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Clinical aspects of histological and hormonal parameters in boys with cryptorchidism

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All patient photos in the thesis are published after written consent from the parents.
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To Aleksander

Contents

Papers included in this thesis

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Hildorf S, Clasen-Linde E, Cortes D, Fossum M, Thorup J. Fertility potential is compromised in 20% to 25% of boys with nonsyndromic cryptorchidism despite orchiopexy within the first year of life. *J Urol* 2020 Apr;203(4):832–840.

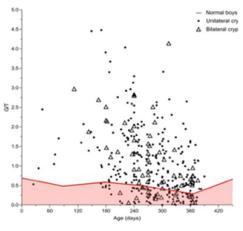
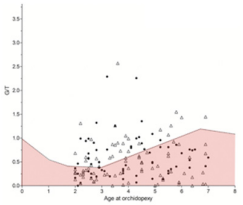
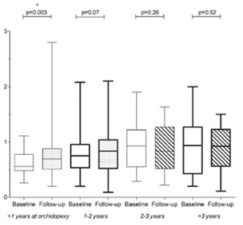
II. Hildorf S, Clasen-Linde E, Fossum M, Cortes D, Thorup J. Fertility potential is impaired in boys with bilateral ascending testes. *J Urol* 2021 Feb;205(2):586–594.

III. Hildorf S, Cortes D, Clasen-Linde E, Fossum M, Thorup J. The impact of early and successful orchidopexy on hormonal follow-up for 208 boys with bilateral nonsyndromic cryptorchidism. *Pediatr Surg Int* 2021 Mar;37(3):339–345.

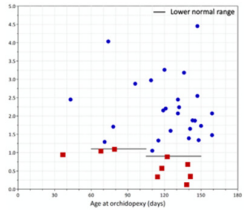
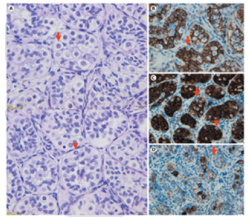
IV. Hildorf S, Hildorf A, Clasen-Linde E, Cortes D, Walther-Larsen S, Li R, Hutson JM, Thorup J. The majority of boys having orchidopexy for congenital nonsyndromic cryptorchidism during minipuberty exhibited normal reproductive hormonal profiles. *Eur J Pediatr Surg* 2022 Feb;32(1):26–33.

V. Hildorf S, Cortes D, Gül M, Dong L, Kristensen SG, Jensen CFS, Clasen- Linde E, Fedder J, Andersen CY, Hoffmann ER, Sønksen J, Fossum M, Thorup J. Parental Acceptance Rate of Testicular Tissue Cryopreservation in Danish Boys with Cryptorchidism. *Sex Dev*. 2019;13(5–6):246–257 (published Oct 20 2020).

Thesis at a glance

Paper	Main questions	Methods	Results	Illustrations	Conclusions
I	What is the fertility potential at the time of surgery among infant boys with cryptorchidism who undergo orchidopexy within the first year of life?	A clinical cohort study with retrospective assessment of testicular biopsies and reproductive hormonal values. Studied: 333 boys aged 34–390 (median age 274) days. 69 (21%) boys had bilateral cryptorchidism.	25% had reduced G/T, 23% lacked Ad spermatogonia, and two boys had no germ cells. Inhibin B significantly correlated with G/T and AdS/T, a total of 70 boys (21%) had inhibin B below 2.5th percentile.		About 20% to 25% of boys with nonsyndromic cryptorchidism possibly have a risk for infertility despite early orchiopexy during the first year of life.
II	Do testes that ascent from the scrotum have a decreased fertility potential?	*as Paper I* Studied: 67 boys aged 2–7.0 (3.8 years) years diagnosed with bilateral ascended testes compared to 86 boys median aged 3.9 years (2–6.9 years) with late referral bilateral congenital cryptorchidism	Same fraction of boys had G/T below lower range (60% vs 66%), lacked Ad spermatogonia (31% vs 34%) and had inhibin B below 2.5th percentile (13% vs 15%, all $p > 0.05$).		The fertility potential was equally impaired in boys with bilateral ascending testes and bilateral congenital cryptorchidism. Ascended testes should be surgically corrected when the diagnosis is settled.
III	Does the hormonal status (inhibin B and FSH) improve at 1-year follow-up in prepubertal boys after surgical correction for bilateral cryptorchidism?	*as Paper I* and a clinical follow-up study. Studied: 208 boys with bilateral cryptorchidism aged 4 months to 9 (1.7) years. The median age at follow-up was 2.7 years.	Inhibin B MoM improved significantly from time of surgery to follow-up. Orchidopexy before 1 year of age expressed the most favourable improvement, as inhibin B MoM significantly increased for this age group ($p < 0.03$). Totally, at 1-year follow-up, inhibin B was below 2.5th percentile in 26% of the boys.		Surgery does matter. At time of follow-up, 26% of these boys with surgery for bilateral cryptorchidism may risk infertility. Some boys could be suspected of an endocrinopathy.

Thesis at a glance *(Continued)*

Paper	Main questions	Methods	Results	Illustrations	Conclusions
IV	Do boys with congential nonsyndromic cryptorchidism have a normal minipuberty?	*as Paper I* Studied: 35 boys aged 37–159 (124) days. Five (14%) boys had bilateral cryptorchidism.	Nine (26%) had G/T below lower range. Two of them could be expected of an endocrinopathy as FSH were below or just above 2.5th percentile. 97% had normal male LH/FSH ratio. 54% (19/35, 95% CI 0.37–0.71) had normal G/T and AdS/T as well as all hormones within normal range.		The majority of boys with congenital nonsyndromic cryptorchidism exhibit normal minipuberty pattern. Few cases could be suspected of an endocrinpathy.
V	Do parents accept experimental cryopreservation? – a pioneer study. Is the rate of acceptance different when offered as an additional procedure vs as part of bilateral orchiopexy?	Offering cryopreservation to parents as an experimental fertility preservation option for their prepubertal boy. Studied: 14 parents of boys with severely reduced fertility potential who were offered cryopreservation as an additional procedure vs 27 parents of boys at time of bilateral orchidopexy.	The acceptance rate to perform testicular tissue cryopreservation was 90% (95% CI 0.77–0.97) with 37 out of 41 parents. No difference between in the two groups 93% (13/14) vs 89% (24/27, $p = 0.68$).		There was a high acceptance rate to perform cryopreservation, even though the procedure efficacy is largely unproven and may only be indicated in about 20% of bilateral cases.

Acknowledgements

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Abbreviations

Ad spermatogonia, Type A dark spermatogonia; AdS/T, Number of Ad spermatogonia per cross-sectional seminiferous tubule; AMH, Anti-Müllerian hormone; CD99, Cluster of differentiation 99 (also referred to as MIC-2); CI, Confidence interval; c-KIT, KIT proto-oncogene; D2-40, Anti-podoplanin M2A antigen (also referred to as M2A); DAZL, Deleted in azoospermia like; DC, Dina Cortes, MD, DMSc.; ECL, Erik Clasen-Linde, MD.; FSH, Follicle-stimulating hormone; G/T, Number of germ cells (gonocytes, spermatogonia, and spermatocytes) per cross-sectional seminiferous tubule; GnRH, Gonadotropin-releasing hormone; hCG, Human chorionic gonadotropin; HE, Hematoxylin and eosin; HPA, Hypothalamic–pituitary gonadal axis; INSL3, Insulin-like 3 hormone; JT, Jørgen Thorup, MD, PhD.; KK, Kolja Peter Kvist, MD.; LH, Luteinizing hormone; LIN28, Lin-28 homolog A; MAGE-A4, Melanoma-associated antigen A4; MoM, Multiple of the median; NANOG, Homeobox protein Nanog; Oct3/4, Octamer-binding transcription factor 3/4 (also known as POU5F1); PAS, Periodic acid-Schiff; PGC, Primordial germ cells; PLAP, Placental alkaline phosphatase; RXFP2, Relaxin/insulin-like family peptide receptor 2; SC/T, Sertoli cell number per cross-sectional tubules SOX9: SRY-box transcription factor 9; SH, Simone Hildorf, MD, PhD student; SRY, Sex-determining region Y; SSC, Spermatogonial stem cell; UTF-1, Undifferentiated embryonic cell transcription factor 1; VASA, Dead-box helicase 4 (also known as DDX4).

Summary

In about 1–4% of full-term boys, one or both testes fail to descend into the scrotum at birth representing one of the most common congenital anomalies. The cryptorchid testis is typically characterized by the decreased number and impaired maturation of germ cells following an increased risk of infertility and testicular cancer in adulthood. Particularly, boys with bilateral cryptorchidism comprise a high risk of infertility with reports of azoospermia in up to 18–46%. To minimize germ cell loss and damage, early surgical correction between 6 and 12 (−18) months of age are now recommended for cryptorchidism. However, the effect of early surgery has sparsely been evaluated. Moreover, many other aspects of cryptorchidism still remain controversial including management strategies for patients with acquired spontaneously ascending testis (ascent of a scrotal testis during infancy) and those suspected of a high risk of later infertility. Hence, a better understanding of pathohistological and hormonal findings of boys with cryptorchidism may change and extend our treatment and management strategies.
This thesis is based on unique data obtained at orchidopexy for valuing the fertility potential with a combined analysis of histological and hormonal parameters including FSH, LH, testosterone, and the Sertoli cell hormone inhibin B. This is very seldom practiced elsewhere.

The PhD project aimed at analyzing the fertility potential in cryptorchid boys who were expected to exhibit good fertility potentials: patients who underwent early orchidopexy according to the Nordic recommendations (Paper I), patients with bilateral acquired ascending testes (Paper II), and patients operated for cryptorchidism during minipuberty (Paper IV). Another aim was to evaluate the hormonal effect of surgery in boys with bilateral cryptorchidism by determining preoperative and postoperative hormones in relation to testicular histopathology (Paper III). Finally, we studied the parental acceptance rate of testicular tissue cryopreservation, an experimental procedure where a small testicular biopsy from a prepubertal boy is stored at −80° C with the prospect of preserving future fertility (Paper V).

In Paper I, we evaluated histological and hormonal data from 333 infant boys with congenital nonsyndromic cryptorchidism (21% bilateral disorder) who underwent orchidopexy within the recommended age. At the time of surgery, 25% of boys had a reduced number of germ cells per cross-sectional tubule (G/T) and 23% of boys lacked type A dark (Ad) spermatogonia, which is suggested to be the male germline stem cell and essential for future fertility. Moreover, 21% of boys had an inhibin B below 2.5th percentile. Consequently, even though cryptorchid boys undergo orchidopexy within the first year of age up to 25% may risk later infertility.
In Paper II, we compared histological and hormonal data from 67 boys with bilateral ascending testes to 86 boys with late-referral congenital bilateral cryptorchidism. The age of these patients was similar ranging between 2 and 7 (median 3.8 vs 3.9) years. The fertility potential within these groups was compromised to almost the same level with mean G/T below normal lower range (60% vs 66%), bilateral absence of Ad spermatogonia (31% vs 35%), and inhibin B below 2.5th percentile (13% vs 15%), implying that at least 30% are at risk of infertility.
This advocates that orchidopexy should be performed of ascending testis immediately after diagnosis and underscores the importance of clinical examinations for acquired cryptorchidism. Especially, boys with retractile testes should be followed regularly, since such testes often undergo ascent.
In Paper III, we studied a cohort of 208 boys with bilateral cryptorchidism (median age 1.7 years) who underwent orchidopexy with fertility potential assessment and 1-year hormonal follow-up. Orchidopexy improved the fertility potential in bilateral cryptorchidism based on inhibin B measurements. Patients who underwent orchidopexy before 1 year of age expressed the most favorable improvement in inhibin B. But around 25% of patients, corresponding to those with low inhibin B at follow-up, may risk future fertility problems despite surgery. In 85% of these patients, no compensatory elevated FSH was observed,

which may suspect a hypothalamus–pituitary–gonadal defect. It is an open question if these patients may benefit from adjuvant gonadotropin treatment after orchidopexy.
In Paper IV, we included 35 consecutive boys with congenital nonsyndromic cryptorchidism (14% bilateral disorder) who underwent orchidopexy during minipuberty before 160 days of age. By assessing the fertility potential, we found G/T to be below the normal lower range of 26% (9/35). Two of these had indications of endocrinopathy as no compensatory FSH could be demonstrated. Totally, 97% exhibited a normal LH/FSH ratio. LH was more often above 97.5th percentile than FSH: 34% vs 3%. Thus, reproductive hormonal profiles of the cryptorchid boys exhibited a normal mini-pubertal pattern.
In Paper V, we examined the parental acceptance rate of testicular tissue cryopreservation. We found that 90% of 41 parents of prepubertal boys with cryptorchidism gave consent to cryopreservation of testicular biopsies during initial orchidopexy or as a second procedure. These parents gave consent despite their knowledge that the efficacy of cryopreserved prepubertal testicular tissue for fertility preservation is largely unproven and that the use of tissue may only be needed in about 20% of boys with bilateral cryptorchidism. The high acceptance rate is important to highlight if cryopreservation should be considered in clinical practice in the future.

In conclusion, this PhD project revealed new and important findings on the risk of later infertility in cryptorchid boys. At the time of surgery within the recommended age range (6–12 months), about 20–25% of infant boys with nonsyndromic cryptorchidism had a compromised fertility potential. Also, about 30% of boys with bilateral ascending testes displayed compromised fertility potential equivalent to that seen in congenital cryptorchid testis at the same age.
Early bilateral orchidopexy, especially before 1 year of age, improved the fertility potential based on follow-up inhibin B measurements, however, around 25% of the patients exhibited low inhibin B at follow-up, and the majority of these had no compensatory increase in FSH.
The present studies contribute to the debate on whether early orchidopexy is sufficient for improving the fertility outcome or if supplemental treatment modalities such as adjuvant hormonal treatment or cryopreservation might be indicated in patients with suspected risk of later infertility. Based on our pioneer study, parents may welcome new aspects of treatment for cryptorchidism. Future multidisciplinary research should expand our knowledge on adult gonadal function after early orchidopexy, test efficacy of adjuvant hormone treatment for those at high risk of later infertility, and develop fertility preservation approaches based on cryopreserved prepubertal testicular tissue.

Dansk Resumé

Kryptorkisme, hvor én eller begge testikler mangler i skrotum, forekommer hos 2–4% af alle fuldbårne drenge og repræsenterer dermed en af de hyppigste medfødte misdannelser indenfor børnekirurgi. Den kryptorke testikel er karakteriseret ved lavt antal og nedsat modning af germinalceller. Som konsekvens heraf har disse drenge en øget risiko for senere infertilitet og testikelcancer i voksenlivet. Risikoen for infertilitet er særlig høj ved bilateral kryptorkisme, hvor azoospermi kan konstateres i op til 18–46% af tilfældene. For at minimere tab og beskadigelse af germinalceller anbefales tidlig korrigerende operation (orkiopeksi) i alderen 6–12 måneder. Effekten af tidlig operation er dog kun sparsomt belyst. Ydermere er mange aspekter af kryptorkisme fortsat omdiskuterede, herunder behandling af drenge med erhvervede ascenderende testikler (ascensus af en skrotal testis i løbet af barndommen) og behandling af drenge med særlig høj risiko for senere infertilitet. En bedre forståelse af histologiske og hormonelle fund hos drenge med kryptorkisme kan muligvis bidrage til udviklingen af nye og individualiseret behandlingsmuligheder. Denne afhandling er baseret på unikke data indsamlet ved orkiopeksi med henblik på en vurdering af fertilitetspotentialet ud fra en analyze af histologiske og hormonelle parametre, herunder follikelstimulerende-hormone (FSH), luteiniserende hormon (LH), testosteron og inhibin B.

Ph.d.-projektet havde til formål at analysere fertilitetspotentialet for tre grupper af kryptorke drenge, der forventedes at have gode prognoser for senere fertilitet: drenge, der har fået foretaget tidlig orkiopeksi i henhold til anbefalingerne (artikel I), drenge med erhvervet bilateral ascenderende testikler (artikel II), samt drenge der er opereret før 6 månedersalderen (artikel IV). Et andet formål var at evaluere den hormonelle effekt af operation hos drenge med bilateral kryptorkisme ved at bestemme præ- og postoperative hormonniveauer og sammenligne dem med den histopatologiske status på operationstidspunktet (artikel III). Endeligt undersøgte vi, hvorvidt forældre til drenge med kryptorkisme ville acceptere kryopræsevering af præpubertalt testikelvæv; en eksperimentel procedure, hvor en lille testikelbiopsi fra en præpubertal dreng nedfryses og opbevares ved −80°C med henblik på bevarelse af fremtidig fertilitet (artikel IV).

I artikel I evaluerede vi histologiske og hormonelle data fra 333 drenge med medfødt ikke-syndromisk kryptorkisme (21% bilateral tilstand), som fik foretaget orkiopeksi indenfor den anbefalede alder. På operationstidspunktet påviste vi, at 25% af drengene havde et nedsat antal af germinalceller per tværskåret tubulus (G/T), og at 23% af drengene manglende type A dark (Ad) spermatogonier, der menes at være stamceller for mandlige kønsceller og dermed er af afgørende betydning for fremtidige fertilitet. Desuden havde 21% af drengene inhibin B under 2,5th percentilen. Ud fra disse fund fandt vi, at selvom orkiopeksi udføres indenfor det første leveår risikerer op mod 25% af drenge stadig senere infertilitet. I artikel II sammenlignede vi histologiske og hormonelle data fra 67 drenge med bilateralt ascenderende testikler med 86 aldersmatchede drenge med medfødt bilateral kryptorkisme, der var henvist i sen alder. Fertilitetspotentialet i de to grupper var nedsat i næsten samme grad. Vi fandt, at den gennemsnitlige G/T var under den normale nedre grænse i 60% vs 66% af tilfældene, et bilateralt fravær af Ad spermatogonier i 31% vs 35% og nedsat inhibin B i 13% vs 15% af tilfældene, hvilket indebærer, at mindst 30% kan være i risiko for infertilitet. Det taler for, at orkiopeksi bør udføres af ascenderende testikler umiddelbart efter diagnosen og understreger betydningen af kliniske undersøgelser af drenge for erhvervet kryptorkisme. Især bør drenge med retraktile testikler følges regelmæssigt, da sådanne testikler har øget risiko for ascensus.
I artikel III undersøgte vi en kohorte af 208 drenge med bilateral kryptorkisme (medianalder 1,7 år), der fik foretaget orkiopeksi med vurdering af fertilitetspotentialet samt etårs opfølgning med hormonværdier. Baseret på inhibin B forbedrede operation fertilitetspotentialet for drenge med bilateral kryptorkisme. Særligt de drenge, der fik foretaget orkiopeksi før etårsalderen, fik betydelig forbedring i inhibin B med signifikant forskel mellem præ- og postoperativ inhibin B. Dog risikerer omkring 25% af drengene,

svarende til dem med lav inhibin B ved opfølgning, fremtidige fertilitetsproblemer trods operation. Hos 85% af disse drenge blev der ikke observeret forhøjet kompenseret FSH, hvilket kan give mistanke om en defekt af hypothalamus-hypofyse-gonadal aksen. Det er fortsat til debat, om disse patienter kan have gavn af supplerende hormonbehandling.
For at vurdere fertilitetspotentialet blandt drenge, der er opereret i minipuberteten (2–5 månedersalderen), blev 35 drenge (14% bilaterale) inkluderet i artikel IV. Vi fandt, at ni drenge havde nedsat G/T, hvoraf to af disse drenge havde nedsat FSH, lavere end eller blot lige over 2,5th percentilen, trods nedsat G/T. Generelt, havde 97% af drengene en normal LH/FSH ratio. LH var oftere højere end 97,5th percentilen end FSH: 34% vs 3%. Langt de fleste drenge havde en normal minipubertetsprofil.
Som et pilotstudie undersøgte vi i artikel V, hvor ofte forældrene accepterede, at deres søn fik foretaget eksperimental kryopræservation af testikelvæv. Vi fandt, at 90% af 41 forældre til drenge med kryptorkisme ønskede kryopræservering af testikelbiopsier, som en sekundær procedure eller i forbindelse med orkiopeksi. Forældrene var fuldt informerede om, at det endnu ikke er afklaret, om kryopræserveret præpubertalt testikelvæv kan anvendes til at erhverve fertilitet som voksen, og at det sandsynligvis kun bliver nødvendig for omkring 20% af drengene, som blev opereret for bilateral kryptorkisme. Den høje acceptrate i dette studie kan få betydning, hvis kryopræservering bliver en del af klinisk praksis i fremtiden.

Ph.d.-projektet præsenterer nye og vigtige fund, der er relevante for vurderingen af senere infertilitsrisiko for drenge med kryptorkisme. Omkring 20–25% af drenge med ikke-syndromisk kryptorkisme, der blev opereret indenfor den anbefalede alder (6–12 måneder), havde et nedsat fertilitetspotentiale. Ydermere blev der påvist et nedsat fertilitetspotentiale hos omkring 30% af drenge opereret for bilateralt ascenderende testikler ligesom hos drenge opereret for medfødt kryptorkisme i tilsvarende alder. Tidlig bilateral orkiopeksi, især før etårsalderen, viste det bedste fertilitetspotentiale vurderet ud fra inhibin B. Dog havde omkring 25% af drengene opereret omkring 1,7 års-alderen, et lavt inhibin B 1 år efter operation, og størstedelen af de drenge havde ikke kompensatorisk forhøjet FSH.
De foreliggende undersøgelser bidrager til debatten om, hvorvidt tidlig orkiopeksi er tilstrækkeligt for at forbedre fertilitetspotentialet, eller om supplerende behandling såsom hormonbehandling eller kryopræservering bør overvejes for drenge med høj risiko for senere infertilitet. Pilotstudiet belyser, at forældre er imødekommende overfor kryopræservering, som kan lede forskningen ind på endnu uprøvede behandlingsmuligheder ved kryptorkisme. Fremtidig multidisciplinær og tværfaglig forskning skal udvide vores viden om fertilitetspotentialet hos voksne mænd efter tidlig orkiopeksi, teste effekten af supplerende hormonbehandling og udvikle nye fertilitetsbevarende teknikker til præpubertale drenge ved kryopræserveret testikelvæv.

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Clinical aspects of histological and hormonal parameters in boys with cryptorchidism

Thesis for PhD degree

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INTRODUCTION—THE SCOPE OF THE PROBLEM

Cryptorchidism (from the Greek "hidden testis") is the failure of descent of one or both testes to the scrotum [1,2]. In the western world, about 2.5% of all boys undergo surgery for cryptorchidism, thus it remains one of the most common surgical procedures performed in boys [3,5,4].

In general, cryptorchidism is considered a mild anomaly, but it can have serious effects on men's health in adulthood as it represents the best-characterized risk factor for infertility and testis cancer. Men with a history of cryptorchidism make up around 20%–27% of azoospermic men [6,7]. Men with former bilateral cryptorchidism have significantly lower paternity rates (65%) in comparison to men with former unilateral cryptorchidism (90%) and control men (93%) [8,9]. In addition, such patients have a greater risk of testicular neoplasia than the general population [10–15].

Nowadays, early surgical correction is the gold standard for cryptorchidism with the prospects of minimizing germ cell loss and damage. The Nordic consensus on the treatment of cryptorchidism advocates for surgery starting at 6 months and preferably performed by 12 months of age [16]. The European Association of Pediatric Urology recommends orchidopexy from 6 months and within the subsequent year, by 18 months at the latest [17,18], whereas the American Urological Association advises orchidopexy between 6 and 18 months of age [2]. However, the effect of early orchidopexy has sparsely been evaluated, and surgery in childhood does not guarantee subsequent fertility and paternity [19-22]. Consequently, an important issue is how to identify prepubertal boys with cryptorchidism at high risk of infertility at surgery and monitor testicular function after surgical treatment. Moreover, it is a challenge to identify and treat boys who develop cryptorchidism after birth.

The intention of this thesis is to increase knowledge of the fertility potential in boys with cryptorchidism. Testicular histology with pathohistological grading together with the status of reproductive hormones obtained at the surgery are markers of importance for future fertility (the fertility potential), whereas the status of reproductive hormones can be followed to monitor testicular function after treatment. This unique data can help clinicians identify those patients with a high risk of infertility, which is the first step to potentially changing our approach and management.

The main treatment goals for boys with cryptorchidism are to give them the ability to father biological children and avoid testicular cancer. However, this might not be achievable by early surgical intervention alone. If there is an underlying endocrinopathy resulting in a low number of germ cells and inadequate germ cell maturation, simply repositioning the testis into the scrotum will not correct the endocrinopathy. Hence, supplementary treatment should be considered for boys with evident hypoplasia of

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germ cells and malfunctioning of the hypothalamic–pituitary–gonadal axis. Moreover, if the boy has a severely decreased number of germ cells, it may be valuable to offer testicular tissue cryopreservation of the early germ cells, while they are still present.

TESTICULAR DEVELOPMENT

Development of germ cells

One may advocate that germ cells are the most unique and precious cell type of the human body. They not only proliferate and differentiate but also are the only cell type to undergo meiosis to produce haploid gametes, having the ability to give rise to each subsequent generation. Testicular cells serve a crucial role in germ cell differentiation and maintenance including the production of reproductive hormones.

The production of the haploid spermatozoa is a highly complex process, in preparation for embryonic life involving the migration of the bipotential primordial germ cells to the formation of fetal, neonatal, and prepubertal germ cells, which further develops from puberty and continues through the renewal and division of spermatogonial stem cells (SSCs) to produce spermatozoa by meiosis.

Prenatal development – The sex-specific development of the male germline initiates around 6–7 weeks post-conception with the expression of sex-determining region Y (SRY) and SRY-box transcription factor 9 (SOX9) in the gonadal somatic cells (pre-Sertoli cells), inducing the formation of seminiferous cords with Sertoli cells [23-25]. With their formation, Sertoli cells start to synthesize anti-Müllerian hormone (AMH), which inhibits Müllerian duct development and thereby prevents female development [24]. At this point, primordial germ cells are settled in the gonadal ridge and are now commonly referred to as gonocytes, where they begin to differentiate and proliferate populating the seminiferous cords along with Sertoli cells. In this thesis, the term gonocyte covers all germ cells after they become residents in the developing testis [24]. Other classifications of gonocytes and fetal germ cells, for example, pro- or prespermatogonia have been proposed [26,27] but no ubiquitous consensus regarding terminology has been reached. Leydig cells begin to appear within the interstitial of the testis around week 9 post-conception [23,24]. They secrete testosterone and other factors critical for germ cell differentiation and testicular development such as insulin-like-3 hormone (INSL3). Surrounding the seminiferous cords lie peritubular myoid cells, factor-secreting muscle cells, that can be recognized at week 12 post-conception [23,24].

During the first trimester, gonocytes are mitotically active forming a quite homogenous cell population expressing markers typical of pluripotent cells including primordial germ cells such as KIT proto-oncogene (c-KIT), octamer-binding transcription factor 3/4 (Oct3/4, also known as POU5F1), lin-28 homolog A (LIN28), homeobox protein Nanog (NANOG), anti-podoplanin M2A antigen (D2-40, also referred to as M2A), and placental alkaline phosphatase (PLAP) [23,24,28,29,30,31,32]. This suggests that gonocytes are quite equivalent to primordial germ cells, both presenting the distinctive morphology of being large circular cells with a prominent nucleus containing one or two nucleoli surrounded by a spherical shape cytosol [26,33]. Gonocytes occupy the center of the lumen-less seminiferous cords and are easy to distinguish from the associated Sertoli cells (Fig. 1B). Based on stereological estimations of 50 fetal human testes, the number of germ cells increased from a mean of 3.700 to 1.417.000 from 5 to 19 weeks post-conception [34].

Toward the end of pregnancy, the majority of fetal germ cells lose mitotic activity together with the pluripotency and fetal markers starting to form different germ cell subpopulations. They transform into fetal spermatogonia which are larger, flattened cells located on the basement membrane [1]. Simultaneously, they begin to express additional germ cell-specific markers such as melanoma-associated antigen A4 (MAGE-A4), dead-box helicase 4 (DDX4; also known as VASA), and deleted in azoospermia like (DAZL) [28,30]. In support of this, Li *et al.* [30] studied testes from 12 human fetuses ranging from 4 to 25 weeks of gestation for single-cell RNA sequencing and grouped fetal germ cells into three clusters: "migrating," "mitotic," and those entering "mitotic arrest." The cluster of the "migrating" fetal germ cells was dominated by the

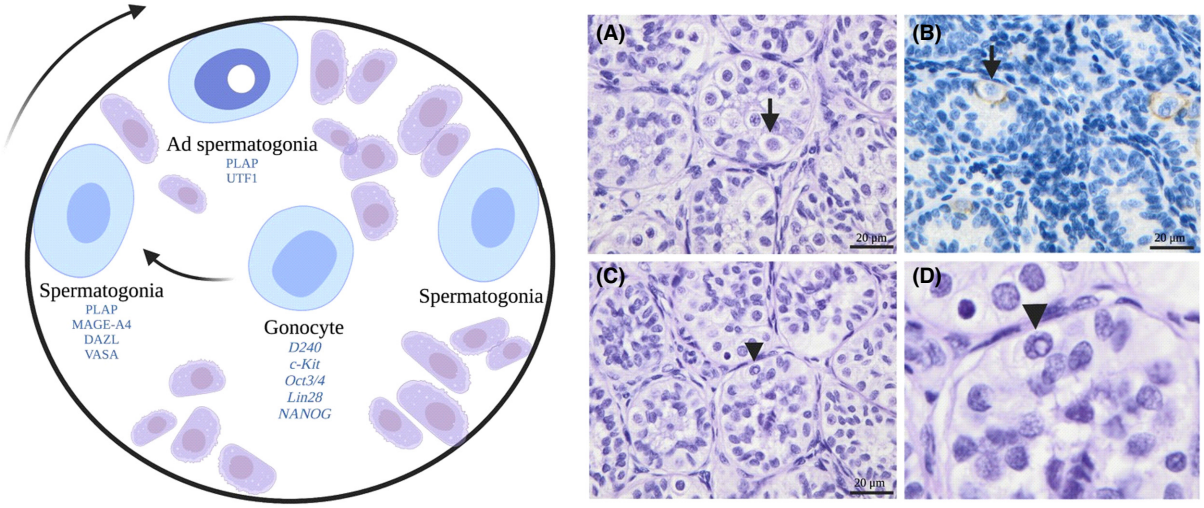

Fig. 1. Germ cell maturation. Schematic illustration of the postnatal germ cell maturational steps through migration, morphological characteristics, and expression profiles. Histologically, the seminiferous tubules in the infant boy does not have a lumen and are filled with Sertoli cells and germ cells. (A) HE-staining of testis biopsy from an infant boy with cryptorchidism illustrating the typical morphological characteristics of spermatogonia, arrow (B) in PLAP-staining, and (C, D) ad spermatogonia, arrowhead.

expression of Oct3/4, NANOG, Sal-like 4 protein 4 (SALL4) whereas the "mitotic" fetal germ cells expressed the same but in addition VASA and DAZL.

From week 9 and onwards, "mitotic arrest" fetal germ cells appeared to have similar expression patterns, but weaker for Oct3/4 and NANOG and stronger for VASA and DAZL in addition to new markers. Importantly, in the testis from an 18-week of gestation fetus, VASA-positive germ cells were distributed more in the peripheral zones of the seminiferous tubules in contrast to the Oct3/4-positive germ cells. Altogether, this supports the notion that male fetal germ cells develop through stages of migration, mitosis, and cell-cycle arrest.

Postnatal development – Postnatally, the germ cells continue to undergo further differentiation and migration toward the basement membrane of the seminiferous tubule. After migration, the morphology of germ cells is distinctly different forming the spermatogonial population, which consists of SSCs and other non-stem cells progenitors (subtypes of spermatogonia, Fig. 1B,C). Three morphologically distinct types of spermatogonia have been classified, namely type A dark (Ad), type A pale, and type B spermatogonia [35-37].

It is believed that gonocyte transformation into type A dark (Ad) spermatogonia is an essential step for the formation of the SSC pool necessity for fertility [38,39]. In other words, Ad spermatogonia are considered to represent the SSC dividing to either self-renew to maintain the SSC pool or give rise to the Ap spermatogonia that may undergo one or more divisions before giving rise to differentiated B spermatogonia [36]. The nucleus of the Ad spermatogonia is homogeneous dense and dark with at least one rarefaction zone (chromatin-free cavity), which is distinguishable from the lightly stained, coarser nuclei from the other two types without the rarefaction zones (Fig. 1A) [36,37,40].

Ad spermatogonia usually appear during minipuberty, sharply increasing in number by the age of 4–5 months and remaining largely quiescent [41,42], until the boy enters puberty where germ cell differentiation begins, truly indicated by a transition capable of meiotic division [24]. Studies have demonstrated that some fetal germ cells seem to remain after birth and along up to the first months of life, as demonstrated by some germ cells positive

for the maintaining markers D2-40, Oct3/4, c-Kit, and NANOG [43-47]. Kvist *et al.* [44,45] found positivity for D2-40 in germ cells up to 6 months of age, Oct3/4 up to age 6 and 9 months, and c-Kit up to 11–16 years during puberty. Gonocytes that fail to migrate to the basement membrane and differentiate normally undergo apoptosis and are cleared from the seminiferous epithelium, which presumably takes place during minipuberty until 1 year of age [37,48,49].

The importance of Ad spermatogonia has been supported by follow-up demonstrating their link with fertility outcomes [39,50]. Ad spermatogonia are sometimes positive for PLAP and undifferentiated embryonic cell transcription factor 1 (UTF1) [51,52], however, no specific marker for only Ad spermatogonia exists [53]. More recent publications including novel omics techniques have made attempts to assign spermatogonial subtypes into a hierarchical organization or functional stages [30,46,54,55], indicating that SSC might have more complex physiology than previously assumed. But despite these efforts to widen the landscape of the testis, no specific marker and unequivocal definition of the prepubertal human SSC have been made. Therefore, the morphological criteria for identifying Ad spermatogonia have been used in the present thesis.

Transformation of spermatogonia to primary spermatocytes usually begins around the age of 3–4 years [37,38]. During childhood, the germ cells express various different markers, such as c-KIT, UTF1, PLAP, MAGE-A4, VASA, and fibroblast growth factor receptor 3 (FGFR3) but normal materials during the prepubertal period are sparse [44,47,55].

The prepubertal testis

Germ cells together with Sertoli cells are most prominent in the prepubertal testis. Sertoli cells appear to have a more constant morphological and immature appearance from birth to 11 years of age, in contrast to Leydig cells that are more scarce and only seldom demonstrable in the light microscopy after 2 years of life [37,42,48].

The prepubertal testis may be regarded as a quiescent organ but serves as a crucial period for germ cell development. Given the highly complex environment necessary for germ cell development requiring delicate cellular arrangement, cell-to-cell interactions, migration, mitosis, and possible endocrine factors, the prepubertal period is highly sensitive and at risk of being disturbed, which consequently can lead to disease and infertility. Moreover, proliferation and differentiation take place in the prepubertal testis as the number of germ cells varies a lot from mid-gestation toward puberty [56].

During the period from week 28 of gestation until around 3 years of life, a rise in the total number of germ cells by a factor of 3 was demonstrated during the first 100 days of life with a maximum at 100–150 days of age and followed by a decreased by a factor of 0.5 [49]. From the same materials, Sertoli cells increased by more than a factor of five during the first 3 months of life to reach a steady density until the onset of puberty where Sertoli cells increased by a factor of 2 [57]. The postnatal escalation of Sertoli cells elongates the seminiferous tubules and results in testicular growth [58,59].

The histological parameter "number of germ cells per cross-sectional tubule (G/T, also referred to as S/T; 'S" stands for spermatogonia) has been used in the quantification of germ cells and correlates with the number of germ cells per cm^3 testicular parenchyma [56]. Hence, G/T is a parameter of numerical density. Based on normal materials, it has been demonstrated that G/T decreases slightly from birth to 3 years of age, with the most rapid decline during the first year, and then increases afterward until 8 or 9 years with a small decline or pause to then increase markedly toward the puberty [37,56,60,61].

The prepubertal testis has two main functions—the production of sex steroids and the establishment and maintenance of the diploid germ cells, essential for male genital differentiation, growth, and future spermatogenesis. At the onset of puberty, the organization of the prepubertal testis changes dramatically as the lumen occurs and Sertoli cells mature and form the blood–testis barrier by compartmentalizing the seminiferous epithelium into basal and ad luminal compartments [1]. These changes enable spermatogenesis.

The hypothalamic–pituitary–gonadal axis

The two pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the pivotal endocrine regulators of these testicular functions controlled by pulsatile secretions of gonadotropin-releasing hormone (GnRH, also known as the luteinizing-releasing hormone, LHRH) from the hypothalamus. This system is coordinating a tightly regulated feedback loop between the hypothalamus, the anterior pituitary, and the testes, the so-called hypothalamic–pituitary–gonadal axis. Evidence suggests that early stimulation of the testis is regulated by the placental human chorionic gonadotropin (hCG) since hypothalamic control of gonadotropic function is not operative until after the first trimester [62,63].

Minipuberty – The hypothalamic–pituitary–gonadal axis is largely quiescent until the onset of puberty, except for two brief transient activations; one during fetal life at mid-gestation and a second activation at 2–5 months postnatally known as the minipuberty [64,65].

During minipuberty, circulating testicular hormones reach high levels as seen in adults [66]. The gonadotropins exert differential targets on the compartments of the testes. LH stimulates Leydig cells that secrete testosterone, along with glycoprotein mediators such as INSL3, whereas FSH presumably targets Sertoli cells to release inhibin B and anti-Mullerian hormone (AMH) [67]. A Danish study including reproductive hormone measurements on 1840 infants (1041 boys and 799 girls) during minipuberty showed that the LH concentrations overcome the FSH concentrations in male infants, the opposite situation occurs in females [68].

It is generally believed that minipuberty is essential to prime germinal epithelium for subsequent germ cell maturation as well as expansion since the transient peak is associated with increased proliferation of Sertoli cells, Leydig cells, and germ cells [42,48,49,57,69]. Of importance, it is believed that minipuberty is essential for the gonocyte transformation into Ad spermatogonia [48,70]. Moreover, the postnatal surge of testosterone is believed to be associated with a rapid increase in penile growth, testicular volume, and anogenital distance [65,71,72]. Despite the presence of high testosterone and FSH, spermatogenesis does not progress since immature Sertoli cells are devoid of androgen receptors. Minipuberty may also have an important impact on

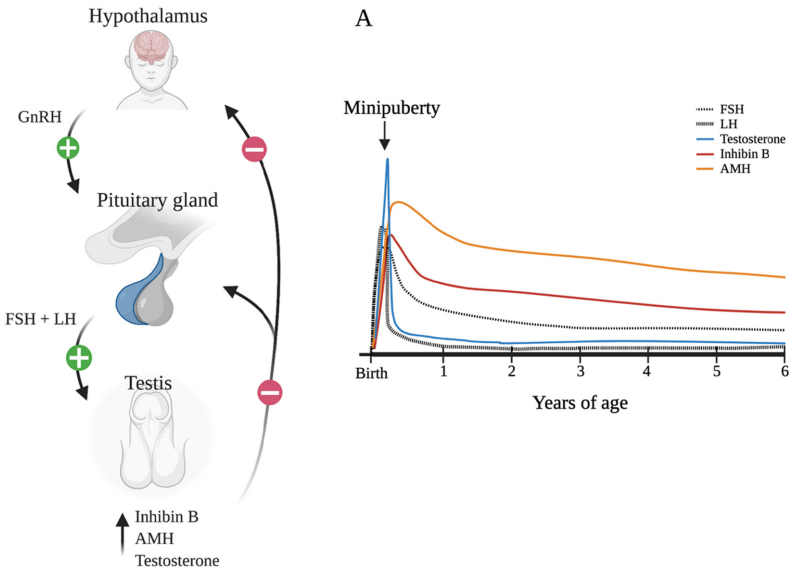

Fig. 2. The hypothalamic–pituitary–gonadal axis. Schematic presentation of the function and regulation of the male hypothalamic–pituitary-gonadal axis. (A) the concentrations of reproductive axis hormones during minipuberty.

secondary sex characteristics and other biological functions [65,73].

After 6 months of age and through childhood, LH and testosterone decrease to very low or undetectable concentrations [66] (Fig. 2). FSH and inhibin B also decrease but remain clearly detectable throughout childhood [66] (Fig. 2). In contrast, AMH remains rather high during childhood [74] until it decreases in puberty [75].

Testicular descent

The normal process of testicular descent is multi-staged and complex involving the interplay of anatomical and hormonal factors [76]. The current theory generally focuses on hormonal aspects and divides testicular descent into two functional phases: the "transabdominal phase" with the migration of the testis from the abdomen to the internal inguinal canal and the "inguinoscrotal phase," where the testis enters the inguinal canal to reach the bottom of the scrotum [24,77,78]. These findings largely originate from animal models, but the main steps can also be demonstrated in humans in accordance with the widely accepted two-phases theory [79,80].

Transabdominal phase – The testes begin their descent around 8–10 weeks of gestation from the lower kidney pole, where they are anchored in position cranially by the cranial suspensory ligament attached to the posterior abdominal wall and caudally by the gubernaculum (also known as the genitoinguinal ligament) (Fig. 3A) [24]. During the transabdominal phase, the descent of the testis is guided by these two ligaments, as the cranial suspensory ligament regresses and the gubernaculum swells thus facilitating descent downwards (Fig. 3A).

According to animal studies, regression of the cranial suspensory ligament is regulated by androgens [81,82]. At the same time, the gubernaculum enlarges by increasing its extracellular matrix, mainly glycosaminoglycans and hyaluronic acid, becoming bulky and gelatinous [83,84].

Since the gubernaculum arises from the testis' caudal segment and attaches close to the future internal inguinal ring, its swelling gradually moves the testis downward [24].

The proliferation and swelling of the gubernaculum are influenced by the Leydig cell-derived INSL3 [82,85,86], a peptide hormone of an insulin-related gene family that can be detected in human male amniotic fluid in the second trimester during the transabdominal phase [87]. The importance of INSL3 has been demonstrated in rodents with depletion of INSL3-receptor (RXFP2) and INSL3 knockout rodents associated with intraabdominal testes and a "feminized" gubernaculum (which is thin and elongated without proliferation and swelling reaction) [82,86,88].

Besides INSL3, the gubernaculum might be controlled by AMH produced by the Sertoli cells, however, direct experimental evidence is lacking. An argument against the role of AMH is the observation that AMH-deficient mice present normal testicular development and descent [89].

The transabdominal phase may be completed as early as 15 weeks of gestation when the testis is positioned close to the internal annular ring, which allows a timely pause for the gubernaculum to undergo extensive remodeling and further enlargement. Regression of the cranial suspensory ligament is important, but not the primary effector of transabdominal descent. Namely, this phase may be independent of androgens but is highly dependent on INSL3 targeting the gubernaculum [24,78].

Inguinoscrotal phase – The subsequent migration through the inguinal canal and further down to the bottom of the scrotum occurs between 25 and 35 weeks of gestation [78-80].

During the transinguinal migration of the testis, the gubernaculum gradually grows and extends through the internal annular ring situated caudally, dilating, and forming the inguinal canal thus allowing passage for the testis (Fig. 3B). Aided by gubernacular protruding and possibly the intraabdominal pressure during enlargement of the abdominal cavity, the testis is pulled downward [78]. During this stage, a specialized peritoneal diverticulum forms the processus vaginalis [24]. Moreover, striated muscle fibers from the abdominal wall and the genitofemoral nerve arising from the lumbar plexus are carried downward with the evagination to form the scrotal sac including the cremaster muscle. Another downward pull-

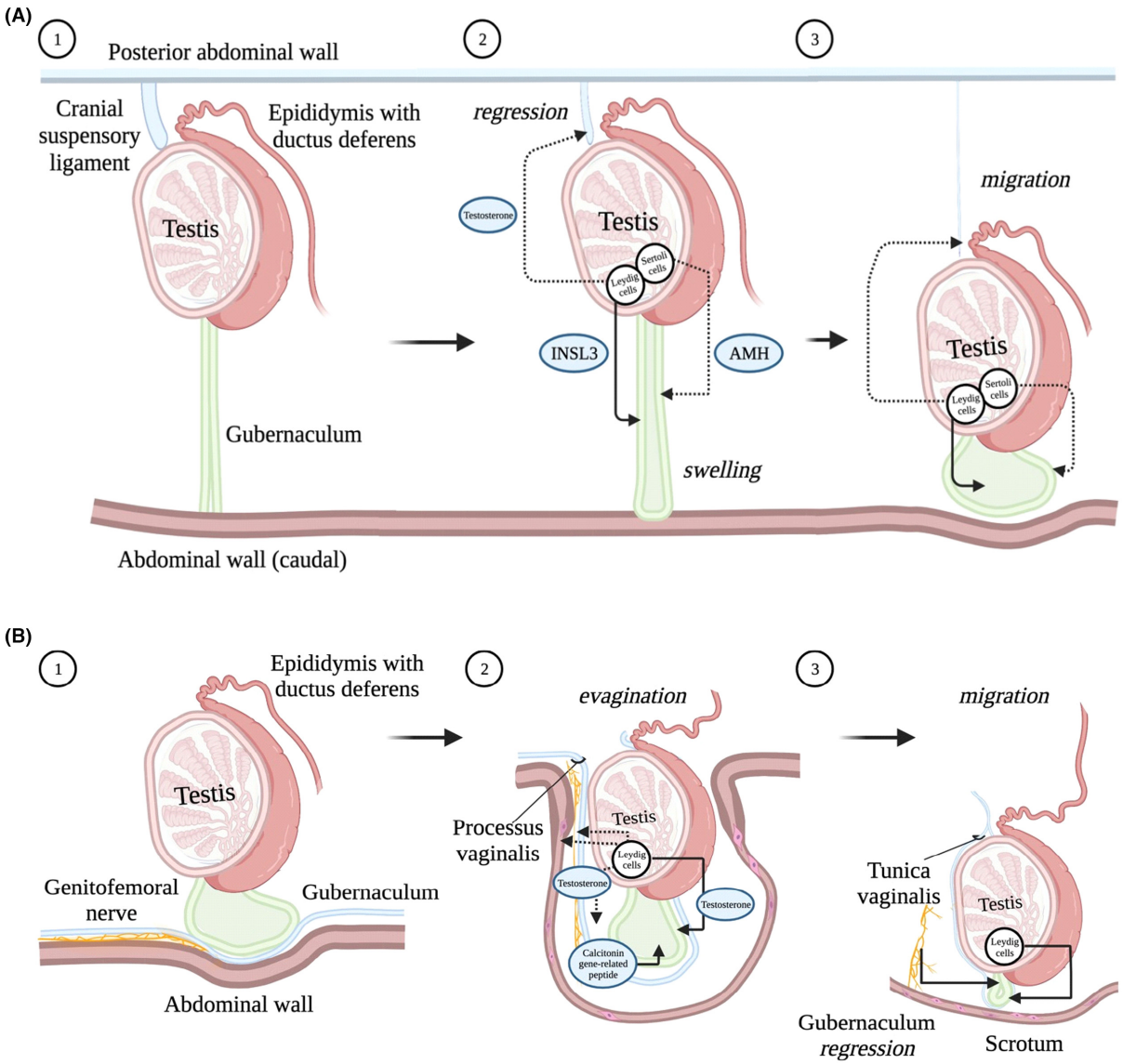

Fig. 3. (A) The normal descent of the testes. Illustration of key elements during the transabdominal phase, which is dependent on insulin-like 3 hormone (INSL3). The solid lines relate to the most evident and essential hormonal signals, whereas the dotted lines represent more uncertain and probably weaker signals. (B) The normal descent of the testes. Illustration of the key elements during the androgen-dependent inguinoscrotal phase.

function has been suggested, evoked by the rapidly growing cauda epididymis involving a fusion between the testis and epididymis [90].

Recent studies have elucidated that the major regulator of inguinoscrotal descent is testosterone *via* possible actions; masculinization of the genitofemoral nerve, stimulation of processus vaginalis needed to constrict the inguinal canal, and remodeling of gubernaculum mediating downward migration [78,91].

In particular, the inguinoscrotal migration is believed to be highly dependent on androgen stimulation of the genitofemoral nerve, which secretes calcitonin gene-related peptide to provide chemotactic directional guidance for the

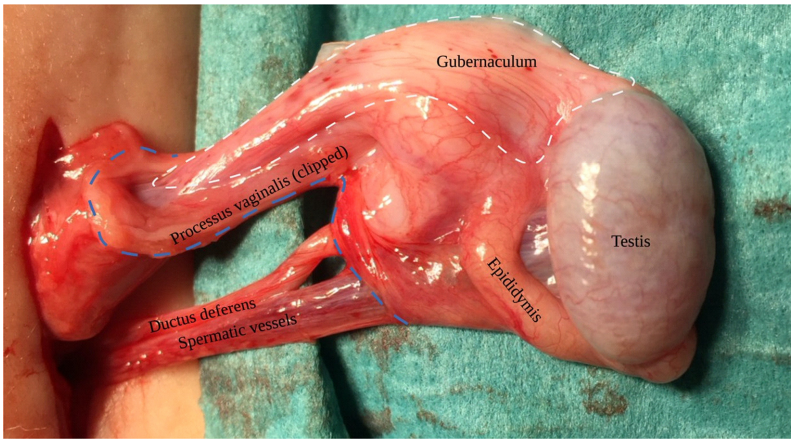

Fig. 4. The gubernaculum. What the gubernaculum can look like at orchidopexy in an 11-months old patient with right-sided cryptorchidism. In this patient, the gubernaculum is a bulky and fibrous structure connected to the caudal pole of both the testis and epididymis, which is covered on all sides by a peritoneum except the posterior where the vessels and ductus deferens pass. (published after consent received from the boy's parents).

gubernacular expansion, growth of the cremaster muscle, and expansion of vaginal processus [91-93].

Testicular descent is completed by regression of the gubernaculum to its final position within the scrotum around 35 weeks of gestation [24]. The human gubernaculum may regress fully but can often clearly be defined at orchidopexy as a remnant or sometimes as a thick conical structure adherent to the base of the testis and epididymis (Fig. 4). During infancy, the cranial portion of the processus vaginalis is usually obliterated resulting in a discrete remnant sac, the vaginal tunic (or tunica vaginalis), but it is not rare for the entire vaginal process to remain patent leaving a connection between the abdominal cavity and the scrotal sac [24].

The essential physiological feature of the scrotal position for the testis refers to a specialized, low-temperature environment (3°–5° cooler than abdominal temperature) with thermoregulation by the cremaster muscle [24,94].

CRYPTORCHIDISM

Cryptorchidism, "undescended testis," "retentio testis," and "maldescended testis" are terms synonymously used to define incomplete

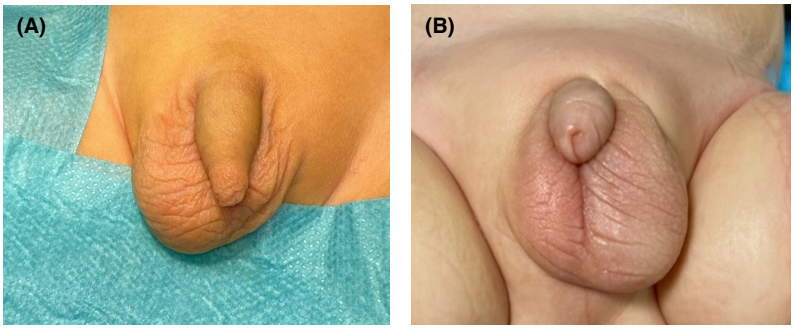

Fig. 5. Cryptorchidism. Two 10-months old boys with unilateral congenital cryptorchidism, (A) left-sided and (B) right-sided and non-palpable, where the testis was located within the inguinal canal verified by laparoscopy. (Published after consent was received from the boy's parents.)

descensus of the testis from an intraabdominal position into the scrotal sac [1]. The term cryptorchidism is from the Greek "kryptos" (κρυπτός) and "orchis" (ὄρχις) meaning "hidden" and "testis," respectively (Fig. 5).

Prevalence

The prevalence of cryptorchidism is age-dependent and can vary between countries and centers as the definition of cryptorchidism differs (Fig. 6), however, a figure of 1–4% at birth in full-term and normal weight (>2500 g) boys are generally accepted [3,5,95,96].

According to a systematic review of 46 studies between 1934 and 2006, the rate of cryptorchidism at birth in term and/or birth weight >2500 g infants varied from 1% to 4.6%, whereas the rate was a lot higher among premature and/or birth weight <2500 g infants varying from 1.1% to 45% [96]. Congenital cryptorchidism may resolve spontaneously during the first months of life probably due to minipuberty [97,98]. Hence, the prevalence is lower among boys at 1 year of age with a prevalence of 1–1.5% in term and/or birth weight >2500 g infants [96]. The prevalence of cryptorchidism increases after 1 year of age due to acquired forms of cryptorchidism with rates as high as 7% around 7 years of age [99] (Fig. 6). At 15 years of age, the prevalence decreases to 1.6–2.2% [96]. Using the most quoted figure of 1–4% at birth, it can be assumed that about 624 Danish boys (with a mean of 2%) were born with cryptorchidism in 2020 (out of 31.175 boys born that year, range between 312 and 1247) (Statistics Denmark, 2020).

Subtypes of cryptorchidism

The diagnosis of cryptorchidism is settled by physical examination with palpation. The physician often distinguishes between a non-palpable testis and a palpable testis. Approximately 70% of cryptorchid testes are palpable [100]. An ultrasound investigation or other imaging is not a part of the current recommendations for diagnosis [2,16,17]. An undescended testis usually lies along the normal

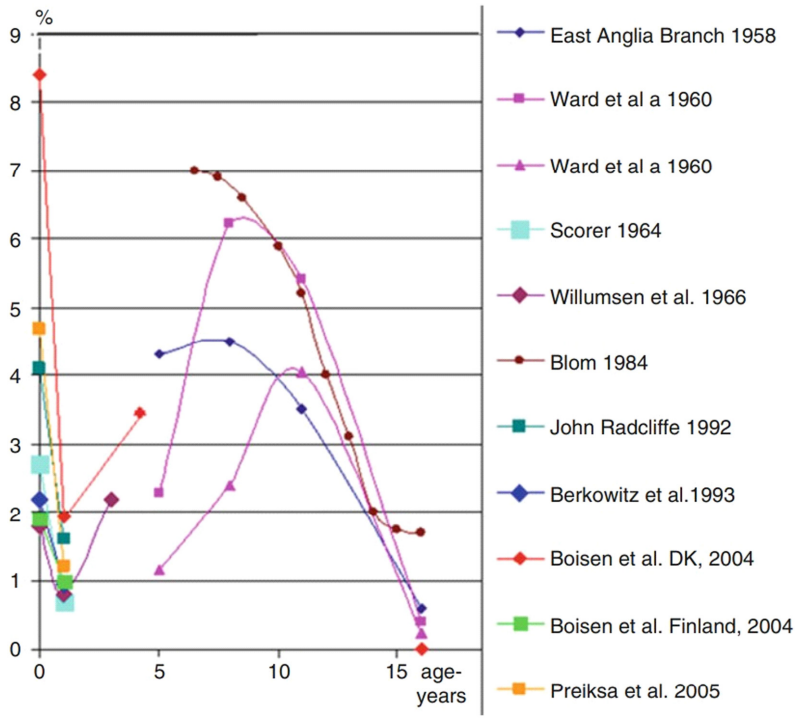

Fig. 6. Prevalence of cryptorchidism. The reported prevalence (%) of cryptorchidism in relation to the age of the boys. Source from Thorup *et al.* [5].

pathway of testicular descensus, see Fig. 7, or can more rarely, be ectopic. Failure of the testis to normally descend can occur bilaterally, which is observed in approximately 23% of operated cases, as reviewed in a total of 2150 boys including seven studies [4].

Congenital cryptorchidism – Congenital cryptorchidism is usually found at the first physical examination of the newborn after birth or at routine pediatric examinations by the general practitioner. The Danish healthcare system is publicly funded and accessible to all Danish citizens with high adherence to the seven pediatric examinations at 5 weeks, 5 months, 1 year, and then every year up to 5 years of age. Any boy suspected of cryptorchidism should be referred to a pediatric surgeon or urologist for final diagnosis and treatment, preferably within the first 6–12 months, or 18 months of life [2,16,17]. Thus, referral already at the recognition of the condition before 5–6 months of age is, in most cases, preferable allowing internal hospital check-up for awaiting spontaneous descent consequently avoiding ill-fated waiting time for surgical treatment [101].

Other subtypes – Cryptorchidism is not solely a congenital disorder since acquired forms diagnosed in infancy and childhood also exist. This is reflexed in the reported bimodal distribution of age at surgery for orchidopexy [102-105].

An "ascending" testis represents an acquired form of cryptorchidism in which a scrotal testis later ascents into a cryptorchid position during infancy (typically after 1–2 years of age) and cannot be manipulated into a stable position in the scrotum anymore [106-108]. The frequency of ascending testis of all operated cases has been reported to range from 20% to 70% [104,106,108,109]. Boys with spontaneously resolved congenital cryptorchidism or retractile testis have a higher rate of ascent. A 32% risk of ascent has been reported among boys with retractile testis [110].

In boys with retractile testis, pronounced (or hyperactive) cremaster reflex may retract testes to the upper part of the scrotum, or suprascrotal, even though the testicular cord allows the testes to be manipulated into the scrotum. It is important to distinguish between ascending testis and retractile testis, since the latter may be a normal variant that can be settled at a stable scrotal position without tension until cremaster reflex is induced.

The existence of acquired forms of cryptorchidism in addition to the congenital form adds to the complexity of the evaluation of cryptorchidism. In addition, some surgeons propose that a high scrotal or "gliding" testis should be regarded as a part of the spectrum of cryptorchidism, which is characterized as a testis that is located below the external ring and can be brought through the scrotal entrance into the upper half of the scrotum but tends to ascend to its original position [111,112]. It refers to an unstable testis where further traction toward the scrotum may cause pain. The gliding testis is not a well-defined subtype and is not mentioned in the guidelines. Finally, secondary testicular ascent can be a complication of inguinal herniorrhaphy or hydrocelectomy resulting in an iatrogenic cryptorchid testis. Presumably, the mobile prepubertal testis may mechanically slip out of its scrotal position and becomes trapped in scar tissue after groin surgery in an abnormal position above the scrotum [113].

Finally, congenital monorchism or absent testis can be identified in around 4–10% of all boys with cryptorchidism [17,100,114]. Congenital monorchism is considered a condition in which an initially normal testis in intrauterine life has subsequently atrophied and disappeared partially (defined as testicular regression syndrome) or completely, a vanished testis [115]. Consequently, a vanished testis is characterized by blind-ending of ductus deferens and vessels (also known as the vanishing testis syndrome) with complete regressed testis, whereas testicular regression syndrome is anatomically characterized by a remnant or nubbin at the terminal end of the spermatic cord with no macroscopically identifiable testicular tissue. The absence of a viable testis is usually unilateral and assumed to be caused by testicular agenesis or a traumatic/catastrophic event such as perinatal testicular torsion or infarction [116]. The contralateral testis is likely to enlarge beyond the normal size for age due to compensatory testicular

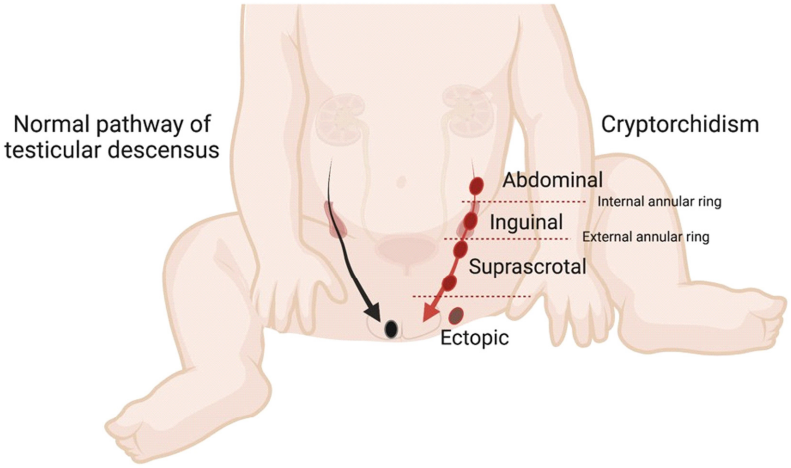

Fig. 7. The different locations of a cryptorchid testis. Illustration of normal pathway of testicular descensus (black) and maldescensus leading to cryptorchidism (red). Cryptorchid testes are classified on the basis of their position along the pathway of descent; high or low intraabdominal, inguinal including close to the internal or external annular ring, suprascrotal, or ectopic involving an abnormal location outside the normal pathway.

hypertrophy [117,118], which also can be reflected by serial sets of inhibin B measurements indicating a functional compensation [115,119]. When evaluating studies on materials of boys with cryptorchidism, it is crucial to be aware of what subtypes are included. Especially when the interpretation of results is generalized.

Etiology and risk factors

The etiology of cryptorchidism is partly unknown but believed to be multifactorial with a combination of anatomical, endocrinological, environmental, and genetic factors. Understanding the relationship between hormonal factors and anatomical structures, such as the cranial suspensory ligament, gubernaculum, and the transinguinal migration, is vital to the comprehension of how cryptorchidism can occur (see background on normal testicular descensus).

Given the crucial importance of Leydig cell hormones in the process of normal testicular descent, disturbance in the hormonal balance might be involved in the etiology of cryptorchidism [120-122]. Congenital hypogonadotropic hypogonadism is a condition characterized by GnRH or gonadotropin deficiency and can result in cryptorchidism lacking sufficient FSH and LH at birth [123,124]. Based on anatomical findings, some claim that cryptorchidism can be caused by disturbances of fusion between the testis and epididymis [90,125], however, clinical data may argue against it [126]. An incomplete disappearance of processus vaginalis can result in a fibrous remnant within the spermatic cord that cannot elongate with age has been a suggested cause for the ascending testis [92,127]. Also, an abnormal gubernaculum insertion site or an endocrine deficiency have been suggested as etiological factors, especially for ascending testis [128-130]. A recent study claim that the etiology of ascending testes involves similar operative findings as to the congenital condition, suggesting that ascending testis is a congenital condition and is first noticeable with the child's growth [131].

A markedly higher incidence of cryptorchidism can be found among boys who are born small for gestational age, which suggests that the intrauterine environment plays an important role in the development of cryptorchidism [105,132,133,134]. Other risk factors of importance relate to overall maternal health and lifestyle characteristics during pregnancy including overweight (during and prepregnancy), smoking, gestational diabetes, intake of paracetamol, and binge drinking episodes [135-140]. Few studies have indicated an increased risk of cryptorchidism among sons

of mothers exposed to pesticides during pregnancy, for example, by farm residence or occupation [141,142]. Moreover, environmental compounds and chemicals mimicking the actions of hormones may contribute to cryptorchidism as they can act as estrogens and antiandrogens affecting the complex descent process [143-145]. In this context, the interaction of environmental and genetic factors may lead to differences in the susceptibility of individuals to potentially adverse effects of the endocrine disruptors [144]. Recently, a potential association between antenatal serum concentrations of phthalate metabolites and male reproductive health in early adulthood has been demonstrated [146], which is supported by studies showing that phthalate exposure in adult men has an adverse impact on testicular function [147,148]. Finally, it has been hypothesized that cryptorchidism can be part of a so-called testicular dysgenesis syndrome, which also comprises hypospadias, testicular germ cell cancer, and a high risk of azoospermia [145]. The founders of this theory propose that testicular dysgenesis syndrome is a result of endocrine-disrupting chemicals in the embryonal programming and gonadal development during fetal life. However, the concept of testicular dysgenesis syndrome as a unitary entity has also been questioned [149-151].

The genetics in cryptorchidism are complex but certain genetic conditions and mutations are associated with cryptorchidism modulating the prevalence of cryptorchidism with interactions in the gene–hormone–environment. For example, the disorder of sexual development and other conditions, for example, mutations in the Kal1 gene (responsible for Kallmann syndrome), in the INSL3 gene or receptor gene (RXFP2), are associated with hypogonadotropic hypogonadism and manifest Leydig cell dysfunction including reduced production of INSL3 and testosterone [124,143]. According to an Italian study, the frequency of genetic alterations was low in a cohort of 600 cases (2.8%, 95% CI 1.7–4.5%) [152]. In this study, Klinefelter syndrome was found in 8 cases (1.3%) as the only chromosomal anomaly, whereas five cases had mutations in the RXFP2 gene (0.8%), two cases in the INSL3 gene, and two cases with androgen receptor gene mutations (0.3%). More complex syndromes including Downs syndrome, Prader Willi, Noonan's syndrome, prune belly syndrome, and persisting Müllerian duct syndrome have cryptorchidism as a typical feature [143]. Furthermore, cryptorchidism can be a part of a caudal field defect linked with other malformations and dysplasia of the kidneys, ureters, and spine from T10 to S5 [153].

Lastly, familial predisposition influences the risk of cryptorchidism. A large population-based study observed increasing concordance rates of cryptorchidism based on family relations: 3.2% (95% CI 2.7–3.6%) in boys with no relation, 3.4% (2.3–4.7%) in paternal half-brothers, 6.0% (4.5–7.7%) in maternal half-brothers, 8.8% (8.3–9.8%) in full brothers, 24.1% (16.–34%) in dizygotic, and 27.3% (16%–41%) in monozygotic twin brothers, suggesting both unknown genetic, environmental, and maternal factors contribute to cryptorchidism risk [136,137].

The multifactorial etiology of cryptorchidism involves a combination of anatomical, endocrinological, environmental, and genetic factors. Largely, cryptorchidism occurs as an isolated condition without an association with other anomalies or syndrome, referred to as nonsyndromic cryptorchidism where the underlying cause cannot be identified [13,143,154].

Treatment

Over the last decades, the recommended age of surgical correction for cryptorchidism has progressively decreased and today early orchidopexy should ideally be performed soon as possible after 6 months of age. In 2007, the Nordic consensus on the treatment of cryptorchidism advocated that orchidopexy for cryptorchidism should be performed between 6 and 12 months of age [16]. According to the American Urological Association (AUA) and European Association of Urology (EAU)/ European Society of Paediatric Urology, the recommended age for orchidopexy is between 6 and 18 months of age [2,17,18], preferably before 12 months in the European guidelines [17,18].

The essence of early corrective intervention is to (1) optimize the potential for fertility and prevent infertility; (2) reduce the risk of

testicular neoplasia; (3) eliminate the risk of testicular torsion; and (4) alleviate cosmetic concerns and in addition to allowing an early diagnosis of neoplasm by self-palpation. Current recommendations for early orchidopexy are based on observational and follow-up studies utilizing various metrics in adulthood including paternity rates, semen samples, testicular volume, and hormonal reproductive concentrations (mainly FSH and inhibin B) as well as histological findings at orchidopexy (mainly germ cell number) advocating that early intervention is favorable for the reproductive function. The only randomized trial regarding age at orchidopexy is the prospective study by [155] of 164 boys with unilateral cryptorchidism who were randomized to undergo orchidopexy at 9 months or 3 years. One-year postoperative, a significantly larger testicular volume and a significantly higher number of germ cells and Sertoli cells per tubule were demonstrated in the early orchidopexy group when compared to the late orchidopexy group.

Hormonal treatment to achieve testicular descent has been used in the past but is not currently recommended [2,16,17,18].

Possible supplementary treatment modalities

Clinically, one of the challenges regarding patients with cryptorchidism is the lack of evidence for the efficacy of treatment adjuvant to surgery.

Adjuvant hormonal treatment – Since cryptorchidism can be associated with endocrinopathy, adjuvant hormonal treatment is relevant to consider. In recent years, there has been considerable interest in the potential gonadotrophic effects of GnRH on the testis, largely because of the potential therapeutic use for improving the fertility potential. This is based on the theoretical rationale that a normal hypothalamic–pituitary–gonadal axis is a prerequisite for germ cell maturation and proliferation, particularly the postnatal stimulation during minipuberty. Hadziselimovic and Herzog [128] observed a significant increase in the number of spermatozoa, increased normal forms, and improved motility of spermatozoa in men who after surgery for cryptorchidism in childhood had received Buserelin for 6 months in childhood compared to those who had not.

The most recent clinical guidelines from the EAU, presented at the annual congress 2021, were in favor of the adjuvant use of GnRH to improve the fertility potential in bilateral cryptorchidism [18]. However, they stated that the identification of those boys who would benefit from adjuvant hormonal treatment is very difficult. The Nordic Consensus group does not recommend medical therapy for improving the fertility potential [16]. The reluctance to recommend adjuvant treatment is related to the past when hormonal treatment was used for testicular descent, which was associated with low success and inflammatory changes in the testes and even reports of reducing the number of germ cells [156,157].

The foregoing results strongly indicate that adjuvant GnRH can be safe with no significant adverse effects reported and efficacious by improving fertility, particularly for those with bilateral cryptorchidism [128,158,159,160,161,162,163] (Table 1).

However, all the studies have limitations and more high-quality randomized clinical trials are needed in the future with specific identification of subtypes and grades of cryptorchidism that will benefit from the hormonal treatment. This is a challenge. In a recent pilot study assessing histology before and after hormone treatment, our group showed that two out of five Kryptocur®-treated boys after surgery for bilateral cryptorchidism demonstrated normalized testicular histology, including the average germ cell count and the Ad spermatogonia count per cross-sectional tubule [161]. Although this sample is very modest, we demonstrated important results in agreement with Hadziselimovic *et al.* [166] indicating that if germ cell count is below 0.2, most cryptorchid boys will develop infertility irrespective of whether they had only surgery or adjuvant hormonal treatment.

Testicular tissue cryopreservation – It is now possible to cryopreserve testicular tissue specimens from prepubertal boys [167-169]. Cryopreservation of testicular tissue for a potential fertility preservation strategy in prepubertal boys has been given a great deal of interest over the last decades, especially with the

Table 1. Hormonal treatment for fertility potential. A list of the most recent studies testing adjuvant GnRH treatment in boys with nonsyndromic cryptorchidism since 2005

Study *Design*	Patients (treated *vs* surgery alone)	Age range	Drug (trade name) and dose	Timing and evaluation	Outcome of hormone treatment	Remarks
Fiala *et al.* [158] *Prospective randomized*	36 unilateral (21 *vs* 15)	2.5–3.5 months at inclusion. 6 months at drug treatment start and orchidopexy within 12 months	Gonadorelin (Kryptocur®) i.n. 0.2 mg (0.1 mL) × 6 every day for 4 weeks	Pre-orchidopexy Concentrations of LH, FSH, testosterone, inhibin B, AMH, penile size, and testicular size were evaluated at 3 months of age and at orchidopexy	No significant effect on hormonal concentrations, penile and testicular size ($p > 0.05$)	No side effects (high dose to infants)
Thorup *et al.* [161] *Prospective case–control*	10 bilateral (5 *vs* 5)	0.6–3.5 years at orchidopexy. Drug treatment started 3 months later Cryopreservation and second biopsy 12 months later	Gonadorelin (Kryptocur®) i.n. 0.2 mg (0.1 mL) × 2 every second day in 16 weeks	Post-orchidopexy Histology before and after: G/T (at least 100) and Ad spermatogonia per cross-sectional tubule (at least 250). Offered cryopreservation 12 months after primary orchidopexy	Improved fertility potential as 3/5 cases normalized, one of whom lacked Ad spermatogonia at inclusion	No side effects None of boys with G/T < 0.2 improved significantly, whether they were treated or not
Vincel *et al.* [162] *Prospective randomized*	10 bilateral (5 *vs* 5)	7–55 (median 20 *vs* 22 between the two groups) months ($p = 0.32$) at orchidopexy. Drug treatment immediately after 10–62 (median 27 *vs* 30) months ($p = 0.18$) at second biopsy	Buserelin (Suprefact®) i.n. 10 µg every second day in 6 months	Post-orchidopexy Histology before and after: G/T (at least 100) and Ad spermatogonia per cross-sectional tubules (at least 100) At surgery, G/T was similar ($p = 0.67$)	Improved fertility potential as G/T increased within the treated group: median 0.11 *vs* 0.43 ($p = 0.03$) compared to no change found in the surgery alone group Ad spermatogonia only appeared after treatment (all lacked at inclusion)	No side effects Unknown and non-authorized dilution of buserelin
Jallouli *et al.* [159] *Prospective randomized*	24 unilateral (12 *vs* 12)	21–110 (median 38) *vs* 12–123 (34.5) months ($p = 0.27$) at the start of drug treatment	Gonadorelin (Kryptocur®) i.n. 1.2 mg every day for 4 weeks	Pre-orchidopexy Histology: Ad spermatogonia per tubule (at least 50)	Improved fertility potential as mean Ad spermatogonia per tubule was greater: 0.88 *vs* 0.49 ($p = 0.002$) Only significance in boys treated after 3 years	No side effects
Zivkovic *et al.* [165] *Prospective case–control*	55 unilateral (32 *vs* 33)	1–7 years at the start of drug treatment	17/32: Buserelin (Suprefact®) i.n. 20 µg every day for 28 days + hCG i.m. 1500 IU every week for 3 weeks 15/32: received hCG i.m. 1500 IU every week for 3 weeks	Pre-orchidopexy Histology: Ad spermatogonia per tubule (at least 50) Testosterone: Before, 14 days after Buserelin®, 24 h after each hCG, and 3 months after orchidopexy	Improved fertility potential as more treated boys had a number of Ad spermatogonia per tubules above 0.1 with 53% *vs* 18% ($p = 0.019$) Those with normal Ad spermatogonia count had better sufficient Leydig cell function by testosterone concentration ($p < 0.003$)	No side effects None-authorized dilution of buserelin

Table 1 *(continued)*

Study *Design*	Patients (treated *vs* surgery alone)	Age range	Drug (trade name) and dose	Timing and evaluation	Outcome of hormone treatment	Remarks
Schwentner *et al.* [160] *Prospective randomized*	42 unilateral and bilateral (21:12 unilateral *vs* 21:9 unilateral)	11–100 (median 32) *vs* 13–100 (47) months ($p = 0.15$) at the start of drug treatment	Gonadorelin i.n. 1.2 mg every day for 4 weeks before orchidopexy	Pre-orchidopexy Histology: Ad spermatogonia per tubule (at least 80)	Improved fertility potential as mean Ad spermatogonia per tubule was greater: 1.05 *vs* 0.52 ($p = 0.007$) Treated boys had 101.9% more spermatogonia per tubule Best effect in bilateral cases (0.96 *vs* 0.56, $p = 0.005$)	No side effects Best effect within the first year

increasing treatment success of pediatric cancers receiving gonadotoxic treatment. This is very valuable, as sperm banking is not an option.

Cryopreserved prepubertal tissue offers the prospect of fertility restoration in adulthood and is currently being offered to prepubertal boys with a high risk of infertility in highly specialized centers worldwide, although as an experimental approach [168]. However, despite significant progress, the strategy remains under development and is far from successful in humans. All steps require anticipation, timely preparation, and careful management, for example, strategies for a favorable injection method for SSC transplantation [170]. This great anticipation emerges from the promising results of fertility restoration by autologous SSC transplantation, testicular tissue grafting, and *in vitro* spermatogenesis that have been achieved in various animal species, all techniques capable of producing healthy offspring [171-173]. Noteworthy, our group has demonstrated that intact testicular tissues from infant cryptorchid boys tolerate cryopreservation and are capable of *in vitro* proliferation maintaining structural and functional characteristics [174,175]. Therefore, we suggest that cryopreservation may be a possible treatment modality for boys with bilateral cryptorchidism with severely impaired fertility potential or after the failure of adjuvant hormonal treatment.

Long-term sequelae

The prime long-term concerns for patients with cryptorchidism relate to the risk of malignancy and infertility. Based on large series of biopsies during the last centuries, it has been shown that cryptorchid testes can be characterized by hypoplasia, abnormal morphology, and dysgenetic features, all leading to an increased risk of disturbance in development and later spermatogenesis [10,13,41,43,48,60,164]. Importantly, cryptorchidism is associated with an abnormal maturation of germ cells involving a diminished formation of Ad spermatogonia [41,43], supported by the prolonged period in which immunohistochemical germ cell markers are found in cryptorchid testes [164]. Thus, gonocyte numbers were higher in the cryptorchid testis during 6–18 months of age and disappeared up to 6 months delayed compared to the contralateral scrotal testis, implying a lack of progression to more mature germ cells [43,176]. The deterioration of the germ cells progresses if the testes are not placed in the scrotum [1]. Cryptorchidism encompasses a wide range of presentations (as reviewed under subtypes) that may have varying impacts on testicular development, function, and long-term sequelae.

Risk of malignancy – Testicular malignancy is a complication of cryptorchidism [10,11,13,177]. In 2013, a large meta-analysis including more than 2 million men with isolated

cryptorchidism in childhood found a relative risk for testicular cancer of 2.9 (95% CI 2.2–3.8) [11]. In 2017, our group reported a standardized incidence ratio of 2.7 (95% CI 1.5–4.3) in a cohort study of 1403 men operated at the median age of 11.8 years for cryptorchidism [13]. Histological changes of progressive degeneration and dysplasia in cryptorchidism may influence the increased risk of malignancy [13]. Cryptorchid boys with chromosomal abnormalities, the disorder of sexual development, and certain syndromes lead to an increased risk of germ cell neoplasia *in situ* [13,178]. Moreover, cases with intraabdominal testis and bilateral cryptorchidism present a greater risk of malignancy [177,179].

In recent years, due to a shift in recommendations for early surgical correction in infancy or before 18 months of life, the figure for the relative risk of malignancy in men with a history of cryptorchidism may be lower. Two large registry studies on the association between age at orchidopexy and testicular malignancy indicated that lower age at orchidopexy reduces the risk of testicular cancer [14,15]. In fact, for every 6 months' delay in orchidopexy after 18 months up to 5.9 years of age, there was a 6% increase in the risk of testicular cancer (HR 1.06, 95% CI 1.03–1.08) [15]. Thus, the risk of developing testicular malignancy will not be eliminated by orchidopexy in former cryptorchid patients, but it appears to reduce the risk [180].

Risk of infertility – Infertility is an unfortunate consequence for men with former cryptorchidism [181,182]. About 20–27% of men with azoospermia (no spermatozoa in the ejaculate) have a history of cryptorchidism [6,7]. In a series of 27 men treated surgically before 2 years of age (22%, 6/27 bilateral cases), 4 had azoospermia and 5 had severe oligospermia (<5×106 sperm cells/ejaculate) accounting for 41% (11/27) of the cohort [39]. The authors indicated better fertility outcomes in boys operated on in the first year compared to those surgically treated during the second year, which agrees with a later study by Feyles *et al.* [20]. Though, they found that 86% (44/53, 11 bilateral cases) had a normal sperm concentration (>15 million/mL) independently of previous hormonal treatment, presurgical position, and uni- or bilaterality when operated within the two first years. Another study including only boys with bilateral cryptorchidism found that even when it is treated before the age of 2 years, only 57% (8/14) had normal sperm concentrations (>20 million/mL, normal forms, and motility) [19]. When comparing semen data within a fertility clinic for couple infertility between men with a history of cryptorchidism (n = 357, the median age at orchidopexy 8.5 years) and without (n = 709), cryptorchidism was inversely correlated with testicular volume ($p < 0.0001$), sperm concentrations ($p < 0.0001$), and endocrine testicular function (higher gonadotropins $p < 0.0001$ and lower testosterone $p = 0.003$) [183]. Ultimately, only 12% (16/135) of men treated for bilateral cryptorchidism had normal sperm concentrations (>15 million/mL). Azoospermia was detected in 62/222 (28%) patients with unilateral and 62/135 (46%) with bilateral cryptorchidism. Thus, the rate of azoospermia (especially for unilateral cases) is much higher in this cohort representing those men in couple infertility treatment.

In a recent Danish cross-sectional study including 6376 men (median age 19 years), of whom 570 (9%) had a history of cryptorchidism, cryptorchidism was significantly associated with impaired semen parameters, except for semen volume, with a 28% (95% CI 20–37) lower sperm concentration than healthy controls [184]. Moreover, the men with a history of cryptorchidism had impaired hormonal function with a 26% lower inhibin B/FSH ratio and a slightly reduced Leydig cell function (6% lower testosterone/LH ratio). The study is excellent by including semen parameters and reproductive hormones in many young men, however, information regarding laterality, age at orchidopexy, and treatment modus (spontaneous descent, hormone treatment, or surgery) was scarce and obtained by retrospective self-reporting, which might have introduced misclassification due to lack of recall accuracy. Nonetheless, no differences between former unilateral and bilateral cases were detected in any of the parameters in available data (unilateral n = 203 *vs* bilateral n = 126).

According to available follow-up studies of men treated for unilateral cryptorchidism, the

reported prevalence of azoospermia ranges from 0% to 14%, the lower rates being quite similar to findings in the general population [9,21,185,186,187,188,189,190]. However, comparison data from the general population also include men with a history of cryptorchidism, and as 2.5% of boys in the western world undergo surgery for cryptorchidism and about 5% exhibit cryptorchidism in at least some years, findings similar to the general population does not exclude the impact of cryptorchidism on azoospermia. Several studies which investigated fertility outcomes in cases of unilateral *vs* bilateral cryptorchidism have generally detected a worse outcome in men with a history of bilateral cryptorchidism [8,21,179,183,188,189,190]. Undoubtedly, untreated bilateral cryptorchidism results in a high, up to 89%, incidence of azoospermia [1,21]. The incidence of azoospermia in men with former bilateral cryptorchidism has been detected between 10% and 46% [19,21,179,187,188,190]. Cryptorchidism is a heterogeneous disease that can cause deleterious effects on reproductive parameters.

Retrospective studies have assessed paternity and use of assisted reproductive technologies rates in cryptorchid boys to evaluate fertility. An Australian population-based study of 350.835 men found that those with former cryptorchidism (n = 7499) had a 21% decreased likelihood of paternity (adjusted hazard ratio 0.79, 95% CI 0.74–0.85), which proved to be related to the age at orchidopexy as a 1% reduction (relative risk 0.99, 95% CI 0.98–0.99) in paternity was found for every 6 months' delay in surgery from 18 months up to 5.9 years of age [15]. Men with former bilateral cryptorchidism had lower paternity rates compared to unilateral cases. This is in agreement with Lee and Coughlin [8] who reported significantly lower paternity among men with former bilateral cryptorchidism (65%, n = 88) in comparison to unilateral (90%, n = 609) and control men (93%, n = 708). Moreover, it appears that men with former cryptorchidism are in need of assisted reproductive technologies more than the general population. Schneuer *et al.* [15] found that the men had a two-times increase in the use of assisted reproductive technologies for male factor infertility than unaffected men (adjusted relative risk 2.26, 95% CI 1.58–3.25). Again, for boys who underwent surgery for cryptorchidism in the age interval 18 months up to 5.9 years of age, a delay in orchidopexy (for every 6 months) increased the risk of needing assisted reproductive technologies by 5% (relative risk 1.05, 95% CI 1.03–1.08).

In this context, the big issue when reviewing and comparing the fertility outcomes of men with the previous orchidopexy in childhood relates to the fact that all materials are selected and comprised of patients with cryptorchidism representing different subtypes of the disease spectrum. In addition, even the definition of infertility, paternity rate, and semen analysis may differ between studies.

The rationale for the fertility potential assessment

In this thesis, assessment of the fertility potential at the time of surgery in childhood is quantitated by histological parameters by G/T and the number of Ad spermatogonia per tubular cross-section (AdS/T) as well as simultaneously FSH, LH, and inhibin B, which is the assessment method of our group [115,135,161,191,192,193,194,195,196,197,198]. Importantly, all parameters are evaluated according to age. The rationale behind assessing the fertility potential assessment is explained in the following paragraph:

G/T – Our group matched histological findings of the testes from 67 patients who had undergone bilateral orchidopexy (median age 13.2 years, range 10.6–15.9 years) with their fertility outcome. Significantly, the mean G/T at the time of surgery was correlated with later sperm concentration, total testicular volume, and FSH in adulthood [199]. A normal mean G/T meant that 100% (95% CI 29–100) of such boys would later have at least 20 million sperm cells/mL. On the contrary, there was a high risk of subsequent infertility if a biopsy revealed less than 1% of the lowest normal age-related G/T. If bilaterally, the risk of later infertility (not possible to fecundate without assisted reproduction) was 100% (95% CI 66–100) as none of nine such patients had more than 1 million sperm cells/mL and lacked normal motility [199], whereas if such finding was

unilateral, the risk of infertility was 73% (95% CI 45–92) since 11 of 15 such patients had less than 2 million sperm cells/mL and lacked normal motility [1].

Our group also matched histological findings of the testes from 65 patients who had undergone unilateral orchidopexy (median age 10.8 years, range 2.9–11.9 years) with their fertility outcome. If a biopsy revealed less than 1% of the lowest normal age-related G/T at surgery, later experience of infertility was suspected by 33% (95% CI 12–62), as five out of 15 men had not more than 5 million sperm cells/mL and lacked normal motility [185]. However, there was no correlation between the age-related G/T and the later sperm concentration [185]. Nonetheless, the age-related G/T was somewhat prognostic for the later fertility, being correlated with total testicular volume in the adult patients [185]. When the semen data from the bilateral and the unilateral cryptorchid patients were pooled, the age-related G/T at the time of surgery correlated with the later sperm concentration [179].

Equivalently, others have reported that G/T at the time of surgery of unilateral and bilateral cryptorchid patients analyzed together correlated to later sperm concentration [19,200,201,202,203]. However, others did not find any correlation between prepubertal histological findings and adult semen parameters [187,204] but they only quantified the "tubular fertility index" defined as the number of tubules with spermatogonia/total number of counted tubules.

AdS/T – The quantification of Ad spermatogonia is regarded as a very important marker for male fertility [50,52,165,205]. Hadziselimovic and Herzog [128] conducted a follow-up investigation of sperm variables in 31 patients who had undergone early orchidopexy before the age of 2 years. If Ad spermatogonia were present in the testicular biopsies in childhood, 17 of 18 (94%, 95% CI 73–100) of the men had a total sperm count of 40 million per ejaculate or greater. On the contrary, despite early successful orchidopexy, if Ad spermatogonia were absent, 12 of 13 (92%, 95% CI 64.0–99.8) had abnormal spermiograms. In their later study from 2008, they confirmed that the number of Ad spermatogonia at the time of orchidopexy correlated significantly with a later sperm concentration of 89 men (78% unilateral) [205].

Kraft *et al.* [50] conducted follow-up sperm analyses of 91 patients with surgery for unilateral and 19 for bilateral cryptorchidism (mean age 7, SD ±3.8 years) and found that in both cases of unilateral and bilateral cryptorchidism, an abnormal AdS/T was significantly correlated with lower sperm density than those with normal AdS/T. Moreover, a normal AdS/T was associated with a normal sperm density in 88% (29/33) of unilateral cases and 100% (2/2) of bilateral cases [206]. Thus, Ad spermatogonia seem crucial for later fertility.

Inhibin B – Inhibin B is a specific biomarker of Sertoli cell function named for its ability to negatively regulate FSH in adulthood [207]. Inhibin B may be interpreted as a marker of testicular function for prepubertal boys [191,192,194,207,208,209]. A study from our group reported that in 40 cryptorchid infant boys aged 4–35 months, inhibin B correlated with Sertoli cell number per cross-sectional tubules [194]. Inhibin B has also been reported to correlate with the testicular volume [58]. Moreover, studies from our group found inhibin B correlated to AdS/T in 94 prepubertal boys (41 bilateral) aged 0.5–13 years [192] and G/T in 40 cryptorchid boys (11 bilateral cases) aged 4–35 months [194]. This is in accordance with the statement, that each Sertoli cell can provide for a fixed number of germ cells [210]. In contrast, a study of 71 cryptorchid boys (17 bilateral cases) aged 7–65 months did not reveal a correlation between inhibin B and AdS/T, but these patients only underwent unilateral testicular biopsy also in cases of bilateral cryptorchidism [211].

Our group has reported that inhibin B was impaired in ranges of about 16–24% and 14–26% of bilateral and unilateral cases, respectively [135,191,192,194,197,198]. Impaired inhibin B was usually identified among patients with decreased number of germ cells and Ad spermatogonia [180,191,192]. Others have also reported lower inhibin B levels in boys with cryptorchidism [212-214]. However, studies of inhibin B in cryptorchid boys have yielded

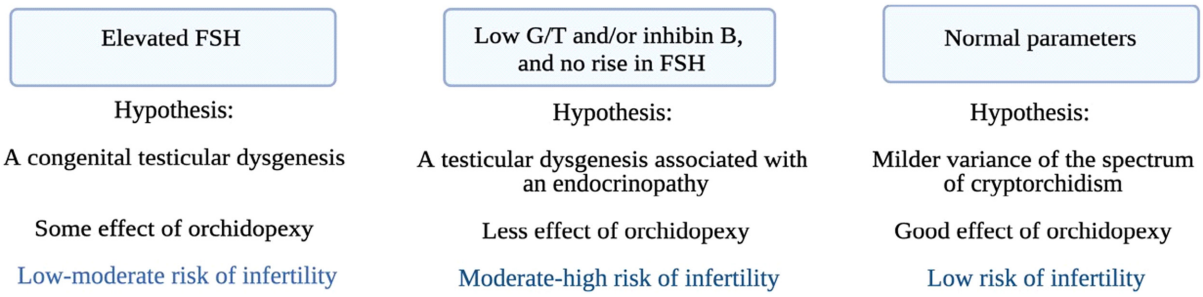

Fig. 8. The fertility potential as a tool to classify cryptorchidism. A hypothesis from our group—that the spectrum of cryptorchidism might comprise different patterns in the fertility potential at the time of surgery.

mixed results, also reporting normal concentrations [215-218]. Although when analyzing some of these studies in detail, impaired inhibin B was found among some boys with bilateral cryptorchidism and in cases with intraabdominal testes [216,217]. Two of the studies pooled unilateral cases with bilateral cases in a modest amount of patient samples and one of them measured inhibin B during minipuberty [215,219].

Our group hypothesizes that inhibin B can add more robustness in identifying patients with a high risk of infertility as reduced histological parameters (G/T and/or AdS/T) have been reported to be accompanied by impaired inhibin B. In a previous study and in preliminary data, we found a high sensitivity of inhibin B below 2.5th percentile for a reduced G/T [191,196].

Furthermore, studies have evaluated pre- and postoperative inhibin B concentrations in prepubertal boys with cryptorchidism, indicating that this hormone may be an important marker of improvement of the Sertoli cells, and thereby possible also the germ cells after surgical correction [115,119,198,219,220, 221,222]. Moreover, adult men who had undergone early orchidopexy had higher inhibin B compared with those who underwent orchiopexy later in life [190,223,224]. A large cross-sectional study of 1.797 fertile men demonstrated that inhibin B is strongly associated with sperm concentrations and sperm counts especially up to a level of 150 pg/mL [225].

FSH – FSH usually remains measurable during childhood, unlike LH [66]. Altered functions of the testis may be reflected by higher FSH suggesting that a subtle Sertoli cell dysfunction might already be manifest at this early stage. Thus, higher FSH in cryptorchid boys compared to boys with normally descended testes has been reported in some studies [214]. Mildly elevated FSH and LH between 2–7.3 IU/L and 0.2–12.2 IU/L, respectively, have been reported in boys with bilateral cryptorchidism aged from 6 months to 9 years [226]. However, this is not the case in all studies [227].

Boys with anorchia (bilateral vanished testes, verified at laparoscopy) having a normal karyotype 46XY present a typical hormonal profile with very high FSH and LH and low or undetectable inhibin B and testosterone [228]. In such cases, FSH concentrations ranged from 41 to 191 UI/L and were much higher than the upper normal range of all ages (including adults) and previously published by Andersson *et al.* [66]. Not even in a series of infant boys with bilateral cryptorchidism undergoing a GnRH stimulation test, did the maximum values of FSH and LH 30 min after stimulation exceed 10.0 and 8.8 IU/L, respectively [229].

In a recent study, inhibin B from birth to 15 years of age and FSH during the first year of life at certain cut-off concentrations appeared to be excellent diagnostic markers of testicular function and for differentiating non-palpable cryptorchid testes and anorchia [230]. In specific, testicular tissue was not present in cases of FSH higher than 28.9 IU/L during the first year of age or FSH higher than 20.3 from 1 to 15 years of age in boys without hypogonadotropic hypogonadism.

The interplay between FSH and inhibin B during childhood is not clearly understood. Our study group has reported that LH was associated with inhibin B in cryptorchid boys

aged 0.5–13 years of age [192], which has been supported in normal boys at 3 months of age [58], also reporting a negative correlation between inhibin B and FSH at that age. We have also previously reported a negative correlation between FSH and inhibin B in bilateral cryptorchid boys [191,231], similar to a previous study [218]. Another study of cryptorchid patients found no correlation between FSH and inhibin B between 9 months and 3 years of age [221]. But these were all unilateral cases. Some evidence suggests that FSH drives inhibin B as administration of recombinant FSH to prepubertal boys aged 12–13 years old and in infants young than 12 months increased inhibin B [232,233].

Lower FSH and higher inhibin B have been reported among men who underwent orchidopexy at 2 years of age than men who had surgery later, which is indicative of a benefit of earlier orchidopexy [223]. Another follow-up study showed a significant positive correlation between G/T in 24 men operated upon for cryptorchidism in childhood to later FSH and LH [187]. Given this and the evidence that the postnatal rise in inhibin B is under the influence of the elevated gonadotropins and that impaired inhibin B has been observed in infants with hypogonadotropic hypogonadism, our group included FSH in our assessment of the fertility potential.

Classification based on fertility potential at the surgery – In prepubertal cases with reduced G/T and AdS/T and/or inhibin B, testicular dysgenesis may exist. If there is no competent elevation of gonadotropins as expected from the feedback mechanism, an endocrinopathy may also co-exist and the impairment of the germinative epithelium may result in infertility. Hadziselimovic [38] first introduced the term prepubertal transient hypogonadotropic hypogonadism for the condition with a decreased number of germ cells, especially reduced number of spermatogonia Ad, and a supposed relative gonadotropin insufficiency.

Our study group has previously classified boys with bilateral nonsyndromic cryptorchidism based on the fertility potential assessed at the time of orchidopexy according to these above-mentioned parameters with the hypothesis that the pathogenesis and effect of surgery might differ (Fig. 8) [197,198].

OBJECTIVES

The main objective of the studies in this PhD thesis is to improve the management of boys with cryptorchidism, including quantification of the fertility potential at the time of surgery by the number of germ cells in combination with the hormonal profile, clarifying the need for supplementary treatment and optimizing identification of those who would benefit from such.

Paper I: To evaluate the fertility potential in boys with nonsyndromic cryptorchidism operated within the first year to clarify the need for eventual supplementary treatment modalities.

Hypothesis: We hypothesized that some boys with cryptorchidism would have compromised fertility potential, despite early and successful orchidopexy.

Paper II: To compare histological and hormonal parameters of the fertility potential in boys with bilateral ascending testes and boys with late-referred bilateral congenital cryptorchidism to clarify if the fertility potential is decreased in boys with bilateral ascending testes.

Hypothesis: We hypothesized that testicular ascent would be associated with a compromised fertility potential.

Paper III: To determine pre- and postoperative concentrations of gonadotropins and inhibin B in boys with bilateral cryptorchidism in relation to testicular histopathology at the time of surgery and 1-year follow-up to evaluate the impact of corrective surgery on hormonal parameters.

Hypothesis: We hypothesized that orchidopexy would improve the fertility potential based on inhibin B and FSH and that the improvement would be related to the classification of the fertility potential at the time of surgery.

Paper IV: To evaluate histological and hormonal parameters of the fertility potential in boys with congenital nonsyndromic

cryptorchidism who underwent orchidopexy during minipuberty to identify patients with possible endocrinopathy.

Hypothesis: We hypothesized that most boys with congenital nonsyndromic cryptorchidism would display normal reproductive hormonal profiles during minipuberty.

Paper V: To report our experience with offering testicular tissue cryopreservation to parents of prepubertal boys with cryptorchidism as an experimental fertility restoration option—a pioneer intervention for future theoretical benefit.

Hypothesis: We hypothesized that most parents would be in favor of cryopreservation, despite the unproven efficacy.

METHODS AND MATERIALS

Ethics

Ethical approval for retrieving and evaluating patient testicular biopsies and hormonal concentrations from orchidopexy was obtained from the Regional Ethics Committee in Copenhagen (No. #H-18063061). For Paper IV, testicular tissue collection for cryopreservation and research use was approved by the same committee (No. #H-2–2012–060.anm.37655).

Patients

Paper I Orchidopexy within the first year of life – This study included all prepubertal boys with cryptorchidism who underwent orchidopexy before 12 months of age in the period January 2010 to April 2019 at the Department of Pediatric Surgery, Rigshospitalet. In total, 446 consecutive treated boys were included. After exclusion of those who had syndromic cryptorchidism, missing data, vanished testis, or had repair of inguinal hernia, the study population consisted of 333 boys. The median age at orchidopexy was 274 days ranging from 34 to 390 days. Sixty-nine (21%, 69/333) boys had bilateral cryptorchidism and 6% (21/333) had at least one intraabdominal testis.

Paper II Ascending testes – This study included all prepubertal boys treated for bilateral ascending testes between January 2011 and October 2018 at our center. The boys' previously confirmed history of testes present in the scrotum was obtained from the documented neonatal check which was either done by a midwife or neonatologist/pediatrician and in some cases also confirmed in the doctoral referral from the general practitioner. A previous scrotal position was also confirmed by the parents at the clinical examination. None had retractile testes at the time of orchidopexy. Any boy with unclear or inadequate history was excluded from this study to minimize sampling error. Included were 67 boys who were otherwise healthy (nonsyndromic) with a median age of 3.8 (range: 2.0–7.0) years old. From the same inclusion period, 86 boys with late referral bilateral congenital nonsyndromic cryptorchidism (median age 3.9, 2.0–6.9 years) were identified as a comparison group. Within the comparison group, 23

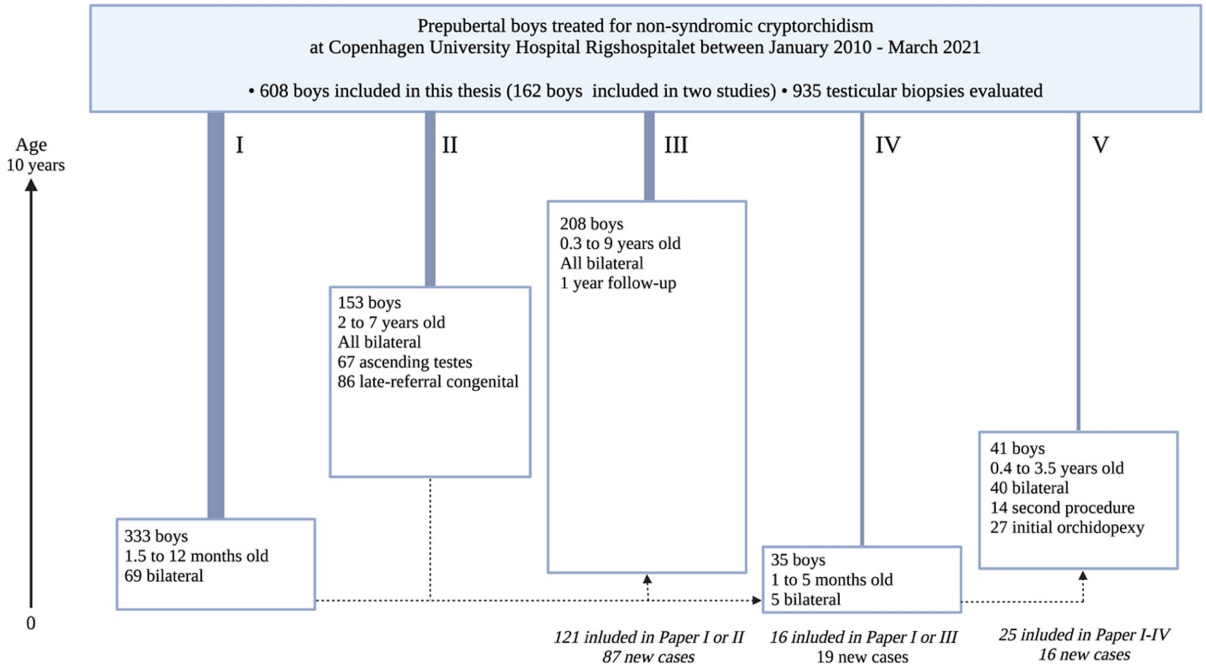

Fig. 9. Summary of patients included. Overview of boys with cryptorchidism included in Papers I–V. The arrows indicate that some boys were included in two studies.

(27%, 23/86) boys had at least one testis positioned within the inguinal canal or above (Fig. 9).

Paper III Follow-up of bilateral cryptorchidism – This study included all consecutive prepubertal boys who underwent bilateral orchidopexy with testicular biopsies and hormonal profile at the time of orchidopexy plus secondary hormonal assessment at 1-year postoperative. Since 2014, a total of 208 boys met the eligibility criteria of having nonsyndromic cryptorchidism, not undergone previous inguinal surgery, and both testes successfully repositioned within the scrotum at clinical examination during follow-up. The median age at orchidopexy was 1 year and 8 months (4 months to 9 years) and at follow-up 2 years and 8 months (13 months to 10 years). Ten (2%, 10/416) testes were located intraabdominal and 90 (22%, 90/416) were positioned within the inguinal canal or above.

Paper IV Orchidopexy during minipuberty – A total of 45 consecutive full-term boys with cryptorchidism underwent orchidopexy before 160 days of age at the Department of Pediatric Surgery, Rigshospitalet. In total, 35 boys with nonsyndromic cryptorchidism with a median age of 124 (37–159) days were included in this study, five (14%, 5/35) of whom had bilateral cryptorchidism. Ten boys were excluded due to syndromic cryptorchidism. Twenty-two (55%, 22/40) boys had at least one testis positioned within the inguinal canal or above. All boys were examined by the same surgeon, JT, with experience in more than 2000 orchidopexies assessing that spontaneous descent would not occur.

Paper V Parental acceptance rate toward cryopreservation – This study included the parents of 41 prepubertal boys aged from 4 to 45 (median age 13) months with cryptorchidism who were offered cryopreservation of a testicular biopsy as part of their son's treatment program at our center as an experimental approach for preserving the fertility. The parents were grouped into two: (A) prepubertal boys who were offered cryopreservation as a secondary procedure after successful orchidopexy and (B) prepubertal boys who were offered cryopreservation at initial orchidopexy.

Group-A boys were carefully selected based on the histological and hormonal assessments from primary surgery indicating a high risk of infertility, identified in the period July 2014 to January 2020. Boys in group A were followed 1 year postoperative by a new hormonal assessment to monitor the testicular function and were included at this time point in case of inadequate hormonal improvement. Five group-A boys were included in a previous publication [161].

Group B-boys were included if they, in the period January 2018 to January 2020, met the following criteria: bilateral congenital nonsyndromic cryptorchidism, younger than 3.5 years at enrollment, and were examined by JT and present PhD student SH. In total, the parents of 27 boys with congenital bilateral cryptorchidism were offered cryopreservation as a part of the initial orchidopexy to avoid another surgical procedure (group B). Seven (26%, 7/27) boys had at least one testis positioned within the inguinal canal or above.

Testicular biopsies

In an open approach, the tunica albuginea is incised about 3–5 mm longitudinal with a No. 11 blade scalpel avoiding any blood vessels (Fig. 10). Next, with carefully and gently squeezing of the testis the protruding seminiferous tubules are excised using an iris scissor.

A small specimen of about 2–3 mm3 (mean weight of 7 mg) is then placed immediately in modified Stieve's fixation (GR fixative; 200 mL 37% formaldehyde, 40 mL acetic acid added to 1 L of 0.05 M phosphate buffer, pH 7.4) for transportation and further preparation at the Department of Pathology at Rigshospitalet. The tunica albuginea is closed with a continuous 5–0 vicryl suture.

Since 1971, it has been a clinical routine practice at Rigshospitalet to obtain testicular biopsies of boys undergoing surgery for cryptorchidism. Testicular biopsy is considered a minor surgical procedure but is not routinely performed during orchidopexy in most centers, mainly because of concerns of damaging the cryptorchid testis [234]. Aside from common surgical risks such as bleeding and infection, which rarely occurs, concerns related to the risk of atrophy, development of microlithiasis, breaching the blood–testis barrier, and formation of antisperm antibodies have been raised. However, no long-term adverse effects have been reported with prepubertal open biopsies including finding no increased rate of microlithiasis or evidence of antisperm antibodies [235,236]. Finally, the prepubertal testis does not have the blood–testis barrier until about 10 years of age [234]. Based on this, our center reason that testicular biopsies are valuable in prepubertal boys with cryptorchidism, as histology can provide valuable information on the risk of malignancy, predicting future fertility, and even potential fertility preservation.

Histological assessment

Immunohistochemistry – The testicular tissue specimens were formalin-fixed and paraffin-embedded in cassettes and cut into serial sets of 2–3-µm sections. Histological sections were stained with hematoxylin and eosin (HE) and incubated with immunohistochemical antibodies against D2-40 (1:25, M3619, Dako, Glostrup, Denmark), CD99/MIC-2 (1:100, 12E7, Dako), and placental-like-alkaline phosphatase (1200, PL8-F6, Biogenex, Fremont, USA). Immunohistochemistry was performed automatically using the Ventana BenchMark ULTRA platform (Ventana Medical Systems, Tucson, USA) with positive and negative control tissue blocks per section for validation of each antibody. All specimens were validated and histopathologically reviewed by an experienced pathologist ECL. Included histological sections were stored as a separate research biobank for the purposes of these studies.

Assessment – In a blinded fashion, the number of germ cells within every cross-sectional seminiferous tubule (G/T) was measured from at least 100 cross-sectioned tubules, as previously described by our group [56,60]. Type Ad spermatogonia were identified based on the morphological criteria exhibiting a rarefaction zone centrally within the nucleus and homogeneous deeper staining of the nucleus [40,43]. Based on examination from at least 250 cross-

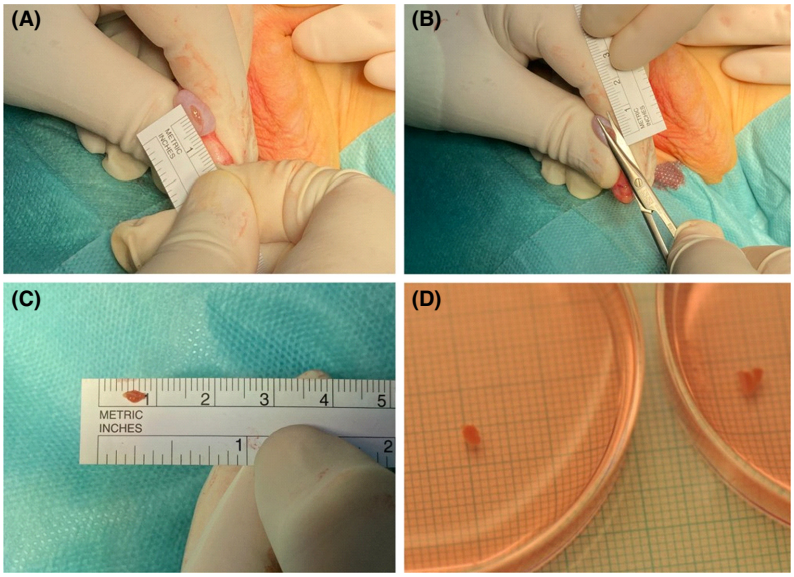

Fig. 10. Testicular biopsies. A testicular biopsy was obtained at the Department of Pediatric Surgery, Rigshospitalet. (A) A longitudinal incision in the tunica albuginea, (B) Excision with a scissor to obtain a biopsy sample, (C, D) A testicular biopsy of approximately 2–3 mm^3. (Published after consent was received from the boy's parents.)

sectioned tubules, the number of type Ad spermatogonia per cross-sectional tubule (AdS/T) was determined. The number of Ad spermatogonia was considered reduced when the count was below 0.01 [41,205,237]. In bilateral cases, both testicular biopsies were assessed and consequently, a mean value was calculated. A total of 935 biopsies were assessed in this thesis (Fig. 8). Cell counting was assisted by an APP for android smartphones (M's Counter 2, Maki System, Inc, Tsuyama, Japan).

Description and discussion of reference material

– The age-related G/T was employed as our histological assessment so that our results could be compared with reported data [1,19,44,50,162,200,201,202,203,205].

Reference for normal lower range which defined the bar for normal age-related G/T values (values below bar were considered reduced) was determined by the same measurements carried out by DC. The normal control material consisted of pairs of testes from 72 males aged 28 weeks of gestation to 18 years of age, including 22 fetuses with scrotal testes who were stillborn or died within the first 3 days of life and testes from 47 boys who had died suddenly and unexpectedly (from meningitis or pneumonia less than 6 days in duration) [56,60]. No other disease was known, and an autopsy revealed no abnormalities. Furthermore, the control material also included 3 testicular biopsies obtained from the scrotal testes of boys who underwent contralateral inguinal herniotomy. A control material according to KK of 69 other normal boys aged up to 2 years (138 autopsy specimens) was included in Paper I [44].

The normal G/T values were similar to other reported G/T values, as reviewed in a meta-analysis including 334 normal boys [61]. However, in normal boys aged 1–3 years old, the normal values from our center are

Fig. 11. Normal lower range of G/T. Reference lower ranges of G/T in the studies included in this thesis based on normal materials [44,56,60,61].

somewhat lower. Since we used the lowest age-related G/T value as reference, one may argue that our findings consequently really underline that when having a reduced G/T, it is truly impaired. The values used for the normal lower ranges within the five studies are almost identical but span over different age periods which makes them differ (Fig. 11). Before each study, a consensus between the PhD student and the supervisors was made to set the lower range of each study population. For Paper IV, we lifted the lower range accordingly to values presented in the meta-analysis by Masliukiate *et al.* [61] excluding DC's values not to set the lower range too low; G/T was set at 1.1 for the age group 60–105 days and 0.9 for the age group 106–150 days (Fig. 11).

Furthermore, this thesis uses histological assessments involving cell counting of HE-stained and immunohistochemical stained sections, which differs from the reference values by DC, that was based on HE- and PAS-stainings. Immunohistochemistry may favor the identification of germ cells [193].

Cryopreservation (specific for Paper V) – In Paper V, a small part of the testicular tissue specimen from each side was collected for cryopreservation (Fig. 10D). The tissue specimens were immediately immersed in McCoy's 5A medium (modified, 22330–021, Gibco, Life Technologies, Paisley, UK) for transportation to the Laboratory of Reproductive Biology at Rigshospitalet. Upon arrival, the tissue specimens underwent cryopreservation by slow freezing to preserve tissue integrity for long-time storage in liquid nitrogen cryo tanks (−196 °C). First, the tissue specimen was placed in a 10 mL plastic tube containing 7 mL freezing solution: 0.1 moL/L sucrose (Merck, Damstadt, Germany), 1.5 moL/L ethylene glycol (Sigma–Aldrich, Merck), 10 mg/mL human serum albumin (200 mg/mL, CSL Behring, King of Prussia, USA) and phosphate-buffered saline. The tissue specimens were equilibrated in the freezing solution for 20 min at 1–2°C (crushed ice) on a tilting table and subsequently transferred individually to 1.8 mL cryovials (Nunc A/S, Roskilde, Denmark) each containing 1 mL of the freezing solution for cryopreservation using a programmable Planer freezer (Kryo 360–1.7).

Hormonal assessment

Blood measurements – At orchidopexy, serum concentrations of the reproductive hormones FSH, LH, testosterone, and inhibin B were collected by venous puncture between 8.00 am and 11.00 am. In Paper III, similar measurements were performed at follow-up. Hormone analyses were performed at the Department of Growth and Reproduction at Rigshospitalet according to the methodology previously described [66,68]. Since our hormone analyses span over 10 years (oldest from January 2010), commercial kits have improved during our study period lowering detection limits and values of the interassay coefficient of variations.

Concentrations of FSH and LH were assessed using a time-resolved immunofluorometric assay (Autodelfia; PerkinElmer, Wallac Oy, Turku, Finland). FSH and LH concentrations were expressed in IU/L with detection limits of 0.05 IU/L (former limit of detection for FSH was 0.06). Interassay coefficients of variations were below 6% (former 8%) in both FSH and LH assays. Concentrations of testosterone were determined with radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corp., Los Angeles, USA) with a detection limit of 0.35 mmol/L (former limit of detection was 0.23 nmol/L). After completion of minipuberty, the testosterone concentration was not detected in any of our patients. Finally, inhibin B was measured by double-antibody enzyme-immunometric assay (Oxford Bio-Innovation, Oxfordshire, UK; later named Serotec Ltd., Oxford, UK) with the detection limit of 3 pg/mL (since 2010; former 18–20 pg/mL). The interassay coefficients of variations for testosterone and inhibin B were below 10% and 18%, respectively.

For Paper IV, additional blood testing for certain diseases, such as human immunodeficient virus-1 and 2, hepatitis B and C, and syphilis, were required according to the Danish National Board of Health (European directive #2004/23/EF) when freezing tissue. The results are not presented.

Assessment – For assessment of the reproductive hormone concentrations, we used the median and 2.5th and 97.5th percentiles of the normal reference values. An impaired concentration of inhibin B was defined as below the 2.5th percentile. For Paper I, an elevated FSH was assessed accordingly to Andersson *et al.* [66] as an above upper range. For Paper III, an FSH of 1.4 IU/L or greater was considered elevated.

Description and discussion of reference material – Reference ranges were determined by the Department of Growth and Reproduction at Rigshospitalet through a unique establishment of in-house analyses of sex- and age-related hormones for growth and reproduction covering infants, children, adolescents, and adults.

This establishment is done in collaboration with Danish population-based health studies providing samples for analysis from a large population of healthy individuals. Sex- and age-related reference material is fundamental for investigating the natural history of a given hormone over different stages of life, as well as differences between boys and girls, and it is an indispensable tool for comparison of hormone changes in abnormal development and screening for pathogenesis. For Papers I–III and V, the exact number of healthy individuals included in the reference material for FSH, LH, testosterone, and inhibin B is unknown. In total, 1041 healthy boys served as reference material providing hormone concentrations during minipuberty for Paper IV, which is described in a study by Johannsen *et al.* [68]. It is valuable, that the hormone analyses included in this thesis were performed at the Department of Growth and Reproduction at Rigshospitalet, which described the reference materials for healthy boys.

Statistical analyses

Statistical analysis was performed using GraphPad Prism version 6 and 8.1 (Graphpad Software, San Diego, USA). Data were presented as medians with ranges. Nonparametric analyses were used as the data, within this thesis, were not normally distributed within this thesis. Differences between groups were investigated with Mann–Whitney *U* test (unpaired), Wilcoxon matched-pairs test

(paired), and Chi-Square test or Fisher exact test for categorical variables. Moreover, the Spearman correlation test was used to measure the degree of association between two variables.

Besides, a multiple of the median (MoM) estimation was used to determine how far the patient's result deviates from the normal median value accordingly to the age of the patient. A two-sided p-value of less than 0.05 was considered significant.

RESULTS

Paper I Orchidopexy within the first year of life

This study provides insight into the fertility potential among boys with nonsyndromic cryptorchidism undergoing early surgery in compliance with the Nordic guidelines [16].

We found that inhibin B significantly correlated with G/T ($p < 0.001$) and AdS/T ($p = 0.02$). Seventy boys (21%, 70/333) had a reduced inhibin B, of whom almost half (46%, 32/70) had reduced G/T. In our series, 83 boys (25%, 83/333) had reduced G/T, defined as a value below the normal lower range, and 92 boys (28%, 92/333) had reduced AdS/T (value below <0.01). G/T was reduced in 67% (62/92) of the boys with reduced AdS/T. Two boys (0.6%, 2/333) with unilateral cryptorchidism had no germ cells in their biopsy, one of whom had impaired inhibin B. A complete absence of Ad spermatogonia was demonstrated in 75 cases (23%, 75/333).

Boys who underwent earlier surgery (before 9 months of age) had significantly better histology and hormonal parameters ($p < 0.005$, except AdS/T among bilateral cases). When comparing boys with bilateral cryptorchidism to those with unilateral cryptorchidism, no significant difference was demonstrated in histology parameters and inhibin B concentrations. Boys with bilateral cryptorchidism had significantly higher FSH (median 0.8, range 0.2–0.4 IU/L) than those with unilateral cryptorchidism (0.6, 0.1–3.5 IU/L, $p < 0.001$). Moreover, we found that boys with non-palpable testes were associated with lower inhibin B, but no significant difference was found.

Paper II Ascending testes

Cryptorchidism is generally considered a congenital anomaly, but some boys born with descended testes later develop cryptorchidism due to the ascent of the testes.

When comparing histological parameters from boys with bilateral ascending testes to age-matched boys with late referral bilateral congenital cryptorchidism, we found that the histopathological status was similar. The mean G/T and AdS/T were not significantly higher in boys with ascending testes ($p = 0.11$ and $p = 0.83$, respectively), even though this patient group had scrotal testes after birth. About 60% (40/67) of the boys with ascending testes had a mean G/T below the normal lower range *vs* 66% (57/86) in the congenital cryptorchid group ($p = 0.40$). One boy with ascending testes aged 2.1 years at surgery had no germ cells in either of the biopsies (1.5%, 1/67) corresponding to three cases (aged 3, 5, and 6.4 years with at least one non-palpable testis) found among the boys with congenital cryptorchidism (3.5%, 3/86, $p = 0.44$). Moreover, it was noted that 31% of the boys (21/67) with ascending testes lacked Ad spermatogonia which was a similar fraction in the comparison group (35%, 30/86). When we analyzed the reproductive hormones at orchidopexy, no significant differences between the two groups were found.

Twenty-tree boys with congenital cryptorchidism having at least one non-palpable testis had a significantly higher frequency of reduced G/T and AdS/T than those with palpable testes (ascending testes and congenital palpable). Moreover, this subgroup had a significantly higher frequency of impaired inhibin B at 35% (8/23) *vs* 13% (9/67, $p = 0.024$) among the boys with ascending testes and 8% (5/63, $p = 0.0021$) of boys with congenital cryptorchidism and palpable cryptorchid testes.

Paper III Follow-up of bilateral cryptorchidism

To monitor the testicular function after successful orchidopexy, we conducted a study evaluating pre- and postoperative concentrations of gonadotropins and inhibin B in relation to histological parameters in boys with bilateral cryptorchidism.

Histologically, G/T was reduced in 50% (104/208) of the boys having a similar reduction observed for AdS/T. Forty-three (21%, 43/208) boys had both parameters reduced.

Preoperative inhibin B was impaired in 23% (47/208) of the boys. In total, 16 boys (8%, 16/208) had all three parameters compromised.

At 1-year follow-up, 26% (54/208) had impaired inhibin B. Of those with impaired preoperative inhibin B, 64% (30/47) still had a low inhibin B at follow-up. Overall, inhibin B improved significantly after surgery when each value was converted into MoM values, where 52% (55/105) of boys exhibited an improvement ($p = 0.034$). Only the youngest age group comprising boys who underwent orchidopexy before 1 year showed a significant difference between pre- and postoperative MoM values when the data was divided into age groups according to age at orchidopexy ($p = 0.003$).

On the basis of histological and hormonal findings at orchidopexy, the boys have subdivided into three groups accordingly to a previously published paper [197]. The first group (group 1) consisted of 32 boys who had elevated FSH, defined as 1.4 IU/L or greater. Of these, around half (47%, 15/32) had reduced G/T. At follow-up, FSH normalized in 20 (63%, 20/32) and 19 boys (59%, 19/32) displayed an increase in inhibin B MoM. Thirty-one percent (10/32) had impaired inhibin B at follow-up, of whom four (40%, 4/10) still had elevated FSH. Of the boys who had reduced G/T at surgery, most boys (60%, 9/15) exhibited an increment of inhibin B as well as a normalization of FSH (60%, 9/15).

A total of 105 boys had reduced G/T and/or impaired inhibin B but no rise in FSH as expected feedback mechanism and were subsequently suspected endocrinopathy, defined as group 2. After successful orchidopexy, only 11% (12/105) had an increment of FSH and yet 31% (31/105) had an impaired inhibin B (*vs* 33% (35/105) at orchidopexy). Thus, half of the group increased inhibin B MoM (52%, 55/105). Of the 105 boys with suspected endocrinopathy at the time of surgery, only 11 (15%, 11/105) boys later exhibited a rise in FSH indicating a functioning hypothalamic–pituitary–gonadal axis, thus at least 94 (89%, 94/105) of these boys in group 2 would possibly benefit from adjuvant hormonal treatment after orchidopexy.

The remaining group (group 3) included 71 boys who at orchidopexy had all parameters within normal range. At follow-up, half of the boys (54%, 38/71) improved inhibin B MoM. Only 15% (11/71) of boys had impaired inhibin B and 10% (7/71) had elevated FSH at follow-up.

Paper IV Orchidopexy during minipuberty

A defective minipuberty is reported in infants with cryptorchidism. To better characterize this phenomenon, we studied the reproductive hormones that normally increase in boys during the first few months of life including boys who were born with cryptorchidism and underwent surgery during the minipuberty period. In addition, repositioning the testes during minipuberty may improve the fertility potential as the germ cells may benefit from the naturally occurring gonadotropin stimulation. Fifty-five percent (22/40) of the undescended testes were non-palpable positioned in the inguinal canal or above, 40% (16/40) were fixed in an annular position at the external inguinal ring, whereas two (5%, 2/40) testes were unable to reach the scrotum from a

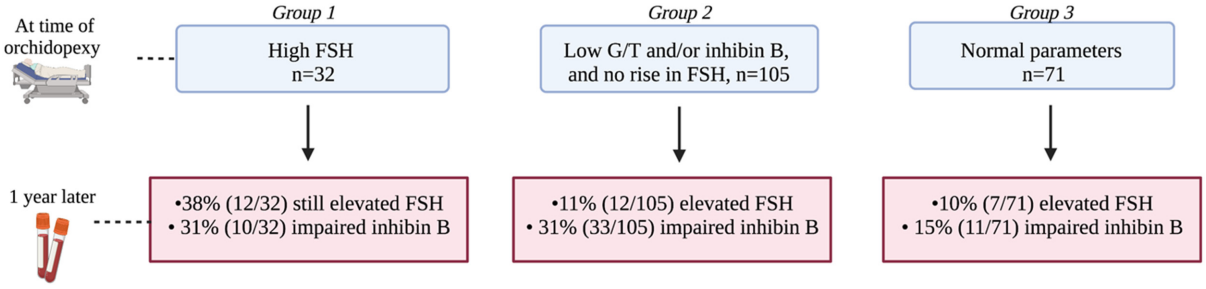

Fig. 12. Overview of Paper III. A schematic overview of the results of the 208 boys with bilateral cryptorchidism stratified into three groups based on the findings at surgery.

suprascrotal position. The processus vaginalis was open in all cases and none had a low scrotal insertion of the gubernaculum.

We found that 54% (19/35, 95% CI 0.37–0.71) of the cryptorchid boys had a normal minipuberty hormonal profile and G/T.

Nine (26%, 9/35) cryptorchid boys had reduced G/T according to age. Of these, 78% (7/9) boys also had impaired Ad spermatogonia, which was a significantly larger proportion when compared with the remaining 26 boys (4%, 1/26) with normal G/T ($p < 0.0001$). All nine boys with reduced G/T had normal testosterone, whereas inhibin B was below 2.5th percentile in only one case (one inhibin B value missing). This one case was a 2-months old boy with a G/T value of 1.05, just below the lower range of G/T. We observed that all boys with reduced G/T had LH above the mean, five of these (14%, 5/35) were even above the 97.5th percentile.

Two boys (25%, 2/8, one FSH value missing) had indications of a relative FSH insufficiency having no sufficient rise in FSH as could be expected as the feedback mechanism due to reduced G/T. Although only one case had an FSH below the 2.5th percentile, the other case with a reduced G/T of 0.58 only had an FSH just above the 2.5th percentile. The remaining six boys (6/8) had FSH above the 97.5th percentile.

All, except for one, displayed a male LH/FSH ratio with higher LH than FSH (33/34) as seen in normal boys in contrast to girls having higher FSH [68]. Of note, 88% (30/34) boys had an LH/FSH ratio above 1.00 and 24% (8/34) had above 97.5th percentile, of whom three had reduced G/T. Normal gonadotropins were found in all boys, except for one boy an LH below 2.5th percentile (case with normal G/T) and two boys with an FSH below 2.5th percentile. For the two boys with low FSH, one had reduced G/T (mentioned above) and the other boy a very high G/T of 4.45, the latter indicating a normal hypothalamic–pituitary–gonadal axis.

Finally, we found that 6 boys (18%, 6/34) had impaired inhibin B, whereas the majority of the boys (85%, 29/34) had an inhibin B below the mean. Overall, we concluded that the reproductive hormones of boys with cryptorchidism exhibited a normal minipuberty pattern, although two cases could be suspected of endocrinopathy.

Paper V Parental acceptance rate toward cryopreservation

This study addresses an important clinical question relating to the acceptability of testicular biopsy for experimental fertility preservation in boys with cryptorchidism with presumed reduced fertility potential. It introduces the state of the art of SSC and prospects of testicular tissue cryopreservation as means to preserve future fertility in prepubertal boys. To date, fertility preservation is an important clinical issue for prepubertal boys and recently there has been a lot of attention to this field, largely stemming from the increasing numbers of boys receiving gonadotoxic treatments.

In line with our findings from Papers I to IV, we reasoned that some boys with bilateral cryptorchidism have implications of a high infertility risk and some can consequently be offered cryopreservation simultaneously to orchidopexy. Thus, we conducted an investigation on the parents' acceptance rate toward cryopreservation.

Parents of 14 boys with cryptorchidism (13 bilateral and one unilateral cryptorchidism) were offered the possibility to cryopreserve a testicular biopsy with a second procedure after successful orchidopexy. Mainly based on histological findings at orchidopexy, these patients were suspected to have a high risk of infertility. Hence, all boys had reduced G/T (median G/T at 0.07, range 0.03–0.37), of whom eight boys (57%, 8/14) completely lacked Ad spermatogonia and three boys (21%, 3/14) had reduced AdS/T (<0.01). Moreover, five boys (36%, 5/14) had impaired inhibin B. Thirteen out of the 14 parents (93%, 13/14) gave consent to cryopreservation agreed to undergo another procedure, well-informed of the surgical risks and the experimental state of procedure. We did not explore the reasons for declining participation.

In another setup, parents of 27 boys were offered cryopreservation as part of initial bilateral orchidopexy. Twenty-four out of 27 (89%, 24/27) boys underwent cryopreservation. Henceforth, from histological assessments, we

found eight boys (29%, 8/27) with reduced G/T and nine boys (33%, 9/27) with reduced AdS/T, of whom four (15%, 4/27) lacked Ad spermatogonia. Seven boys (26%, 7/27) had impaired inhibin B. Therefore, based on the criteria from group A, 8 (29%, 8/27) boys from group B achieved cryopreservation and were spared one more surgical procedure.

The parental acceptance rate was similar between the two groups with rates of 93% and 89%, respectively ($p = 0.68$). In conclusion, the acceptance rate toward a new experimental treatment procedure among parents of Danish boys with bilateral cryptorchidism was high since 90% (95% CI 0.77–0.97) of the parents accepted.

DISCUSSION

General considerations—Key findings compared to other studies

The new findings in this thesis are

I. Despite early and successful orchidopexy before 1 year of age, a considerable fraction of boys with nonsyndromic congenital cryptorchidism may risk infertility based on histological and hormonal findings at surgery.
II. Boys with bilateral ascending testes share the same histopathological impairment as bilateral congenital cryptorchidism and should therefore be treated as soon as possible after diagnosis. Consequently, retractile testes are important to follow since they have a greater risk of ascent.
III. Orchidopexy does improve inhibin B in some boys with bilateral cryptorchidism which might be reflected in histology. However, a fraction of boys with bilateral cryptorchidism can be suspected of endocrinopathy because of testicular dysgenesis at surgery (defined as reduced G/T and/or impaired inhibin B) but no compensatory gonadotropin stimulation as expected.
IV. Most boys with congenital cryptorchidism display a normal minipuberty, however, a few cases can be suspected of endocrinopathy.Sub conclusion: Early and successful orchidopexy might not be a sufficient treatment strategy to improve fertility for all boys with cryptorchidism, especially boys with bilateral cryptorchidism or those with an endocrinopathy, and it would therefore be relevant to investigate supplementary treatment modalities.
V. The majority of parents of boys with bilateral cryptorchidism accept cryopreservation for their son's testicular biopsy, even though it in its current form holds no promise about future fertility preservation.

Assessing the fertility potential in prepubertal boys – To date, there is a consensus that cryptorchidism should be surgically corrected as soon as possible after 6 months of age and within the subsequent year, or by 18 months at the latest [2,16,17,18]. However, we still do not have proof to what extent the fertility potential is improved by early treatment, especially when the condition is bilateral and there is endocrinopathy such as a hypofunction of the hypothalamic–pituitary–gonadal axis. Is early orchidopexy a sufficient treatment for improving the fertility potential in all cases, probably not, and what other treatment strategies should be offered? To finally prove that early orchidopexy has been successful, normal sperm outcome and achieved paternity in adulthood need to be verified in large, prospective, long-term follow-up studies, which we currently lack for patients undergoing surgery according to the guidelines. Based on the findings in this thesis and literature review, we assume that early treatment is not sufficient in all cases, and we speculate if supplementary treatment is indicated for some groups of patients. In the era of precision medicine, it becomes increasingly important to understand the heterogeneity of a disease like cryptorchidism to design proper individualized treatments.

By assessment of the fertility potential at orchidopexy with testicular histology and reproductive hormones (inhibin B, FSH, LH, and testosterone), the clinicians can achieve data on testicular state and function that can help to determine the risk of later infertility and clarify the need for supplementary treatment. Besides our group, others support the

concept that testicular biopsies can serve as a prognostic tool for the fertility potential [50,200,201,202,203,205,238,239]. Supplement of hormonal assessment may reveal if there is a testicular dysfunction by impaired inhibin B to the dysgenesis verified by histology, and if the abnormal testis is compensated by elevated FSH and LH exploring the function of the hypothalamic–pituitary–gonadal axis [197].

This thesis was designed to increase the knowledge of the fertility potential in boys with cryptorchidism by evaluating present unique data: testicular biopsies and reproductive hormones, which are very seldom practiced elsewhere. To the best of our knowledge, only a few studies have published histological and hormonal data on boys with cryptorchidism [211,221,240]. Overall, the papers included in the thesis revealed a compromised fertility potential at the time of surgery that was found in about 20–30% of boys with bilateral cryptorchidism (Papers I–V, Table 2—assessed by one or more parameters). For the 264 unilateral cases included in Paper I, we found that 24% had reduced G/T, 30% had reduced AdS/T, and 20% impaired inhibin B, which was similar to the unilateral young cases in Paper IV (except for inhibin B, impaired in only 13%).

Even a considerable fraction of the boys who underwent orchidopexy before 6 months (Paper IV), 9 months (Paper I), and 1 year (Papers I, III, and IV) had reduced fertility potential, which is in accordance with a study in fetal cryptorchid testes of which 23% had a reduced G/T [60]. Thus, testicular dysgenesis may be a congenital condition in some cases. This fitted well with other histological findings demonstrating a marked depletion of germ cells in prepubertal patients with cryptorchidism [43,48,237,241,242].

Different from other studies, this thesis included both G/T and AdS/T and combined the parameters with hormonal profiles. Moreover, our studies included large cohorts of boys who underwent early orchidopexy with the distinction between different subtypes of cryptorchidism. Surgery for cryptorchidism at Rigshospitalet is performed at a median age of 34 months for boys, who were operated on before 15 years of age within the period 2015–2019 (Paper II). This is earlier than in most other European centers [105,243,244,245].

Lastly, our findings support stressing early orchidopexy since those who underwent orchidopexy before 9 months (Paper I) and 1 year (Paper III) had better parameters, as also other groups have reported on with different effect outcomes and metrics [1,20,21,155,190,242,246,247]. The odds for germ cell depletion increased with increasing age at orchidopexy, with boys undergoing orchidopexy between 13 and 24 months having an odds ratio of 3.9 compared to those having surgery before 1 year of life ($p = 0.0007$) [248]. For boys undergoing orchidopexy between 25 and 96 months, the odds ratio for germ cell depletion increased to 8.3 and for boys older than 8 years of age, the odds roughly doubled reaching a ratio of 16.8. These are in accordance with findings from our group [231].

In conclusion, we speculate that a combined assessment of testicular biopsies and hormonal status is a cornerstone upon which a clinician can formulate a treatment plan for prepubertal boys with cryptorchidism, including if supplementary hormonal treatment or cryopreservation is indicated.

Assessing hormones in boys with cryptorchidism – The pathogenesis of cryptorchidism continues to be debated but recent evidence suggests endocrine involvement and deficiencies in the interplay of the hypothalamic–pituitary–gonadal axis. It appears that abnormal germ cell development in cryptorchidism may not only be a result of congenital dysgenesis but may be associated with an endocrine imbalance and perturbation in germ cell maturation [38,41,205]. Testing for gonadotropins and inhibin B are important attributes to the assessment of testicular biopsies in boys with cryptorchidism at the time of surgery.

Papers I, II, and IV gave an insight into the fractions of boys at risk of infertility at the time of surgery but cannot answer the question to what extent the fertility potential would be normalized after surgical correction. If there is an underlying endocrinopathy, simply placing the testis into the scrotum by early surgery will not correct the endocrinopathy. Conversely, the effect of surgery might depend on the classification of

Table 2. Summary of histological and hormonal findings. Overview of the findings in Papers I–V

	Paper I	Paper II		Paper III		Paper IV	Paper V	
	Orchidopexy before 1 year	Ascending	Congenital	Orchidopexy	Follow-up	Minipuberty	Group A	Group B
No. patients (no. unilateral)	333 (264)	67 (0)	86 (0)	208 (0)		35 (30)	14 (1)	27 (0)
Median age (range)	274 (34–390) days	3.8 (2–7) years	3.9 (2–6.9) years	1.7 (0.3–9) years	2.7 (1.1–10) years	124 (37–159) days	16 (6–45) months	12 (4–41) months
Bilateral absence of germ cells	0% (0/69); 0.7% (2/264) unilateral cases with absence in one biopsy	1.5% (1/67)	3.5% (3/86)	1.4% (3/208)		0% (0/5)	0% (0/13)	0% (0/27)
Median G/T (range)	0.87 (0–4.48)	0.50 (0–2.29)	0.37 (0–2.57)	0.44 (0–2.97)		1.71 (0.12–4.45)	0.07 (0.03–0.37)	0.56 (0.03–1.7)
Reduced G/T; below lower range	25% (83/333); 28% (19/69) bilateral cases	60% (40/67)	66% (57/86)	50% (104/208)		26% (9/35)	100% (14/14)	30% (8/27)
Median AdS/T (range)	0.155 (0–0.222)	0.007 (0–0.14)	0.006 (0–0.25)	0.017 (0–0.214)		0.024 (0–0.118)	0.001 (0–0.015)	0.015 (0–0.134)
Reduced AdS/T; below 0.01	28% (92/333)	64% (43/67)	55% (47/86)	24% (50/208)		23% (8/35)	79% (11/14)	33% (9/27)
Bilateral absence of Ad spermatogonia	16% (11/69); 24% (64/264) unilateral cases with absence in one biopsy	31% (21/67)	34% (29/86)	13% (26/208)		6% (2/35)	57% (8/14)	15 (4/27)
Median inhibin B (range)	174 (57–489)	80 (17–176)	76 (7–227)	114 (17–377)	85 (7–314)	291 (122–478)	109 (17–300)	136 (44–249)
Impaired inhibin B; below 2.5th percentile	21% (70/333)	13% (9/67)	15% (13/86)	23% (47/208)	26% (54/208)	16% (6/34)*	36% (5/14)	26% (7/27)
Median FSH (range)	0.65 (0.05–4.01)	0.7 (0.15–31)	0.7 (0.12–26.4)	0.79 (0.17–4.01)	0.7 (0.05–4.79)	1.29 (0.19–2.75)	0.8 (0.4–1.4)	0.64 (0.27–2.5)

[1]One boy missing value.

fertility potential which could reflect different pathogenesis (Table 2).

In Paper III, we aimed to link histology at the time of surgery to the evaluation of the reproductive hormones at surgery and at 1-year follow-up to identify the fraction of boys with bilateral cryptorchidism that suffers some sort of endocrinopathy. When data from orchidopexy revealed reduced GT and/or impaired inhibin B (testicular dysgenesis) with no rise in gonadotropins, and follow-up testicular hormones and gonadotropins not improved, we reasoned that such boys with bilateral cryptorchidism could be suspected as having an endocrinopathy, which others have termed prepubertal transient hypogonadotropic hypogonadism. Such patients will have a high risk of later infertility (Fig. 12). Surprisingly, we found that half (105/208) of the boys with bilateral cryptorchidism fulfilled these criteria at the time of surgery. Of these with reduced G/T, 11 boys had normal inhibin B at the time of surgery but exhibited an impaired inhibin B below 2.5th percentile 1 year postoperative indicating, in this regard, no benefit of surgery (Fig. 12). Moreover, of the 35 boys who had impaired inhibin B at the time of surgery (20 of whom also reduced G/T), 66% (22/35) had persistent impairment of inhibin B (15 of those with reduced G/T) 1 year postoperative. Ninety-three (89%, 93/105) had no elevation of FSH at 1-year which could suggest an endocrinopathy. Although inhibin B MoM improved by surgery in this group (median MoM 0.60 *vs* 0.70), this was not significant.

Despite that no significant difference could be demonstrated in the boys with elevated FSH, the trend in this group was stronger as inhibin B MoM increased from 0.56 to 0.74 and 59% (19/32) of boys increased inhibin B MoM. In the total material, 54 (26%, 54/208) had impaired inhibin B at follow-up (*vs* 17% at surgery), 12 of whom had increased in FSH.

The distinction between cryptorchid boys with a competent hormonal compensation of the testicular dysgenesis responding to reduced G/T *vs* those with elevated FSH reflecting a reaction to the severity of G/T and worse fertility potential is a challenge (Fig. 13).

These findings are in agreement with the classification from Thorup *et al.* [197] who characterized boys with bilateral cryptorchidism into groups of low, intermediate, and high risk for infertility based on the same parameters. Interpretation of such findings is difficult, and more studies with follow-up examinations in adulthood, are necessary to estimate the true existence and subsequently the frequency of these subtypes based on the fertility potential at the time of surgery.

Based on low Ad spermatogonia count at the time of orchidopexy (less than 0.01), a study classified 18 out of 89 men operated for cryptorchidism as a high-risk infertility group, ten of whom were bilateral [205]. In adulthood, the mean sperm count was 8.9 million per ejaculate (95% CI 3–14.9) and 22% (4/18) experienced azoospermia, thus this group of patients exhibited significantly worse sperm counts than patients with Ad spermatogonia in the testicular biopsies at the time of surgery for cryptorchidism. Moreover, at least 70% within the high-infertility group experienced a relative FSH insufficiency in adulthood. These findings further support that cryptorchidism may be associated with endocrinopathy. Since gonocyte transformation into Ad spermatogonia occurs during minipuberty, this research group suggests that boys with cryptorchidism suffer from a so-called diminished minipuberty [48,70,205,237,249].

In Paper IV, we found that 9 out of 35 (26%) had a reduced G/T already in the minipubertal period. Of these, seven (7/9, 78%) boys displayed normal minipuberty hormone concentrations, thus reflecting a competent gonadotropin feedback mechanism. The remaining two cases with reduced G/T could be suspected of a subtle endocrinopathy together with testicular dysgenesis (one unilateral boy had FSH below and the other one unilateral FSH just above 2.5th percentile) accounting for 6% (2/35) of the total material. These two patients would most likely benefit from hormonal treatment. Of note, our findings showed that most boys with congenital cryptorchidism had a normal minipuberty pattern. This is supported by a case–control study that failed to identify any significant differences in hormones between 26 healthy controls and 20 boys with non-syndromic cryptorchidism at age of 2 months [215]. Another case–control study of 43 cryptorchidism boys *vs* 113 controls found significantly lower testosterone in the age between 1 and 6 months (mean testosterone 1.8 *vs* 2.6 nmol/L), however, no sign of insufficiency of either LH or FSH was found [227]. In a separate study, without any hormonal assessment, authors postulate that the incidence of a defective minipuberty in boys with unilateral cryptorchidism is at least one-third (even up to 50%) with the assumption that the formation of Ad spermatogonia is linked to the minipuberty stimulation [249]. However, this is obscure since no hormone samples were evaluated.

Another way to assess the endocrine function of the hypothalamic–pituitary–gonadal axis is by stimulation tests with GnRH in which a blunted FSH and LH response suggest hypogonadotropic hypogonadism either due to pituitary or hypothalamic function [67]. However, conflicting results of hormonal changes after stimulation in cryptorchid boys have been published. Blunted LH responses after stimulation with GnRH have been found in boys with cryptorchidism, whereas peak FSH values were not significantly different from control values [250,251]. Others could not demonstrate a functional insufficiency of the hypothalamic–pituitary–gonadal axis during the first year of life [252]. In a study conducted at our department, 35 boys with bilateral cryptorchidism aged 9 months to 3.7 years had a GnRH stimulation test with 100 mg buserelin (Suprefact®) and blood samples for LH and FSH at t:0 and t:30 min [229]. Sixteen had normal G/T above 0.38 and 19 had severely reduced G/T below 0.2. Most patients had a normal response with normal LH and FSH and only 23% (8/35, 4 from each group) had an insufficient rise in LH and/or FSH. Therefore, an endocrinopathy such as a so-called prepubertal transient hypogonadotropic hypogonadism (mild variance of hypogonadotropic hypogonadism) may exist in some boys with cryptorchidism in early life which could cause long-term impairment of fertility in adulthood. There is evidence to support that cryptorchidism is associated with decreased androgen production during childhood [227].

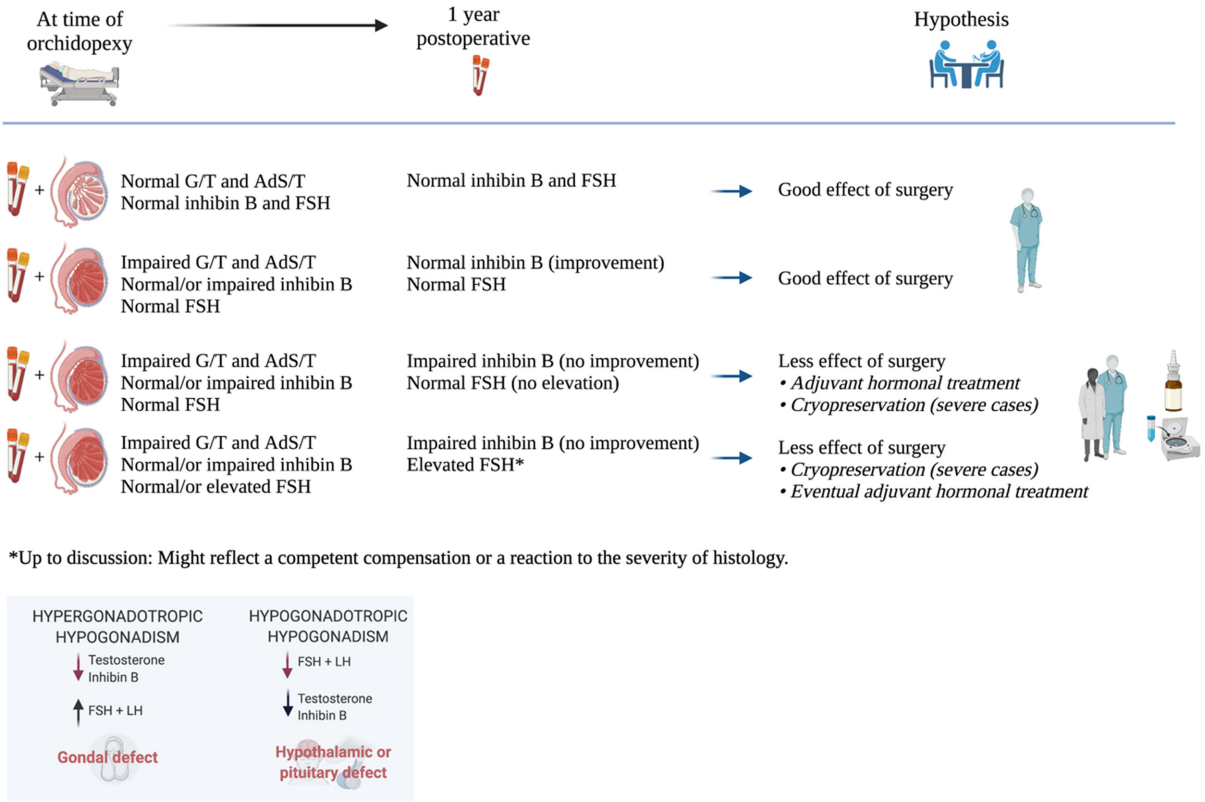

Fig. 13. Discussion—how to assess hormones when evaluating the effect of orchidopexy?. An overview of how clinicians could use histological and hormonal parameters to clarify the use of adjuvant hormonal treatment.

To date, the potential mechanisms of hypogonadism in cryptorchidism are still elusive [124].

From the papers included in this thesis, in addition to other literature, it is clear there is a reduction in the number of germ cells and a defect in the transformation of the gonocytes to Ad spermatogonia in cryptorchid testis, even though the testes were in the scrotum during the minipuberty period. More work should focus on possible prevention strategies to reduce this deterioration and diminishment of germ cell maturation. Adjuvant hormonal treatment around the time of orchidopexy has been shown to improve fertility potential (Table 1), whereas one study was able to demonstrate the long-term favorable effect on future fertility [128]. Although there might be room for hormonal treatment in the management of cryptorchidism, long-term studies on adjuvant treatment are still warranted to fully recommend this as routine practice.

Cryopreservation may be an option in some cases of cryptorchidism [161]. The final study in the thesis (Paper V) demonstrated that offering cryopreservation to boys with bilateral cryptorchidism is acceptable to 90% (95% CI 0.77–0.97) of parents. The experimental nature and unknown future utility of using cryo-stored testicular tissue to preserve fertility do not appear to influence the acceptance. We propose that boys with cryptorchidism are potential candidates for cryopreservation, specifically those with a bilateral disorder with implications of a severely compromised fertility: reduced germ cell count below 0.2 and/or no Ad spermatogonia with impaired inhibin B and no compensatory elevation of FSH (Fig. 13). Germ cells should be present in testicular biopsies. Moreover, to avoid repeated surgery with biopsy, some parents may choose biopsy for cryopreservation at the time of bilateral orchidopexy, well informed that the procedure may only be truly indicated in

around 20% of the cases [161]. Besides us, and to the best of our knowledge, only one other group has offered cryopreservation of testicular tissue to boys with bilateral cryptorchidism (n = 19) [169]. The authors also offered cryopreservation for parents of 14 prepubertal boys with cancer and found similar acceptance rates between the two groups (78.9% *vs* 78.6%). These rates of acceptance were similar to what was found in a study by [253,254] for boys facing gonadotoxic treatment, whereas our acceptance rate was slightly higher.

Assessing different subtypes of cryptorchidism – Many surgeons agree that testicular position should be screened among boys during childhood to recognize boys with testicular ascent, who should be referred for orchidopexy [2,16,17,18]. Despite these guidelines, surprisingly little is known about the fertility potential in these patients. We reported that ascending testes have similar adverse findings on the testicular histology as seen in boys with congenital cryptorchidism (Paper II), as reported in two comparative studies [130,255]. Our study focused on bilateral cases and included hormonal data. We found similar histological findings as Rusnack *et al.* [130]. It may be perceived as a high number of bilateral ascending testes cases in our inclusion period. However, in accordance with our data, a prospective study following a well-defined birth cohort, demonstrated that 63% of boys with ascending testis presented a bilateral ascent [108].

Ascending testis merely represents a part of the spectrum of cryptorchidism [103,107] and it is essential to analyze the subtypes of cryptorchidism separately since the etiology, treatment, and outcomes might differ between them.

The ascending testis complicates the interpretation of study findings on men with a history of cryptorchidism for several reasons. First, some studies may not have examination data available at birth and rely on self-reporting, especially if adult men are studied, which makes the distinction between ascending testes and congenital cryptorchidism more difficult. Moreover, ascending testes must be distinguished from retractile testis, which might be challenging. Boys may not be aware of ascending testis, and therefore the onset of acquired cryptorchidism is often uncertain, which results in a delay in treatment.

Brakel et al. [256] performed andrological evaluations including scrotal ultrasound, reproductive hormones, paternity, and semen analysis of 53 healthy men serving as controls, 62 men treated for congenital bilateral cryptorchidism, and 65 men with former bilateral ascending testis (with spontaneously descent or orchidopexy). They found that men with ascending testis more often had significant abnormal testicular consistency, smaller testes, lower semen concentration, and less motility compared to controls. Four (6%, 4/65) men had azoospermia. Except for better testicular consistency in the former ascending testes, no significant differences were found in comparison with men with former congenital cryptorchidism (median age at orchidopexy 3 years). Furthermore, no difference was found between men with ascending testes in whom spontaneous descent was successful and those who underwent orchidopexy (median age at orchidopexy 13.2 years). Unfortunately, information on how long the ascended testes were retained at a cryptorchid position was not known, thus lacking in to disclose whether the fertility potential would be improved by immediate surgery at diagnosis, which should be subject to future studies. Promm *et al.* [255] found that boys with ascending testis undergoing orchidopexy later than 9 years of age had worse histological parameters, however, we have not found this in our cohort (Paper II).

In agreement with others, we suggest that this descent-ascent pattern may be caused by an endocrinopathy [97,130,257]. A Danish-Finnish cohort study found an association between Leydig cell function and testicular position assessed by the testicular distance to the pubic bone [257]. The authors reported a physiological small testicular ascent that occurs after 3–18 months of life (in cryptorchid and normal boys alike) possibly due to the peak and subsequent postnatal decline in the activity of the hypothalamic–pituitary–gonadal axis during infancy. This is in agreement with the high prevalence of testicular ascent at 1 year of age found in a large British prospective cohort study [97]. Moreover, the British cohort study reported a reduced penile growth

during infancy in boys with ascending testis, which may be associated with reduced early postnatal androgen activity [97]. Based on our study, the reproductive hormones in our cohort of boys whose testes ascended did not differ from the congenital cases at the time of surgery, but we did not have hormonal concentrations from these boys during minipuberty. Nonetheless, our findings highlight the importance of further studies on the etiology of ascending testes and the long-term effects. If boys with ascending testis suffer from endocrinopathy, adjuvant hormonal treatment could be a possibility for these boys.

Unilateral vs bilateral disorder – A study revealed that in boys with unilateral cryptorchidism, the scrotal testis is affected in 71% with a reduced G/T and 75% had impaired transformation into Ad spermatogonia [205]. However, the age at orchidopexy ranged between 1 and 16 years and it was not clear how many patients underwent early surgery. A more recent study, including 319 boys treated for unilateral cryptorchidism with simultaneous biopsy of the contralateral scrotal testis, found that 48% of the cryptorchid testes and 21% of contralateral testis had no Ad spermatogonia [237]. In total, 11% of the cases had no Ad spermatogonia. The median age at orchidopexy was 39 (range 5–192) months, whereas 58 boys underwent orchidopexy after 18 months of age. These findings may indicate that unilateral cryptorchidism can be a bilateral disorder.

In Paper I, we could not find significant differences in any parameter between bilateral and unilateral cases, except for FSH which was higher in bilateral cases ($p < 0.001$). This is in agreement with Verkauskas *et al.* [211] who also reported that unilateral and bilateral cryptorchidism showed similar tubular fertility index (number of tubules with germ cells), Ad/T, and hormones (FSH, LH, and inhibin B) in a cohort of 71 boys (24% bilateral) aged 7–65 months. From Paper IV, the two boys with reduced G/T who could be suspected of an FSH insufficiency were unilateral cases and both had a reduced AdS/T. However, we had no histology from the contralateral testis and inhibin B was within the normal range for one of the boys (the other one missing inhibin B). Moreover, we included one unilateral case in Paper IV, after orchidopexy as the cryptorchid testis revealed no Ad spermatogonia and a severely reduced G/T of only 0.03, which was close to 1% of normal age-related G/T at 13 months of age. At cryopreservation 19 months later, both testicular biopsies exhibited the same reduction of G/T at 0.023 and 0.038 emphasizing that unilateral cryptorchidism can affect both testes. So far, five boys with unilateral cryptorchidism with reduced G/T in their undescended testis and impaired inhibin B (a highly selected group) have undergone bilateral testicular biopsies as part of the cryopreservation program at our department (one case included in Paper V; four cases after January 2020). Four of them (80%, 4/5) revealed reduced G/T in the contralateral descended testis, one of them with no germ cells bilaterally (unpublished data).

Our histological and hormonal findings suggest that cryptorchidism may be more than a simple anomaly of testicular position as cryptorchidism may be associated with an endocrinopathy (as a possible subtle hypofunction of the hypothalamic–pituitary–gonadal axis) that may contribute to impairment of testicular development in both testes, even when one testis descend to the scrotum. This is in agreement with our department's previous follow-up studies showing that in cases of less than 1% of normal age-related G/T in the biopsy from the undescended testes in unilateral cryptorchidism at 2–12 years of age, there was a 33% risk of later infertility [1,185]. Moreover, some authors did not reveal any differences in semen data between unilateral and bilateral cases [20,184], however, this might be due to the modest number of samples.

In a cohort study of 225 men with a history of cryptorchidism as the sole etiopathogenetic factor for non-obstructive azoospermia, the frequency of no germ cells between men with bilateral and unilateral cryptorchidism was similar (50% *vs* 43%, $p = 0.30$) [258]. However, these men were selected based on non-obstructive azoospermia and 64% (145/225) in this cohort had a history of bilateral cryptorchidism. In an epidemiological study including 21 cases with unilateral cryptorchidism and testicular cancer, cancer occurred in the

contralateral scrotal testis in 7 cases, thus carrying a risk of bilateral neoplasia (relative risk 1.9 in the descended *vs*3.9 in the undescended testis) [177]. These findings suggest that a history of unilateral cryptorchidism can, in a few cases, affect both testes in adulthood.

Boys with unilateral cryptorchidism who undergo surgery have a lower risk of infertility to around 5–10% than in bilateral cases [186,259]. There is evidence of similar, inhibin B increased significantly in boys with unilateral congenital cryptorchid boys after follow-up [115,119,221,222].

Strengths and limitations

The main strength of this thesis was the assessment of the fertility potential including both histological and hormonal parameters of many young prepubertal boys operated for nonsyndromic cryptorchidism, which to the best of our knowledge is not routinely practiced elsewhere. Rigshospitalet has the largest department of pediatric surgery at a national level, treating around half of the cryptorchidism cases in Denmark and the participation rate in hormonal follow-up is high.

Another important strength is that the hormonal parameters included in this thesis were measured at the Department of Growth and Reproduction, Rigshospitalet, that have unique hormone reference ranges. Age-related reference materials are essential for investigating the natural history of hormones over different stages of life, as well as the sex differences, and it is an indispensable tool for comparison of hormone changes in abnormal development and screening for pathogenesis. The retrospective nature of the studies is a limitation. However, the retrospective revision through journals secured that the included boys had no syndromes or other associated anomalies, henceforth the blinded assessment of histological sections and hormonal parameters was conducted. It also allowed for a large patient cohort as patients have had hormonal samples in addition to testicular biopsies since the year 2010.

A limitation in our studies is related to normal values of germ cells, including Ad spermatogonia, due to the sparse normal material both at Rigshospitalet and in the literature. Although histology preparations were all done in the same laboratory, our normal control tissues included a modest sample size. We emphasized some difficulties: (1) our values (used in Papers I, II, III, and V) were in the lowest normal values compared to others according to a meta-analysis [61], (2) counting on HE and PAS stained sections (as done in control material) in general revealed fewer germ cells than immunohistochemical sections (as done in this thesis), and finally, (3) counting germ cells on autopsy material is more difficult than counting on tissue that is fixed just after it left the body. Therefore, we modified our lowest normal value in Paper IV.

Furthermore, the lowest normal value for Ad spermatogonia is mainly defined accordingly to clinical practice by others, a stronger parameter would be fertility parameters such as paternity and sperm analyses. However, as of today, our department lack follow-up data from men who have had testicular biopsies measured also by Ad spermatogonia in childhood.

When obtaining testicular biopsies to assess the fertility potential, the biopsy may not reflect the total testicular parenchyma and in unilateral cases only mirror the undescended (diseased) testis. Because of the heterogeneity of the germ cells in the undescended testes, we quantitated the whole biopsy with at least 100 and 250 cross-sectional tubules for G/T and AdS/T, respectively, which is higher than other studies [41,50,187,202,238,241]. Biopsies with fewer transversal tubules were excluded.

As hormones during the first months of life undergo developmental changes, interpretation of concentrations observed in cryptorchid boys must be done with caution. The interpretation of FSH in the thesis is a limitation. We interpreted FSH as elevated when it was higher than the 97.5th percentile of normal distribution, in accordance with our previous methodology in adult patients [1,185,199,259]. FSH in childhood is poorly understood, and the determination of a high or low FSH needs to be elucidated. We did not have normal percentiles for FSH in Papers I, II, III, and V, which is why we sat in the upper range accordingly to the age as described by Andersson *et al.* [66] (Paper I) and 1.4 or greater (Paper III). Because some of these boys in the young age

group were only 0.3 years old, we cannot reject that some may exhibit a normal physiological FSH level since they underwent orchidopexy early, close to the minipuberty, and therefore should not belong to this group.

CONCLUDING REMARKS

By better understanding the histological and hormonal aspects of cryptorchidism, we can strive to improve our ability to predict fertility potential. In this thesis, we gained insights into the histological and hormonal parameters in boys with cryptorchidism.

We found that up to 20–25% of boys with nonsyndromic cryptorchidism (unilateral and bilateral) who underwent surgery within the first year of age risk impaired fertility in adulthood based on findings from surgery. We showed that minipuberty often is normal in boys with nonsyndromic congenital cryptorchidism, as only 6% had testicular dysgenesis and lacked a compensatory FSH stimulation (two unilateral cases).

In boys with bilateral cryptorchidism who underwent orchidopexy at the median age of 1.7 years (0.3–9 years of age), we found that about 51% of such boys had reduced G/T and/or impaired inhibin B with no rise in FSH at the time of surgery. At follow-up 1 year later, 31% of these still had or decreased in inhibin B below the 2.5th percentile. Overall, 26% of all the bilateral cases might have a risk of infertility corresponding to those with impaired inhibin B at follow-up. Moreover, we learned that ascended testes exhibit compromised fertility potential similar to what was found among boys with congenital cryptorchidism.

These findings suggest that cryptorchidism may be associated with an endocrinopathy, such as a subtle hypofunction of the hypothalamic–pituitary–gonadal axis, that contributes to impairment of testicular development, probably even when the cryptorchid testis is unilateral. We, therefore, suggest that some boys with cryptorchidism may benefit from supplementary hormonal treatment. In cases with a very low number of germ cells and/or no effect of supplementary hormonal treatment, cryopreservation of testicular tissue may be indicated.

In a pioneer study, we found that about 90% of parents of bilateral cryptorchid boys accepted cryopreservation of testicular tissue, even though they were not promised that it could be of use or truly indicated in adulthood, as its efficacy has not been proved and we believe bilateral cryptorchidism may only risk infertility in about 20%.

PERSPECTIVES AND FUTURE STUDIES

Our study group finds that it is now possible to a certain extent to distinguish between cases of cryptorchidism wherein the condition may be part of a congenital defect or may be associated with an endocrinopathy [161,197]. For those patients with endocrinopathy, adjuvant treatment with GnRH holds a promising therapeutic potential for optimizing the fertility potential [159-161] Table 1. A prospective randomized clinical trial to assess the efficacy and safety of postoperative GnRH treatment for boys with bilateral cryptorchidism is in progress at the Department of Pediatric Surgery, Rigshospitalet. With a placebo-controlled, double-blind setup, we will include boys with bilateral cryptorchidism (6–36 months of age) with verified testicular dysgenesis (severely reduced age-related G/T but above 0.2) with indications of hypothalamus-pituitary-gonadal hypofunction (impaired FSH and/or LH).

Half of the patients will receive GnRH agonist (Kryptocur®) administrated by nasal spray 0.2 mg/0.1 mL x 2 every second day (3 mL/month) or placebo during a 4-months period. After the completed treatment regime, a second surgery will be performed to collect testicular biopsies for cryopreservation in addition to the hormonal profile. Herein, we aim to present a concise analysis of an extended RNA profiling of Sertoli cells upon treatment of GnRH using the Nanostring GeoMx platform (NanoString Technologies, Inc., Seattle, USA) at the Department of Pathology, Rigshospitalet.

The transcriptome-wide findings will be analyzed in relation to the traditional quantitative assessment of germ cells and Ad spermatogonia. A successful result from that study will add evidence-based knowledge to apply well-indicated adjuvant hormone treatment for a

hopefully well-selected patient group who will need it to preserve fertility, alongside other recent observations [260].

Loss of germ cells and SSCs is critical in prepubertal boys who suffer from testicular failure due to for example cryptorchidism or gonadotoxic therapy, as there is no option of sperm cryopreservation due to sexual immaturity [167]. Moreover, recent studies have shown that early germ cells have more complex physiology than previously anticipated and that we do not have sufficient markers to identify them properly (as described in the background section) [53]. Studies should focus on the developmental landscapes of male germ cells in the prepubertal testis from patients with cryptorchidism and normal testes, if available. In a manuscript under preparation, we try to distinguish different phenotypic subpopulations of early germ cells based on molecular marker profiles by multiplex analysis with immunofluorescence markers. Focusing on further analysis of gonocytes and Ad spermatogonia, single-cell RNA sequencing techniques allow us to resolve intercellular relationships and unveil the existence of heterogeneity, even in a seemingly homogeneous population. Encouraged by three reports [30,46,55], we want to improve the characterization at additional state embedding spermatogonia into clusters by single-cell RNA sequencing based on the assumption that many subtypes of spermatogonia exist. In summary, these studies will increase our knowledge about early germ cell subpopulations in normal and cryptorchid testes. This will bring new expectations for the accuracy and precision of tissue-based evaluation of future fertility, particularly when quantification of SSCs can be made by identifying histologic markers.

The gained knowledge of the characterization of prepubertal germ cells (gonocytes, spermatogonia, and Ad spermatogonia) can improve the prospects for the promising attempt to produce human haploid germ cells *in vitro* or *in vivo* by autologous transplantation.

Currently, male reproductive function is at the center of new developments and different fertility preservation strategies for prepubertal boys are under investigation, also at the Laboratory of Reproductive Biology at Rigshospitalet. The research at Rigshospitalet is based on a multidisciplinary translational network of hospital departments (mainly urology and pediatric surgery), the National cryopreserving center (Laboratory of Reproductive Biology, Rigshospitalet), and the University of Copenhagen centered around the Fertility Restoration Consortium and a Clinical Academic Group within regenerative medicine, urogenital surgery, and fertility named CAG-SURF. We are currently exploring if colonization of human gonocytes and SSCs directly transplanted to seminiferous tubules in busulfan-sterilized mice is feasible. The preliminary analysis shows that without *in vitro* propagation, naturally enriched human testicular cells settled on the basal membrane of seminiferous tubules and survived in the recipient mouse testes at least for 2 months, demonstrating that human gonocytes and SSC were capable of colonization (unpublished data).

For more than two decades, early orchidopexy has been considered the most effective therapy for preventing infertility. Despite this, the evidence of fertility outcomes in early surgically treated cryptorchidism is very scarce. Large prospective, follow-up studies with andrological evaluations including semen analysis and paternity rates are warranted and are hopefully already on its way. We hope to contribute and by 2026, we will be able to assess at least 35 young men who underwent orchidopexy before 2 years of age due to bilateral cryptorchidism.

REFERENCES

1. Cortes D. Cryptorchidism – aspects of pathogenesis, histology and treatment. Scand J Urol Nephrol Suppl. 1998;196:1–54.
2. Kolon TF, Herndon CD, Baker LA, Baskin LS, Baxter CG, Cheng EY, et al. Evaluation and treatment of cryptorchidism: AUA guideline. J Urol. 2014;192(2):337–45.
3. Barthold JS, Gonzalez R. The epidemiology of congenital cryptorchidism, testicular ascent and orchiopexy. J Urol. 2003;170(6 Pt 1):2396–401.
4. Thorup J, Cortes D. The incidence of maldecended testes in Denmark. Pediatr Surg Int. 1990;5:2–5.
5. Thorup J, Cortes D. Surgical Management of Undescended Testis - timetable and outcome: a debate. Sex Dev. 2019;13(1):11–9.
6. Fedder J, Crüger D, Oestergaard B, Petersen GB. Etiology of azoospermia in 100 consecutive nonvasectomized men. Fertil Steril. 2004;82(5):1463–5.
7. Olesen IA, Andersson AM, Aksglaede L, Skakkebaek NE, Rajpert-de Meyts E, Joergensen N, et al.

Clinical, genetic, biochemical, and testicular biopsy findings among 1,213 men evaluated for infertility. Fertil Steril. 2017;107(1):74–82.
8. Lee PA, Coughlin MT. Fertility after bilateral cryptorchidism. Evaluation by paternity, hormone, and semen data. Horm Res. 2001;55(1):28–32.
9. Miller KD, Coughlin MT, Lee PA. Fertility after unilateral cryptorchidism. Paternity, time to conception, pretreatment testicular location and size, hormone and sperm parameters. Horm Res. 2001;55(5):249–53.
10. Giwercman A, Bruun E, Frimodt-Møller C, Skakkebaek NE. Prevalence of carcinoma in situ and other histopathological abnormalities in testes of men with a history of cryptorchidism. J Urol. 1989;142(4):998–1001.
11. Lip SZ, Murchison LE, Cullis PS, Govan L, Carachi R. A meta-analysis of the risk of boys with isolated cryptorchidism developing testicular cancer in later life. Arch Dis Child. 2013;98(1):20–6.
12. Møller, H. and H. Evans (2003). Epidemiology of gonadal germ cell cancer in males and females. Apmis 111(1): 43–46.
13. Osterballe L, Clasen-Linde E, Cortes D, Engholm G, Hertzum-Larsen R, Reinhardt S, et al. The diagnostic impact of testicular biopsies for intratubular germ cell neoplasia in cryptorchid boys and the subsequent risk of testicular cancer in men with prepubertal surgery for syndromic or non-syndromic cryptorchidism. J Pediatr Surg. 2017;52(4):587–92.
14. Pettersson A, Richiardi L, Nordenskjold A, Kaijser M, Akre O. Age at surgery for undescended testis and risk of testicular cancer. N Engl J Med. 2007;356(18):1835–41.
15. Schneuer FJ, Milne E, Jamieson SE, Pereira G, Hansen M, Barker A, et al. Association between male genital anomalies and adult male reproductive disorders: a population- based data linkage study spanning more than 40 years. Lancet Child Adolesc Health. 2018;2(10):736–43.
16. Ritzen EM, Bergh A, Bjerknes R, Christiansen P, Cortes D, Haugen SE, et al. Nordic consensus on treatment of undescended testes. Acta Paediatr. 2007;96(5):638–43.
17. Radmayr C, Dogan HS, Hoebeke P, Kocvara R, Nijman R, Silay S, et al. Management of undescended testes: European Association of Urology/European Society for Paediatric Urology Guidelines. J Pediatr Urol. 2016;12(6):335–43.
18. Radmayr, C., G. Bogaert, H. S. Dogan, J. M. Nijman, Y. F. H. Rawashdeh, M. S. Silay, R. Stein, S. Tekgül (2021). European Association of Urology Guidelines. Presented at the Annual Congress Milan 2021. Guidelines Office, Arnhem, Netherlands.
19. Engeler DS, Hosli PO, John H, Bannwart F, Sulser T, Amin MB, et al. Early orchiopexy: prepubertal intratubular germ cell neoplasia and fertility outcome. Urology. 2000;56(1):144–8.
20. Feyles F, Peiretti V, Mussa A, Manenti M, Canavese F, Cortese MG, et al. Improved sperm count and motility in young men surgically treated for cryptorchidism in the first year of life. Eur J Pediatr Surg. 2014;24(5):376–80.
21. Hadziselimovic F, Herzog B. Importance of early postnatal germ cell maturation for fertility of cryptorchid males. Horm Res. 2001a;55(1):6–10.
22. Lee PA. Fertility after cryptorchidism: epidemiology and other outcome studies. Urology. 2005;66(2):427–31.
23. Ostrer H, Huang HY, Masch RJ, Shapiro E. A cellular study of human testis development. Sex Dev. 2007;1(5):286–92.
24. Schoenwolf GC, Bleyl SB, Brauer PR, Francis-West PH. Larsen's Human Embryology. Philadelphia, PA: Elsevier; 2021.
25. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, et al. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature. 1990;346(6281):240–4.
26. Culty M. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today. 2009;87(1):1–26.
27. Stukenborg JB, Kjartansdóttir KR, Reda A, Colon E, Albersmeier JP, Söder O. Male germ cell development in humans. Horm Res Paediatr. 2014;81(1):2–12.
28. Gaskell TL, Esnal A, Robinson LL, Anderson RA, Saunders PT. Immunohistochemical profiling of germ cells within the human fetal testis: identification of three subpopulations. Biol Reprod. 2004;71(6):2012–21.
29. Guo F, Yan L, Guo H, Li L, Hu B, Zhao Y, et al. The transcriptome and DNA methylome landscapes of Human primordial germ cells. Cell. 2015;161(6):1437–52.
30. Li L, Dong J, Yan L, Yong J, Liu X, Hu Y, et al. Single-cell RNA-seq analysis maps development of Human germline cells and gonadal niche interactions. Cell Stem Cell. 2017;20(6):891–2.
31. Rajpert-De Meyts E, Hanstein R, Jørgensen N, Graem N, Vogt PH, Skakkebaek NE. Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. Hum Reprod. 2004;19(6):1338–44.
32. Sonne SB, Herlihy AS, Hoei-Hansen CE, Nielsen JE, Almstrup K, Skakkebaek NE, et al. Identity of M2A (D2-40) antigen and gp36 (Aggrus, T1A-2, podoplanin) in human developing testis, testicular carcinoma in situ and germ-cell tumours. Virchows Arch. 2006;449(2):200–6.
33. Gondos B, Hobel CJ. Ultrastructure of germ cell development in the human fetal testis. Z Zellforsch Mikrosk Anat. 1971;119(1):1–20.
34. Mamsen LS, Lutterodt MC, Andersen EW, Byskov AG, Andersen CY. Germ cell numbers in human embryonic and fetal gonads during the first two trimesters of pregnancy: analysis of six published studies. Hum Reprod. 2011;26(8):2140–5.
35. Clermont Y, Leblond CP. Differentiation and renewal of spermatogonia in the monkey, *Macacus rhesus*. Am J Anat. 1959;104:237–73.
36. Clermont Y. Renewal of spermatogonia in man. Am J Anat. 1966;118(2):509–24.
37. Paniagua R, Nistal M. Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat. 1984;139(Pt 3):535–52.
38. Hadziselimovic F. Cryptorchidism – ultrastructure of normal and cryptorchid testis development. Berlin Heidelberg: Springer-Verlag; 1977.
39. Hadziselimovic F, Herzog B. The importance of both an early orchidopexy and germ cell maturation for fertility. Lancet. 2001b;358(9288):1156–7.

40. Roosen-Runge EC, Barlow FD. Quantitative studies on human spermatogenesis. I Spermatogonia Am J Anat. 1953;93(2):143–69.
41. Hadziselimovic F, Emmons LR, Buser MW. A diminished postnatal surge of ad spermatogonia in cryptorchid infants is additional evidence for hypogonadotropic hypogonadism. Swiss Med Wkly. 2004;134(25–26):381–4.
42. Huff DS, Hadziselimovic F, Snyder HM, Blyth B, Duckett JW. Early postnatal testicular maldevelopment in cryptorchidism. J Urol. 1991;146(2 Pt 2):624–6.
43. Huff DS, Fenig DM, Canning DA, Carr MG, Zderic SA, Snyder HM 3rd. Abnormal germ cell development in cryptorchidism. Horm Res. 2001;55(1):11–7.
44. Kvist K, Clasen-Linde E, Langballe O, Hansen SH, Cortes D, Thorup J. The expression of markers for intratubular germ cell neoplasia in Normal infantile testes. Front Endocrinol. 2018;9:286.
45. Kvist K, Hildorf S, Clasen-Linde E, Cortes D, Thorup J. Germ cells positive for PLAP and c-kit in 11-16 year old normal boys with ongoing spermatogenesis. Pediatr Surg Int. 2020;36(10):1249–54.
46. Sohni A, Tan K, Song HW, Burow D, de Rooij DG, Laurent L, et al. The neonatal and adult Human testis defined at the single-cell level. Cell Rep. 2019;26 (6):1501–17.
47. Vigueras-Villaseñor RM, Cortés-Trujillo L, Chávez-Saldaña M, Vázquez FG, Carrasco-Daza D, Cuevas-Alpuche O, et al. Analysis of POU5F1, c-kit, PLAP, AP2γ and SALL4 in gonocytes of patients with cryptorchidism. Acta Histochem. 2015;117(8):752–61.
48. Hadziselimovic F, Thommen L, Girard J, Herzog B. The significance of postnatal gonadotropin surge for testicular development in normal and cryptorchid testes. J Urol. 1986;136(1 Pt 2):274–6.
49. Müller J, Skakkebaek NE. Fluctuations in the number of germ cells during the late foetal and early postnatal periods in boys. Acta Endocrinol. 1984;105 (2):271–4.
50. Kraft KH, Canning DA, Snyder HM 3rd, Kolon TF. Undescended testis histology correlation with adult hormone levels and semen analysis. J Urol. 2012;188(4 Suppl):1429–35.
51. Hildorf, S., D. Cortes, E. Clasen-Linde, J. B. Georgesen, P. S. Nielsen and J. Thorup (2021b). Digital multiplexing technique to identify germ cell subtypes in cryptorchid testes. Presented the 2 September at: 22nd Annual Congress of the European Paediatric Surgeons Association, Athens, Greece. Abstract No. 12 (unpublished work).
52. Thorup J, Kvist K, Clasen-Linde E, Petersen BL, Cortes D. The relation between adult dark spermatogonia and other parameters of fertility potential in cryptorchid testes. J Urol. 2013;190(4 Suppl):1566–71.
53. von Kopylow K, Spiess AN. Human spermatogonial markers. Stem Cell Res. 2017;25:300–9.
54. Guo J, Grow EJ, Yi C, Mlcochova H, Maher GJ, Lindskog C, et al. Chromatin and single-cell RNA-seq profiling reveal dynamic signaling and metabolic transitions during Human Spermatogonial stem cell development. Cell Stem Cell. 2017;21(4):533–46.
55. Guo J, Grow EJ, Mlcochova H, Maher GJ, Lindskog C, Nie X, et al. The adult human testis transcriptional cell atlas. Cell Res. 2018;28(12):1141–57.
56. Cortes D. Histological versus stereological methods applied at spermatogonia during normal human development. Scand J Urol Nephrol. 1990;24(1):11–5.
57. Cortes D, Muller J, Skakkebaek NE. Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. Int J Androl. 1987;10(4):589–96.
58. Main KM, Toppari J, Suomi AM, Kaleva M, Chellakooty M, Schmidt IM, et al. Larger testes and higher inhibin B levels in Finnish than in Danish newborn boys. J Clin Endocrinol Metab. 2006;91 (7):2732–7.
59. Müller J, Skakkebaek NE. Quantification of germ cells and seminiferous tubules by stereological examination of testicles from 50 boys who suffered from sudden death. Int J Androl. 1983;6(2):143–56.
60. Cortes D, Thorup JM, Beck BL. Quantitative histology of germ cells in the undescended testes of human fetuses, neonates and infants. J Urol. 1995;154 (3):1188–92.
61. Masliukaite I, Hagen JM, Jahnukainen K, Stukenborg JB, Repping S, van der Veen F, et al. Establishing reference values for age-related spermatogonial quantity in prepubertal human testes: a systematic review and meta-analysis. Fertil Steril. 2016;106 (7):1652–7.
62. Choi J, Smitz J. Luteinizing hormone and human chorionic gonadotropin: distinguishing unique physiologic roles. Gynecol Endocrinol. 2014;30(3):174–81.
63. Huhtaniemi IT, Korenbrot CC, Jaffe RB. HCG binding and stimulation of testosterone biosynthesis in the human fetal testis. J Clin Endocrinol Metab. 1977;44(5):963–7.
64. Forest MG, Cathiard AM, Bertrand JA. Evidence of testicular activity in early infancy. J Clin Endocrinol Metab. 1973;37(1):148–51.
65. Lanciotti L, Cofini M, Leonardi A, Penta L, Esposito S. Up-to-date review about Minipuberty and overview on hypothalamic-pituitary-gonadal Axis activation in fetal and neonatal life. Front Endocrinol. 2018;9:410.
66. Andersson AM, Toppari J, Haavisto AM, Petersen JH, Simell T, Simell O, et al. Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. J Clin Endocrinol Metab. 1998;83(2):675–81.
67. Brook CGD, Dattani MT. The Management of Growth Disorders. In Brook CGD, Dattani MT, eds. Handbook of clinical pediatric endocrinology. New York, US: John Wiley & Sons; 2012:70–98.
68. Johannsen TH, Main KM, Ljubicic ML, Jensen TK, Andersen HR, Andersen MS, et al. Sex differences in reproductive hormones during mini-puberty in infants with Normal and disordered sex development. J Clin Endocrinol Metab. 2018;103(8):3028–37.
69. Codesal J, Regadera J, Nistal M, Regadera-Sejas J, Paniagua R. Involution of human fetal Leydig cells. An immunohistochemical, ultrastructural and quantitative study. J Anat. 1990;172:103–14.
70. Hadziselimovic F, Zivkovic D, Bica DT, Emmons LR. The importance of mini-puberty for fertility in cryptorchidism. J Urol. 2005;174(4 Pt 2):1536–9.
71. Boas M, Boisen KA, Virtanen HE, Kaleva M, Suomi AM, Schmidt IM, et al. Postnatal penile length and growth rate correlate to serum testosterone levels: a

longitudinal study of 1962 normal boys. Eur J Endocrinol. 2006;154(1):125–9.
72. Priskorn L, Petersen JH, Jørgensen N, Kyhl HB, Andersen MS, Main KM, et al. Anogenital distance as a phenotypic signature through infancy. Pediatr Res. 2018;83(3):573–9.
73. Busch AS, Ljubicic ML, Upners EN, Fischer MB, Kolby N, Eckert-Lind C, et al. Cohort profile: the COPENHAGEN Minipuberty study-a longitudinal prospective cohort of healthy full-term infants and their parents. Paediatr Perinat Epidemiol. 2021;35 (5):601–11.
74. Aksglaede L, Sorensen K, Boas M, Mouritsen A, Hagen CP, Jensen RB, et al. Changes in anti-Mullerian hormone (AMH) throughout the life span: a population-based study of 1027 healthy males from birth (cord blood) to the age of 69 years. J Clin Endocrinol Metab. 2010;95(12):5357–64.
75. Rey R, Lordereau-Richard I, Carel JC, Barbet P, Cate RL, Roger M, et al. Anti-mullerian hormone and testosterone serum levels are inversely during normal and precocious pubertal development. J Clin Endocrinol Metab. 1993;77(5):1220–6.
76. Sarila G, Hutson JM, Vikraman J. Testicular descent: a review of a complex, multistaged process to identify potential hidden causes of UDT. J Pediatr Surg. 2021;57:479–87.
77. Hutson JM. A biphasic model for the hormonal control of testicular descent. Lancet. 1985;2(8452):419–21.
78. Hutson JM, Southwell BR, Li R, Lie G, Ismail K, Harisis G, et al. The regulation of testicular descent and the effects of cryptorchidism. Endocr Rev. 2013;34(5):725–52.
79. Barteczko KJ, Jacob MI. The testicular descent in human. Origin, development and fate of the gubernaculum Hunteri, processus vaginalis peritonei, and gonadal ligaments. Adv Anat Embryol Cell Biol. 2000;156:1–98.
80. Benzi TSCG, Costa WS, Sampaio FJ, Favorito LA. Inguinoscrotal stage of testicular descent: analysis in 217 human fetuses. J Pediatr Urol. 2020;16(2):198–204.
81. van der Schoot P, Elger W. Androgen-induced prevention of the outgrowth of cranial gonadal suspensory ligaments in fetal rats. J Androl. 1992;13 (6):534–42.
82. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, et al. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. Mol Endocrinol. 1999;13(5):681–91.
83. Heyns CF, de Klerk DP. The gubernaculum during testicular descent in the pig fetus. J Urol. 1985;133 (4):694–9.
84. Heyns CF, Human HJ, Werely CJ, De Klerk DP. The glycosaminoglycans of the gubernaculum during testicular descent in the fetus. J Urol. 1990;143 (3):612–7.
85. Kubota Y, Nef S, Farmer PJ, Temelcos C, Parada LF, Hutson JM. Leydig insulin-like hormone, gubernacular development and testicular descent. J Urol. 2001;165(5):1673–5.
86. Tomiyama H, Hutson JM, Truong A, Agoulnik AI. Transabdominal testicular descent is disrupted in mice with deletion of insulinlike factor 3 receptor. J Pediatr Surg. 2003;38(12):1793–8.
87. Bay K, Cohen AS, Jørgensen FS, Jørgensen C, Lind AM, Skakkebaek NE, et al. Insulin-like factor 3 levels in second-trimester amniotic fluid. J Clin Endocrinol Metab. 2008;93(10):4048–51.
88. Nef S, Parada LF. Cryptorchidism in mice mutant for Insl3. Nat Genet. 1999;22(3):295–9.
89. Behringer RR, Finegold MJ, Cate RL. Müllerian-inhibiting substance function during mammalian sexual development. Cell. 1994;79(3):415–25.
90. Hadziselimovic F, Kruslin E. The role of the epididymis in descensus testis and the topographical relationship between the testis and epididymis from the sixth month of pregnancy until immediately after birth. Anat Embryol. 1979;155(2):191–6.
91. Amann RP, Veeramachaneni DN. Cryptorchidism in common eutherian mammals. Reproduction. 2007;133(3):541–61.
92. Clarnette TD, Hutson JM. The genitofemoral nerve may link testicular inguinoscrotal descent with congenital inguinal hernia. Aust N Z J Surg. 1996;66 (9):612–7.
93. Ng YH, Farmer PJ, Sourial M, Southwell BR, Hutson JM. Growth of the rat gubernaculum in vitro and localisation of its growth Centre. J Pediatr Surg. 2009;44(2):422–6.
94. Sutthoff-Lorey, G. and K. L. Waag (1990). Die Prae- und intraoperative Gewebtemperatur Messung Bei Maldescensus Testis. Maldescensus Testis. J. W. H. F. Schier. Berlin, W Zuckschwerdt Verlag.
95. Cortes D, Kjellberg EM, Breddam M, Thorup J. The true incidence of cryptorchidism in Denmark. J Urol. 2008;179(1):314–8.
96. Sijstermans K, Hack WW, Meijer RW, van der Voort-Doedens LM. The frequency of undescended testis from birth to adulthood: a review. Int J Androl. 2008;31(1):1–11.
97. Acerini CL, Miles HL, Dunger DB, Ong KK, Hughes IA. The descriptive epidemiology of congenital and acquired cryptorchidism in a UKinfant cohort. Arch Dis Child. 2009;94(11):868–72.
98. Wenzler DL, Bloom DA, Park JM. What is the rate of spontaneous testicular descent in infants with cryptorchidism? J Urol. 2004;171(2 Pt 1):849–51.
99. Blom K. Undescended testis and time of spontaneous descent in 2516 schoolboys (in Danish). Ugeskr Laeger. 1984;146(8):616–7.
100. Smolko MJ, Kaplan GW, Brock WA. Location and fate of the nonpalpable testis in children. J Urol. 1983;129(6):1204–6.
101. Kuiri-Hänninen T, Koskenniemi J, Dunkel L, Toppari J, Sankilampi U. Postnatal testicular activity in healthy boys and boys with cryptorchidism. Front Endocrinol. 2019;10:489.
102. Bergbrant S, Omling E, Bjork J, Hagander L. Cryptorchidism in Sweden: a Nationwide study of prevalence, operative management, and complications. J Pediatr. 2018;194:197–203.
103. Fenton EJM, Woodward AA, Hudson IL, Marschner I. The ascending testis. Pediatr Surg Int. 1990;5:6–9.
104. Hack WW, Meijer RW, Van Der Voort-Doedens LM, Bos SD, De Kok ME. Previous testicular position in boys referred for an undescended testis: further explanation of the late orchidopexy enigma? BJU Int. 2003;92(3):293–6.

105. Omling E, Bergbrant S, Persson A, Björk J, Hagander L. How boys and testicles wander to surgery: a nationwide cohort study of surgical delay in Sweden. BMJ Paediatr Open. 2020;4(1):e000741.
106. Mayr J, Rune GM, Holas A, Schimpl G, Schmidt B, Haberlik A. Ascent of the testis in children. Eur J Pediatr. 1995;154(11):893–5.
107. Villumsen A, Zachau-Christensen B. Spontaneous alterations in position of the testes. Arch Dis Childh. 1966;41:198–200.
108. Wohlfahrt-Veje C, Boisen KA, Boas M, Damgaard IN, Kai CM, Schmidt IM, et al. Acquired cryptorchidism is frequent in infancy and childhood. Int J Androl. 2009;32(4):423–8.
109. Rabinowitz R, Hulbert WC Jr. Late presentation of cryptorchidism: the etiology of testicular re- ascent. J Urol. 1997;157(5):1892–4.
110. Agarwal PK, Diaz M, Elder JS. Retractile testis – is it really a normal variant? J Urol. 2006;175(4):1496–9.
111. Hack WW, Sijstermans K, van der Voort-Doedens LM, Meijer RW, Haasnoot K. The high scrotal ("gliding") testis revised. Eur J Pediatr. 2007;166(1):57–61.
112. Lais A, Caterino S, Talamo M, Nahom A, Bagolan P, Ferro F. The gliding testis: minor degree of true undescended testis? Eur J Pediatr. 1993;152(Suppl 2):S20–2.
113. Eardley I, Saw KC, Whitaker RH. Surgical outcome of orchidopexy. II. Trapped and ascending testes. Br J Urol. 1994;73(2):204–6.
114. Turek PJ, Ewalt DH, Snyder HM, Stampfers D, Blyth B, Huff DS, et al. The absent cryptorchid testis: surgical findings and their implications for diagnosis and etiology. J Urol. 1994;151(3):718–20.
115. Hildorf S, Clasen-Linde E, Cortes D, Fossum M, Thorup J. Serial inhibin B measurements in boys with congenital monorchism indicate compensatory testicular hypertrophy in early infancy. Eur J Pediatr Surg. 2021c;32(1):34–41.
116. Pirgon Ö, Dündar BN. Vanishing testes: a literature review. J Clin Res Pediatr Endocrinol. 2012;4(3):116–20.
117. Huff DS, Snyder HM, Hadziselimovic F, Blyth B, Duckett JW. An absent testis is associated with contralateral testicular hypertrophy. J Urol. 1992;148(2 Pt 2):627–8.
118. Koff SA. Does compensatory testicular enlargement predict monorchism? J Urol. 1991;146(2 Pt 2):632–3.
119. Thorup J, Kvist K, Clasen-Linde E, Hutson JM, Cortes D. Serum inhibin B values in boys with unilateral vanished testis or unilateral cryptorchidism. J Urol. 2015a;193(5):1632–6.
120. Barthold JS, Kumasi-Rivers K, Upadhyay J, Shekarriz B, Imperato-Mcginley J. Testicular position in the androgen insensitivity syndrome: implications for the role of androgens in testicular descent. J Urol. 2000;164(2):497–501.
121. Bay K, Main KM, Toppari J, Skakkebæk NE. Testicular descent: INSL3, testosterone, genes and the intrauterine milieu. Nat Rev Urol. 2011;8(4):187–96.
122. Elder JS, Isaacs JT, Walsh PC. Androgenic sensitivity of the gubernaculum testis: evidence for hormonal/mechanical interactions in testicular descent. J Urol. 1982;127(1):170–6.
123. Boehm U, Bouloux PM, Dattani MT, de Roux N, Dodé C, Dunkel L, et al. Expert consensus document: European consensus statement on congenital hypogonadotropic hypogonadism - pathogenesis, diagnosis and treatment. Nat Rev Endocrinol. 2015;11(9):547–64.
124. Rodprasert W, Virtanen HE, Mäkelä JA, Toppari J. Hypogonadism and cryptorchidism. Front Endocrinol. 2019;10:906.
125. Rachmani E, Zachariou Z, Snyder H, Hadziselimovic F. Complete testis-epididymis nonfusion anomaly: a typical association with cryptorchid testis. Urol Int. 2012;89(3):355–7.
126. Elder JS. Epididymal anomalies associated with hydrocele/hernia and cryptorchidism: implications regarding testicular descent. J Urol. 1992;148(2 Pt 2):624–6.
127. Clarnette TD, Rowe D, Hasthorpe S, Hutson JM. Incomplete disappearance of the processus vaginalis as a cause of ascending testis. J Urol. 1997;157(5):1889–91.
128. Hadziselimovic F, Herzog B. Treatment with a luteinizing hormone-releasing hormone analogue after successful orchiopexy markedly improves the chance of fertility later in life. J Urol. 1997;158(3 Pt 2):1193–5.
129. Haid B, Silay MS, Radford A, Rein P, Banuelos B, Oswald J, et al. Late ascended testes: is non-orthotopic gubernacular insertion a confirmation of an alternative embryological etiology? J Pediatr Urol. 2019;15(1):71.e71–6.
130. Rusnack SL, Wu HY, Huff DS, Snyder HM 3rd, Zderic SA, Carr MC, et al. The ascending testis and the testis undescended since birth share the same histopathology. J Urol. 2002;168(6):2590–1.
131. Alchoikani N, Ashour K. Ascending testis: a congenital predetermined condition. J Pediatr Urol. 2021;17(2):192.e191–3.
132. Akre O, Lipworth L, Cnattingius S, Sparen P, Ekbom A. Risk factor patterns for cryptorchidism and hypospadias. Epidemiology. 1999;10(4):364–9.
133. Biggs ML, Baer A, Critchlow CW. Maternal, delivery, and perinatal characteristics associated with cryptorchidism: a population-based case-control study among births in Washington state. Epidemiology. 2002;13(2):197–204.
134. Jensen MS, Wilcox AJ, Olsen J, Bonde JP, Thulstrup AM, Ramlau-Hansen CH, et al. Cryptorchidism and hypospadias in a cohort of 934,538 Danish boys: the role of birth weight, gestational age, body dimensions, and fetal growth. Am J Epidemiol. 2012;175(9):917–25.
135. Hildorf S, Clasen-Linde E, Dong L, Cortes D, Thorup J. Impaired serum inhibin-B and number of germ cells in boys with cryptorchidism following heavily gestational maternal smoking. J Pediatr Surg. 2018;54(4):809–14.
136. Jensen MS, Rebordosa C, Thulstrup AM, Toft G, Sørensen HT, Bonde JP, et al. Maternal use of acetaminophen, ibuprofen, and acetylsalicylic acid during pregnancy and risk of cryptorchidism. Epidemiology. 2010a;21(6):779–85.
137. Jensen MS, Toft G, Thulstrup AM, Henriksen TB, Olsen J, Christensen K, et al. Cryptorchidism concordance in monozygotic and dizygotic twin

brothers, full brothers, and half-brothers. Fertil Steril. 2010b;93(1):124–9.
138. Kjersgaard C, Arendt LH, Ernst A, Lindhard MS, Olsen J, Henriksen TB, et al. Lifestyle in pregnancy and cryptorchidism in sons: a study within two large Danish birth cohorts. Clin Epidemiol. 2018;10:311–22.
139. Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C, et al. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. Hum Reprod. 2011;26(1):235–44.
140. Zhang L, Wang XH, Zheng XM, Liu TZ, Zhang WB, Zheng H, et al. Maternal gestational smoking, diabetes, alcohol drinking, pre-pregnancy obesity and the risk of cryptorchidism: a systematic review and meta-analysis of observational studies. PLoS One. 2015;10(3):e0119006.
141. Andersen HR, Schmidt IM, Grandjean P, Jensen TK, Budtz-Jørgensen E, Kjaerstad MB, et al. Impaired reproductive development in sons of women occupationally exposed to pesticides during pregnancy. Environ Health Perspect. 2008;116 (4):566–72.
142. Weidner IS, Møller H, Jensen TK, Skakkebaek NE. Cryptorchidism and hypospadias in sons of gardeners and farmers. Environ Health Perspect. 1998;106 (12):793–6.
143. Foresta C, Zuccarello D, Garolla A, Ferlin A. Role of hormones, genes, and environment in human cryptorchidism. Endocr Rev. 2008;29(5):560–80.
144. Giwercman A, Rylander L, Lundberg Giwercman Y. Influence of endocrine disruptors on human male fertility. Reprod Biomed Online. 2007;15(6):633–42.
145. Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod. 2001;16(5):972–8.
146. Hart RJ, Frederiksen H, Doherty DA, Keelan JA, Skakkebaek NE, Minaee NS, et al. The possible impact of antenatal exposure to ubiquitous phthalates upon male reproductive function at 20 years of age. Front Endocrinol. 2018;9:288.
147. Joensen UN, Frederiksen H, Blomberg Jensen M, Lauritsen MP, Olesen IA, Lassen TH, et al. Phthalate excretion pattern and testicular function: a study of 881 healthy Danish men. Environ Health Perspect. 2012;120(10):1397–403.
148. Thurston SW, Mendiola J, Bellamy AR, Levine H, Wang C, Sparks A, et al. Phthalate exposure and semen quality in fertile US men. Andrology. 2016;4 (4):632–8.
149. Akre O, Richiardi L. Does a testicular dysgenesis syndrome exist? Hum Reprod. 2009;24(9):2053–60.
150. Bonde JP, Flachs EM, Rimborg S, Glazer CH, Giwercman A, Ramlau-Hansen CH, et al. The epidemiologic evidence linking prenatal and postnatal exposure to endocrine disrupting chemicals with male reproductive disorders: a systematic review and meta-analysis. Hum Reprod Update. 2016;23(1):104–25.
151. Thorup J, McLachlan R, Cortes D, Nation TR, Balic A, Southwell BR, et al. What is new in cryptorchidism and hypospadias—a critical review on the testicular dysgenesis hypothesis. J Pediatr Surg. 2010;45(10):2074–86.
152. Ferlin A, Zuccarello D, Zuccarello B, Chirico MR, Zanon GF, Foresta C. Genetic alterations associated with cryptorchidism. JAMA. 2008;300(19):2271–6.
153. Cortes D, Thorup JM, Beck BL, Visfeldt J. Cryptorchidism as a caudal developmental field defect. A new description of cryptorchidism associated with malformations and dysplasias of the kidneys, the ureters and the spine from T10 to S5. APMIS. 1998;106(10):953–8.
154. Virtanen HE, Toppari J. Epidemiology and pathogenesis of cryptorchidism. Hum Reprod Update. 2008;14(1):49–58.
155. Kollin, C., B. Karpe, U. Hesser, T. Granholm and E. M. Ritzen (2007). Surgical treatment of unilaterally undescended testes: testicular growth after randomization to orchiopexy at age 9 months or 3 years. J Urol 178(4 Pt): 1589–1593; discussion 1593.
156. Cortes D, Thorup J, Visfeldt J. Hormonal treatment may harm the germ cells in 1 to 3-year-old boys with cryptorchidism. J Urol. 2000;163(4):1290–2.
157. Dunkel L, Taskinen S, Hovatta O, Tilly JL, Wikström S. Germ cell apoptosis after treatment of cryptorchidism with human chorionic gonadotropin is associated with impaired reproductive function in the adult. J Clin Invest. 1997;100(9):2341–6.
158. Fiala, V., Z. Valova, M. Drlik, J. Sedlacek, Z. Dite, R. Kocvara and M. Kalousova (2021). The effect of neoadjuvant hormonal treatment in cryptorchid boys. Presented at: 5th joint meeting of the European Society for Paediatric Urology (ESPU) and The Societies for Pediatric Urology (SPU). Abstract No. S14-5 (unpublished work) Lissabon, Portugal (virtually).
159. Jallouli M, Rebai T, Abid N, Bendhaou M, Kassis M, Mhiri R. Neoadjuvant gonadotropin-releasing hormone therapy before surgery and effect on fertility index in unilateral undescended testes: a prospective randomized trial. Urology. 2009;73(6):1251–4.
160. Schwentner C, Oswald J, Kreczy A, Lunacek A, Bartsch G, Deibl M, et al. Neoadjuvant gonadotropin-releasing hormone therapy before surgery may improve the fertility index in undescended testes: a prospective randomized trial. J Urol. 2005;173 (3):974–7.
161. Thorup J, Clasen-Linde E, Dong L, Hildorf S, Kristensen SG, Andersen CY, et al. Selecting infants with cryptorchidism and high risk of infertility for optional adjuvant hormonal therapy and cryopreservation of germ cells: experience from a pilot study. Front Endocrinol. 2018b;9:299.
162. Vincel B, Verkauskas G, Bilius V, Dasevicius D, Malcius D, Jones B, et al. Gonadotropin-releasing hormone agonist corrects defective mini-puberty in boys with cryptorchidism: a prospective randomized study. Biomed Res Int. 2018;2018:4651218.
163. Zivkovic D, Bica DT, Hadziselimovic F. Relationship between adult dark spermatogonia and secretory capacity of Leydig cells in cryptorchidism. BJU Int. 2007;100(5):1147–9.
164. Thorup J, Clasen-Linde E, Li R, Reinhardt S, Kvist K, Vikraman J, et al. Postnatal germ cell

development in the cryptorchid testis: the key to explain why early surgery decreases the risk of malignancy. Eur J Pediatr Surg. 2018a;28(6):469–76.
165. Zivkovic D, Hadziselimovic F. Development of Sertoli cells during mini-puberty in normal and cryptorchid testes. Urol Int. 2009;82(1):89–91.
166. Hadziselimovic F. Opinion: comment on evaluation and treatment of cryptorchidism: AUA/AAP and Nordic consensus guidelines. Urol Int. 2016;96 (3):249–54.
167. Jensen CF, Dong L, Gul M, Frode M, Hildorf S, Thorup J, et al. Fertility preservation in boys facing gonadotoxic cancer therapy. Nat Rev Urol. 2021;19 (2):71–83.
168. Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, et al. A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod. 2015;30(11):2463–75.
169. Sadri-Ardekani H, McLean TW, Kogan S, Sirintrapun J, Crowell K, Yousif MQ, et al. Experimental testicular tissue banking to generate spermatogenesis in the future: a multidisciplinary team approach. Methods. 2016;99:120–7.
170. Gul M, Hildorf S, Dong L, Thorup J, Hoffmann ER, Jensen CFS, et al. Review of injection techniques for spermatogonial stem cell transplantation. Hum Reprod Update. 2020;26(3):368–91.
171. Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, et al. Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. Science. 2019;363(6433):1314–9.
172. Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell. 2012;11(5):715–26.
173. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011;471(7339):504–7.
174. Dong L, Kristensen SG, Hildorf S, Gul M, Clasen-Linde E, Fedder J, et al. Propagation of Spermatogonial stem cell-like cells from infant boys. Front Physiol. 2019;10:1155.
175. Kvist K, Thorup J, Byskov AG, Høyer PE, Møllgård K, Yding Andersen C. Cryopreservation of intact testicular tissue from boys with cryptorchidism. Hum Reprod. 2006;21(2):484–91.
176. Huff DS, Hadziselimovic F, Snyder HM, Blythe B, Ducket JW. Histologic maldevelopment of unilaterally cryptorchid testes and their descended partners. Eur J Pediatr. 1993;152(Suppl 2):S11–4.
177. Møller H. Epidemiological studies of testicular germ cell cancer (thesis). London: King's College; 2001.
178. Berney DM, Looijenga LH, Idrees M, Oosterhuis JW, Rajpert-De Meyts E, Ulbright TM, et al. Germ cell neoplasia in situ (GCNIS): evolution of the current nomenclature for testicular pre-invasive germ cell malignancy. Histopathology. 2016;69(1):7–10.
179. Cortes D, Thorup JM, Visfeldt J. Cryptorchidism: aspects of fertility and neoplasms. A study including data of 1,335 consecutive boys who underwent testicular biopsy simultaneously with surgery for cryptorchidism. Horm Res. 2001;55(1):21–7.
180. Hutson J, Thorup J, Beasley S. Descent of the testis. Switzerland: Springer International Publishing; 2016.
181. Lee PA, O'Leary LA, Songer NJ, Coughlin MT, Bellinger MF, LaPorte RE. Paternity after unilateral cryptorchidism: a controlled study. Pediatrics. 1996;98(4 Pt 1):676–9.
182. Lee PA, O'Leary LA, Songer NJ, Coughlin MT, Bellinger MF, LaPorte RE. Paternity after bilateral cryptorchidism. A controlled study. Arch Pediatr Adolesc Med. 1997;151(3):260–3.
183. Rohayem J, Luberto A, Nieschlag E, Zitzmann M, Kliesch S. Delayed treatment of undescended testes may promote hypogonadism and infertility. Endocrine. 2017;55(3):914–24.
184. Koch T, Hansen AH, Priskorn L, Petersen JH, Carlsen E, Main KM, et al. A history of cryptorchidism is associated with impaired testicular function in early adulthood: a cross- sectional study of 6376 men from the general population. Hum Reprod. 2020;35 (8):1765–80.
185. Cortes D, Thorup JM, Lindenberg S. Fertility potential after unilateral orchiopexy: an age independent risk of subsequent infertility when biopsies at surgery lack germ cells. J Urol. 1996;156(1):217–20.
186. Gracia J, Sánchez Zalabardo J, Sánchez García J, García C, Ferrández A. Clinical, physical, sperm and hormonal data in 251 adults operated on for cryptorchidism in childhood. BJU Int. 2000;85(9):1100–3.
187. Mayr J, Pusch HH, Schimpl G, Reitinger T, Sorantin E, Mayr-Koci M. Semen quality and gonadotropin levels in patients operated upon for cryptorchidism. Pediatr Surg Int. 1996;11(5–6):354–8.
188. Moretti E, Di Cairano G, Capitani S, Scapigliati G, Baccetti B, Collodel G. Cryptorchidism and semen quality: a TEM and molecular study. J Androl. 2007;28(1):194–9.
189. Okuyama A, Nonomura N, Nakamura M, Namiki M, Fujioka H, Kiyohara H, et al. Surgical management of undescended testis: retrospective study of potential fertility in 274 cases. J Urol. 1989;142 (3):749–51.
190. Trsinar B, Muravec UR. Fertility potential after unilateral and bilateral orchidopexy for cryptorchidism. World J Urol. 2009;27(4):513–9.
191. Cortes D, Thorup J, Hogdall E, Norgaard-Pedersen B, Petersen BL, Hogdall C. The relation of germ cells per tubule in testes, serum inhibin B and FSH in cryptorchid boys. Pediatr Surg Int. 2007a;23 (2):163–9.
192. Cortes D, Clasen-Linde E, Hutson JM, Li R, Thorup J. The Sertoli cell hormones inhibin-B and anti Mullerian hormone have different patterns of secretion in prepubertal cryptorchid boys. J Pediatr Surg. 2016;51(3):475–80.
193. Dong LH, Hildorf S, Clasen-Linde E, Kvist K, Cortes D, Thorup J, et al. Postnatal germ cell development in cryptorchid boys. Asian J Androl. 2020;22 (3):258–64.
194. Hildorf S, Dong L, Thorup J, Clasen-Linde E, Yding Andersen C, Cortes D. Sertoli cell number correlates with serum inhibin B in infant cryptorchid boys. Sex Dev. 2019;13(2):74–82.
195. Hildorf S, Cortes D, Thorup J, Clasen-Linde E, Hutson J, Li R. During infancy low levels of follicle-stimulating hormone may result in high rate of germ

cell apoptosis. J Pediatr Surg. 2021a;56(12):2399–406.
196. Hildorf, S., E. Clasen-Linde, M. Fossum, D. Cortes and J. Thorup (2021d). The positive predictive value of using FSH and inhibin B serum levels to diagnose gonadotropin insufficiency in bilateral cryptorchid boys is high. Presented the 18 September at: 5th joint meeting of the European Society for Paediatric Urology and The Societies for Pediatric Urology, Lissabon, Portugal (virtually). Abstract No. S14-2 (unpublished work).
197. Thorup J, Petersen BL, Kvist K, Cortes D. Bilateral undescended testes classified according to preoperative and postoperative status of gonadotropins and inhibin B in relation to testicular histopathology at bilateral orchiopexy in infant boys. J Urol. 2012;188 (4 Suppl):1436–42.
198. Thorup J, Clasen-Linde E, Thorup SC, Cortes D. Pre- and postoperative status of gonadotropins (FSH and LH) and inhibin-B in relation to testicular histopathology at orchiopexy in infant boys with unilateral undescended testes. J Pediatr Urol. 2015b;11 (1):21–5.
199. Cortes D, Thorup J. Histology of testicular biopsies taken at operation for bilateral maldescended testes in relation to fertility in adulthood. Br J Urol. 1991;68(3):285–91.
200. Cendron M, Keating MA, Huff DS, Koop CE, Snyder HM, Duckett JW. Cryptorchidism, orchiopexy and infertility: a critical long-term retrospective analysis. J Urol. 1989;142(2 Pt 2):559–62.
201. Hadziselimovic F, Hecker E, Herzog B. The value of testicular biopsy in cryptorchidism. Urol Res. 1984;12(3):171–4.
202. Nistal M, Riestra ML, Paniagua R. Correlation between testicular biopsies (prepubertal and postpubertal) and spermiogram in cryptorchid men. Hum Pathol. 2000;31(9):1022–30.
203. Rusnack SL, Wu HY, Huff DS, Snyder HM, Carr MC, Bellah RD, et al. Testis histopathology in boys with cryptorchidism correlates with future fertility potential. J Urol. 2003;169(2):659–62.
204. Gracia J, Sánchez J, García C, Pueyo C, Ferrández A. What is the relationship between spermatozoa per mililiter at adulthood and the tubular fertility index at surgical age for patients with cryptorchidism. Pediatr Surg. 1998;33:594–6.
205. Hadziselimovic F, Hoecht B. Testicular histology related to fertility outcome and postpubertal hormone status in cryptorchidism. Klin Padiatr. 2008;220(5):302–7.
206. Cortes D. Editorial comment. J Urol. 2012;188(4 Suppl):1435.
207. Valeri C, Schteingart HF, Rey RA. The prepubertal testis: biomarkers and functions. Curr Opin Endocrinol Diabetes Obes. 2013;20(3):224–33.
208. Esposito S, Cofini M, Rigante D, Leonardi A, Lucchetti L, Cipolla C, et al. Inhibin B in healthy and cryptorchid boys. Ital J Pediatr. 2018;44(1):81.
209. Wilson V, Thorup J, Clasen-Linde E, Cortes D, Hutson JM, Li R. Gonocyte transformation in congenital undescended testes: what is the role of inhibin-B in cell death? Pediatr Surg Int. 2019;35(11):1309–16.
210. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology. 1988;122(3):787–94.
211. Verkauskas G, Malcius D, Eidukaite A, Vilimas J, Dasevicius D, Bilius V, et al. Prospective study of histological and endocrine parameters of gonadal function in boys with cryptorchidism. J Pediatr Urol. 2016;12(4):231–8.
212. Gaudino R, Cavarzere P, Camilot M, Teofoli F, Zampieri N, Tatò L. Prepubertal serum inhibin B in cryptorchid infants and in monorchid boys with compensatory testicular hypertrophy. Fertil Steril. 2008;90(6):2217–21.
213. Hamdi SM, Almont T, Galinier P, Mieusset R, Thonneau P. Altered secretion of Sertoli cells hormones in 2-year-old prepubertal cryptorchid boys: a cross-sectional study. Andrology. 2017;5 (4):783–9.
214. Suomi AM, Main KM, Kaleva M, Schmidt IM, Chellakooty M, Virtanen HE, et al. Hormonal changes in 3-month-old cryptorchid boys. J Clin Endocrinol Metab. 2006;91(3):953–8.
215. Barthold JS, Manson J, Regan V, Si X, Hassink SG, Coughlin MT, et al. Reproductive hormone levels in infants with cryptorchidism during postnatal activation of the pituitary-testicular axis. J Urol. 2004;172 (4 Pt 2):1736–41.
216. Christiansen P, Andersson AM, Skakkebaek NE, Juul A. Serum inhibin B, FSH, LH and testosterone levels before and after human chorionic gonadotropin stimulation in prepubertal boys with cryptorchidism. Eur J Endocrinol. 2002;147(1):95–101.
217. Kubini K, Zachmann M, Albers N, Hiort O, Bettendorf M, Wölfle J, et al. Basal inhibin B and the testosterone response to human chorionic gonadotropin correlate in prepubertal boys. J Clin Endocrinol Metab. 2000;85(1):134–8.
218. Raivio T, Dunkel L. Inverse relationship between serum inhibin B and FSH levels in prepubertal boys with cryptorchidism. Pediatr Res. 1999;46(5):496–500.
219. Irkilata HC, Yildirim I, Onguru O, Aydur E, Musabak U, Dayanc M. The influence of orchiopexy on serum inhibin B level: relationship with histology. J Urol. 2004;172(6 Pt 1):2402–5.
220. Irkilata HC, Dayanc M, Kibar Y, Musabak U, Yesilkaya E, Oral E, et al. Effect of scrotal incision orchiopexy on serum inhibin B levels and comparison with classic inguinal orchiopexy. Urology. 2008;72(3):525–9.
221. Kollin C, Stukenborg JB, Nurmio M, Sundqvist E, Gustafsson T, Soder O, et al. Boys with undescended testes: endocrine, volumetric and morphometric studies on testicular function before and after orchidopexy at nine months or three years of age. J Clin Endocrinol Metab. 2012;97(12):4588–95.
222. Ma D, Yao ZG, Guo YP, Wu RF. Dynamic changes in serum inhibin B levels in 6- to 24-month-old children receiving cryptorchidism surgery. Int J Gen Med. 2021;14:1173–7.
223. Coughlin MT, Bellinger MF, Lee PA. Age at unilateral orchiopexy: effect on hormone levels and sperm count in adulthood. J Urol. 1999;162(3 Pt 2):986–8.
224. de Gouveia Brazao CA, Pierik FH, Erenpreiss Y, de Jong FH, Dohle GR, Weber RF. The effect of

cryptorchidism on inhibin B in a subfertile population. Clin Endocrinol. 2003;59(1):136–41.
225. Jorgensen N, Liu F, Andersson AM, Vierula M, Irvine DS, Auger J, et al. Serum inhibin-b in fertile men is strongly correlated with low but not high sperm counts: a coordinated study of 1,797 European and US men. Fertil Steril. 2010;94 (6):2128–34.
226. Grinspon RP, Gottlieb S, Bedecarrás P, Rey RA. Anti-Müllerian hormone and testicular function in prepubertal boys with cryptorchidism. Front Endocrinol. 2018;9:182.
227. Pierik FH, Deddens JA, Burdorf A, de Muinck Keizer-Schrama SM, Jong FH, Weber RF. The hypothalamus-pituitary-testis axis in boys during the first six months of life: a comparison of cryptorchidism and hypospadias cases with controls. Int J Androl. 2009;32(5):453–61.
228. Thorup J, Petersen BL, Kvist K, Cortes D. Bilateral vanished testes diagnosed with a single blood sample showing very high gonadotropins (follicle-stimulating hormone and luteinizing hormone) and very low inhibin B. Scand J Urol Nephrol. 2011;45(6):425–31.
229. Thorup J, Cortes D. Cryptorchidism: aspects of pathogenesis. J Pediatr Surg Spec. 2008;2:18–23.
230. Jespersen K, Ljubicic ML, Johannsen TH, Christiansen P, Skakkebaek NE, Juul A. Distinguishing between hidden testes and anorchia: the role of endocrine evaluation in infancy and childhood. Eur J Endocrinol. 2020;183(1):107–17.
231. Cortes D, Petersen BL, Thorup J. Testicular histology in cryptorchid boys – aspects of fertility. J Pediatr Surg Spec. 2007b;1:32–5.
232. Lambert AS, Bougneres P. Growth and descent of the testes in infants with hypogonadotropic hypogonadism receiving subcutaneous gonadotropin infusion. Int J Pediatr Endocrinol. 2016;2016:13.
233. Raivio T, Toppari J, Perheentupa A, McNeilly AS, Dunkel L. Treatment of prepubertal gonadotrophin-deficient boys with recombinant human follicle-stimulating hormone. Lancet. 1997;350(9073):263–4.
234. Faure A, Bouty A, O'Brien M, Thorup J, Hutson J, Heloury Y. Testicular biopsy in prepubertal boys: a worthwhile minor surgical procedure? Nat Rev Urol. 2016;13(3):141–50.
235. Cortes D, Brandt B, Thorup J. Direct mixed antiglobulin reaction (MAR) test in semen at follow-up after testicular biopsy of maldescended testes operated in puberty. Z Kinderchir. 1990;45(4):227–8.
236. Patel RP, Kolon TF, Huff DS, Carr MC, Zderic SA, Canning DA, et al. Testicular microlithiasis and antisperm antibodies following testicular biopsy in boys with cryptorchidism. J Urol. 2005;174 (5):2008–10.
237. Verkauskas G, Malcius D, Dasevicius D, Hadziselimovic F. Histopathology of unilateral cryptorchidism. Pediatr Dev Pathol. 2019;22(1):53–8.
238. Nistal M, Paniagua R, Riestra ML, Reyes-Múgica M, Cajaiba MM. Bilateral prepubertal testicular biopsies predict significance of cryptorchidism-associated mixed testicular atrophy, and allow assessment of fertility. Am J Surg Pathol. 2007;31(8):1269–76.
239. Spinelli C, Morelli G, Cito G, Bertocchini A, Ghionzoli M, Pucci A, et al. Correlation between testicular volume and histological findings in children with unilateral cryptorchidism: potential impact on future fertility. Urologia. 2022;89(2):292–97.
240. Vigueras-Villaseñor RM, Ramírez Cuevas Z, Chávez-Saldaña M, Landero-Huerta DA, Cuevas Alpuche JO, Arceo Olaiz RA, et al. Disorders in the function of Sertoli cells and arrest in the differentiation of gonocytes in patients with cryptochidism and microlithiasis. J Pediatr Surg. 2021;56(10):1886–93.
241. Kogan SJ, Tennenbaum S, Gill B, Reda E, Levitt SB. Efficacy of orchiopexy by patient age 1 year for cryptorchidism. J Urol. 1990;144(2 Pt 2):508–9.
242. Wolffenbuttel, K. P., R. Kranse, H. J. A. Stoop, J. P. Scheepe, J. van den Hoek and L. H. J. Looijenga (2018). Tubular fertility index (TFI) in correlation with testis position, consistency, epididymal anatomy and age at orchidopexy in 237 boys with cryptorchidism. Presented at: 29th congress of the European Society for Paediatric Urology (abstract no. S9-33, unpublished work). Helsinki, Finland.
243. Jay MA, Arat A, Wijlaars L, Ajetunmobi O, Fitzpatrick T, Lu H, et al. Timing of paediatric orchidopexy in universal healthcare systems: international administrative data cohort study. BJS Open. 2020;4 (6):1117–24.
244. Springer A, Huber C, Reck CA, Fengler D, Horcher E. Delayed referral despite appropriate knowledge in cryptorchidism as a cause of delayed orchidopexies in Austria. Klin Padiatr. 2010;222(4):248–51.
245. Zvizdic Z, Islamovic B, Milisic E, Jonuzi A, Vranic S. Changing Trends in the Referral and Timing of Treatment for Congenital Cryptorchidism: A Single-Center Experience from Bosnia and Herzegovina. J Pediatr Surg. 2020;55(9):1965–68.
246. Park KH, Lee JH, Han JJ, Lee SD, Song SY. Histological evidences suggest recommending orchiopexy within the first year of life for children with unilateral inguinal cryptorchid testis. Int J Urol. 2007;14 (7):616–21.
247. Tseng CS, Chiang IN, Hong CH, Lu YC, Hong JH, Chang HC, et al. Advantage of early orchiopexy for undescended testis: analysis of testicular growth percentage ratio in patients with unilateral undescended testicle. Sci Rep. 2017;7(1):17476.
248. Tasian GE, Hittelman AB, Kim GE, DiSandro MJ, Baskin LS. Age at orchiopexy and testis palpability predict germ and Leydig cell loss: clinical predictors of adverse histological features of cryptorchidism. J Urol. 2009;182(2):704–9.
249. Bilius V, Verkauskas G, Dasevicius D, Kazlauskas V, Malcius D, Hadziselimovic F. Incidence of high infertility risk among unilateral cryptorchid boys. Urol Int. 2015;95(2):142–5.
250. Gendrel D, Roger M, Chaussain JL, Canlorbe P, Job JC. Correlation of pituitary and testicular responses to stimulation tests in cryptorchid children. Acta Endocrinol. 1977;86(3):641–50.
251. Job JC, Gendrel D, Safar A, Roger M, Chaussain JL. Pituitary LH and FSH and testosterone secretion in infants with undescended testes. Acta Endocrinol. 1977;85(3):644–9.
252. De Muinck Keizer-Schrama SM, Hazebroek FW, Drop SL, Degenhart HJ, Molenaar JC, Visser HK. Hormonal evaluation of boys born with undescended testes during their first year of life. J Clin Endocrinol Metab. 1988;66(1):159–64.

253. Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, et al. An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod. 2010;25(1):37–41.
254. Ginsberg JP, Li Y, Carlson CA, Gracia CR, Hobbie WL, Miller VA, et al. Testicular tissue cryopreservation in prepubertal male children: an analysis of parental decision-making. Pediatr Blood Cancer. 2014;61(9):1673–8.
255. Promm M, Schroder A, Neissner C, Eder F, Rosch WH, Schroder J. Acquired cryptorchidism: more harm than thought? J Pediatr Urol. 2016;12(4):231–6.
256. van Brakel J, Kranse R, de Muinck Keizer-Schrama SM, Hendriks AE, de Jong FH, Hack WW, et al. Fertility potential in a cohort of 65 men with previously acquired undescended testes. J Pediatr Surg. 2014;49(4):599–605.
257. Koskenniemi JJ, Virtanen HE, Wohlfahrt-Veje C, Löyttyniemi E, Skakkebaek NE, Juul A, et al. Postnatal changes in testicular position are associated with IGF-I and function of Sertoli and Leydig cells. J Clin Endocrinol Metab. 2018;103(4):1429–37.
258. Barbotin AL, Dauvergne A, Dumont A, Ramdane N, Mitchell V, Rigot JM, et al. Bilateral versus unilateral cryptorchidism in nonobstructive azoospermia: testicular sperm extraction outcomes. Asian J Androl. 2019;21(5):445–51.
259. Cortes D, Thorup J, Lindenberg S, Visfeldt J. Infertility despite surgery for cryptorchidism in childhood can be classified by patients with normal or elevated follicle-stimulating hormone and identified at orchidopexy. BJU Int. 2003;91(7):670–4.
260. Hadziselimovic F, Verkauskas G, Stadler MB. Molecular clues in the regulation of mini-puberty involve neuronal DNA binding transcription factor NHLH2. Basic Clin Androl. 2021;31(1):6.

