore, large quantitative and qualitative changes take place in testicular tissue during infancy, childhood and puberty[15,16]. Extensive systematic reviews suggest that exposure to mixtures of synthetic endocrine disruptors during these sensitive periods may interfere with male reproduction[17–19], but large uncertainties remain[20]. Undescended testes and reduced testicular volume are well described risk factors of testicular germ-cell cancer[14,21–23], and correlate with reduced semen quality[24–26]. This thesis aims to elucidate the biology behind these two biomarkers in order to better characterise the links between perinatal development and adult reproductive function.

Firstly, we analysed the changes in testicular position from birth to prepuberty, and correlated these changes with hormonal data describing Sertoli and Leydig cell function at three months of age during minipuberty. Secondly, we assessed the association between congenital cryptorchidism and the exposure to persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs, or 'dioxins') and polybrominated diphenyl ethers (PBDEs). Finally, testicular growth during puberty was followed up among boys with and without congenital cryptorchidism, and the agreement between ultrasonography, Prader orchidometer and ruler was tested.

2 REVIEW OF LITERATURE

2.1 The overview of male reproductive system

2.1.1 Structure and function of male reproductive organs

The male reproductive system refers to a tract of organs containing two testes, two epididymides, two efferent ducts (*vasa deferentia*), penis and accessory sex glands including seminal vesicles and prostate, all of which are involved in the production, processing and transport of the sperm from testes to the female reproductive tract and thus facilitate the survival of human species through generations[27]. Although this thesis focuses exclusively on function and development of testes and its importance, malfunction of the reproductive system distally from testes remains a frequent cause of male infertility[28].

Testicular histology is illustrated schematically in Figure 1. Testicular tissue consists of seminiferous tubules and interstitium that lies between the tubules. Seminiferous tubules intersect in the *rete testis* and the tubular fluid is drained towards the epididymides. Seminiferous tubules consist of peritubular myoid cells, Sertoli cells and germ cells[27]. Sertoli cells extend both to the basal membrane and to the lumen of the seminiferous tubule, and connect with each other with tight junctions [29]. This cytological architecture provides a habitat for germ cells and forms a blood-testis-barrier[29]. The blood-testis-barrier shelters the haploid cells from the immune system, and permits the formation of the unique microenvironment for germ cells[30].

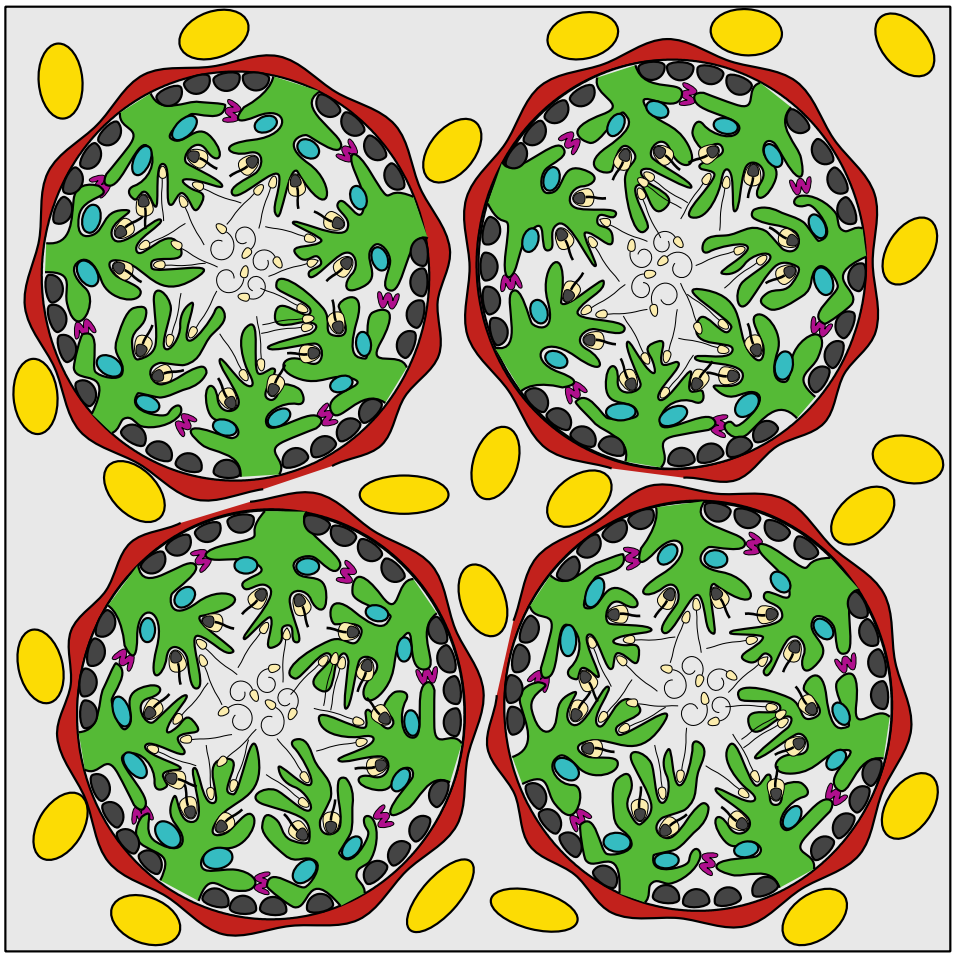

Figure 1 Schematic representation of human testicular histology

A simplified figure showing the location of human testicular Sertoli cells (green), which form seminiferous tubules. Seminiferous tubules are lined by peritubular myoid cells (red). Sertoli cells house spermatogonia (black) in their basal compartment and spermatocytes (turquoise) and spermatids in the crypts and projections in adluminal compartment. During spermatogenesis, spermatozoa are released into the tubular lumina. Leydig cells (yellow) are scattered in the interstitium between seminiferous tubules.

2.1.2 Steroidogenesis and spermatogenesis

The two main functions of the testes are steroidogenesis, i.e. the production of sex steroids including androgens and spermatogenesis i.e. the production of spermatozoa. These two functions are linked, as intratesticular androgen concentrations are indispensable for normal spermatogenesis[31]. As testosterone is produced in Leydig cells, intratesticular testosterone concentration is >100-fold compared to peripheral circulation[32].

An overview of steroidogenesis is presented in Figure 2. During steroidogenesis, cholesterol is transformed into an active steroid hormone such as testosterone within mitochondrial membranes or in its proximity[33]. Within testis, only Leydig cells express *P450SCC*, a key rate-limiting enzyme, and are thus steroidogenic. Within Leydig cells, the presence of 17βHSD3 and 3βHSD2 is essential for testosterone synthesis. Furthermore, due to the absence of P450c11 and P450c21, Leydig cells do not normally produce glucocorticoids[33]. Testosterone is converted into a more potent androgen, dihydrotestosterone (DHT), by 5α-reductase isoenzymes 1 and 2 (5α-R1 and 5α-R2) in target organs, whereas those two enzymes are not expressed in Leydig cells[33]. Leydig cells also show some P450ARO activity that metabolises testosterone to estradiol[33].

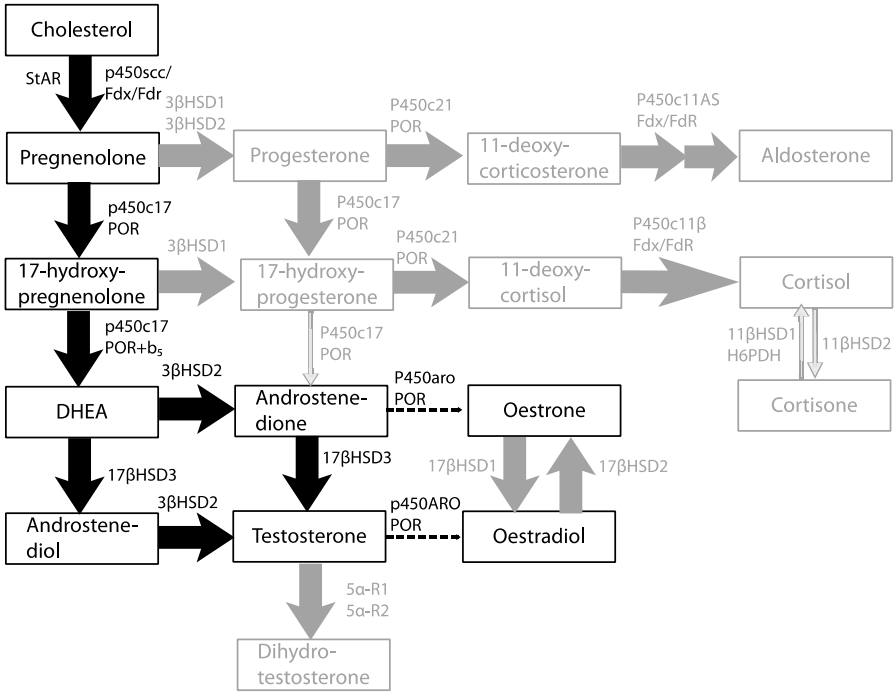

Figure 2 Overview of steroidogenesis

Modified from Miller & Auchus 2011[33]. Black arrows, boxes and text indicate steps, intermediate products and enzymes, respectively, that occur in testicular Leydig cells. Black dashed arrows indicate steps that occur to a lesser degree. Grey-shaded arrows, boxes and text denote steps, intermediate products and enzymes, respectively, that occur in other steroidogenic cells or tissues.

In the process of spermatogenesis, haploid spermatozoa are produced from the diploid spermatogonia via spermatocytes and spermatids in the germinal epithelium[27,34]. The process of spermatogenesis consists of three steps: commitment of spermatogonia to spermatogenesis, meiosis and spermiogenesis[34].

2.1.3 Hypothalamic-pituitary-testicular axis

The function of both Sertoli and Leydig cells is regulated by the hypothalamic-pituitary-testicular axis (HPT-axis, illustrated in Figure 3). In primates, neurons in the infundibular region of the forebrain release kisspeptin, which increases the pulsatile secretion of gonadotropin-releasing hormone (GNRH) from the hypothalamus[35].

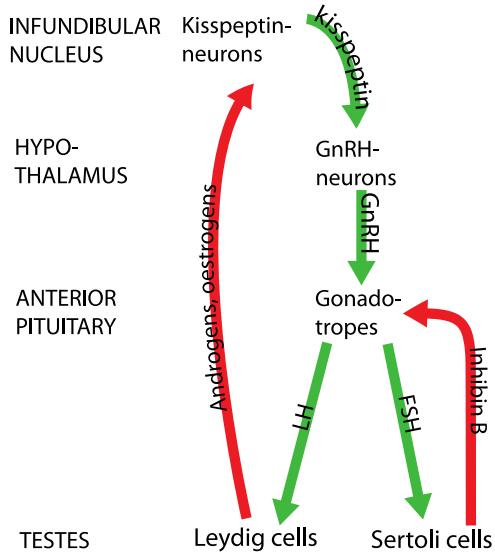

Figure 3 Hypothalamic-pituitary-testicular axis

Modified from Pinillla at al. 2012[35]. In infundibular nucleus in forebrain, neurons secrete kisspeptin, which excites the pulsatile release of GNRH from GNRH-neurons in the hypothalamus. GNRH stimulates pituitary gonadotropes to secrete LH, which stimulates Leydig cells and FSH, which in turn stimulates Sertoli cells. Stimulated Leydig cells produce androgens and estrogens, inhibiting the release of kisspeptin from kisspeptin neurons. Sertoli cells secrete inhibins (in humans predominantly inhibin B), which further inhibits the FSH secretion from pituitary gonadotropes.

Downstream in the HPT-axis, GNRH induces the release of follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary gland[36]. FSH binds to FSH-receptors (FSHR) in Sertoli cells, which stimulates the production of inhibins[37,38]. LH stimulates testosterone production in testicular Leydig cells by binding to the luteinising hormone / choriogonadotropin receptor (LHCGR)[39].

Androgens and aromatase-converted estrogens exert a negative feedback on kisspeptin-neurons in the infundibular region of the forebrain, thereby decreasing GNRH pulses and thus circulating FSH and LH[35,40–42]. *In vitro* and *in vivo* experiments on non-human primates suggest that inhibins, in humans mostly inhibin B, are also involved in the negative feedback on the pituitary gland, especially in terms of FSH[41].

2.1.4 Developmental insights into adult testicular function

Large enough amount of sperm with normal morphology must be produced from the testicular germ cells to father a child[1–3]. In addition, a failure in the regulation of the developing germ cells may lead to testicular germ-cell cancer[43], and it it is well established that Sertoli cells are pivotal in sperm production and germ cell regulation[43–46].

In rodents, Sertoli cells seem to proliferate mostly during puberty and not in adulthood[45], although a recent study revealed that a small number of cells in transitional zone close to the *rete testis* may also divide in adulthood[47]. In humans, Sertoli cells seem to proliferate only during perinatal and pubertal development and not in adulthood[44,48]. During development, substantial quantitative and qualitative changes take place in Sertoli cells, which are regulated in part by Leydig cells and peritubular cells[15,49–51]. Development of the testis from fetal period to adulthood is therefore reviewed next with special reference to these three cell lineages.

2.2 Testicular development

2.2.1 From bipotential gonad to prenatal testis

The first macroscopic sign of the gonads during fetal development is the ridge-like paired structure on the ventromedial surface or the mesonephros at E10–10.5 (embryonic day 10–10.5) in mice and approximately at 5–6 weeks post conception (wpc) in humans[52–54]. During that time developing putative ovaries and testes are indistinguishable, and with experimental manipulation of the necessary growth factors either of them may develop into an ovary or testis[55–58]. Furthermore, the developing foetus has both paramesonephric ducts, i.e. Müllerian ducts, the precursors of the female reproductive tract, and mesonephric ducts, i.e. Wolffian ducts, the precursors of the male reproductive tract[59,60]. Primordial germ cells migrate from the yolk sac via hindgut to the genital ridges where they arrive approximately at 5–6 wpc in humans[52,61].

The origins and the interrelationship between the testicular cell lineages are shown in Figure 4. Gonadal somatic cells differentiate into Sertoli cells at 7–8 wpc in humans (E10.5 in mice[52,62,63]) when they start to express the sex-determining region Y (*SRY*) gene located in the Y-chromosome[64–66]. This invokes the expression of *SOX9*, an indispensable downstream player that amplifies the signal of *SRY* expression, and is itself sufficient to commit the gonad to testicular development if overexpressed[55–58]. SOX9 initiates and maintains a complex downstream network

of genes that promotes and maintains the commitment of the testicular development and suppresses the ovarian development[67].

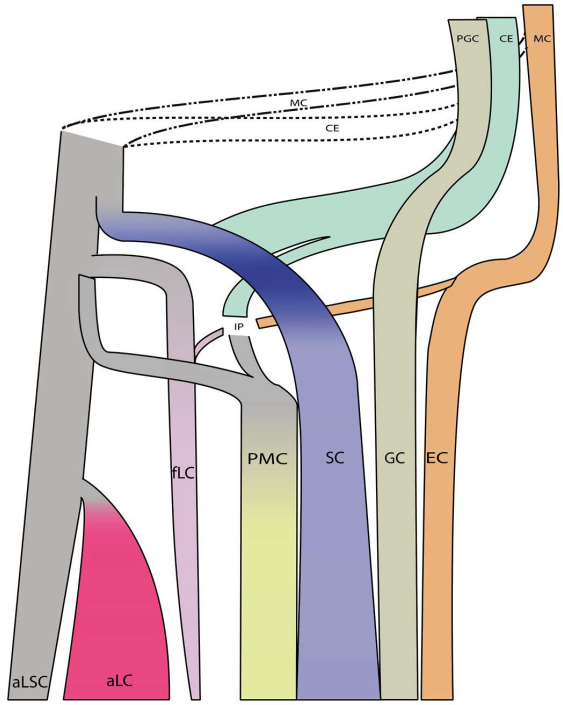

Figure 4 Origins and interrelationships between testicular cell lineages

PGC = primordial germ cells, CE = coelomic epithelial cells, MC = mesonephric cells, IP = interstitial precursors, ALSC = adult Leydig stem cells, fLC = fetal Leydig cells, aLC = adult Leydig cells, PMC = peritubular myoid cells, SC = Sertoli cells, GC = germ cells, EC = endothelial cells.

Sertoli-endothelial cell interaction organises the testis by promoting the formation of testicular cords, the primitive seminiferous tubules that lack lumina[68,69]. Testicular cords then elongate, which increases the width of the genital ridge and changes the macroscopic form of the testis from ridge-like to more ellipsoid[70]. Sertoli cells also initiate the development of other cell lineages by inducing Leydig cell and peritubular myoid cell differentiation[71–75]. However, other factors that are expressed mainly in the interstitium including peritubular myoid cells are also needed for Leydig cell differentiation[76–79]. Leydig cells can be recognised at 9 wpc (E12.5–13.5 in mice) and peritubular myoid cells at 12 wpc (E13.5 in mice)[62,74,80].

Cells of the coelomic epithelium seem to be the precursors of the Sertoli, peritubular myoid cells and at least a subpopulation of the fetal Leydig cells[81]. Some fetal Leydig cells may also originate from the mesonephric epithelium or mesonephric border[82,83], or from the testicular steroidogenic cells that migrate from the adrenogonadal primordia to the testis[84,85].

Inactivating mutations in genes involved in the early testicular determination can cause severe disorders of sex development[55,76,86]. These syndromes often manifest not only in testes, but also in other organ systems such as the skeletal system in campomelic dysplasia and the central nervous system in X-linked lissencephaly with abnormal genitalia (XLAG) syndrome[55,76].

2.2.2 Expansion of Sertoli cells and adult Leydig stem cells during masculinisation programming window

Fetal Leydig cells start to secrete androgens gradually after testis determination. Although testosterone can be detected in human fetal testis already at gestational week 6, a clear increase in testosterone can be demonstrated between 8 and 14 gestational weeks (E15.5 in rats) suggesting endogenous testosterone secretion from the fetal testis[87,88].

Experimental studies on rats have shown that the development of the reproductive tract is especially vulnerable during this window of E15.5–E18.5 when androgen concentrations increase[51]. Exposure to androgen receptor (AR) inhibitor flutamide during this window reduced semen quality and increased the risk of testicular maldescent and hypospadias, even though testicular descent takes place later during development[51]. Thus, this period can be called 'masculinisation programming window' (MPW)[51]. Exposure to dibutyl phthalate (750 mg/kg/day), which decrease intratesticular testosterone concentrations by inhibiting steroidogenic enzymes in rat[89,90], or flutamide during MPW also decreased anogenital distance (AGD)[51,91]. AGD is a sex-dimorphic trait that is usually roughly twice longer in males compared to females both in laboratory animals and in humans and is considered a read-out of androgen levels during MPW[92,93].

At and after MPW (E17.5–E21.5 in rats), intensive proliferation of Sertoli cells takes place, which results in 11-fold increase in Sertoli cell numbers (in rats) and elongation and coiling of the testicular cords[94,95]. In mice, this second wave of Sertoli cell proliferation and coiling of the testicular cords was reduced between E15.5 and E19.5 after targeted inactivation of activin A gene in fetal Leydig cells or the gene of its receptor *Smad4* in Sertoli cells[94]. Furthermore, rats with inactivating mutation in AR or that were exposed to phthalates slightly after MPW (E19.5–E21.5), had a reduced perinatal number of Sertoli cells[95–98]. This decrease in Sertoli cell number may be caused by decreased proliferation of Sertoli cells, or increased breakdown of the existing seminiferous tubules and Sertoli cell apoptosis[99].

Recent studies have confirmed the prenatal existence of adult Leydig stem cells (ALSCs) in rodents[100–102]. These cells originate from the same precursor cells as at least a subpopulation of fetal Leydig cells[101–103]. While fetal-type Leydig cells regress and do not have a clear function postnatally[100], the adult-type Leydig cell population originates from ALSCs[100–102]. The number of ALSCs increases 17-fold between E17.5 and E19.5, and their proliferation was reduced by approximately 40% after the reduction of intratesticular testosterone concentrations by daily gavage of 500 mg/kg/day of dibutyl phthalate between E13.5 and E21.5[101]. Furthermore, this decline was reflected in reduced testosterone levels and higher LH concentration in adulthood despite the normal Leydig cell numbers[101].

Exposure to dibutyl phthalate (750 mg/kg/d) during this window also increases the focal dysgenesis of the testicular microarchitecture by causing central aggregation of Leydig cells, malformed testicular cords and ectopic Sertoli cells that are located outside testicular cords in mice[91].

2.2.3 Masculinisation of reproductive tract and testicular descent

Müllerian ducts gradually regress due to secretion of Anti-Müllerian hormone (AMH) from the testicular Sertoli cells between E13 and E18 in mice[104–106]. Simultaneously, androgens secreted from the testis stabilise the Wolffian ducts, which are the precursors of *vasa deferentia*, ejaculatory ducts and seminal vesicles. The Wolffian ducts regress on the level of gonads before E17 during ovarian development[106]. The same female-type development occurs in mice lacking a functional AR, and in patients with gonadal aplasia or complete androgen insensitivity[59,107–109].

Testes start their descent towards the bottom of the scrotum in an area close to kidneys[60]. Early descent of testes is caused by the descent of the anlage of the diaphragm[60]. From early on, a cone-like gelatinous structure named *gubernaculum testis* (Latin for ‘rudder of testis’) starts to form at the conjunction of the mesonephros and the ventral abdominal wall, where the internal ring of the inguinal canal is formed[60]. Although initially the gubernaculum is not in contact with the testis but the mesonephros, eventually the testis slides on top of the gubernaculum during the involution of the Müllerian structures and attaches to it[60]. Consequently, the gubernaculum keeps the testis close to the internal opening of the inguinal canal, and may also actively guide the testis towards the inguinal canal[60,110,111].

The gubernaculum undergoes a massive swelling reaction which widens the inguinal canal before transinguinal testicular descent at around gestational week 20[60]. Swelling of gubernaculum and enlargement of the gubernacular bulb is prevented

in INSL3 (insulin-like 3) knock-out mice, which disrupts transabdominal testicular descent[112,113]. After that, the tip of the abdominal part of the gubernaculum bulges to the inguinal canal and the *processus vaginalis* elongates towards the scrotum through the inguinal canal and anteriorly envelopes the gubernaculum[60,110,111]. Under the guidance of the gubernaculum, the testis and epididymis rapidly migrate through inguinal canal mostly between gestation weeks 23 and 28, while among some boys testes are still intra-abdominal at gestation week 34[110].

In humans, gubernaculum then regresses to form a scrotal ligament[60]. The regression of gubernaculum is primarily androgen-induced and important for full testicular descent, as demonstrated by impaired transinguinal testicular descent in mice with ubiquitous or gubernaculum-specific inactivation of AR or human patients with an inactivating AR mutation[114,115]. Finally, *processus vaginalis* closes[60].

In addition to the changes described above, elongation of *vas deferens* and testicular blood vessels is needed for full testicular descent[116]. Furthermore, calcitonin gene-related peptide (CGRP) secreted from the genitofemoral nerve plays a role in guiding testicular descent to the scrotum by secreting chemotactic signals and/or by inducing rhythmic contractions of the gubernaculum in rodents[117]. An *in vitro* experiment suggests that CGRP is involved in the obliteration of the *processus vaginalis* in humans[118], while no further data exists to judge whether or not CGRP is involved in testicular descent in humans.

2.2.4 Role of hypothalamus and pituitary in prenatal and postnatal testicular development

Unlike in adulthood, the hypothalamus and the pituitary do not appear to control testicular function or development early during fetal development. Postnatally, normal testicular morphology including both Sertoli and Leydig cells has been reported in studies of GNRH-deficient hpg-mice[119,120], mice lacking functional LHβ[121], FSHβ[122], LH receptor (LHR)[123], FSHR[124,125], or the whole functioning pituitary[126]. Furthermore, case reports suggest that the inactivating mutations in *LHβ*, *FSHβ* or *FSHR* genes do not cause severe disorders of sex development[127–130].

However, testicular Leydig cells are stimulated by human chorionic gonadotropin (hCG) secreted from the placenta and present in high concentrations especially early during pregnancy in humans[131,132]. This seems important for the testicular development, as patients with a completely inactivating mutation in the *LHCGR*, which encodes the shared receptor for LH and hCG, present with a severe disorder of sex development and milder mutations with hypospadias or micropenis[39,133].

2.2.5 Minipuberty

Serum and urine reproductive hormone concentrations including FSH, LH, testosterone, inhibin B and AMH start to rise shortly after birth and peak between 1 and 3 months postnatally[134–138]. This postnatal surge in reproductive hormones is often called minipuberty of infancy, or simply minipuberty. It has been proposed to be triggered by the withdrawal of the negative feedback from the placenta, since towards the end of the third trimester the peak in placental oestrogen concentrations coincide with the decrease in gonadotropin concentrations during the fetal period[139].

Binding of testosterone to sex-hormone binding globulin (SHBG) increased after birth in an early study[136,140], and a decrease, rather than an increase, in testosterone was first demonstrated in saliva postnatally[141]. Thus, the significance and bioactivity of the testosterone levels was initially questioned. However, in a small study serum testosterone concentration and free androgen index correlated with the androgen bioactivity, suggesting that testosterone is indeed active[142]. Furthermore, clear penile and testicular growth was reported during minipuberty, and the penile growth correlated with minipubertal testosterone levels indicating that the peak in androgen levels is biologically significant[143–145]. Finally, reproductive hormone concentrations were associated with the severity of acne and sebaceous gland hypertrophy during minipuberty in a Finnish study[146].

In terms of testicular descent, the majority of cases of cryptorchidism resolve spontaneously during minipuberty[147–151]. In addition, a small study and a case report suggest that boys with congenital cryptorchidism due to hypogonadotropic hypogonadism rarely show testicular descent before supplementation with hCG and FSH or testosterone[152,153]. Serum androgen bioactivity in three-month-old boys with at least one suprascrotal or higher testis (N=16) was non-measurable. In contrast, 26 of the 55 boys who had bilaterally intrascrotal testes (including high scrotal testes) had measurable serum androgen bioactivity[142]. These data suggest that minipuberty may contribute to testicular descent postnatally. However, cryptorchid boys with spontaneous testicular descent seem to show a high rate of testicular ascent later on, suggesting that the effect of minipuberty on testicular descent may be transient[154,155].

Sertoli cell proliferation peaks during minipuberty based on studies of both human cadavers and experimental studies of primates[48,156–159]. This elongates the seminiferous tubules, and results in testicular growth as mentioned above[143,145,160]. Spermatogenesis is not initiated despite the high intratesticular testosterone concentrations[48,145,157,160], probably because ARs are not yet present in immature Sertoli cells[161,162].

In terms of germ cell development, the last gonocytes (often also called prespermatogonia), move from their initial position in the middle of the seminiferous cord to contact the Sertoli cells during minipuberty[43,163]. It has been proposed that adequate hormonal stimulation, androgen in particular, during minipuberty is needed for differentiation of the testicular gonocytes into adult dark spermatogonia, which appears to be a key prognostic step for semen quality in adulthood especially among boys with bilateral cryptorchidism[164–166]. Furthermore, according to Hadziselimovic and Huff, the transformation of gonocytes into adult dark spermatogonia was impaired postnatally among 10 of 12 patients with androgen insensitivity syndrome[167]. However, the published histological observations of patients with androgen insensitivity syndrome or failure of androgen biosynthesis due to enzyme defects in other groups or animal models with AR knock-out do not entirely line up with these observations[168–171]. Furthermore, neonatal suppression of minipuberty with GNRH-antagonists did not appear to reduce the differentiation of the gonocytes to spermatogonia in marmosets[172]. Thus, the significance of both minipuberty and androgens in this respect remains open.

Minipuberty also seems to contribute to masculinization of other organ systems. A small Danish-Finnish study showed that the linear growth rate was slower among patients with congenital hypogonadotropic hypogonadism than in control population during minipuberty[173]. In addition, a large Finnish study revealed that the sexdimorphic difference in height became apparent during minipuberty and the growth velocity was related to serum testosterone concentrations[174]. Minipubertal urine androgen concentrations and minipubertal penile growth also predicted later male-type play behaviour[175,176], suggesting that minipuberty may play a role in the masculinization of the brain.

2.2.6 Childhood and prepuberty

After minipuberty, HPT-axis is silenced by circulating makorin ring finger protein 3 (MKRN3)-levels and possibly other non-gonadal factors[177,178]. During the same interval, Sertoli cells also mature and get prepared for the onset of spermatogenesis. The proportion of Sertoli cells expressing AR gradually increases[161,162], rendering them capable to respond to androgen stimulation later during puberty. This maturation may be influenced by thyroid hormone, FSH and androgens[15].

Before the onset of spermatogenesis during puberty, testicular germ cells are predominantly spermatogonia[156]. A recent meta-analysis suggests that the density of spermatogonia per cross-section or volume decreases until three years, moderately peaks approximately at 6-7 years of age, and remains stable until the substantial increase during the onset of puberty[179]. However, as these calculations do not take

into account the changes in testicular size and seminiferous tubule length, some of the decrease early during childhood may be caused by the spreading of the germ cells due to growth of the seminiferous tubules[179].

2.2.7 Puberty

2.2.7.1 Testicular growth as a marker of pubertal development

In boys, pubertal transition from adolescence to adulthood is marked by substantial changes in size, body composition and voice break. After pioneering work by James Tanner, Andrea Prader and their co-workers, pubertal development has been tracked either by visual inspection of the genitals and pubic hair and comparison with five and six, respectively, stages of development i.e. 'Tanner stages'[180], or the measurement of testicular volume by Prader orchidometer, a set of 12 rotational ellipsoids with a fixed ratio of length to width developed by Prader[181]. During the pubertal development, a wide inter-individual variation at the age of attainment of these developmental milestones emerges[180,182,183].

Based on examinations with orchidometer, Prader and his co-workers noticed that the testicular growth mostly takes place during the year after the testicular volume has reached the size of 5 ml by orchidometer[182]. They further noticed that the period of the fastest testicular growth takes place approximately at the age of 13–14 years, roughly one year before the pubertal growth spurt in stature, and roughly at Tanner pubic hair stage 3 (P3)[182].

A larger longitudinal study confirmed that after the testes reach the volume of ≥3 ml by orchidometer, further growth is noted during the first 6 months in 72 %, during 12 months in 90 % and during 24 months in 100 % of the boys[183]. These findings were replicated by another study in the USA[184]. Furthermore, testicular volume of 3 ml (or 4 ml) is two standard deviations (SD) larger than the mean of prepubertal orchidometer-measured testicular volume[185,186], which implies that a boy with a testicular volume of ≥3 ml by orchidometer has shown pubertal testicular growth with an approximate probability of 97.5%, based on mathematical properties of the normal distribution[16]. Thus, the age at the attainment of ≥3 ml in testicular volume by orchidometer has been conventionally regarded as the first reliable marker of the onset of puberty, and as the clinical definition of the early puberty. However, the attainment of >3ml[187,188], and even >4 ml[189] have also been suggested to mark the onset of puberty in previous studies and textbooks. Furthermore, the calliper-measured testicular length of >25 mm has been equated with pubertal onset[189,190], possibly because it is approximately the length of the 3 ml orchidometer bead[189].

Despite the strong evidence listed above for the use of the Prader orchidometer in the estimation of pubertal onset, it has a high intra and interobserver variation [191]. Unlike ultrasonography, it also systematically overestimates testicular volume compared to water displacement[192]. Testicular volume measured by the Prader orchidometer and by ultrasonography correlate, suggesting that the measurements by the two methods are not entirely random[25,193–195].

A cross-sectional Dutch study utilising ultrasonography showed that median pubertal testicular growth during puberty follows a sigmoid curve[186]. However, inferences on testicular growth pattern of an individual child cannot be made based on cross-sectional data because of the large inter-individual variation in the timing of the pubertal onset, as admitted by the authors of the study[186]. Thus, e.g. it is very likely that the median testicular growth curve is less steep than that of each individual.

2.2.7.2 Pubertal growth in testicular tissue

On tissue level, the fastest testicular growth during puberty results from the increase in seminiferous tubule diameter due to expansion of germ cells during the onset of spermatogenesis[48,156,158,160,196,197]. These changes are preceded by maturation of Sertoli cells, including changes such as an increase in expression of ARs, morphological changes, formation of tight junctions between Sertoli cells, reduced expression of AMH and the changes in secretion of inhibin B[15,161,162,198,199].

2.2.7.3 Reactivation of the hypothalamus-pituitary-gonad axis during puberty

During the pubertal transition, the activity of the hypothalamic GNRH-pulse generator slowly increases causing pulsatile secretion of LH and FSH[178,200]. These LH and FSH pulses, and the consequential increase in circulating testosterone concentrations, are initially much more pronounced nocturnally[201–205]. Along the pubertal development, amplitudes and frequency of FSH and LH pulses increase, and concentrations of these reproductive hormones become more evident also during daytime[201,203–205].

2.2.7.4 Relationship between reproductive hormones and testicular growth

In primates, puberty can be induced precociously by stimulation with GNRH or LH and FSH[158,196], and among human patients with hypogonadotropic hypogonadism either by pulsatile administration of GNRH or a combination of FSH and hCG[206–208].

In treatment of hypogonadotropic hypogonadism, hCG alone is often enough to induce testicular growth, spermatogenesis and fertility[209]. In parallel, constitutively activating mutation in LHCGR usually manifests as precocious puberty between 1 and 4 years of age[210], whereas pubertal development was apparently normal in two case reports of activating FSHR-mutation[211,212]. Thus, LH but not FSH, may independently initiate puberty in primates.

The findings of optimising, but not indispensable role of FSH are largely echoed in studies on receptor knock-out mice, in which *Fshβ* or *Fshr* knock-out mice display reduced testicular growth but normal fertility[122,124,125,213]. This seems to be the case in humans as well based on observations on men homozygous for inactivating mutation in the *FSHR*, among whom the downstream signalling is completely blocked[130,133,214,215]. Those men produced sperm and, although their testicular volume and semen quality were reduced, some of them had even fathered children[130]. Intriguingly, in the presented few case reports inactivating mutations in the *FSHβ* gene seem to always result in infertility[216–219]. This discrepancy cannot be fully explained.

Mice and men with ubiquitous inactivating mutation in AR have female external genitalia and do not show testicular descent or progress beyond spermatocytes in spermatogenesis[107,114,168]. Mice with completely inactivated *Lhβ* gene or *Lhr* lack virilisation during puberty, and show Leydig cells hypoplasia and decreased seminiferous tubules size along with a spermatogenic arrest[121,123]. Testicular biopsies reveal a similar effect on spermatogenesis in humans with *LHβ* mutation[127–129]. However, in humans the disruption of LHCGR produces a more severe disorder of sex development, since it is shared with LH and hCG during fetal period[133].

The similar reproductive phenotype between the LH and androgen deficient mice described above suggest that the effects of LH in adulthood are mediated via intratesticular androgens, which are normally present in concentrations >100-fold higher compared to serum[32]. Experimental studies on mice with testicular cell-specific AR ablation have shown that Leydig cells, Sertoli cells and peritubular cells are all important targets of androgen signalling[49,220–223]. Targeting of AR in any of the three led to markedly impaired spermatogenesis, while complete block in spermatogenesis was observed only in Sertoli and Leydig cell-specific ablations of AR[49,220–223]. A qualitatively complete spermatogenesis with motile spermatozoa can be initiated with prolonged stimulation with testosterone alone in immature crab-eating macaques[224] and in *hpg* rats[225], which lack functional GNRH due to a spontaneous gene mutation[107]. However, administration of exogenous testosterone does not seem to induce spermatogenesis in human patients with hypogonadotropic hypogonadism[226,227]. This is very likely because high enough intratesticular

testosterone levels cannot be reached without the risk of serious side-effects, such as myocardial infarction and arrhythmia[228].

Despite the preceding theoretical discussion of the relative roles of FSH and LH, they display a high degree of synergy in practice. The above-mentioned macaques, who entered puberty precociously due to chronic testosterone stimulation, had severely reduced testicular volumes and their electro-stimulated ejaculates were too small to even allow the quantification of sperm concentration[224]. Therefore, they were very likely infertile[224]. After an 11-day-stimulation with FSH and/or LH, cells more advanced than stem spermatogonia were noticed only among immature rhesus monkeys treated with FSH, and primary spermatocytes only in monkeys treated with LH and FSH[196]. Among humans, augmentation of the conventional hCG treatment of hypogonadotropic hypogonadism with FSH may increase the odds for the induction of spermatogenesis[229,230]. An alternative sequential treatment scheme, in which FSH is initially utilised to maximise the number of Sertoli cells and spermatogonia followed by the induction of spermatogenesis by FSH and hCG, has also yielded some promising results[207,208,231]. Although both approaches are yet experimental, they highlight the importance of FSH for full pubertal reproductive development.

2.2.7.5 Completion of pubertal development

Along with the progress of pubertal testicular growth and development, sperm production starts usually early in puberty, and is detectable in urine approximately at the age of 14 (spermaturia), slightly before peak height velocity[232,233]. Average testicular volume at spermarche is 11.5 ml measured by the Prader orchidometer, and Tanner pubic hair stage is 2–3[232]. However, some subjects have spermaturia already at testicular volumes of 5–8 ml or even <5 ml[233]. In adulthood, an average testicular volume is approximately 24–25 ml measured by the orchidometer and 13–16 ml by ultrasonography[24,25,186]. It has been proposed that the discrepancy between orchidometer and ultrasonography may partially result from the inclusion of surrounding tissue including the epididymis and skin[234]. When the growth in testicular volume has ended, the pubertal transition to adulthood is complete and testes enter the period of maintenance.

2.3 Congenital cryptorchidism

2.3.1 Definitions

'Undescended testis', 'cryptorchidism', 'retentio testis' and 'maldescensus testis' are used somewhat interchangeably when referring to a testis that is not positioned in the scrotum at birth. Cryptorchidism can be unilateral (in 1/2 to 2/3 of the cases) or bilateral[147,149,150], and may persist postnatally or recover spontaneously during minipuberty[147–151]. Cryptorchidism can present as a part of a syndrome such as Klinefelter syndrome, Noonan syndrome or Down syndrome, or as an isolated non-syndromic form[235].

Scorer measured the distance from the midpoint of the testes to the pubic tubercle among 3 500 new-born infants[148]. Although he did not actually report the measurements, he made a remark that 'testis is usually lying in the scrotum at a distance of between 5 and 8 cm from the pubic crest'[148]. Thus, he defined testes positioned within < 4 cm (2.5 cm in preterm infants) from the pubic bone as undescended. He also discussed the spectrum of testicular positions between abdominal and fully descended testes, describing the abdominal, inguinal, suprascrotal and high scrotal testes.

A succeeding British cohort study undertaken at John Radcliffe Hospital modified the original Scorer criteria (shown in Figure 5) faithfully following Scorer's description of degrees of descent and considered only low-scrotal testes normal[147]. However, these two criteria agreed in 99.3% of all examinations, and in 87.7% of the examinations when either of the two examinations suggested that the boy had at least one undescended testis[147]. The modified criteria have been adopted by most of the large prospective cohort studies[149,150,236–238].

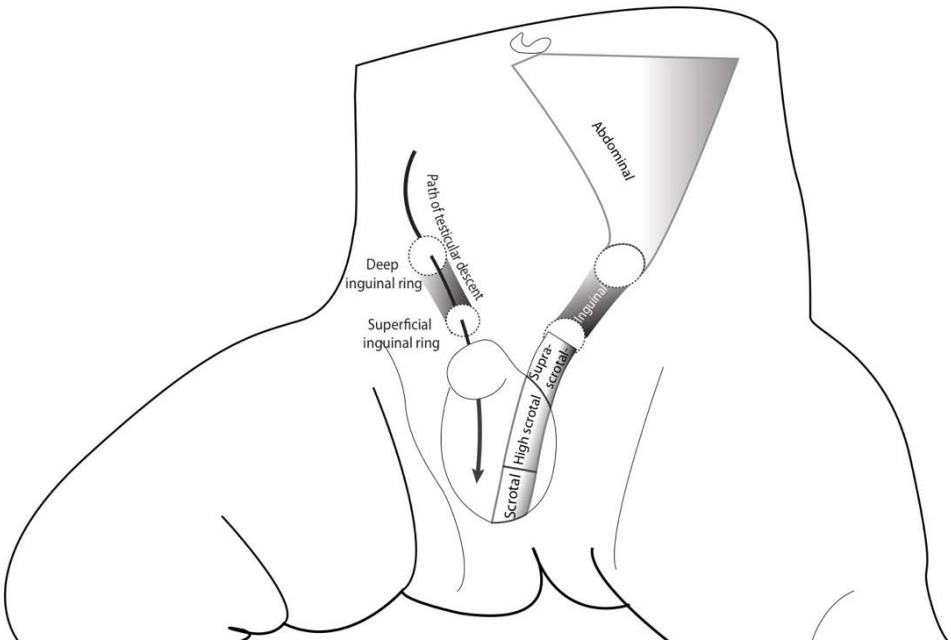

Figure 5 Testicular position by John Radcliffe Hospital Cryptorchidism Study Group criteria

Substantially less is known about normal testicular position later during childhood. In two longitudinal studies, new cases of undescended testis were recognised later during childhood among those boys who had normally descended testes at birth[150,239]. This 'testicular ascent' is also often called 'acquired cryptorchidism' or 'acquired undescended testis'[240].

Among some boys, pronounced cremaster reflex may draw testis from the scrotum during the examination, even though the testicular cord allows testes to be manipulated into scrotum without tension[241]. This 'retractile testis' is generally considered a normal variant[117,149,240], although a follow-up with repeated examinations of testicular position is commonly recommended to recognise testicular ascent[239,242,243].

2.3.2 Prevalence and trends

Prospective population-based studies with similar and clearly stated definitions of cryptorchidism provide the most robust estimate of prevalence and the possible temporal and geographical differences in the prevalence of congenital cryptorchidism[244]. In studies published in the 21st century, the prevalence of cryptorchidism was lowest at birth in Finland (2.4%) and highest in Denmark (9.0%), whereas Lithuania (5.7%), the United Kingdom (5.9%) and Italy (6.9%) are between these

two extremes[149,150,236,238]. Temporally, an increasing trend is apparent in Denmark and the United Kingdom, where multiple such studies exist[147–150,245].

2.3.3 Risk factors

Various risk factors for cryptorchidism have been reported, although many of them do not seem very consistent across studies[246]. This underscores the possible interrelationship between many of these risk factors and possible differences in the methods of collecting risk factor data (e.g. registries, interviews or questionnaires), or ascertainment of the cases of cryptorchidism (maternally reported, prospectively observed or based on retrospective data of cryptorchidism or orchidopexy).

Some of these risk factors relate to overall characteristics of the parents including paternal and maternal age[247,248], maternal health during pregnancy including preeclampsia and maternal gestational diabetes[247–250], lifestyle during pregnancy including smoking[250,251], alcohol consumption[252,253] and possibly caffeine intake[254]. In addition, the use of mild analgesics during pregnancy, paracetamol (acetaminophen) in particular, was associated with the risk of cryptorchidism in three studies[255–257]. Among obstetric data, low birth weight[238,248], breech presentation[238,248] and low placental weight are risk factors for cryptorchidism[247,258]. Furthermore, a shorter length of gestation and a lower weight for gestational age were both associated with a higher risk of cryptorchidism in four observational studies[147,238,247,248]. Among other congenital malformations, hypospadias and inguinal hernia are more common in boys with congenital cryptorchidism than in non-cryptorchid boys[147,247,248].

2.3.4 Consequences and treatment

According to a recent meta-analysis, non-syndromic cryptorchidism is associated with a risk ratio of 2.9 of testicular germ-cell cancer compared to general population[259]. In addition, especially men with a history of bilateral cryptorchidism seem to have elevated circulating FSH and reduced inhibin B concentrations and paternity rates in adulthood[260]. In contrast, men with a history of unilateral cryptorchidism appear to have normal paternity rates[26], but nevertheless display a pattern of Sertoli cell dysfunction including reduced testicular volume and serum inhibin B and elevated serum FSH[26,261]. However, in these studies men were operated for congenital cryptorchidism later than currently suggested, which may affect the testicular function in adulthood (see below).

Previous observational studies utilising various metrics including paternity rates, semen and hormonal analyses (FSH and inhibin B) in adulthood as well as analyses of histological sections taken in surgery (orchidopexy, i.e. surgical fixation of the testes to the scrotum) suggest that an early orchidopexy is beneficial for the reproductive function[262–264]. In the only randomised-controlled trial so far, unilaterally cryptorchid boys who were operated at 9 months showed testicular catch-up growth between 9 months and 2 years compared to the controls who were scheduled for orchidopexy at 3 years[265], whereas no catch-up growth was evident after orchidopexy among the latter group[266].

A Swedish registry study based on 16 983 men and a meta-analysis including five studies on the association between age at orchidopexy and testicular germ-cell cancer indicated that lower age at orchidopexy may reduce the risk of testicular germ-cell cancer[267,268]. However, the results could not be replicated in the Danish national registry including 21 488 men operated for cryptorchidism[269]. Thus, it remains uncertain whether the early orchidopexy reduces the risk of testicular germ-cell cancer.

The adverse postnatal development in cryptorchidism is largely thought to result from prolonged exposure to higher temperatures compared to the scrotum[270]. In prepubertal mice Leydig cells, steroidogenesis is subnormal at 37 degrees *in vitro* and in non-scrotal testicular position *in vivo*[271]. However, circulating testosterone concentrations did not consistently differ between cryptorchid boys and controls during minipuberty in most of the recent studies[142,272–275], suggesting that such a postnatal effect on steroidogenesis is not sufficient enough to explain the pathogenesis and adverse postnatal reproductive development in congenital cryptorchidism. Thus, it has been proposed that cryptorchidism is a sign of a primary testicular dysgenesis (reviewed in 2.3.6).

2.3.5 Etiology of congenital cryptorchidism

The geographical differences and the relatively rapid increase in prevalence of congenital cryptorchidism suggest that environmental factors play a salient role in the etiology of congenital cryptorchidism[147–150,245]. In addition, a recent large genome-wide association study failed to identify a reproducible association with genetic loci and cryptorchidism[276]. Finally, a Danish registry-based study of 1 024 500 Danish boys showed that the concordance of cryptorchidism is higher in maternal vs paternal half-brothers (6.0% vs 3.4%), higher in dizygotic twin brothers vs brothers (24.1% vs 8.8%), but comparable in dizygotic vs monozygotic twin brothers (24.1% vs 27.3%)[277]. These results suggest that the maternal intrauterine

milieu seems to determine the risk of cryptorchidism, although genetic factors, especially maternal, may contribute as well.

2.3.5.1 Postnatal hypothalamus-pituitary-Leydig cell axis

As described in section 2.2, INSL3 and testosterone secreted from fetal Leydig cells are indispensable for testicular descent in mice[112–115]. In humans, INSL3 was measurable in amniotic fluid and displayed a high degree of sex-dimorphism[278]. The role of mutations in *INSL3*[279–281] or its receptor *RXFP2* (relaxin/insulin-like family peptide receptor 2)[280,282] in the etiology cryptorchidism seems limited in humans, although the latter was more common among cryptorchid boys vs controls in an Italian study[282]. Nevertheless, INSL3 concentrations in cord blood differed between cryptorchid and healthy boys at birth[283,284], whereas serum LH/INSL3-ratio, but not INSL3 concentrations, differed between cryptorchid boys and controls at three months[284]. Thus, dysfunction of INSL3/RXFP2 signalling may be commonly involved in pathogenesis of congenital cryptorchidism also in humans. However, it is more likely modulated by environmental factors instead of simple loss-of-function mutations[235].

Testicular descent is disrupted in humans in complete androgen insensitivity syndrome, which is caused by the inactivation of *AR* due to a gene mutation[109,114]. Small Dutch (43 cryptorchid boys and 113 controls) and Finnish studies (11 cryptorchid boys and 23 boys with scrotal or high scrotal testes), reported lower concentrations of total testosterone among cryptorchid boys compared to controls at the age of 1–6 months and three months, respectively, and the latter study also observed a lower androgen bioactivity among 16 cryptorchid boys (N=64, including 9 boys with high scrotal testes)[142,272]. However, testosterone concentrations at birth or at the age of three months did not differ between congenitally cryptorchid and healthy boys in majority of the larger studies[273–275,283]. Thus, testosterone concentrations seem generally high enough to permit testicular descent among most cryptorchid boys.

Cryptorchidism is very common among patients with congenital hypogonadotropic hypogonadism, especially in boys with Kallman syndrome (48%) and in familial cases of hypogonadotropic hypogonadism (71%)[285]. Supplementation of boys with hypogonadotropic hypogonadism with recombinant LH and FSH can induce testicular descent[152]. However, hypogonadotropic hypogonadism is rare and thus not a very common cause of cryptorchidism.

Serum LH concentrations at three months were higher among Finnish cryptorchid boys (N=88) vs controls (N=300) in our Danish-Finnish cohort, whereas the difference was similar but not statistically significant in a smaller Danish cohort (34

cryptorchid boys and 399 controls)[274]. Dutch (41 cryptorchid boys and 113 controls) and American cohorts (20 cryptorchid boys and 26 controls) reported no association between LH and cryptorchidism, but they may have lacked statistical power due to smaller study sizes[272,273]. An older Dutch study undertaken before the era of immunofluorometric assays did not observe an association between cryptorchidism and LH, possibly because it lacked the analytical power due to the tenfold lower sensitivity of the previous generation of LH assays[274,275]. In terms of placental regulation of fetal Leydig cell function, low placental weight was a risk factor of cryptorchidism in a Danish registry-based study[258], and a recent study showed that low hCG levels may predispose to cryptorchidism[286].

2.3.5.2 Postnatal hypothalamus-pituitary-Sertoli cell axis

During childhood, serum inhibin B originates mostly from testicular Sertoli cells and correlates with testicular volume[143,199]. Experimental manipulation of Sertoli cell number by unilateral orchiectomy in adult rhesus monkeys or administration of GNRH agonists in rats is reflected in decreased serum inhibin B concentrations[287,288], and testicular volume and spermatogenic function in adulthood correlate with serum inhibin B[289]. Thus, serum inhibin B is considered a marker of Sertoli cell function in childhood[290,291]. Recently, circulating AMH derived from Sertoli cells has been increasingly used as a biomarker of immature Sertoli cell function among prepubertal boys[291,292].

In our Danish-Finnish cohort, serum inhibin B levels were reduced and FSH elevated postnatally at the age of three months among Finnish boys with congenital cryptorchidism (N=88) compared to controls (N=388)[274]. However, in the smaller Danish cohort only the difference in FSH was significant between cryptorchid boys (N=34) and controls (N=433)[274]. Similar to the results with LH, smaller Dutch and American cohorts did not observe any difference in serum inhibin B or FSH levels between cryptorchid and healthy boys[272,273]. Boys with congenital cryptorchidism had lower serum AMH levels in three independent studies[293–295], although in these studies most of the subjects were 1–2 years old or even older. Thus the possible adverse effect of the abnormal testicular position cannot be ruled out[265,266,296].

In conclusion, cryptorchidism seems to be associated with a variable degree of primary Sertoli and possibly Leydig cell dysfunction. However, this dysfunction may be compensated by higher FSH and LH concentrations, and is apparent only in larger studies. The possible causes of this primary testicular dysgenesis are discussed next.

2.3.6 Testicular dysgenesis syndrome as a cause of congenital cryptorchidism

In an article published in 2001, Skakkebæk et al. proposed that several reproductive disorders including cryptorchidism are manifestations of an entity called testicular dysgenesis syndrome (TDS)[297]. In addition to cryptorchidism, these hallmarks of TDS would include the incomplete fusion of the urethral folds (hypospadias), testicular germ-cell cancer and declined semen quality[13,298].

In humans, TDS hypothesis is supported by a similar pattern in the prevalence cryptorchidism[244] and hypospadias[244,299–301] in prospective population-based cohort studies in Northern Europe and prevalence of testicular germ-cell cancer in registry-based data[9,10]. Furthermore, a similar pattern was apparent in semen quality especially in the early 2000s when multiple studies were published within a short time frame[302].

Finally, associations have been reported between cryptorchidism and hypospadias[147,247], cryptorchidism and testicular germ-cell cancer[259,303,304], cryptorchidism and declined fertility[260,305], hypospadias and testicular germ-cell cancer[303,304] and testicular germ-cell cancer and reduced semen quality[306,307], but not between isolated hypospadias and reduced semen quality[308].

Although all human evidence for TDS so far has been observational, a discovery that experimental reduction of intratesticular testosterone concentrations by developmental dibutyl phthalate exposure (500–750 mg/kg/d) reproduces the TDS hallmarks (except for testicular germ-cell cancer) in rats in high incidence, has allowed elaboration on the biological background of the TDS hypothesis[91,309]. There are no reports of testicular germ-cell cancer in rats[310]. However, the histological changes observed in this model resemble those seen in human patients with germ-cell neoplasia *in situ*, a precursor lesion of the invasive testicular germ-cell cancer, which is thought to have a prenatal origin[91].

These histological changes appear as central aggregation of Leydig cells and appearance of ectopic Sertoli and germ cells within prenatal testis. Postnatally, these clusters of ectopic Sertoli cells start to form seminiferous tubules that later appear anastomotic and occasionally contain intratubular Leydig cells[91,99]. Histological characterisation of the ectopic Sertoli cells suggests that they do not originate from *de novo* transformation of the precursors of Sertoli or Leydig cells, but possibly from rupture of seminiferous tubules[99]. However, these changes can currently be visualised only histologically, which prevents longitudinal analyses and thus the conclusive proof that these changes indeed develop across time.

A case report of the three related patients, who inherited a hemizygous mutation in *AR* gene, presents an example of TDS[311]. In the affected individuals, a single

point mutation reduced the ability of DHT to induce AR-mediated activation of a reporter gene by 50% compared to wild type AR within physiological concentrations of DHT. All the three subjects had high circulating LH and testosterone. Two of the subjects who were adults had high circulating concentrations of FSH, and low inhibin B, indicating a Sertoli cell dysfunction. They also had a very low sperm concentration, and developed testicular germ-cell cancer. One of the two had a history of unilateral transient cryptorchidism and the other had undergone surgery for hypospadias. The third patient was only 15 at the clinical work-up, and thus the full analysis of adult reproductive phenotype was not possible, however he had a mild glanular hypospadias.

2.3.7 Etiology, prevalence and treatment of acquired cryptorchidism

Compared to congenital cryptorchidism, substantially less is known about acquired cryptorchidism. Based on longitudinal cohort studies, the incidence of acquired cryptorchidism is high (up to 4%) at the age of 12 months, and lower (0.6–1.3%) later until the age of three years, depending on the criteria used[150,239]. Cross-sectional Dutch data suggests that the prevalence of acquired cryptorchidism is 1.1–2.2% between 6 and 13 years of age[312].

Especially boys with spontaneously resolved congenital cryptorchidism or retractile testes have a high rate of testicular ascent[154,155]. Thus, it has been proposed that testicular ascent may be a late presentation of a 'borderline' case of congenital cryptorchidism, which becomes evident as a boy grows[313]. Alternatively, a fibrous remnant of the processus vaginalis may hinder the growth of the spermatic cord and thus cause testicular ascent[116,117]. There is also some indirect evidence that subnormal androgen concentrations may predispose to acquired cryptorchidism. Testicular ascent was more common among boys with hypospadias in retrospective data[314,315], and a prospective longitudinal cohort suggests that boys who later acquired cryptorchidism had reduced penile growth during minipuberty[150]. Thus, low prenatal or postnatal androgen concentrations might be associated with acquired cryptorchidism.

The optimal approach to the treatment of acquired cryptorchidism remains unknown, and no randomised-controlled trials exist to judge whether orchidopexy is necessary. However, current American and Nordic guidelines recommend orchidopexy[242,243], whereas Dutch researchers advocate a conservative 'wait and see' policy and only recommend operation if the testis does not show a spontaneous descent during puberty[313].

2.4 Effects of the endocrine disrupting chemicals on testicular growth and descent

2.4.1 Overview of the endocrine disruptors

International Panel on Chemical Safety (ICPS) defined an endocrine disruptor in 2002 as *'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.'*[316]. This definition has been widely used, and was adhered to e.g. by the successive systematic reviews by World Health Organization (WHO)/United Nations Environment Programme (UNEP)[19], and the Endocrine Society[17].

Currently, approximately 85,000 synthetic chemicals have been registered for the use in the US under Toxic Substances Control Act (TSCA)[317], and at least 9 868 of those are still in active commercial use according to the US Environmental Protection Agency non-confidential TSCA inventory of chemical substances updated in June 2017[318]. In terms of reproductive toxicity, a quantitative structure-activity modelling revealed that 800–2 560 known synthetic chemicals may interfere with binding of the AR[319].

Recent studies estimated that the annual economic burden of the exposure to endocrine disruptors is 163 billion Euros in the European Union (EU) (217 billion US dollars, 1.28% of the total gross domestic product), and 340 billion US dollars in the USA (2.33% of the gross domestic product)[18,320,321]. The burden is caused both by costs of treatments due to increased morbidity and lost productivity, and is predominantly caused by impaired neurodevelopment, although reproductive toxicity plays a role as well[320]. The transatlantic difference in financial burden is thought to be mostly driven by the differences in exposure to polybrominated diphenyl ethers due to differences in fire safety standards[321].

2.4.2 Toxicological special characteristics of endocrine disruptors

According to a widely-accepted dogma of toxicology that the dose makes a poison, a quote often attributed to a controversial 16th century physician Paracelsus[322], suggests that each substance is toxic at high doses and safe below a certain threshold[323]. The recent research in environmental toxicology suggest that this assumption is not valid in terms of reproductive toxicology of environmental endocrine disruptors, unless a few additional caveats are considered.

Firstly, the susceptibility of an individual to adverse effects of endocrine disruptors varies with age. As reviewed in the previous section, experimental studies suggest that in terms of reproductive toxicity male foetuses are the most susceptible during gestational weeks 8-14[51]. Although the same substances may induce only a minor reversible effective in adulthood with same doses per bodyweight, exposure during this interval can result in irreversible changes.

Secondly, the principle of additivity strongly suggests that even exposure that itself is 'safe' may present a hazard for health and well-being when combined with other exposures[324]. In an *in vivo* or *in vitro* experiment, the adverse effects or receptor binding observed after the exposure to endocrine disruptors may greatly decrease with the smaller doses, and usually become indistinguishable of that in the controls. However, exposure to multiple anti-androgenic endocrine disruptors with similar modes of action in these 'safe' concentrations yielded significant adverse effects, which could be predicted by the dose-response curves of the individual chemicals[325,326]. Further studies demonstrated that the shared mode of action (e.g. antiandrogens versus inhibitors of testosterone synthesis)[327,328] or even shared toxicological signalling pathway (e.g. dioxin-induced induction of AhR signalling versus phthalate-induced inhibition of testosterone synthesis) is not required to cause additive adverse effects in the common downstream target organs[329]. In addition, some authors argue that most toxic substances display non-monotonic dose-response curves and thus low-dose actions cannot be considered safe based on the lack of effects in high doses[330]. However, this remains controversial[331].

Thirdly, the susceptibility may vary substantially between individuals because of genetic polymorphisms involved in the metabolism of endocrine disruptors[332], or due to genetic or lifestyle factors related to the baseline health effects that predispose to a given illness such as infertility[333]. Thus, the adverse health effects after exposure may increase approximately linearly without a clear threshold in a population even if in each individual the mechanism or mode of action was threshold-dependent[333].

2.4.3 Characteristics, trends and exposure routes of persistent organic pollutants

Polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs, or more loosely 'dioxins'), polychlorinated biphenyls (PCBs) and polybrominated biphenyl diethers (PBDEs) are synthetic chemicals that are highly lipophilic. They bioaccumulate in the ecosystem, and have long biological half-lives. These chemicals, along with other chemicals such as organochlorine pesticides, are called persistent organic pollutants (POPs)[334,335].

There are altogether 210 PCDD/F, 209 PCB and 209 PBDE congeners[336–338]. However, only 130 PCB congeners are likely to occur in commercial products[337]. PCDD/Fs can be grouped from mono to octa-chlorinated dibenzo-*p*-dioxin/furan (CDD/F) and PBDEs congeners from mono to deca-brominated diphenyl ethers (BDEs) based on the number of the chlorine or bromine atoms, respectively[336,338]. Although a similar naming scheme of mono to decachlorobiphenyl exists for PCBs, IUPAC (International Union of Pure and Applied Chemistry) numbering of PCB congeners from 1 to 209 (e.g. PCB1–3 are monochlorodiphenyls, PCB105–127 are pentachlorodiphenyls, and PCB209 is a decachlorodiphenyl), is more commonly used[339].

PCBs are thermally stable, and were thus originally manufactured for use in electrical equipment such as dielectric and heat-exchange fluids in capacitors and transformers since 1930[337]. PBDEs were intentionally manufactured and used as flame retardants in textiles and electrical devices[338]. PBDE congeners with larger number of bromine atoms can debrominate, thus forming less brominated congeners[340]. In contrast, PCDD/Fs were not intentionally produced, but mostly formed during combustion in industrial processes and incineration of waste[336].

The concentrations of PCBs and PCDD/Fs were alarmingly high in humans in the 1980s[341–343]. This hazard slowly provided an impetus for international action to reduce the production of these chemicals. The use of PCBs was phased out in the US in 1979[344] and the use and marketing of PCBs were heavily restricted in the European Community in 1985[345]. In 2004, the UNEP-backed intergovernmental Stockholm convention entered into force, effectively banning production, import, export and use of PCBs and nine other persistent organic pollutants in participant states with some exemptions, and obliged the participant states to take steps to reduce emissions of PCDD/F congeners[334,346]. The concentrations of PCBs and PCDD/Fs have thus gradually declined[341–343].

In terms of PBDEs, an EU directive banned the production of penta and octaBDE in 2003 but allowed the production and use of decaBDE to continue temporarily while the risk posed to the population was evaluated[347]. For a while, the ban of decaBDEs as flame retardants in polymers in electrical devices was exempted in an EU directive on grounds of the alleged technical impracticality of the alternative flame retardants[348], until EU Court of Justice ruled against the exemption in 2010[349]. Tetra, penta, hexa and heptaBDE congeners were considered persistent organic pollutants in the revision of Stockholm convention in 2009, and decaBDEs in May 2017[335], thus effectively ending the global production and use of PBDEs in the long-term.

Human exposure to of PBDE congeners increased substantially from 1970s until the late 1990s or early 2000s in Northern Europe[350]. Concentrations of PBDEs

have stabilised or slightly decreased since the early 2000s[350–353]. Recent studies suggest that the PBDE congeners with lower number of bromine atoms have clearly declined, while higher brominated congeners have remained stable or even increased[354,355]. This may reflect the slower process of legislative action against decaBDE compared to tetra and pentaBDE.

Despite the eventual success in global regulation, these contaminants are present in the ecosystem for years to come due to their toxicological profile. Human infants are predominantly exposed via lactation[356,357], although especially in terms of PBDEs the exposure via indoor dust and air is substantial since they leach to the indoor environment also from electrical devices[358]. In adulthood, most of the exposure occurs via lipid-rich foods of animal origin including dairy products, red meat and fish[359,360].

2.4.4 Evidence for reproductive toxicity of PDBEs

Although toxicity of PBDEs was initially recognised in the context of thyroid development and neurodevelopment[361,362], many recent animal and human studies have reported reproductive effects of exposure to PBDEs.

DE-71, a commercial mixture containing predominantly penta and tetraBDEs bind the AR and increased the basal, but not hCG-stimulated, testosterone levels in rat adult Leydig cells *in vitro* [363,364]. In parallel, among adult human sport fishers, serum levels of PBDE49 (a tetraBDE), correlated positively with serum testosterone[365], whereas a European multicentre study did not observe any association between adulthood reproductive hormone concentrations and serum levels of PBDE47 (a tetraBDE)[366].

However, exposure of the adult castrated and testosterone-treated rats to DE-71 showed a decrease in androgen-dependent organ weights[364,367]. Furthermore, prenatal or perinatal exposure to PBDE47 (a tetraBDE) or PBDE99 (a pentaBDE) reduced AGD, testicular weight and semen quality compared to controls in rats, but the serum levels of testosterone did not differ in adulthood[368–370]. In contrast to tetra or pentaBDEs, both experimental and human evidence suggests an inverse correlation between exposure to decaBDE (PBDE209) and serum testosterone concentrations[371,372]. These results indicate that the toxicological profiles of PBDE congeners may vary, and highlights the increased susceptibility during the important perinatal period for reproductive development.

An experimental study evaluated the association between testicular descent and prenatal exposure to PBDEs (PBDE99, a pentaBDE), and it did not report any

delay[368]. In humans, however, our previous Danish-Finnish study observed an association between cryptorchidism and the levels of PBDEs in breast milk, and a recent Canadian study between congenital cryptorchidism and levels of PBDEs in paediatric hair[373,374]. The analysis of placental samples in the same Danish-Finnish cohort showed lower concentrations of PBDEs compared to breast milk samples and a lack of association between cryptorchidism and PBDEs[373].

2.4.5 Evidence for reproductive toxicity of PCDD/Fs

In general, most of the toxicity of PCDD/Fs is believed to occur via genomic pathway by binding the aryl hydrocarbon receptor (AhR)[375], which forms a dimer with the AhR nuclear translocator protein (ARNT) and translocates to the nucleus[376]. This dimer then upregulates the transcription of various target genes[377]. In addition to this genomic pathway, an alternative non-genomic pathway of dioxin toxicity has been described, in which the actions may take place without the binding of AhR to ARNT[378].

There are substantial species and strain differences in susceptibility to acute toxic effects of dioxins including lethality and wasting syndrome[379–381]. These differences may be explained by polymorphisms in *Ahr* gene[382], as experimental targeting of *Ahr* renders the mice resistant to dioxin-induced teratogenesis as well as liver and thymus toxicity[383–385]. However, it also interferes with various aspects of development including liver, spleen, thymus and female reproductive development, suggesting that AhR has also a physiological function[383,386,387].

Based on expert panel opinion on *in vivo* and *in vitro* data, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the strongest activator of AhR, while other PCDD/F congeners and some PCB congeners possess smaller relative effect potentials[388,389]. Together with an assumption of additivity, this allows calculation of a WHO-recommended 2,3,7,8-TCDD toxic equivalent quantity (WHO-TEq) of mixtures such as human samples, which contain multiple PCDD/F and PCB congeners that are structurally similar to 2,3,7,8-TCDD and bind AhR[388,389]. This is done by multiplying concentrations of each congener with their respective WHO-recommended toxic equivalency factor and summing up the products.

Testicular descent is delayed or disrupted after prenatal exposure to 2,3,7,8-TCDD in rats and swine[390,391]. In humans, the estimated paternal occupational exposure to 2,3,7,8-TCDD contaminated chlorophenol during pregnancy was associated with an increased risk of undescended testis among offspring of sawmill workers

in British Columbia[392]. Furthermore, the milk from Danish, but not Finnish, mothers of cryptorchid boys had a higher sum and WHO-TEq of 17 PCDD/Fs compared to controls[393].

The delay in testicular descent after exposure to PCDD/Fs seems to be caused by reduced androgen action. Multiple reports confirm that the exposure to 2,3,7,8-TCDD reduces the AGD[394–396], suggesting that the net effect of the prenatal dioxin exposure is antiandrogenic [51]. Some studies have also found evidence of reduction in fetal testicular and plasma androgen levels[394,397], and reduced weights of androgen-dependent accessory sex glands after exposure to 2,3,7,8-TCDD[394–396,398]. The decrease in testosterone levels may be caused by a reduction in transcription of steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes P450SCC and 3βHSD1[397]. Structural variation in AhR seems to affect the changes in fetal testosterone levels after exposure 2,3,7,8-TCDD in some[399], but not all studies[400,401]. Polymorphisms in *Ahr* gene also seem to affect the susceptibility to reduced semen quality after 2,3,7,8-TCDD exposure[400].

Both experimental animal studies and observational human studies illustrate that the reproductive toxicity of PCDD/Fs is not limited to cryptorchidism. Following an industrial accident in Seveso, Italy, in 1976, population living near the factory was exposed to high concentrations of 2,3,7,8-TCDD. Compared to controls, male offspring of the exposed mothers had reduced semen quality in adulthood[402]. Unlike breastfed boys, male offspring of highly exposed mothers who were formula-fed had normal semen quality[402]. Boys who were 1–9 years old at the time of the accident also had reduced semen quality compared to controls, whereas boys who were 10–17 years had normal semen quality in adulthood[403]. In addition, a recent Russian Children's Study in Chapaevsk, Russia showed that peripubertal serum PCDD WHO-TEQ was associated with lower sperm concentration but not with motility[404]. Due to the long half-lives of these congeners, these high peripubertal levels of dioxins may represent the exposure during childhood.

2.4.6 Evidence for reproductive toxicity of PCBs

Generally, the toxicity of most PCB congeners is thought to occur via AhR as in PCDD/Fs[405]. Thus, the reproductive toxicity could be inferred from the studies utilising 2,3,7,8-TCDD. However, some PCB congeners are also antiandrogenic in vitro[406]. One study showed that the gestational exposure of rats to PCBs slightly delayed testicular descent[407]. Another report showed that prenatal exposure to PCBs decreased AGD and seminal vesicle weight compared to controls, but increased testicular size and sperm production in adulthood[408]. The authors attributed this surprising finding to the possible induction of hypothyroidism[408], which delays

the Sertoli cell maturation and thus expands the window of prepubertal Sertoli cell proliferation[15].

Exposure to PCBs has been well studied in humans. A large US study did not find evidence for an association between congenital cryptorchidism and concentrations of 11 PCB congeners in maternal serum[409]. Neither a French nor a Faroese study found a difference between cryptorchid boys and controls in seven PCB congeners in cord blood or three PCB congeners in umbilical cord samples, respectively[237,410]. Furthermore, the French study and a Finnish study did not find an association between congenital cryptorchidism and PCB concentrations in breast milk[237,393], whereas among Danes the sum of PCBs was lower in milk of the mothers of cryptorchid boys compared to their controls[393]. Finally, a German study did not observe a difference between the levels of PCBs in adipose tissue of cryptorchid boys versus controls[411].

These studies suggest that the association between congenital cryptorchidism and exposure to PCBs seems rather weak. However, similar to PCDD/Fs, there is evidence for impaired sperm morphology among men exposed to high levels of PCBs and PCDFs *in utero* or during childhood after the accidental rice oil poisoning in Taiwan in 1978–1979, although the sperm concentrations did not differ[412,413].

3 AIMS OF THE STUDY

1. To examine postnatal changes in testicular position and their relationship with the serum reproductive hormone and IGF-I concentrations at the age of three months (study I.).

2. To explore the relationship between the exposure to PCBs, PCDD/Fs and PBDEs and the risk of congenital cryptorchidism (studies II. and III.)

3. To elucidate the dynamics of the pubertal testicular growth among boys with and without a history of congenital cryptorchidism (study IV.).

4. To compare ultrasonography, Prader orchidometer and ruler in estimation of testicular volume and pubertal onset (study IV.).

4 MATERIALS AND METHODS

4.1 Subjects

4.1.1 The Danish-Finnish birth cohort

A Danish-Finnish birth cohort was originally designed by professor Niels E. Skakkebæk, professor Jorma Toppari and professor Katharina M. Main to compare the prevalence of congenital cryptorchidism and hypospadias between Denmark and Finland[149,299,300]. In both countries, only those families in which the upcoming parents and grandparents were born and raised within the country and had not lived abroad for more than three years (mothers) or 10 years (grandparents and fathers) were included. This was done to obtain a genetically well characterised population and minimise the effect of environmental variability on the outcomes of the study.

In Denmark, all the eligible families who had a Danish surname were contacted during the first trimester of the pregnancy, and 22% (N = 2 229) of the eligible families agreed to participate. The enrolled mothers gave birth to 1 072 live-born boys. However, one boy later requested to have all his examinations excluded, after which data of 1 071 subjects were available[149,239]. In Finland, 24% of all the eligible families (N=2 728) agreed to participate and the enrolled mothers gave birth to 1 494 live-born sons.

In addition to the cohort of prenatally recruited boys, in Turku University Hospital, Finland, neonatologists, paediatric surgeons, nurses and midwives were asked to refer all the cases of congenital cryptorchidism or hypospadias to the study personnel for the verification of the diagnosis[149]. This was done in order to form a separate total hospital cohort, which was used to validate that the prevalence of congenital cryptorchidism was similar also among those who declined to participate in the prenatally recruited cohort. Both cohorts provided comparable rates of congenital cryptorchidism among full-term boys (1.9% in the prenatally recruited cohort, 2.1 % in the total hospital cohort)[149].

In both countries, a boy was defined cryptorchid if one or both testes were in high scrotal, suprascrotal, inguinal or non-palpable position per modified Scorer criteria, whereas retractile testes were considered a normal variant[149]. In the Danish cohort, the prenatally recruited boys were examined at birth (expected date of delivery among preterm boys), three months after the expected date of delivery, at 18 months, at 36 months and later once during childhood approximately at the age of 7 years (range 4.5–9.7 years). In the Finnish cohort, all the participants were

invited to an examination at birth (preterm cryptorchid boys also at the expected date of delivery) and at three months (preterm boys three months after the expected date of delivery). Postterm boys were examined at the age of three months in the Finnish cohort and three months after the expected date of delivery in the Danish cohort. In Finland, all the cryptorchid boys in both the prenatally recruited cohort and the total hospital cohort were invited to be examined at the age of 18 months. However, for funding reasons, only two matched controls of the cryptorchid boys and every 10th boy without cryptorchidism or hypospadias were invited for an examination at the age of 18 months. The matching criteria were date of birth (± 14 days), parity, gestational age (±7 days), maternal smoking during pregnancy (yes or no) and maternal diabetes mellitus (yes or no). Although the enrolment of the boys to the prenatally recruited cohort ended 1.1.2000, the boys with congenital cryptorchidism and hypospadias were still recruited postnatally in the paediatric and obstetric departments (during 1.1.2000–28.2.2002). Non-cryptorchid boys were therefore recruited as matched controls in the obstetric department with the above-mentioned matching criteria during the same interval.

Various biomarkers have been compared between the cryptorchid and non-cryptorchid boys and/or Finnish and Danish boys based on this cohort, including cord-blood and serum INSL3 concentrations at birth and three months[284], serum reproductive hormone concentrations at three months[274], as well as testicular size and penile length during childhood[143,144]. Furthermore, we have assessed the exposure to environmental or pharmacological agents with known or hypothesised toxic properties[252,255,373,393,414–418]. In the Danish cohort, also IGF-I (insulin-like growth factor 1) concentrations have been reported[419].

All the studies included in this thesis are at least partially based on the Danish-Finnish birth cohort. The interrelationship between the study populations used in the studies are schematically illustrated in Figure 6, and explained in the following paragraphs.

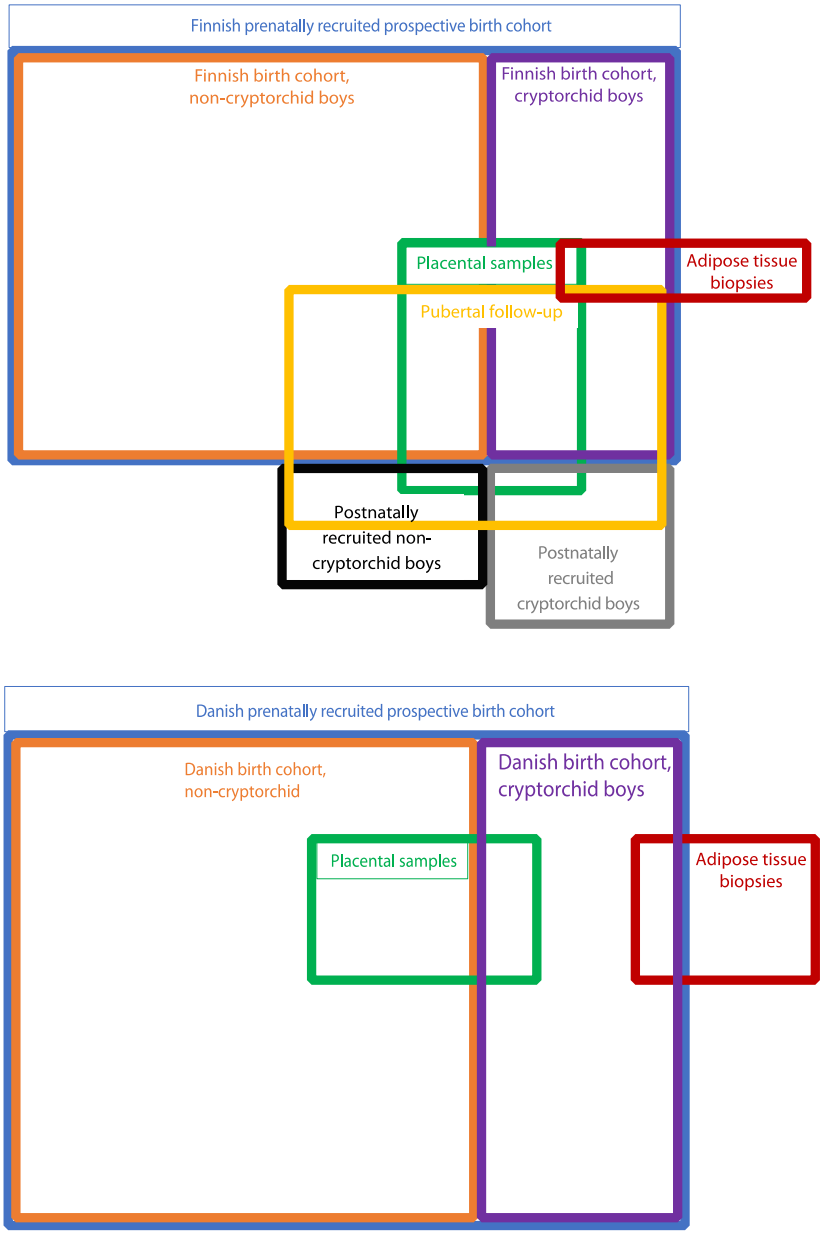

Figure 6 Interrelationship between study populations in studies I.-IV.

Antenatally recruited prospective birth cohort (blue) took place in Turku University Hospital, Finland in 1997–1999 and in Rigshospitalet, Denmark 1997–2001. The antenatally recruited birth cohorts included both cryptorchid (orange) and non-cryptorchid boys (violet). In addition, obstetric and paediatric departments reported the suspected cases of congenital cryptorchidism (grey) to our study group for ascertainment of the diagnoses in Turku University Hospital in 1997–2002. Matched controls for cryptorchid boys born in 2000–2002 (black) were recruited in the obstetric department in Turku University Hospital. All cryptorchid boys, their matched controls and every 10th non-cryptorchid subject in the prenatally recruited prospective birth cohort were invited to a pubertal follow-up (yellow). Placental samples (green) were available from some subjects of the prenatally recruited prospective birth cohort in both countries. Adipose tissue biopsies were taken from boys operated for cryptorchidism, inguinal, abdominal or umbilical hernia (red) in the departments of paediatric surgery in Turku University Hospital and Rigshospitalet.

4.1.2 Testicular distance to pubic bone in Danish-Finnish birth cohort (study I.)

In the first study of this thesis, all the 1 071 and 1 494 prenatally recruited Danish and Finnish boys, respectively, in the Danish-Finnish birth cohort, were included. However, only 1060 and 1485 were included to the analyses due to missing data (explained below in section 4.3).

4.1.3 Placental exposure assessment of PCBs and dioxins in cryptorchid and healthy boys (study II.)

The association between congenital cryptorchidism and exposure to dioxins and PCBs was studied in a nested case-control study within the Danish-Finnish cohort by analysing the placental samples. Placentas of the mothers of 56 Finnish cryptorchid boys and 56 non-cryptorchid matched controls were included (matched for date of birth [±2 weeks], parity, gestational age [±1 week] and maternal smoking during pregnancy [yes or no]). An attempt was made to match the cases and controls for maternal diabetes (yes/no). However, this did not entirely succeed as maternal diabetes was much more common among the mothers of the cryptorchid boys. Thus, maternal diabetes was more frequent among cryptorchid boys versus controls (10 vs 0, $p<0.001$) in the Finnish study sample.

In the Danish cohort, all the 39 available placentas of the mothers of cryptorchid subjects were analysed and 129 control placentas were randomly selected from mothers of non-cryptorchid boys.

4.1.4 PCB, dioxin and PBDE levels in adipose tissue of cryptorchid and non-cryptorchid boys (study III.)

The subjects of the third study were recruited from the departments of paediatric surgery both in Rigshospitalet in 2004–2005 and Turku University Hospital in 2002–2006. In the Finnish subset, only boys who were less than five years old were recruited. The Danish subset did not have such a criterion for age, but only boys who fulfilled the inclusion criteria of the birth cohort in terms of parents' and grandparents' residential history were included.

The boys who were operated for congenital cryptorchidism were regarded as cases, whereas the boys who were operated for inguinal hernia, hydrocele or abdominal hernia were regarded as controls. Among the cryptorchid cases 2 Danish and 12

Finnish boys were referred to the departments of paediatric surgery from the Danish-Finnish birth cohort. Six of these 12 Finnish cryptorchid boys later participated in the pubertal follow-up (described next).

4.1.5 Longitudinal testicular growth in the Finnish puberty follow-up (study IV.)

All the 165 prenatally or postnatally recruited cryptorchid boys in the Finnish cohort, and 306 controls (including matched controls and every 10th boy in the cohort who did not have hypospadias or congenital cryptorchidism), who lived close to Turku area were invited to participate in a pubertal follow-up at the age of 8.5 years. These children were then examined once every six months until no further testicular growth was observed based on ultrasonography. In total, 52 cryptorchid cases (31.5%) and 65 controls (21.2%) agreed to participate. One cryptorchid case and one control were excluded due to precocious puberty.

4.2 Background data

Lengths of gestation were calculated from dates of birth to estimated dates of conception based on routine gestational ultrasonography examinations if available, or the last period of menstruation if not. Boys born before gestational week 37+0 were regarded as preterm, between 37+0 and 42+0 as term and after 42+0 as post-term. Weight for gestational age (WGA) percentile was calculated based on gestational age (GA) and birth weight and national growth references[420,421].

In study III., parents of the Finnish subjects who were operated filled in a questionnaire on the possible predictors of the exposure to POPs (maternal smoking during pregnancy, maternal age, previous breastfeeding, length of gestation, duration of exclusive or partial breastfeeding, age at operation and gestational diabetes during pregnancy). Obstetric data including weight before and after pregnancy as well as birth weight were obtained from the electronic medical records. In Denmark, the version of the questionnaire that contained also questions on obstetric data were mailed to the parents in 2013, and an attempt was made to interview all those parents who did not submit their answer.

4.3 Clinical examinations and sampling

The timeline of the clinical examinations and sampling, and the overlap of data that were used in studies I., II. and IV. are shown in Figure 7. During a visit, height,

weight and testicular distance to pubic bone (TDP) were measured. TDP was defined as the distance between the superior margin of the pubic symphysis and the upper pole of the testis, and was measured to the closest millimetre after traction was applied towards the pathway of descent without causing pain or discomfort to the child. A schematic representation of the TDP measurement is shown in Figure 8. TDP was quantified with a ruler in the Finnish cohort, whereas a calliper was used in the Danish cohort. Only examinations carried out by researchers (N=13) who examined more than 10 subjects were included, which led to the inclusion of 7178 examinations (99.6% of all examinations). Interobserver variability in the TDP measurement was assessed in interobserver workshops in both countries.

TDP was not recorded in 165 Danish and 46 Finnish examinations, possibly due to a non-scrotal testicular location in 47 and 36 examinations, respectively. The reason for the lack of TDP examination was not known in 118 and 10 examinations. Further seven Danish and one Finnish examinations were excluded because of a prior orchidopexy.

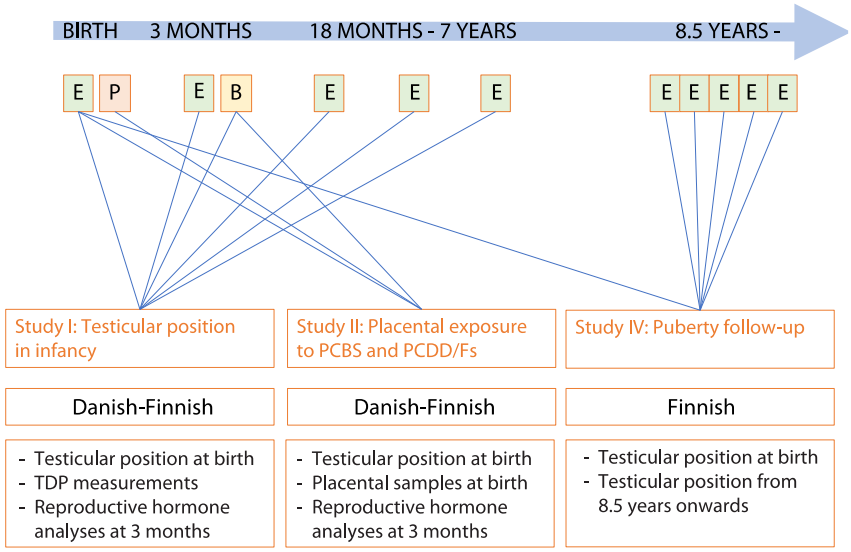

Figure 7 Timeline of clinical examinations and sampling in studies I., II. and IV.

E = examination, P = placental sample, B = blood sample.

At birth, families were asked to donate the placenta to our research team. The placentas were frozen and stored in a freezer at -20 °C. During the visit at the age of three months, an attempt to draw a venous blood sample from a baby was made if the parents gave their consents. In the study I., an adipose tissue biopsy was taken from the incision area during the operation and frozen for later analyses.

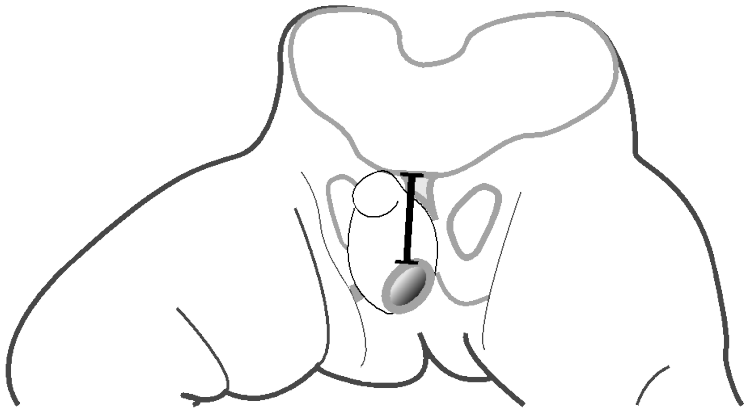

Figure 8 Schematic representation of TDP measurement

Testicular distance to pubic bone (TDP) was quantified using a ruler after a testis was gently pulled towards the bottom of a scrotum without causing pain or discomfort.

In the pubertal follow-up, a similar examination protocol included the measurement of height and weight, as well as the examination of testicular volume using Prader orchidometer[181] and ultrasonography (Aloka Prosound 6, linear probe 5–13 MHz and Aloka SSD-500, linear probe, 7.5 MHz; Hitachi Aloka Medical). Furthermore, testicular length and width were measured with a plastic ruler by palpating the edges of a testis between the thumb and the index finger and measuring the distance between fingers with the ruler. Testicular volume was calculated from the ultrasonography and ruler measurements using the ellipsoid formula (π x length x width x width/ 6) and the Lambert's formula (0.71 x length x width x width)[422].

Pubertal development was assessed using Tanner staging with the following additional criteria to ensure the consistency between examiners[423]. G1: testicular length by ruler < 20 mm, G2: testicular length by ruler > 20 mm, G3: penile length by ruler > 6 cm and at least 0.5 cm growth since the last visit, testicular length > 30 mm by ultrasonography, G4: basal width of the penis > 20 mm.

4.4 Hormonal analyses

In the study I., blood samples were analysed in Rigshospitalet, Department of Growth and Reproduction, Copenhagen, Denmark. All samples were analysed in sets that contained samples of both cryptorchid and non-cryptorchid boys from both countries to minimise the inter-assay variation. The laboratory technicians were blinded to the country and the case-control status in terms of cryptorchidism.

SHBG, LH and FSH were analysed by time-resolved immunofluorometric assays (Delfia, Wallac Inc., Turku, Finland)[274]. INSL3 was measured by a time-resolved

immunofluorometric assay which was developed in co-operation between University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark; Department of Andrology and Institute for Hormone and Fertility Research, University Hospital Hamburg-Eppendorf, Germany; and School of Molecular and Biomedical Science, University of Adelaide, Australia[424]. IGF-I was analysed with a radioimmunoassay, following the method of Bang et al. with modifications reported in the original report[419,425], and testosterone was measured with a radioimmunoassay (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA). Inhibin B was analysed with a double-antibody enzyme immunometric assay (Oxford Bio-Innovation, Oxford, UK)[274]. The limits of detection (LODs) as well as inter and intra-assay coefficients of variation (CVs) are given in Table 1.

Table 1 Assay variability and limits of detection for analyses of reproductive hormones.

	LOD	*Intra-assay CV*	*Inter-assay CV*
LH	0.05 IU/l	<5%	<5%
FSH	0.06 IU/l	<6%	<5%
INSL3	0.05 ng/ml	<8%	<11%
Testosterone	0.23 nmol/l	<10%	<10%
inhibin B	20 pg/ml	<15%	<18%
IGF-I	21 ng/ml	3.9%	8.7%

CV = coefficient of variation.

4.5 Exposure assessment

Both the placental samples and the adipose tissue biopsies were sent to Department of Health Protection, in Kuopio, Finland (previously known as Department of Environmental Health) for assessment of POPs. Placentas were analysed for 37 PCDD/F and 17 PCB congeners, and adipose tissue samples for 37 PCDD/F, 17 PCB and 14 PBDE congeners. The full list of the quantified PCB, PCDD/F and PBDE congeners is given in Table 2. The samples were weighed, homogenised and spiked with internal standards for PCDD/F, PCB and PBDE congeners, and analysed using high-resolution gas chromatography-mass spectrometry as previously described[360,426–428].

Table 2 List of all analysed toxicants (studies II. and III.)

PCDD/Fs	***Dioxin-like PCBs***	***Non-dioxin-like PCBs***	***PBDEs*[A]**
2,3,7,8-tetraCDF	PCB18	PCB77	PBDE28
2,3,7,8-tetraCDD	PCB28/31	PCB81	PBDE47
1,2,3,7,8-pentaCDF	PCB33	PCB105	PBDE66
2,3,4,7,8-pentaCDF	PCB47	PCB114	PBDE71
1,2,3,7,8-pentaCDF	PCB49	PCB118	PBDE75
1,2,3,4,7,8-hexaCDF	PCB51	PCB123	PBDE77
1,2,3,6,7,8-hexaCDF	PCB52	PCB126	PBDE85
2,3,4,6,7,8-hexaCDF	PCB60	PCB156	PBDE99
1,2,3,7,8,9-hexaCDF	PCB66	PCB157	PBDE100
1,2,3,4,7,8-hexaCDD	PCB74	PCB167	PBDE119
1,2,3,6,7,8-hexaCDD	PCB99	PCB169	PBDE138
1,2,3,7,8,9-hexaCDD	PCB101	PCB189	PBDE153
1,2,3,4,6,7,8-heptaCDF	PCB110		PBDE154
1,2,3,4,7,8,9-heptaCDF	PCB122		PBDE183
1,2,3,4,6,7,8-heptaCDD	PCB128		
octaCDF	PCB138		
octaCDD	PCB141		
	PCB153		
	PCB170		
	PCB180		
	PCB183		
	PCB187		
	PCB194		
	PCB206		
	PCB209		

[A]Only in study III. CDD = chlorinated dibenzo-*p*-dioxin, CDF = chlorinated dibenzo-*p*-furan, BDE = brominated diphenyl ether.

Recoveries of all the internal standards were >60% and limits of quantification (LOQ) were set at 3:1 signal to noise ratio. Cross-sample contamination was analysed by measuring procedural blank samples, which had substantially lower concentrations than all the actual samples. Concentrations <LOQ in placentas were set nil in study II., while in study III. they were considered ½ LOQ. CVs of in-house control placental tissue samples were 5.6% for dioxin WHO-TEq and 13% for PCB WHO-TEq. CVs of in-house control adipose tissue samples were 3.3%, 7.2% and 8.9% for sum of PCDD/Fs, sum of PCBs and total-TEq, respectively.

4.6 Statistical analyses

4.6.1 Analysis of background and baseline data

Differences between the cryptorchid and non-cryptorchid boys in the continuous background data described in the section 4.2 were compared with independent samples T-test when the data were normally distributed. When appropriate, a logarithmic or square root transformation were used to better meet the assumption of normality. When the assumption of normality was violated even after the logarithmic and square root transformations, a Mann-Whitney U-test was used instead. When the background or baseline variable was categorical, the differences were compared with a Chi Square test when the group sizes exceeded ten in all groups, and with a Fisher's exact test if not.

4.6.2 Predictors of postnatal testicular position

TDP during childhood was modelled using linear mixed-effect models. Subject and examiner were included as random effects. The serial dependence between residual errors across time within each subject was modelled using an empirical correlation structure, unless stated otherwise. Denominator degrees of freedom were computed using a Kenward-Roger approximation.

Age was regarded as a categorical fixed effect in all models. In Model 1, trends in TDP from birth to 18 months were analysed in the combined data (Finland and Denmark) including age, GA, WGA percentile and country of origin as fixed effects. Furthermore, interactions between age and country, age and GA as well as age and WGA were added as fixed effects, allowing the country difference and the associations between GA & TDP and WGA & TDP to vary with age. In Models 2 and 3, associations between TDP & penile height (Model 2) as well as TDP & height and TDP & penile length (Model 3) were added as fixed effects in addition to the fixed effects in the previous model. The effects of height and penile length were allowed to vary with age in Models 2 and 3. As a sensitivity analysis, Models 1–3 were reanalysed including only Danish data and excluding preterm boys or boys with congenital or acquired cryptorchidism.

In Model 4, we aimed to examine the effect of reproductive hormones and IGF-I on testicular descent after birth. Thus, only examinations at 3 and 18 months were included, and the results were adjusted for TDP at birth by adding it as a fixed effect. IGF-I concentration, testosterone/LH-ratio and inhibin B/FSH-ratio, their respective interactions with age and all covariates of Models 1–3 except for penile

length were added as fixed effects. In a subgroup of 248 boys who had INSL3 and TDP measurements available, Model 4 was repeated using the INSL3/LH-ratio as a fixed effect instead of testosterone/LH-ratio. In that model, only subject was added as a random effect and the compound symmetry covariance structure was used. As a sensitivity analysis, also Model 4 was also reanalysed excluding the subjects who were preterm or who had congenital or acquired cryptorchidism.

4.6.3 Placental concentrations of PCBs and PCDD/Fs and risk of congenital cryptorchidism

The difference in placental levels of PCDD/Fs and PCBs between Danish and Finnish controls was tested in a linear regression model with and without adjusting for confounders (maternal age, maternal smoking, maternal diabetes, body mass index, parity, GA and the date of delivery) using backward selection. The final model included only country, maternal age, maternal smoking, parity, body mass index and the date of delivery as covariates.

The association between congenital cryptorchidism and the placental levels of PCBs and PCDD/Fs was tested separately in both countries. In the Finnish cohort, the association between the exposure and the risk of congenital cryptorchidism was tested by applying an unconditional logistic regression model, which included maternal age and body mass index as covariates. In the Danish cohort, the association was tested with a logistic regression model using backward stepwise selection. In the first model, the previously known predictors of POP exposure (maternal age, parity, body mass index, date of childbirth) and the risk factors of congenital cryptorchidism (prematurity, WGA category [small/appropriate/large for GA] and GA) were added as covariates. However, only GA remained in the model after the exclusion of covariates that did not contribute to the model.

Finally, serum reproductive hormone concentrations at the age of three months were modelled on placental PCDD/F or PCB WHO-TEqs, the exact age at blood sampling and cryptorchidism (yes/no) by a linear regression separately in both countries.

4.6.4 Adipose tissue concentrations of POPs and risk of congenital cryptorchidism

The unadjusted differences between the cryptorchid and non-cryptorchid boys in total-TEq and in sums of PCBs, PCDD/Fs and PBDEs were tested with independent samples T-tests. Our main goal was to assess whether the prenatal exposure to

POPs was associated with congenital cryptorchidism, which is a birth defect by definition. Therefore, we attempted to refine our analyses by using a two-stage approach. In the first stage, the postnatal variability in POP concentrations was minimised by modelling the POP concentrations on the age at adipose tissue biopsy, duration of breastfeeding and country of origin in multivariable linear regression models. The residuals of the first stage were regarded as the best estimates of the prenatal exposure to POPs. In the second stage, associations between the risk of cryptorchidism and the residuals of the first stage of analyses were tested using a logistic regression.

4.6.5 Testicular growth in puberty

The difference in the age at the attainment of testicular volume of >3 ml by orchidometer between cryptorchid and healthy boys was compared with an independent samples T-test. The longitudinal pubertal testicular growth patterns by ultrasonography were modelled with two nonlinear mixed-effect models using statistical package 'nlme' in R statistical environment version 3.2.3[429]. In both models, mean testicular volume was modelled among controls and bilaterally cryptorchid boys. Among unilaterally cryptorchid boys, the volume of the descended testis was modelled in the first model and the volume of the undescended testis in the second model.

The nonlinear mixed-effect modelling was based on the assumption that the pattern of testicular growth follows a sigmoidal four-parameter logistic growth curve in puberty. Thus, the pubertal testicular growth of each child, and the typical growth in the population can be described with a mathematical formula including four parameters (prepubertal testicular volume, postpubertal testicular volume, age at mid-puberty and a scale parameter), mathematically expressed as

$$Testicular\ volume\ (x) = Vol_{prepub} + \frac{Vol_{postpub} - Vol_{prepub}}{1 + e^{\frac{xmid - x}{scale}}}$$

where x = age in years, Vol_{prepub}=asymptote prepubertal testicular volume, $Vol_{postpub}$ = asymptote postpubertal testicular volume, x_{mid} = age at mid-puberty when half of the pubertal testicular growth is reached and scale = scale parameter, describing the width of the interval of the fastest growth. The alterations in the growth curve caused by changes in the parameters are illustrated graphically in Figure 9.

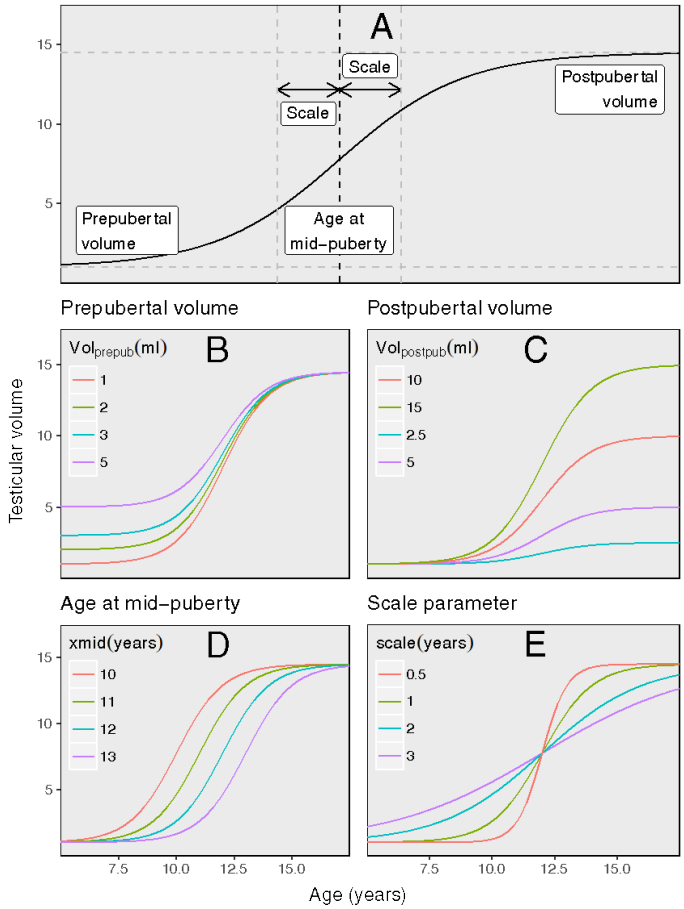

Figure 9 Illustration of four-parameter logistic growth curve

A: A schematic representation of the four parameters. B–E: Illustrations on how a change in each parameter affects the overall pattern of the growth curve if the other parameters remain fixed. E.g. if the scale parameter increases from 0.5 to 1, the pubertal testicular growth lasts longer. The figure is modified from Sadov S, Koskenniemi JJ, Virtanen HE, Perheentupa A, Petersen JH, Skakkebaek NE, Main KM, Toppari J. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. J. Clin. Endocrinol. Metab. 2016;101(6):2570–7, and is reproduced with permission from the Oxford University Press.

The nonlinear mixed-effect models estimated the mean values of these four parameters in the population (fixed effects), as well as their inter-individual variability (random effects). The variance in the residuals of the models was modelled on power of the fitted values. The model fitted the data well based on graphical analyses.

4.6.6 Comparison between methods to assess onset of puberty

The agreement between the orchidometer (volume > 3 ml) and the ruler (testicular length > 25 mm) cut-offs, which are used in the estimation of the onset of puberty, was tested with cross-tabulation. The agreement between testicular volumes measured by ultrasonography and orchidometer was tested using a Bland-Altman analysis[430], with the modification that the difference between the two measurements was plotted against volumes by ultrasonography instead of the mean of the two methods. The correlation between testicular volumes measured by the ultrasonography and the orchidometer was estimated using a Passing-Bablok regression[431]. For computational reasons, only a randomly selected subset including 50% of the total study sample was used in the Passing-Bablok regression. The regression was repeated with the remaining 50% of the study sample, and the results did not differ (data not shown).

4.7 Ethics statement

All the studies were performed in accordance with Helsinki II. declaration. The parents of the subjects in the Danish-Finnish birth cohort gave a written informed consent before they entered the study. Parents of the subjects who entered the adipose tissue biopsy study or pubertal follow-up gave an informed written consent, and an assent was obtained from the subjects of the pubertal follow-up. The study protocols were approved by the Danish Data Protection Agency (1997-1200-074/ 2005-41-5545), the local Danish Ethics Committee (KF 01-030/97/ KF 01276357) and the joint ethics committee of Turku University hospital and the University of Turku (7/1996, 6/2001, 12/2004).

5 RESULTS

5.1 Testicular position and reproductive hormones at age of three months (study I.)

5.1.1 Study population characteristics and interobserver variability in TDP

The study population characteristics are shown in Table 3. The interobserver coefficient of variation in TDP was estimated based on 88 paired examinations in Denmark and 12 in Finland. There were no differences in the interobserver variability in TDP between three, 18 and 36 months in the larger Danish data, and the measurements were thus pooled (data not shown). The interobserver coefficient of variation was 2.9% in the Finnish cohort and 7.8% in the Danish cohort.

Table 3 Study population characteristics (study I.)

	Denmark	Finland
N	1060	1485
Preterm/Post-term	59 (6%) / 67 (6%)	67 (5%) / 53 (4%)
SGA[A]/LGA[B]	38 (4%) / 33 (3%)	26 (2%) / 33 (2%)
GA (weeks)	39.9 ± 1.8	39.8 ± 1.6
Congenital cryptorchidism	85 (8%)	27 (1.8%)
Acquired cryptorchidism	12 (1%)[C]	–

The numbers are given as N (%) or mean ±SD. [A]Defined as a WGA below the 2.5th percentile based on the national growth references. [B]Defined as a WGA above the 97.5th percentile based on the national growth references. [C]Subjects with a testis in a high scrotal or higher position at two consecutive visits.

5.1.2 Changes in TDP in early childhood

The changes in TDP during childhood are summarised in Table 4. In Model 1, TDP at birth was 61.4 mm and increased by 18.5 mm (95% confidence interval [95% CI] 18.1,18.9 mm, p<0.0001) between birth and three months. TDP decreased by 5.0 mm (95% CI -4.5,-5.5 mm, p<0.0001) between three and 18 months.

The changes in TDP from birth to 3 months and 3 to 18 months were slightly smaller when analyses were restricted to the Danish data (16.8 mm [95% CI 16.1,17.4 mm] and -5.2 mm [95% CI -4.6,-5.8 mm], respectively). TDP remained stable in the Danish cohort from 18 to 36 months (95% CI for an increase -0.4,0.9

mm, p=0.40). A further small but significant decrease of 4.6 mm (95% CI -3.8,-5.4 mm) was observed from 36 months to 7 years.

Table 4 TDP from birth to prepuberty (study I.)

Age	N	TDP (mm) Combined data	Change (mm) Combined data	TDP (mm) Danish cohort	Change (mm) Danish cohort	Country difference (mm)
Birth	1 001	61.4 (60.9, 61.9)	NA	62.1 (61.2, 63.1)	NA	-0.3 (-0.3,1.0)
3 months	959	79.9 (79.4, 80.4)	+18.5 (+18.1,+18.9)	78.9 (78.0, 79.9)	+16.8 (+16.1,+17.4)	-3.0 (-3.8, -2.2)
18 months	858	74.9 (74.3, 75.5)	-5.0 (-4.5,-5.5)	73.7 (72.9, 74.5)	-5.2 (-4.6, -5.8)	-3.3 (-4.3, -2.3)
36 months	752	NA	NA	74.0 (73.1, 74.9)	+0.3 (+0.9, -0.4)	NA
7 years	452	NA	NA	69.4 (68.1, 70.6)	-4.6 (-3.8, -5.4)	NA

NA = not applicable. Model-based means and changes in TDP in Model 1 adjusting for GA and WGA. Model also included the country of origin as a fixed effect when both countries were included. Ninety-five percent confidence intervals are given in parentheses.

5.1.3 Predictors of TDP in combined data

Overall in the model, country of origin, WGA and GA were significantly associated with TDP ($p<0.0001$, $p<0.0001$, $p<0.0001$, respectively). Furthermore, country-difference in TDP ($p<0.0001$), and the association between WGA & TDP ($p<0.0001$) and GA & TDP ($p<0.0001$) varied with age. Compared to Denmark, TDP was 3.0 mm larger in Finland (standard error [SE]=0.4 mm, $p<0.0001$) at the age of three months and 3.3 mm larger (SE = 0.5 mm, $p<0.0001$) at 18 months. WGA and GA were significantly associated with TDP at birth ($p<0.0001$ and $p<0.0001$, respectively), at the age of three months ($p<0.0001$ and $p<0.0001$, respectively) and 18 months ($p<0.0001$ and $p=0.0003$, respectively).

Country of origin, WGA and GA ($p<0.0001$, $p=0.0003$, $p<0.0001$, respectively) and their interactions with age ($p<0.0001$, $p=0.01$, $p<0.0001$, respectively) remained significant after the inclusion of height and penile length as fixed effects (Models 2 and 3, respectively). Height was a strong predictor of TDP ($p<0.0001$) in Model 2, and the association did not vary with age between birth and the age of 18 months ($p=0.34$). In Model 3, penile length was also significantly associated with TDP ($p<0.0001$) in an age-invariant fashion ($p=0.68$).

5.1.4 Predictors of TDP in Danish cohort

Associations between the predictors of TDP from birth to 18 months were largely similar when only the Danish data were included. TDP was associated with WGA and GA ($p<0.0001$ and $p<0.0001$, respectively), and the associations varied with age ($p<0.0001$ and $p<0.0001$, respectively). The Danish data suggested that WGA was significantly associated with TDP also at the age of 36 months and 7 years ($p=0.0003$ and $p<0.0001$, respectively). GA was significantly associated with TDP at birth ($p<0.0001$), but not at the age of three months ($p=0.20$) or later.

In agreement with the combined data, associations between TDP & height and TDP & penile length were also significant between birth and 18 months in Models 2 and 3. However, evidence was found for an age-dependent variability in the association between height and TDP ($p<0.0001$) and penile length and TDP ($p<0.0001$). Height did not correlate with TDP at 36 months ($p=0.08$) and 7 years ($p=0.22$) in Model 2, and penile length did not correlate with TDP at 36 months or 7 years ($p=0.54$ and $p=0.14$, respectively) in Model 3.

5.1.5 Associations between reproductive hormones and TDP

In Model 4 (only analysed using combined data), TDP at birth predicted significantly the later testicular position in the whole model ($p<0.0001$), and fixed effects of the previous models except the GA remained significant. IGF-I and ratios of testosterone/LH and inhibin B/FSH were significantly associated with TDP overall during early childhood ($p=0.001$, $p=0.0009$, $p=0.005$, respectively), and their effects varied with age ($p=0.006$, $p=0.03$, $p=0.02$, respectively). Interestingly, IGF-I and testosterone/LH-ratio were significant predictors of TDP only at the age of three months ($p<0.0001$ and $p=0.0001$, respectively), but not at the age of 18 months ($p=0.34$ and $p=0.16$, respectively). In contrast, an inverse pattern was observed for inhibin B/FSH-ratio, which was significant at the age of 18 months ($p=0.0003$), but not at the age of 3 months ($p=0.41$).

5.1.6 Sensitivity analyses

The results in Models 1–4 did not differ when preterm boys or boys with congenital or acquired cryptorchidism were excluded (data not shown).

5.2 Associations between cryptorchidism and placental levels of PCDD/Fs and PCBs (study II.)

The characteristics of the case and control groups are described in Table 5. Gestational diabetes was significantly more common among the mothers of the Finnish cryptorchid boys (10 vs 0, $p=0.001$) than the mothers of the controls, and the Danish cryptorchid boys had a shorter length of gestation compared to the controls (median difference of 7 days, $p<0.001$). The Danish case group also had a higher proportion of boys who experienced a spontaneous testicular descent during minipuberty compared to the Finnish case group ($p<0.001$). There were no significant differences between the case and control groups in Denmark or Finland in maternal age, body mass index, smoking, maternal diabetes (type 1 or type 2), parity or WGA. The cryptorchid boys had a slightly smaller birthweight compared to the controls in the Danish cohort (3.45 vs 3.63 kg, $p=0.04$), whereas there was no such difference in the Finnish cohort.

Table 5 Study population characteristics (study II.)

	Finland (case)	*Finland (control)*	*p*	*Denmark (case)*	*Denmark (control)*	*p*
Total N	56	56		39	129	
Maternal age (years)	29.1 (19.2–42.3)	28.1 (19.9–38.5)	0.35	29.5 (25.7–45.7)	31.0 (19.8–42.5)	0.72
Body mass index (kg/m2)	23.1 (17.7–38.5)	22.3 (17.4–32.1)	0.07	21.5 (17.8–36.1)	22.1 (17.4–37.6)	0.20
smoking (yes/no)	10/46 (22%)	7/49 (14%)	NA	11/28 (39%)	41/87 (47%)	0.65
Gestational diabetes (yes/no)	10/46 (22%)	0/55 (0%)	0.001	0/39 (0%)	2/127 (2%)	~1
Parity (N)			NA			0.90
1	31 (55%)	31 (55%)		26 (67%)	81 (63%)	
2	19 (34%)	19 (34%)		10 (26%)	36 (28%)	
≥3	6 (11%)	6 (11%)		3 (8%)	12 (9%)	
Length of gestation (weeks)	39.9 (36.6–42.4)	40 (36.4–42.0)	NA	39.4 (27.9–42)	40.4 (36.0–42.7)	<0.001
Preterm (N)	1 (2%)	3 (5%)		6	6 (5%)	
SGA[A] (N)	3 (5%)	0 (0%)		2 (5%)	5 (4%)	
Birthweight (kg)	3.63 (2.51–4.66)	3.54 (2.84–4.73)	0.81	3.45 (0.75–4.75)	3.63 (2.29–5.68)	0.04
Cryptorchid at 3 months (N)	33 (59%)	0 (0%)		8 (21%)	0 (0%)	
Blood sample at 3 months (N)	35 (63%)	44 (78%)		25 (64%)	88 (68%)	

NA = not applicable. [A]Defined as WGA below the 2.5th percentile based on national growth references[420,421]. The table is reproduced from Virtanen HE, Koskenniemi JJ, Sundqvist E, Main KM, Kiviranta H, Tuomisto JT, Tuomisto J, Viluksela M, Vartiainen T, Skakkebaek NE, Toppari J. Associations between congenital cryptorchidism in newborn boys and levels of dioxins and PCBs in placenta. Int. J. Androl. 2012;35(3):283–93 with a permission from John Wiley and Sons.

When adjusting for the confounders, the sum of PCDD/Fs did not differ between the Finnish and the Danish cohort (medians 118 vs 124 pg/g fat, respectively), whereas sum of PCBs (38.9 vs 52.3 ng/g fat, p<0.001, respectively) and total-TEq was smaller in the Finnish than in the Danish cohort (10.6 vs 13.0 pg/g, p<0.001, respectively). No significant associations were noted between the risk of cryptorchidism and the sum of 17 PCDD/F congeners, the sum of 37 PCB congeners, PCB-TEq, PCDD/F-TEq or combined WHO-TEq of both PCBs and PCDD/Fs (total-TEq).

Among the six individual PCDD/F and 21 PCB congeners that were detectable in all samples, only the concentration of PCB126 was associated with an increased risk of cryptorchidism in the Danish cohort (p=0.01). In contrast, none of the congeners were associated with the risk of cryptorchidism in the Finnish cohort. The

PCB WHO-TEq was positively associated with the serum LH concentrations at the age of 3 months (β=0.47, p=0.01) in the Finnish cohort, whereas there was no such a difference in the Danish cohort.

5.3 Associations between cryptorchidism and levels of PCDD/Fs, PCBs and PBDEs in adipose tissue (study III.)

The study population characteristics are given in Table 6. The mothers of the cryptorchid boys were slightly older than the mothers of the controls (p=0.04) and another borderline significant difference was observed in the length of gestation (p=0.04). Otherwise, there were no differences in the background data between the groups.

Table 6 Population characteristics (study III.)

	Cases ***N (%) or mean ±SD***	***Controls*** ***N (%) or mean ±SD***	***p***
Total N	44	38	
Maternal age (years)	32.3 ± 4.9	29.6 ± 6.3	0.04
Mother's weight before pregnancy (kg)	68.6 ± 16.7	68.6 ± 19.7	0.92
Parity			0.24
0	23 (56%)	15 (41%)	
1	7 (17%)	13 (35%)	
2	9 (22%)	6 (16%)	
3	2 (5%)	3 (8%)	
Months breastfed in previous pregnancies	3.4 ± 5.8	6.2 ± 8.8	0.51
Maternal smoking during pregnancy	5 (18%)	2 (9%)	0.47
Gestational diabetes	8 (21%)	4 (13%)	0.58
Length of gestation (weeks)	39.4 ± 2.9	38.2 ± 3.0	0.04
Birth weight (g)	3460 ± 680	3170 ± 770	0.07
Duration of exclusive breastfeeding (months)	2.7 ± 2.1	2.8 ± 2.1	0.87
Total duration of breastfeeding (months)	5.8 ± 4.5	7.1 ± 5.7	0.26
Age at operation (years)	2.3 ± 1.0	2.9 ± 2.2	0.53

The table is modified from Koskenniemi JJ, Virtanen HE, Kiviranta H, Damgaard IN, Matomäki J, Thorup JM, Hurme T, Skakkebaek NE, Main KM, Toppari J. Association between levels of persistent organic pollutants in adipose tissue and cryptorchidism in early childhood: a case-control study. Environ. Health 2015;14(1):78 with a permission from copyright holders.

Sums of PCDD/Fs, PCBs and PBDEs as well as total-TEq among the cryptorchid and the healthy Danish and Finnish subjects are given in Table 7. The unadjusted comparisons of the sums of PCDD/F, PCB and PBDE or total-TEq did not yield any significant differences between the cryptorchid and the non-cryptorchid subjects.

Table 7 Adipose tissue concentrations of POPs (study III.)

	Cases ***median (range)***	***Controls*** ***median (range)***	p^A
Finland (N)	30	29	
Sum of PCDD/Fs (pg/g)	113 (42–320)	78 (28–1300)	0.25
Sum of PCBs (ng/g)	69 (13–390)	80 (11–570)	0.97
Sum of PBDEs (ng/g)	7.0 (1.6–64)	5.41 (1.3–86)	0.42
Total-TEq (pg/g)	7.4 (3.2–41)	5.43 (2.7–64)	0.85
Denmark (N)	14	9	
Sum of PCDD/Fs (pg/g)	89 (22–260)	83 (22–160)	0.83
Sum of PCBs (ng/g)	180 (17–700)	160 (13–520)	0.56
Sum of PBDEs (ng/g)	3.9 (1.6–31)	5.7 (3.5–9.4)	0.45
Total-TEq (pg/g)	19 (3.4–56)	13 (2.6–42)	0.73

[A]For the difference between cases and controls. The table is modified from Koskenniemi JJ, Virtanen HE, Kiviranta H, Damgaard IN, Matomäki J, Thorup JM, Hurme T, Skakkebaek NE, Main KM, Toppari J. Association between levels of persistent organic pollutants in adipose tissue and cryptorchidism in early childhood: a case-control study. Environ. Health 2015;14(1):78 with a permission from the copyright holders.

The results of the linear regression are shown in Table 8. Based on R^2s of the linear regression model, the duration of breastfeeding explained a substantial share of the total sum of PCDD/Fs (27%, $p<0.0001$), the sum of PCBs (44%, $p<0.0001$) and total-TEq (48%, $p<0.0001$), but not the sum of PBDEs (5%, $p=0.70$). In contrast, the country of origin significantly predicted only the sum of PCBs (8%, $p=0.006$) and the sum of PBDEs (10%, $p=0.007$). Age at the operation did not significantly predict total-TEq or any sum of POPs.

Table 8 Adjustments for postnatal exposure to POPs using multiple linear regression (study III.)

		Sum of PCBs	*Sum of PCDD/Fs*	*Sum of PBDEs*	*Total-TEq*
Country	***β (95% CI)***	1.20 (0.79-1.81)	0.61 (0.43-0.87)	0.53 (0.33-0.83)	1.13 (0.81-1.57)
	p	0.38	0.006	0.007	0.46
	R^2	<0.01	0.08	0.10	<0.01
Age at the operation	***β (95% CI)***	1.00 (0.99-1.01)	1.00 (0.99-1.01)	1.01 (1.00-1.02)	1.00 (0.99-1.00)
	p	0.83	0.73	0.27	0.27
	R^2	<0.01	<0.01	0.02	<0.01
Duration of breastfeeding	***β (95% CI)***	1.16 (1.12-1.21)	1.08 (1.05-1.12)	1.01 (0.97-1.05)	1.13 (1.10-1.17)
	p	<0.001	<0.001	0.70	<0.001
	R^2	0.44	0.27	0.05	0.48

Modified from Koskenniemi JJ, Virtanen HE, Kiviranta H, Damgaard IN, Matomäki J, Thorup JM, Hurme T, Skakkebaek NE, Main KM, Toppari J. Association between levels of persistent organic pollutants in adipose tissue and cryptorchidism in early childhood: a case-control study. Environ. Health 2015;14(1):78.

In the adjusted analyses, the risk of cryptorchidism was significantly linked to the sum of PCCD/Fs (odds ratio [OR] 3.69, [95% CI 1.45–10.9], p=0.01) and total-TEq (OR 3.21 [95% CI 1.29–9.09], p=0.02), whereas neither the sum of PCBs (OR 1.92 [95% CI 0.98–4.01], p=0.07) nor the sum of PBDEs (0.86, [95% CI 0.47–1.54], p=0.61) were associated with the risk of cryptorchidism.

5.4 Pubertal testicular growth among boys with and without congenital cryptorchidism (study IV.)

Table 9 presents reproductive hormone levels, penile length and testicular volume among cryptorchid and non-cryptorchid subjects in infancy who later participated in the puberty follow-up versus those who declined to participate. The participating controls had a lower INSL3 concentration at the age of three months (p=0.003), a smaller testicular volume both at birth (p=0.002) and at 3 months (p<0.001) than the non-participants. Similarly, the cryptorchid boys who decided to participate had a higher FSH concentration at the age of three months compared to the non-participants (p=0.009). No other differences were observed between the participants and the non-participants.

Table 9 Analyses of potential participation bias in pubertal follow-up (study IV.)

	Cryptorchid		*p*	*Non-cryptorchid*		*p*
Participated	Yes	No		Yes	No	
N	50	143		63	1420	
Testosterone (nmol/l)	4.3	3.7	0.21	3.6	3.5	0.82
Calculated free testosterone (pmol/l)[A]	25.7	22.1	0.09	20.5	21.2	0.65
INSL3 (ng/ml)	0.11	0.13	0.23	0.10	0.15	0.003
LH (IU/l)	2.4	2.1	0.31	1.9	1.8	0.84
Inhibin B (pg/ml)	436	437	0.97	477	462	0.54
Estradiol	18.0	20.4	0.36	24.1	20.4	0.23
FSH (IU/l)	2.0	1.6	0.009 [C]	1.3	1.4	0.34
SHBG (nmol/l)	149	148	0.90	151	147	0.57
Penile length at birth (mm)	32.5	33.0	0.46	33.9	34.6	0.12
Penile length at three months (mm)	36.7	37.1	0.64	36.3	36.8	0.41
Penile length at 18 months (mm)	39.9	40.6	0.22	39.4	40.0	0.38
Testicular volume at birth (ml)[B]	0.09	0.09	0.81	0.11	0.14	0.002
Testicular volume at 3 months (ml)[B]	0.18	0.16	0.15	0.17	0.21	<0.001[C]
Testicular volume at 18 months (ml)[B]	0.17	0.18	0.42	0.18	0.19	0.28

[A]Calculated with the Vermeulen's formula. [B]Calculated using the ellipsoid formula. [C]Tested with a Mann-Whitney U-test, otherwise with independent samples T-test.

There were no differences between the cryptorchid and non-cryptorchid boys in anthropometric measurements, body mass index or testicular volume based on Prader orchidometer at the first visit at the age of 8.5 years. Similarly, none of the above differed significantly between the groups at the first visit when testicular volume was >3 ml.

The model-based estimates of the testicular growth pattern in controls are illustrated in Figure 10. Among controls, the prepubertal testicular volume by ultrasonography was 0.22 ml and the postpubertal volume was 10.2 ml based on the non-linear mixed-effect modelling. Half of the testicular growth was attained by the age of 13.4 years, and the scale parameter describing the duration of the fastest growth was 0.73 years, indicating that approximately 46% of the growth was reached during the period of fastest growth between 12.7 and 14.1 years. On average, testicular volume doubled between 8.5 and 10.6 years, 10.6 and 11.5 years, 11.5 and 12.2 years, 12.2 and 12.9 years and 12.9 and 14.0 years.

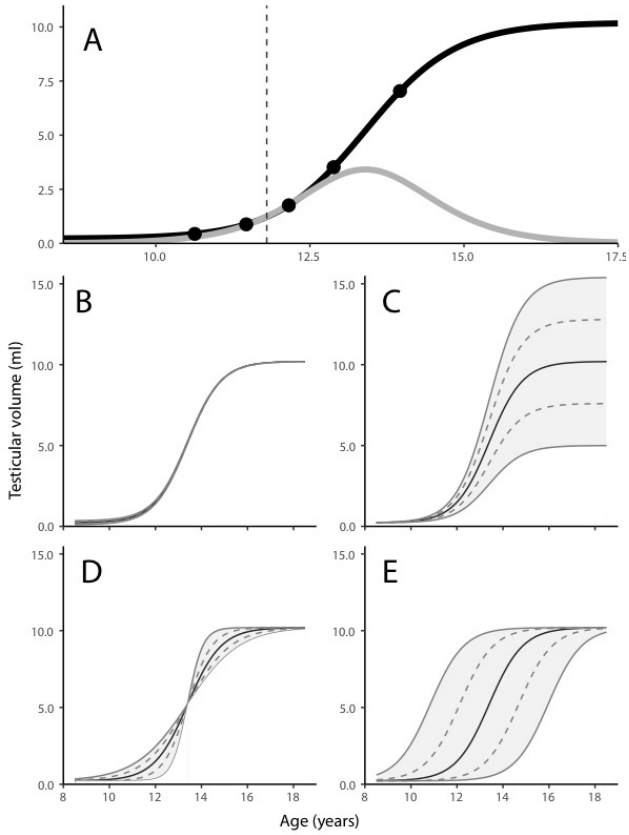

Figure 10 Testicular growth patterns in controls

A: Typical testicular growth pattern during puberty in controls (black curve) and testicular growth rate ml/year (grey curve, first derivative of the mean curve). Black points mark the age when testicular volume was doubled from prepuberty or previous black point. Dashed line indicates the mean age of attainment of testicular volume > 3 ml by orchidometer.
B–E: Variability in modelled prepubertal (B) and postpubertal (C) testicular volumes, scale parameter (D) and age at mid-puberty (E) in controls. Grey-shaded areas represent the ±2 SD and dashed curves ±1 SD. The figure was originally published in Koskenniemi et al.[16] and it is available online: http://journals.lww.com/co-endocrinology/.

SDs of the random effects were 0.07 ml (prepubertal testicular volume), 2.60 ml (postpubertal testicular volume), 1.3 years (age at mid-puberty) and 0.20 years (scale parameter). This indicates that the 95% reference ranges among controls were 0.10–0.34 ml, 5.0–15 ml, 10.8–16.0 years and 0.3–11 years, respectively.

The comparison of pubertal testicular growth patterns between the control testes, the descended and undescended testes of unilaterally cryptorchid boys and the bilaterally cryptorchid boys are shown in Figure 11. Prepubertally, the descended testes of the unilaterally cryptorchid and monorchid boys were slightly but significantly larger than the testes of controls (0.05 ml, 95% CI [0.01, 0.09], p=0.008 and 0.14 ml, 95% CI [0.05, 0.22], p=0.002, respectively). The prepubertal volume of the undescended testes of the bilaterally or unilaterally cryptorchid boys did not

differ from the control testes. Postpubertally, the modelled volumes of the undescended testes of the unilaterally cryptorchid boys (–3.6 ml, 95% CI [-5.0, -2.2 ml], p<0.001) and the modelled testicular volume among the bilaterally cryptorchid boys (-2.4 ml 95% CI [-4.4, -0.5 ml], p=0.01) were significantly smaller than the testicular volume of the controls. In contrast, there was no difference in the modelled postpubertal testicular volume between the control testes and the contralateral descended testes of the unilaterally cryptorchid boys (95% [-0.3, 2.9], p=0.11). None of the other parameters (scale parameter or age when half of the testicular growth was attained) describing the shape of the growth pattern differed between the controls and the bilaterally or unilaterally cryptorchid boys.

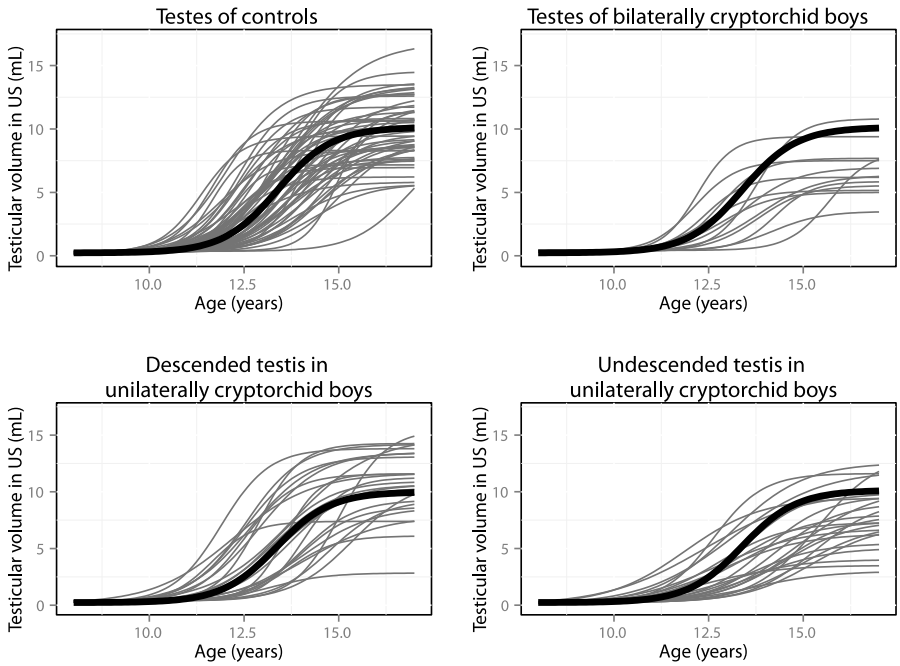

Figure 11 Modelled testicular growth patterns in cryptorchid and non-cryptorchid boys

The modelled typical testicular growth curve among controls is indicated with thick solid lines.

5.5 Comparison between ultrasonography, orchidometer and ruler

Figure 12 shows the Passing-Bablok regression between testicular volumes measured by the orchidometer and ultrasonography (using the ellipsoid and Lambert's formula), and the modified Bland-Altman analyses. The systematic disagreement between orchidometer and ultrasonography increased by 1.04 ml and 0.27 per each ml in the ultrasonography when the ellipsoid formula and the Lambert's formula,

respectively, were used. Furthermore, the width of the 95% limit of agreement increased with age especially when using the ellipsoid formula. Thus, the 95% limits of agreement between the orchidometer and ultrasonography were 5–20 ml and 0–7.5 ml at the testicular volume of 10 ml when the ellipsoid and Lambert's formula, respectively, were used.

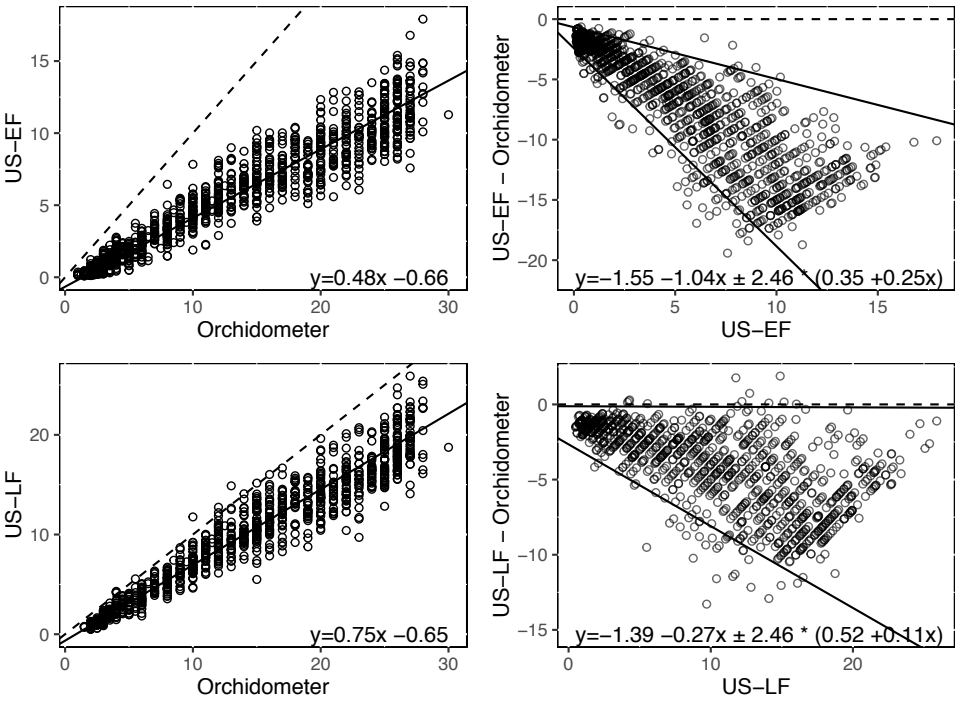

Figure 12 Agreement between orchidometer and ultrasonography

US-EF = testicular volume (ml) by ultrasonography using the ellipsoid formula. US-LF = testicular volume by ultrasonography (ml) using the Lambert's formula. Dashed lines represent a perfect agreement between the two measurements. Regression analyses for the left-hand panel were done using the Passing-Bablok regression[431], and the analyses in the right-hand panel using the modified Bland-Altman analyses[430]. The figure is reproduced from Sadov S, Koskenniemi JJ, Virtanen HE, Perheentupa A, Petersen JH, Skakkebaek NE, Main KM, Toppari J. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. J. Clin. Endocrinol. Metab. 2016;101(6):2570–7 with a permission from the Oxford University Press.

The comparison of the testicular length by ruler versus orchidometer cut-offs as a measurement of the testicular size showed that all the testes that had greater length than 30 mm by ruler were also >3 ml by orchidometer. In addition, all the testes 22 mm or less in length were ≤3 ml by orchidometer. The majority (91%, 516/566) of the testes with a length of 21–25 mm were ≤3 ml, whereas among lengths of 26–30 mm by ruler, 83% (263/318) of the testes were also >3 ml. However, the analysis of the agreement per strata of 1 mm testicular length suggested that the proportion of testes >3 ml was only approximately 50% at 25 mm, and the 95% positive predictive value was reached approximately at 28 mm.

6 DISCUSSION

6.1 Concept and definition of congenital and acquired cryptorchidism

Current diagnostic criteria of congenital cryptorchidism are largely based on the landmark publication by Scorer in 1964[147,148]. Although some German surgeons had suggested an association between cryptorchidism and testicular cancer already in the beginning of the 19th century[432], it is worth noticing that Scorer did not mention that a failure in the testicular descent would lead to subfertility or malignancy in his publications[148,433], and was more concerned about the possible reascent or the associated inguinal hernia[434]. Our study is the first large-scale longitudinal study to actually report the measurements of testicular distance. Our results suggest that 95% of the TDP measurements were within the reference range of 45.7–80.5 mm at birth, a figure closely matching the Scorer's remark.

Although two small cross-sectional studies have previously reported TDP measurements, they did not include repetitive examinations during minipuberty. Thus, we were the first to observe a clear postnatal descent and the succeeding ascent in testicular position among the cryptorchid and normal boys alike. The use of such a continuous metric of testicular descent may present some additional insights on the definition, pathogenesis and significance of acquired cryptorchidism.

Initially, the cases of undescended testis among prepubertal boys were assumed to be of congenital origin, and their ascent later during childhood was controversial. However, cross-sectional and prospective longitudinal cohorts have verified the existence of this entity[150,239,312].

Testicular ascent is more common among boys who have a history of spontaneously resolved congenital cryptorchidism[154,155]. Our results indicate that this group of boys may have a high testicular position to begin with, and the 'spontaneous descent' – in fact a normal phenomenon that takes place among all boys during minipuberty – might not be sufficient to attain a low enough position before the normal 'physiological' testicular ascent. A similar interpretation has been presented previously, although those reviews proposed that testicular ascent was caused by the growth in body size[117,313].

Our results suggest that this physiological postnatal descent-ascent pattern may be caused by the peak and the subsequent decline in the activity of the hypothalamic-pituitary-gonadal axis. In our data, testosterone/LH-ratio, inhibin B/FSH-ratio and IGF-I were all positively associated with TDP. This fits well with the results of a

small French study, which showed that the testicular descent was frequently compromised among boys with hypogonadotropic hypogonadism, and the substitution of these boys with FSH and hCG resulted in the testicular descent[152]. Our results also agree with a British cohort, which reported that the boys whose testes later ascended had a reduced penile growth during minipuberty, indicating that the reduced androgen action increases the risk of acquired cryptorchidism[150].

Based on our results, it can be speculated that high enough levels of reproductive hormones may be needed during minipuberty to form a consolidation of testicular descent, and prevent the later testicular ascent. However, causative inferences cannot be drawn because of the observational nature of our study.

When the original birth cohort was conducted, minipuberty was thought to take place at the age of three months[134]. However, a later longitudinal study showed that the peak in reproductive hormone levels is noticed already during the first month of life both in preterm and term infants[135]. Thus, we may have underestimated the concentrations of the reproductive hormones in our study especially among the preterm infants who were examined three months after the expected date of delivery. Furthermore, the analysis of the circulating testosterone at the time of the study was done using a contemporary radioimmunoassay instead of mass-spectrometry, which is currently considered the gold standard, especially among samples with low testosterone concentrations[435]. However, the results did not change when the preterm infants were excluded, and the possible loss of sensitivity and accuracy should decrease, rather than increase, the possibility to discover significant associations.

6.2 Role of persistent organic pollutants in pathogenesis of cryptorchidism

The prevalence of the male reproductive disorders such as cryptorchidism, hypospadias, declined semen quality and testicular germ-cell cancer exhibit a clear temporal and geographical variation. Until recently, the prevalence of these four male reproductive disorders in Denmark was among the highest and in Finland among the lowest reported[9–11,149,299,300]. In our previous study, we applied a partial least squares analysis to model the exposure to 121 endocrine disruptors in breast milk in the Danish-Finnish cohort[415]. In that study, the Finnish and Danish and subjects formed two perfectly distinguishable clusters, indicating a clear difference between the countries in the exposure pattern to endocrine disruptors[415]. When analysed using a more conventional analysis, the concentration of 58 of these chemicals in breast milk samples was significantly different between countries (6 after correction for multiple testing), and concentrations of 54 of the toxicants were

higher in Denmark (6 of 6 after correction for multiple testing), suggesting a heavier exposure in Denmark[415]. Given this background, it seems plausible that exposure to endocrine disruptors may contribute to the adverse trends in male reproductive disorders.

In terms of persistent organic pollutants, the two studies included in this thesis reported that the unadjusted levels of PCBs and PCDD/Fs in placenta, and the unadjusted levels of PCBs, dioxins and PBDEs in adipose tissue were not associated with an increased risk of congenital cryptorchidism. However, when the concentrations of PCBs and PCDD/Fs in adipose tissue were adjusted for various confounders including the duration of breastfeeding, country of origin and age at operation, a significant association between the risk of congenital cryptorchidism and the sum of PCBs, sum of PCDD/Fs and the total-TEq emerged. In contrast, the adjusted levels of PBDEs were not associated with the risk. Furthermore, the adjusted placental levels of PCBs and PCDD/Fs were not associated with the risk of congenital cryptorchidism. On the first impression, the epidemiological studies on associations between congenital cryptorchidism and placental[373], breast milk[237,373,393], adipose tissue[411], cord blood or tissue[237,410], maternal serum[409] and paediatric or maternal hair[374] concentrations of POPs may seem contradictory and confusing. On a closer inspection, exploiting the accumulated toxicological understanding, a tentative pattern appears.

The maternal breast milk concentrations of PBDEs were higher among the mothers of the cryptorchid boys compared to the mothers of the controls in our previous Danish-Finnish cohort, while the concentration of PBDEs measured in the placentas in the same cohort did not differ[373]. The lower placental concentration of PBDEs suggests that a placenta might not be as good a proxy of the gestational levels of PBDEs[373]. A recent Canadian study using maternal hair as a proxy for the prenatal PBDE exposure also noticed a difference in the levels of PBDEs between cryptorchid and healthy boys[374]. The lack of association between cryptorchidism and PBDE concentrations in the adipose tissue in our study does not contradict these results because our cohort of boys were not exactly new-borns and were very likely postnatally exposed to a large and highly variable amount PBDEs via indoor dust[357,358]. For instance, even though breastfeeding is usually considered the primary source of exposure to PBDEs among infants[357,358], the duration of breastfeeding did not correlate with the levels of PBDEs in our study.

The positive association between cryptorchidism and the concentrations of PCDD/Fs in adipose tissue fit well with our previous finding that the levels of PCDD/Fs in the breast milk of the cryptorchid boys were higher compared to the controls when the analyses were not corrected for multiple testing[393]. In contrast, the exposure-outcome relationship between PCBs and congenital cryptorchidism

does not seem at all coherent. Many studies using various proxies including maternal serum[237,409], cord blood[237], adipose tissue[411], (umbilical) cord samples[410], milk[237] and placenta have not reported an association between PCB concentrations and cryptorchidism. However, the concentrations of PCBs in breast milk were arguably associated with the risk of cryptorchidism in the Finnish cohort (when the correction for multiple testing was not considered), while in the Danish cohort they seemed to decrease the risk of cryptorchidism[393]. In our study, we observed an association between the adjusted adipose tissue concentrations of PCBs and the risk of cryptorchidism.

The variable associations between the exposure to PCBs and the risk of cryptorchidism suggest that instead of being causative themselves, they might act as a sentinel to exposure to other POPs. Speculatively, the exposure-outcome relationship between PCBs and cryptorchidism could also be more complex. In addition to the AhR pathway, PCBs are connected to GNRH and arachidonic acid pathways[393]. The GNRH and arachidonic acid pathways might be beneficial for testicular descent based on human observations of the boys with hypogonadotropic hypogonadism and experimental studies on lipocalin-type prostaglandin D_2 (a downstream metabolite of the arachidonic acid) deficient mice, which both have an increased risk of congenital cryptorchidism[285,436]. As a more simple explanation, our observed positive association might merely be a chance positive finding.

By design, observational epidemiological studies can only establish associations, and may be hampered by various confounders. Thus, experimental studies are needed to evaluate the causation especially in toxicology, since the randomised-controlled trials are out of question due to the ethical reasons. As reviewed in 2.4.4–2.4.6, there is some evidence to support the claims of reproductive toxicity of PCBs, PCDD/Fs and PBDEs.

However, it is difficult to draw conclusions on whether the doses used in experimental studies are relevant for the current exposure among humans for various reasons. Firstly, none of the toxicological studies reviewed in 2.4.4–2.4.6 measured the body burden of the substances after exposing the experimental animals to POPs. Thus, even though the dosage of the POPs were known, in order to translate these results to humans, variability in the amount of lipid, binding affinity to CYP1A2 in liver and the rate of metabolism and excretion have to be taken into account[437]. Secondly, as reviewed in 2.4.5, the susceptibility to 2,3,7,8-TCDD is greatly influenced by the structural variability in the AhR receptor. Thus, it is difficult to judge e.g. how to relate the doses used in the studies to the body burden of a pregnant woman. Applying various assumptions, an expert panel concluded that the long-term tolerable daily intake (TDI) of dioxins and dioxin-like compounds is 1–4 pg/kg body weight[437].

A recent study revealed that up to 10–12.5% of the 3-year-old Finnish toddlers exceeded the limit of 4 pg/kg and up to 58–68% the limit of 1 pg/kg based on analyses of food records[438]. Thus, the levels of environmental exposure may be close to the LOAEL based on experimental studies. However, as TDI was designed as the safety limit for the long-term exposure[437], the cumulative exposure to dioxins during the life-time is not likely to pose a large threat for health and well-being provided that the exposure is below TDI later during adolescence and adulthood[438].

In June 2017, the last commercially used PBDE congener joined PCBs in the list of banned chemicals in Stockholm convention, which also obliges the countries to take steps to reduce the emissions of PCDD/Fs[335]. The levels of PCBs, PCDD/Fs and the less brominated PBDE congeners have declined substantially, while the concentrations of more brominated PBDE congeners have remained stable. Furthermore, the discovery of the physiological role of the AhR signalling in e.g. haematopoiesis and immunity has led even some respected toxicologists to provocatively conclude that the fear of dioxins may be more harmful than the exposure to dioxins at the current low levels[439]. Therefore, these substances represent an eventual success story of the environmental regulation, and the exposure to PCBs, PCDD/Fs and PBDEs do not seem a plausible explanation for the increase in the prevalence of male reproductive disorders including congenital cryptorchidism.

6.3 Pubertal testicular development in boys with and without congenital cryptorchidism

In this thesis, the first longitudinal follow-up of pubertal development utilising serial measurements of testicular ultrasonography was presented. This allowed the use of a nonlinear mixed-effect model, which provided novel insights on testicular growth. We showed that the half of the testicular growth was typically reached at the age of 13.4 years, and the growth was the most intensive between the 12.7 and 14.1 years. Furthermore, our model provided a description of the inter-individual variability in the longitudinal testicular growth patterns, displaying little variation in the prepubertal testicular volume, while a substantial variability was observed in the postpubertal testicular volume and the timing of the testicular growth.

As described in 2.2.7.1, the attainment of a testicular volume of ≥3 ml is considered an evidence-based marker of the onset of puberty. Such a distinction between prepuberty and puberty is needed in epidemiological studies where a long follow-up may not be feasible and in clinical practice where predictive markers are needed to influence the treatment or follow-up. However, our results indicate that the onset

of puberty takes place gradually. In our study, the testicular growth slowly accelerated already years before reaching the testicular volumes corresponding to >3 ml by Prader orchidometer.

In our longitudinal pubertal follow-up, we showed that congenital cryptorchidism was associated with reduced testicular growth and post-pubertal testicular volume. This is in agreement with the previous cross-sectional data[261]. Based on our findings and the results of the previous cross-sectional studies showing elevated FSH and reduced inhibin B in adulthood after cryptorchidism[26,261], it seems that cryptorchid boys often have a Sertoli cell dysfunction. This Sertoli cell dysfunction may translate to subfertility, since the Sertoli cell number and immaturity is associated with reduced semen quality and the risk of testicular germ-cell cancer[13,43]. However, further studies with collection of semen samples from the same cohort are needed to confirm this.

6.4 Comparison between ultrasonography, Prader orchidometer and ruler

Prader orchidometer overestimates the testicular volume compared to ultrasonography and water displacement[192] and has a poor reproducibility[191]. Unsurprisingly, many previous studies have shown that the measurements by ultrasonography and orchidometer correlate[25,193–195]. However, even a highly significant statistical correlation between two measurements does not guarantee a clinically acceptable agreement[430]. Our study is the first to report 95% limits of agreement using Bland-Altman analysis, and showed a surprisingly large disagreement between the Prader orchidometer and ultrasonography. Both the systematic disagreement as well as the width of the 95% limits of agreement between ultrasonography and Prader orchidometer increased with age especially when the ellipsoid formula was used. This suggests that the precision of the orchidometer is relatively poor especially with larger testicular volumes. When reviewing the figures of the previous method comparison studies, this disagreement seems apparent in many[192–195,440,441], but not all previous studies[25,191,442].

This large disagreement may result from the inclusion of the surrounding tissue including scrotal skin and the epididymis, when examining with a Prader orchidometer. As illustrated in Figure 13, the difference in testicular length between 2 and 3 ml orchidometer beads is approximately 3 mm. However, the difference between 25 and 20 ml (3.5 mm) is almost the same. Thus, the inclusion of the surrounding tissue when measuring orchidometer may explain the increase in the width of the 95% limit of agreement. In practice, testes become less consistent with increasing size, which may complicate the measurement of the size and introduce

more variability. Furthermore, at least in childhood, the testicular width increases while the testicular height (a measurement in the third dimension perpendicular to the testicular width and length) remains fixed[443]. This indicates that the assumption that all testes resemble an ellipsoid of revolution with identical width and height (the ellipsoid formula) or with identical width and height and a fixed ratio between length and width (the Prader orchidometer) may be inaccurate when calculating the testicular volume.

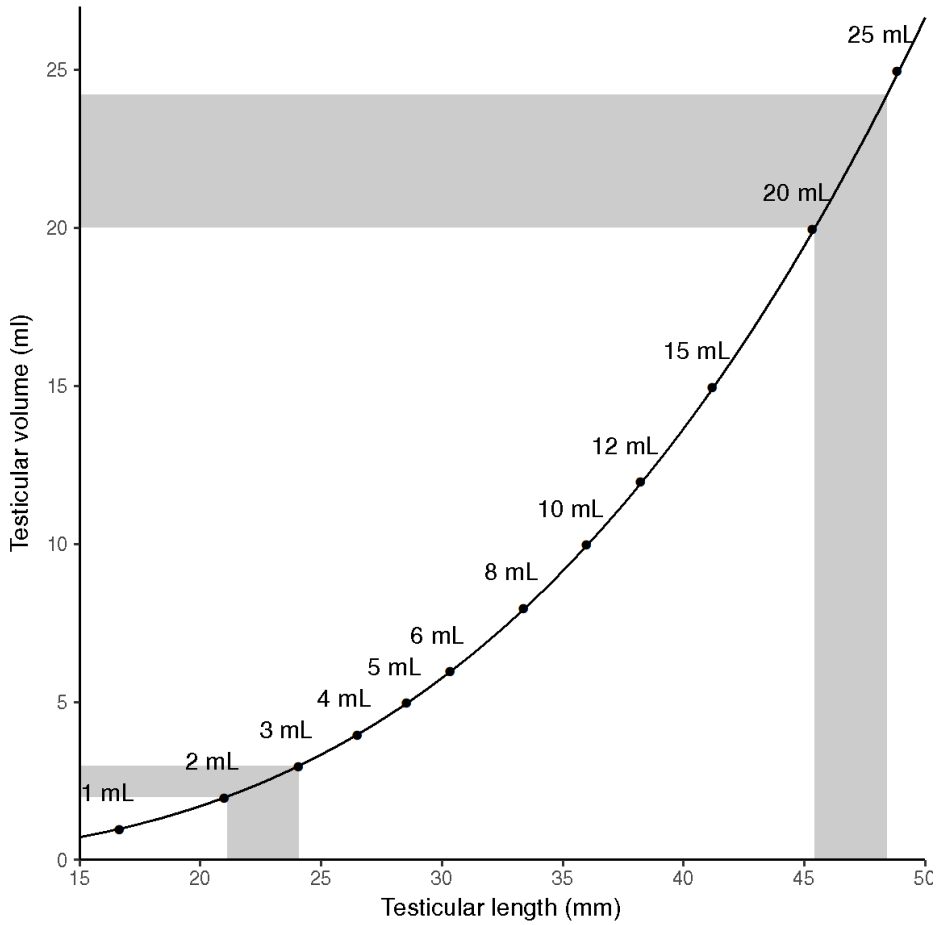

Figure 13 The relationship between testicular length and volume

The black curve represents the testicular volume as a function of testicular length assuming that the testis is an ellipsoid of revolution and that the ratio length/width is 1.567 as described in the original description of the Prader orchidometer[181]. The grey-shaded areas indicate an increase in testicular length by 3 mm from the testicular size of 2 ml and 20 ml, and the corresponding increase in testicular volume. The inclusion of the same amount of surrounding tissue in the measurement of testicular volume results in larger discrepancy between orchidometer and true testicular volume (or ultrasonography which does not include the surrounding tissue) when the testicular size is larger than when the testicular size is small.

Even though our results suggest that the orchidometer may be inaccurate especially with larger testicular sizes, there is nevertheless strong evidence to justify the use of the orchidometer (and the ≥3 ml cut-off) in the estimation of the pubertal onset, as reviewed above in section 2.2.7.1. Thus far, no such a clear cut-off values have been presented for ultrasonography, possibly because the measurement with ultrasonography gives a continuous and a more precise value. Our study reported that when the testicular volume by orchidometer was 3 ml, 95% of the testes measured with ultrasonography were within 0.75±0.28 ml when using the ellipsoid formula and 1.68±0.38 when using the Lambert's formula. Thus, testicular volumes of >1.3 ml and >2.4 ml by ultrasonography using the ellipsoid or Lambert's formula, respectively, suggest that the onset of puberty has been reached with a likelihood of 97.5% if the measurement error is assumed negligible. Similarly, a cross-sectional Dutch study reported that the average testicular volume by ultrasonography at the age when 4 ml by orchidometer was attained was 1.4 ml using the ellipsoid formula[186].

However, these two approaches do not present any benefit compared to the use of the orchidometer alone. In terms of estimating testicular volume, orchidometer can hardly be considered a gold standard. Our results suggest that the true testicular volume among prepubertal boys resides within 0.15–0.29 ml, as measured by ultrasonography. Furthermore, clear testicular growth was noted years before volumes corresponding to 3 ml by orchidometer were reached. It seems likely that the 'prepubertal' testicular growth is a marker of an imminent acceleration of the pubertal maturation. If so, it could aid in the differential diagnostics of hypogonadotropic hypogonadism and constitutional delay in growth and puberty (CDGP)[444]. Thus, further longitudinal studies evaluating both hormonal data and testicular volume by ultrasonography especially among boys with CDGP and hypogonadotropic hypogonadism are warranted.

Although a ruler or a sliding calliper were originally used in the measurement of the testicular volume using the ellipsoid or Lambert's formula, the Prader orchidometer gained popularity since its description in 1966[181]. However, the orchidometer is not always available among general practitioners, and thus the evidence from previous studies using 3 ml cut-off for Prader orchidometer (approximately 24 mm) has been equated with 20 mm or 25 mm testicular length measured by ruler in Finland and elsewhere[190,445]. Our study suggests that a testicular length cut-off of 30 mm (but not 20 or 25 mm) agrees reasonably well with the orchidometer, and may provide an estimate of the imminent onset of puberty, at least in experienced hands. However, it remains unknown whether our findings can be generalised to examiners who may have less experience in the measurement of the testicular length by a ruler.

7 CONCLUSIONS

1. The function of the Sertoli and Leydig cells during postnatal minipuberty contributes to form a consolidation of the testicular descent, which may protect from acquired cryptorchidism during the subsequent testicular ascent in childhood when the activity of hypothalamus and pituitary declines.

2. The prenatal exposure to PCDD/Fs and PCDD/F-like PCBs may predispose to congenital cryptorchidism. However, the relevance of these two groups of toxicants in the pathogenesis of congenital cryptorchidism in future is limited by the successful global environmental regulation.

3. The testicular growth during puberty is a rapid phenomenon and is reduced among boys with a history of congenital cryptorchidism. This indicates that they suffer from a mild Sertoli cell dysfunction, which may predispose them to reduced fertility and testicular germ-cell cancer.

4. A surprisingly large disagreement exists between testicular volume measured by ultrasonography and by Prader orchidometer, suggesting that the ultrasonography cannot be replaced with the Prader orchidometer without a significant loss of precision. However, the use of the orchidometer in the estimation of pubertal onset is backed by a wealth of evidence, whereas no such data for the use of ultrasonography has been presented.

ACKNOWLEDGEMENTS

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