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Sergey Sadov

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To my family

ABSTRACT

Sergey Sadov
Development of male reproductive health in puberty and young adulthood.

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Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2018.

The deterioration of male reproductive health is a worrying global trend in respect to the individual fertility of the man. Modern knowledge suggests the etiological role for early testicular damage and harmful effects of environmental factors.

This thesis is based on the analysis of 3 longitudinal cohorts. In the first pubertal study, we compared pubertal growth of testis between groups of boys with and without a history of congenital cryptorchidism. In the second pubertal study, we assessed the influence of a low-saturated-fat and low-cholesterol dietary intervention on hormonal status and growth during puberty in boys and girls. In the longitudinal study on adult men, changes in semen quality from 19 to 29 years of age were analysed.

Congenitally undescended testes demonstrated poor growth during puberty resulting in reduced postpubertal size. The timing of pubertal onset did not differ between boys with and without a history of congenital cryptorchidism indicating the normal functioning of central regulatory mechanisms of puberty. We provided validation data on the use of ruler measurements in denoting of pubertal onset. Reference data on ultrasonographic testicular volumes defining the onset of puberty was also presented. The growth and hormonal status during puberty was not influenced by a low-saturated-fat and low-cholesterol dietary intervention. However, our study did not provide data on postmenarcheal hormones in girls. Longitudinal follow-up of semen quality during young adulthood showed that almost full individual spermatogenic potential is reached at the age of 19 years. Simultaneously, we observed slight improvement in the percentage of motile sperm and percentage of morphologically normal spermatozoa as the men got older.

Keywords: reproductive health, testis, cryptorchidism, dietary intervention, puberty, semen quality.

TIIVISTELMÄ

Sergey Sadov
Lisääntymisterveyden kehitys murrosikäisillä pojilla ja nuorilla aikuisilla miehillä.

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

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Miesten lisääntymisterveyden heikkeneminen on huolestuttava maailmanlaajuinen ilmiö. Syyksi on epäilty kivisten vauriota sikiökaudella ja haitallisia ympäristövaikutuksia.

Tämä väitöstyö perustuu kolmen pitkittäisen kohortin analyysiin. Ensimmäisessä murrosikäisten tutkimuksessa vertasimme kiveksen kasvua pojilla, joilla oli tai ei ollut taustalla synnynnäistä piilokiveksisyyttä. Toisessa murrosikäisten tutkimuksessa arvioimme, miten tyydyttyneen rasvan ja kolesterolin saannin väheneminen ruokavaliossa vaikutti veren sukupuolihormonipitoisuuksiin ja murrosiän etenemiseen. Aikuisten miesten pitkittäistutkimuksessa analysoitiin siemennesteen laadun muutoksia 19-29 ikävuosina.

Synnynnäisesti laskeutumattomien kivesten postpubertaalinen tilavuus oli pienempi verrattuna normaalisti laskeutuneisiin kiveksiin, mikä viittaa piilokiveksisyyteen liittyvään varhaiseen kivesvaurioon. Murrosiän ajoituksessa ryhmien välillä ei todettu eroja, mikä osoittaa sentraalisten murrosikään liittyvien mekanismien toimineen normaalisti. Osoitimme tavallisen viivoittimen yhtä luotettavaksi välineeksi murrosiän alkamisen toteamiseen, kuin Praderin orkidometri. Selvitimme myös ultraäänen avulla lasketut kiveksen tilavuusarvot murrosiän eri vaiheessa. Tyydyttyneen rasvan ja kolesterolin saannin väheneminen ruokavaliossa ei vaikuttanut hormonaaliseen tasapainoon ja murrosiän etenemiseen. Tytöillä hormonituloksia ei analysoitu menarken jälkeen, koska näytteenottoa ei ollut ajoitettu kuukausikierron mukaan. Tuloksemme osoittavat ravitsemusintervention turvallisuuden puberteettikehityksen suhteen. Nuorten miesten seurantatutkimuksessa siittiöiden tuotanto ei lisääntynyt merkitsevästi 19 ikävuoden jälkeen. Samanaikaisesti kuitenkin siittiöiden liikkuvuus ja rakenne hiukan paranivat.

Avainsanat: lisääntymisterveys, kives, piilokiveksisyys, ravitsemusinterventio, murrosikä, siemennesteen laatu.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AHR	Aryl hydrocarbon receptor
AMH	anti-Müllerian hormone
cAMP	Cyclic adenosine monophosphate
AR	Androgen receptor
BMI-SDS	Body Mass Index–Standard Deviation Score
CGRP	Calcitonin gene-related peptide
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHH	Desert hedgehog
DHT	Dihydrotestosterone
DISC	The Dietary Intervention Study in Children
DMRT1	Doublesex and mab-3 related transcription factor 1
Dyn	Dynorphin
EDC	Endocrine disrupting chemical
FGF9	Fibroblast growth factor 9
FOXL2	Forkhead box L2
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GCNIS	Germ cell neoplasia in situ
GDNF	Glial cell derived neurotrophic factor
GFN	Genitofemoral nerve
GnRH	Gonadotropin-releasing hormone
GPR54	G protein-coupled receptor 54
HAS	The Hormone Ancillary Study
hCG	Human chorionic gonadotropin
HOXA10	Homeobox protein Hox-A10
HPG	Hypothalamic–pituitary–gonadal axis
IGF-1	Insulin-like growth factor 1
INSL3	Insulin-like peptide 3
INSL3R	Receptor for insulin-like peptide 3
KIT	Stem cell factor receptor
KNDy	Kisspeptin/Neurokinin B/Dynorphin A neurons
LDL-C	Low-density lipoprotein cholesterol
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
MKRN3	Makorin RING finger 3
MRI	Magnetic resonance imaging
NICHD	National Institute of Child Health and Human Development
NHANES III	The Third National Health and Nutrition Examination Survey Study
NKB	Neurokinin B
NPY	Neuropeptide Y
PBBs	Polybrominated biphenyls

PCOS	Polycystic ovary syndrome
PDGF	Platelet-derived growth factor
PGD2	Prostaglandin D2
PHV	Peak height velocity
PTGDS	Prostaglandin-H2 D-isomerase
RFRP-3	RFamide-ralated peptide-3
RSPO1	R-Spondin 1
SCF	Stem cell factor
SF1	Steroidogenic Factor 1
SHBG	Sex Hormone-Binding Globulin
SOX9	SRY-box Containing Gene 9
SRY	Sex determining region of the Y-chromosome
STRIP	The Special Turku Coronary Risk Factor Intervention Project
TAC3	Tachykinin 3
TACR3	Tachykinin receptor 3
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDS	Testicular dysgenesis syndrome
TTP	Time-to-pregnancy
WHO	The World Health Organization
WNT4	Wingless-type MMTV Integration Family Member 4
WT1	Wilms Tumor 1

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I. Sergey Sadov*, Jaakko J. Koskenniemi*, Helena E. Virtanen, Antti Perheentupa, Jørgen H. Petersen, Niels E. Skakkebaek, Katharina M. Main, and Jorma Toppari. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. Journal of Clinical Endocrinology and Metabolism, 2016 Jun;101(6):2570-7. **Shared first author*

II. Sergey Sadov, Helena E. Virtanen, Katharina M. Main, Anna-Maria Andersson, Anders Juul, Antti Jula, Olli T. Raitakari, Katja Pahkala, Harri Niinikoski, Jorma Toppari. Low saturated fat and low cholesterol diet does not alter pubertal development and hormonal status in adolescents. Acta Paediatrica. doi:10.1111/apa.14480

III. Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510.

1. INTRODUCTION

The deterioration of semen quality is a common worldwide trend in the 20th century.[1,2] Finland is a country with traditionally good semen quality. However, Finland also experienced a decrease in sperm counts during first years of the 21st century.[3] Testicular dysgenesis syndrome theory postulates, that poor semen quality, testicular cancer, cryptorchidism, hypospadias and androgen insufficiency in adult life are connected via common pathogenesis. All these symptoms are related to each other as risk factors. Testicular damage during fetal life was suggested as a common underlying mechanism.[4] Simultaneously, this phenomenon emphasizes the importance of prospective longitudinal studies, which cover a period from prenatal life to adulthood, for the understanding of precise etiological factors underlying the continuous deterioration of reproductive health.

Normal pubertal development is crucial for adequate reproductive status in adulthood.[5] We decided to continue the follow-up of Finnish boys, who participated in the study on the prevalence of congenital cryptorchidism at infancy[6], throughout their puberty. Since the volume of testis correlates positively with spermatogenic potential[7], our analysis was focused on pubertal testicular growth. We analysed growth of the testes in boys with and without history of congenital cryptorchidism. Moreover, we aimed to provide some validation and reference data on the using of ruler and ultrasonography as tools for the denoting of pubertal onset and measurement of testicular growth.

The Special Turku Coronary Risk Factor Intervention Project (STRIP) provided worthy data on beneficial effects of low-saturated-fat and low-cholesterol dietary counselling during childhood ja adolescence.[8,9,10,11,12,13] Since such a dietary intervention could, potentially, alter hormonal status[14,15], we decided to perform an analysis of pubertal hormones in a subcohort of STRIP participants.

Studies on semen quality trends have been mainly carried out among young adult men (19-30 years of age).[16,17] The natural changes in semen quality during young adulthood are not known well. Thus, we decided to perform a longitudinal follow-up of men from 19 to 29 years of age to analyse natural changes in semen quality during this period. Potential shifts in semen quality during young adulthood should be noticed. Otherwise, they may bias observations on historical trends of semen quality or country differences in respect to reproductive health, when different age groups of young adult men are compared (e.g. 19 vs 29 years of age).

2. REVIEW OF LITERATURE

2.1 Embryonal development of reproductive system

The bipotential gonads develop at the site of genital ridge on the mesonephros during the 5th and 6th post-fertilization weeks. Fetus has two pairs of unipontential genital ducts: mesonephric (Wolffian) and paramesonephric (Müllerian) duct. Wollfian ducts give rise to the epididymides, vasa deferentia and seminal vesicles in the male, and Müllerian ducts develop to the Fallopian tubes, uterus and upper part of vagina in the female. In the male, the Müllerian ducts undergo nearly full degeneration except for small cranial portions which give rise to the appendix testis. The migration of primordial germ cells to the indifferent gonad from the wall of the yolk sac is almost complete at the 6th week after fertilisation.[18,19]

In the seventh week, the gonads of the embryo start their differentiation into a testis or ovary. The first sign of this process in male is forming of the primary solid testicular cords, when Sertoli cells are aggregating around the germ cells. The pubertal canalization of the testicular cords leads to the forming of seminiferous tubules. Sertoli cells originate from the coelomic epithelium of the genital ridge. Fetal Leydig cells are recruited from mesenchymal cells of gonadal ridge during the 9th or 10th week by pre-Sertoli cells (future Sertoli cells) signaling.[18,19]

In male embryos, anterior part of cloaca (urogenital sinus) forms prostatic and membranous urethra, prostatic gland and utricle, and bulbourethral glands. In females, urogenital sinus forms membranous urethra, urethral and paraurethral glands, and greater vestibular glands.[18,19]

The external genitalia develop from three indifferent protuberances after the 12th post-fertilization weeks: the genital tubercle, urogenital folds and labioscrotal swellings. In males, growing genital tubercle eventually forms glans penis, corpora cavernosa and corpus spongiosum of the penis. Fusion of the urogenital folds gives rise to the penile urethra and the ventral part of the penis. Labioscrotal swellings form the scrotum. In females, the genital tubercle, urogenital folds and labioscrotal swellings give rise to the clitoris, labia minora and labia majora respectively.[18,19]

Prenatally gonads are anchored by two ligaments. The cranial suspensory ligament connects gonads to the diaphragm and regresses in male fetuses before the week 13, while the testis remains anchored to the internal inguinal ring by the gubernaculum.[18,19]

Male development is dependent on presence of chromosome Y. Testicular formation occurs under complex multi-genetic control. The key genes providing gonadal differentiation to testis are SRY gene and SOX9.[20,21] Numerous other genes and intercellular signalling factors are also required for normal development of testis in embryo i.e. WT1, SF1, GATA4, DAX1, FGF9, and PTGDS.[22,23,24] Ovarian formation and maintenance requires expression of WNT4, beta-CATENIN, DAX1 and FOXL2 genes.[23]

SRY gene expression starts in pre-Sertoli cells which play crucial role in embryonal testicular development.[22] The regulatory factors secreted by Sertoli cells (e.g., DHH and PDGF) are obligatory for differentiation of Leydig and peritubular myoid cells and compartmentalization of the testis.[18,25] AMH is another essential hormone produced by Sertoli cell. Degeneration of Müllerian ducts in male fetuses take place under influence of this hormone. In the absence of AMH action in the female, Müllerian ducts persist and form female-typical internal reproductive organs.[26]

Testosterone produced by Leydig cells is crucial for normal differentiation of Wolffian ducts in the male. After conversion of testosterone into dihydrotestosterone (DHT) by 5-alpha-reductase in peripheral tissues, DHT stimulates development of external reproductive organs and prostate. INSL3 is a hormone secreted by the Leydig cells, and it is essential for the development of gubernaculum.[26]

2.2 Testicular descent

In fetal testicular descent two major phases are distinguished: the transabdominal and the inguinoscrotal. In humans, the transabdominal phase starts at 8th gestational week and ends at around 15th week of gestation, and the inguinoscrotal phase starts at around 25th gestational week and ends normally before birth. Thus, during fetal period testis descends from an intraabdominal location into the bottom of the scrotum.[19]

During transabdominal phase the testis and epididymis move over the genital ducts in caudal direction to the internal inguinal ring where they are anchored by the gubernaculum. Its extrainguinal portion shortens mainly by swelling at its caudal base (bulb).[27] INSL3 signalling plays a central role in transabdominal phase. The swelling of gubernacular bulb,

cord shortening and formation of muscular layers of gubernaculum are INSL3 dependent events.[19,28] INSL3 is detectable in amniotic fluid only during pregnancies with male fetus (highest level observed at around 15th week of gestation) and INSL3 receptor is expressed in human gubernaculum.[28,29] The second driver of testicular descent in this phase is regression of the cranial suspensory ligament. This regression is an androgen dependent event.[27] In females, cranial suspensory ligament and gubernaculum will form the suspensory ligament of the ovary and round ligament of the uterus, respectively.[18] The direct hormonal participation of AMH in human testicular descent is still controversial. AMH participates in masculinization of the gubernaculum, hence, this hormone should also influence transabdominal testicular descent.[19,27]

Inguinoscrotal phase of testicular descent is mainly androgen-dependent. During this phase the complex of gubernaculum, testis and epididymis descends through the inguinal canal to the scrotum. The swelling and shortening of gubernaculum ensures the dilatation of inguinal canal and final descent of the gonad.[27,30] The increase of intra-abdominal pressure is another factor securing testicular movement in caudal direction.[31] Processus vaginalis covers the gonadal complex on the anterior side. Finally, the gubernaculum shrinks to the scrotal ligament.[18] According to the rodent studies, the important role in normal migration of gubernaculum during the second phase belongs to the genitofemoral nerve and its neurotransmitter, calcitonin gene-related peptide. In humans, the role of GNF/CGRP action remains uncertain.[32] Interaction between androgen and INSL3 signalling also suggested influence of both INSL3 and testosterone on gubernacular androgen receptor-positive cells and, hence, testicular descent during inguinoscrotal phase.[19] The disturbance of testicular descent takes place more often during inguinoscrotal phase, because only less than 10 % of surgically treated patients have testis in an intra-abdominal location.[33]

2.3 Cryptorchidism

In mature newborn boys testicular descent is normally completed. Cryptorchidism means the absence of one or both testes from the normal position in the scrotum with various degrees of severity. It is the most common birth defect of male genitalia. The prevalence of cryptorchidism in full-term boys at birth varies from 2% to 8%.[34,6] Cryptorchidism could be unilateral or bilateral and congenital or acquired. The acquired cryptorchidism means ascent of the testis into a cryptorchid position after normal scrotal position at birth. More than half of cryptorchid testes seen in childhood are explained by testicular ascent after birth. The spontaneous descent of previously cryptorchid testis can also be reversible (recurrent cryptorchidism).[34,35]

Clinical classification of the testicular position (modified from Boisen et al, 2004) is presented in Table 1.[6]

Table 1. Classification of testicular position.

Modified from Boisen et al, 2004[6]

Non-palpable	The testis is in inguinal, abdominal, or ectopic position. Verification of position requires surgical exploration.
Inguinal	The testis stays inside the inguinal canal. Localisation of the testis can be verified by palpation or/and ultrasound.
Suprascrotal	The testis is palpated above the scrotum and upper pole of the testis is located close to the external opening of the inguinal canal. Usually, the manipulation of the testis from the inguinal canal is required.
High scrotal	The testis stays at the upper part of the scrotum (habitually or after manipulation).
Normal-retractile	The testis can be manipulated to the bottom of the scrotum and persists in this position for a while without further traction.
Normal-scrotal	The localisation of midpoint of the testis is at or below the border between the upper and lower segment of the scrotum.

The trends in prevalence of cryptorchidism are a controversial issue. According to some studies, the cryptorchidism rates have increased since the 1950s. However, after 1985 some registry-based data indicated decreasing occurrence of cryptorchidism.[34] Significant geographic differences in the incidence of congenital cryptorchidism were also observed. The incidence rate of cryptorchidism in Finland was almost 4-fold lower than in Denmark.[6] The high concentrations of the reproductive hormones during the first months of life may lead to spontaneous descent of testis in up to 74 % of cryptorchid boys,[36] thus the incidence of cryptorchidism at 3 months of age is lower than at the birth. However, after the first year of life, the prevalence of cryptorchidism rises again due to testicular ascent.[34]

The aetiology of cryptorchidism is largely unexplained and supposed to be multifactorial. Common conditions associated with cryptorchidism are low birth weight (<2.5 kg), being

small for gestational age, and preterm delivery. Other risk factors associated with cryptorchidism are family history of cryptorchidism and rare genetic variants.[34,37] Boys with positive family history of undescended testes show an almost 4-fold increased risk to be cryptochid.[38] To date, 14 candidate genes involved in the development of cryptorchidism were identified (e.g. AHR, AR, ARNT2, HOXA10, INSL3, INSL3R).[37] Gestational diabetes was also reported to be a risk condition for cryptorchidism.[39]

Due to the hormonal dependence of testicular descent, endocrine disrupting chemicals were proposed as one of causal factor for cryptorchidism.[40] There is emerging human-study based evidence, supporting the role of EDCs in the disruption of testicular descent. Many pesticides, polybrominated diphenyl ethers, phthalates etc. are known endocrine disruptors with estrogenic and antiandrogenic effects.[41,42] In humans, numerous factors complicate the assessment of prenatal exposure effects of single chemical: mixed and low-dose nature of EDC exposure in real life, different speed of metabolism and excretion of single chemicals, different timeframes of exposure during the prenatal period etc. To protect human beings from adverse EDC effects further cost effective studies and novel approaches in this field are needed.[42]

Seasonality in the incidence of cryptorchidism was observed, e.g. in Finland the incidence of cryptorchidism was significantly higher in the spring than other seasons among both preterm and term boys.[37,43]

The clinical diagnosis of cryptorchidism is based on palpation of the scrotum. The position or existence of non-palpable testis has to be verified by further surgical evaluation (e.g. laparoscopy). If both testes are not palpable, hormonal evaluation is needed for differentiating cryptorchidism from anorchia. The rise of testosterone after hCG-stimulation indicates the existence of testicular tissue. Inhibin B and AMH can also be used as testicular markers. Imaging (ultrasound, MRI) is not a routine in evaluation of cryptorchid patients. There is no imaging technique that could conclusively determine the existence and position of nonpalpable testis and prevent unnecessary surgical intervention.[44]

Cryptorchidism is associated with numerous long-term consequences such as impaired endocrine function, poor semen quality and testicular cancer.[34] At 3 months of age cryptorchid boys have lower inhibin B levels and higher FSH than control boys. Inhibin B and FSH levels reflect the function of Sertoli cells, and this finding is important even if long-term consequences of this phenomenon are not clear yet.[45] Observations in men who underwent orchidopexy during childhood demonstrated an inverse correlation between the

age at orchidopexy and inhibin B/testosterone levels. Furthermore, FSH levels correlate positively with the age at orchiopexy. Thus, human data suggests that cryptorchidism itself and late orchidopexy have an unfavourable effect on the function of both Leydig and Sertoli cells.[46,47] Cryptorchidism, especially bilateral, is associated with lower paternity rates and poor semen quality, and early orchiopexy ensures more favourable fertility outcome in adulthood.[48,49,50,51] Furthermore, histological data indicate that cryptorchidism causes bilateral damage, even if patient has only unilateral disease. Impaired transformation of Ad spermatogonia to Ap spermatogonia was found in 70% of the scrotal testes in patients with unilateral cryptorchidism.[52]

Older review indicates that in 28 % of men, the adult size of a former cryptorchid testis is abnormal.[48] Taskinen and Wikström found that the adult size of a cryptorchid testis after orchidopexy was almost half smaller compared to the normally descended testis in former unilaterally cryptorchid men. The smaller size of the cryptorchid testis is an important phenomenon, because the association between adult testicular size and fertility is well established.[53] The age at orchidopexy correlates negatively with testicular size in adulthood.[50] To our knowledge, there are no previous longitudinal studies on testicular growth during puberty and timing of puberty in cryptorchid boys.

Cryptorchidism is a well-known risk factor for testicular cancer. Undescended testis is associated with testicular cancer in approximately 5 % of cases[34] and the overall calculated relative risk of testicular germ cell tumors is 4.8 (95% CI 4.9-5.7).[54] Bilaterally cryptorchid patients have a higher risk of testicular cancer than unilaterally cryptorchid. Men with uncorrected cryptorchidism also have a high risk for testicular cancer. Germ cell neoplasia in situ (GCNIS) is a premalignant condition for the testicular germ cell cancer. Malignant transformation begins from a developmental arrest of gonocytes. A prevalence of GCNIS in adult men with a history of cryptorchidism is approximately 2.9%. In general, screening of GCNIS by biopsy of the maldescended testis at the time of orchidopexy can be recommended only in children with intra-abdominal testis, abnormal external genitalia or/and diagnosed abnormal karyotype, because these individuals have higher risk of testicular neoplasia.[34,55]

The Nordic consensus statement does not recommend hormonal treatment of cryptorchidism by human chorionic gonadotrophin (hCG) or luteinizing hormone releasing hormone (LHRH). This type of treatment is not sufficiently efficient and has numerous side effects (acute inflammatory changes in the testis, germ cell apoptosis, reduction in the number of germ cells and, reduction in the testicular volume in adulthood).[56] Early orchiopexy is a

treatment of choice for both types of cryptorchidism (congenital and acquired). Congenital cryptorchidism requires surgery at the age of 6–12 months. If an undescended testis found at any age after 6 months, the patient has to be referred for surgery without delay. The annual follow-up throughout childhood is recommended for the cryptorchid patients after spontaneous descendent of the testis, due to very significant risk for testicular reascent.[57]

2.4 Testicular dysgenesis syndrome

During last decades rapid negative changes in the male reproductive health was observed. The influence of environmental and life-style factors in interaction with genetic background is the most reasonable aetiological explanation for this phenomenon. Cryptorchidism, hypospadias, impaired spermatogenesis, and testicular cancer can be related to each other as a risk factors. According to the testicular dysgenesis syndrome hypothesis, they could be the symptoms of one underlying entity with a fetal origin. Fetal endocrine disruption by environmental oestrogenic and anti-androgenic compounds could be a possible cause of TDS.[4,41]

2.5 Minipuberty

During embryonic development gonadotropin-releasing hormone (GnRH) neurons arise from the epithelium of the medial olfactory bulb and migrate across the nasal mesenchyme into the forebrain.[58] The fetal hypothalamic GnRH pulse apparatus is morphologically mature and well functional by the end of the first trimester of pregnancy. The high circulating levels of placenta-derived estrogens during pregnancy inhibit the GnRH pulse generator of the fetus as well as secretion of pituitary gonadotrophins towards the end of gestation.[59]

The fall of placenta-derived steroids (especially estrogens) during the first few days after birth leads to temporary activation of the hypothalamic–pituitary–gonadal axis. In humans, this phase of development is called minipuberty. In boys, serum FSH and LH levels are high during first 3 months of life and decline by about age 6 months to the low levels that are present until the onset of puberty. During minipuberty in male the concentration of serum testosterone reflects the pattern of LH secretion. A peak of testosterone secretion is reached at about 3 months of age with a subsequent decline to prepubertal values by 6–9 months of age. Minipuberty is also associated with increases in the levels of SHBG, INSL-3, inhibin B and AMH. This hormonal surge results in penile growth and about 6-fold

increase in testicular volume due to increase in seminiferous tubule length, reflecting the rapidly expanding Sertoli cell population.[59,60] Simultaneously, the number of neonatal Leydig cells increases, while the fetal Leydig cells undergo regression. The neonatal Leydig cells continue functioning until the onset of puberty.[61] In boys the total number of germ cells reaches maximum up to 100 days of age and decreases after that.[62] After mini-pubertal hormonal surge testicular volume decreases slightly towards the second year of life and then slowly increases until the pubertal acceleration of testicular growth.[63,64]

In girls, minipuberty leads to maturation of ovarian follicles and increase in estradiol levels due to elevated gonadotrophin levels. Inhibin B and AMH levels also transiently increase, since ovarian granulosa cells produces these hormones. After minipuberty in females FSH levels remain elevated for a longer period than in boys, until 3-4 years of age.[59]

2.6 Puberty in boys and girls

2.6.1 Hormonal changes in puberty

The increased secretion of sex steroids during puberty is a result of two independent physiological processes: gonadarche and adrenarche. Mechanism of adrenarche is an increase in adrenal androgen secretion due to growth and differentiation of the zona reticularis, the innermost zone of the adrenal cortex. The main adrenal sex steroids are dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulphate (DHEAS). The rise in adrenal steroid production is recognized at around 6–8 years of age.[65] Adrenarche does not require functional gonads and it is not linked to increased levels of gonadotrophins or ACTH.[66] However, ACTH acts as a permissive factor in adrenarche.[67] The puberty associated estrogens or androgens increase after the rise of DHEAS. The levels of DHEAS increase particularly during the second decade of life with higher levels in males than in females.[68] The onset of adrenarche is a complex prosses with multifactorial triggering, e.g. the body mass related hormones (insulin and leptin) are suggested for the role of possible launching signals. Low birth size and preterm birth are associated with adrenal hyperandrogenism. However, the association with clinical premature adrenarche is a controversial issue.[69] Clinical signs of adrenarche are the development of axillary and pubic hair (pubarche), adult type body odor, oily hair, acne and comedones, accelerated growth. These signs are normally seen in girls after the age of 8 years and in boys after 9 years of age. Premature adrenarche can be associated with hyperinsulinism, dyslipidemia, and later-appearing ovarian hyperandrogenism.[65,70]

Gonadarche means the pubertal activation of HPG axis and results in the achievement of fertility. After the first period of activity during infancy, the hypothalamic GnRH pulse generator is suppressed until puberty. The increased hypothalamic pulsatile secretion of gonadotropin-releasing hormone is a fundamental regulatory mechanism of the pubertal onset.[71]

The neural complex of several hundred GnRH neurons that controls pituitary gonadotrophin secretion is located in the medial basal hypothalamus and in the preoptic area of hypothalamus.[72] The mechanisms of juvenile suppression of hypothalamic GnRH pulse generator remain elusive especially in humans, since the majority of studies were performed using laboratory animals (rodents and primates). The major inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and GABAergic neurons is suggested to be the key players in braking of the GnRH pulse generator during the juvenile pause.[73,74] Neuropeptide Y (NPY), RFamide-ralated peptide-3 (RFRP-3), the higher concentrations of neuroestradiol also suppress GnRH neurons and, probably, participate in juvenile braking mechanisms.[73,75,76] The relatively low input of excitatory amino acid neurotransmitters (e.g. glutamate) could emphasize the GABA provided suppressive environment from infancy to puberty.[77]

The mechanism of reactivation of the hypothalamic GnRH pulse system during puberty is complex and largely unknown. The defective embryonal migration of GnRH neurons to the hypothalamus (Kallmann syndrom) causes congenital hypogonadotropic hypogonadism and results in the failure of puberty.[78] In humans, gonadotropin-releasing hormone receptor mutations are not a rare cause of isolated hypogonadotropic hypogonadism.[79] One of the key pubertal neuromodulators is kisspeptin. Kisspeptin neurons are located in the preoptic area and arcuate nucleus of hypothalamus and they stay in close interconnection with GnRH neurons. The role of kisspeptin as upregulator of GnRH pulse system is well documented. In humans, the loss of function of kisspeptin receptor (GPR54) leads to development of isolated hypogonadotropic hypogonadism.[80] The subgroup of kisspeptin cells which coexpress neurokinin B (NKB) and dynorphin (Dyn) are called KNDy neurons. NKB stimulates secretion of kisspeptin and Dyn inhibits it. The failure of puberty and severe gonadotropin deficiency was observed among patients with mutations in genes encoding neurokinin B and its receptor (TAC3 and TACR3).[81] Altogether the KNDy system carries out the fine regulation of secretory activity of GnRH neurons and it is imperative for normal pubertal development and initiation of puberty.[82,83]

During pubertal onset, the levels of GnRH pulsatility inhibitors (GABA, NPY, RFRP-3) gradually decrease and excitatory glutamate tone increases. The new balance of inhibitory and excitatory substances seems to contribute in the reactivation of GnRH pulse neurones (upstream of the KNDy system).[82] Most recently proposed gatekeeper of pubertal HPG axis activation is the makorin RING finger 3 (MKRN3) gene and protein. MKRN3 mutations were found in 46 % of cases with familial idiopathic central precocious puberty.[84] The activation of HPG axis is associated with a decline in serum levels of MKRN3 in girls and boys prior to the onset of puberty.[85,86] However, the exact mechanism of MKRN3 action is not known. In humans, the onset of puberty is not dependent of gonadal endocrine secretion. Simultaneously, aromatase presents in the hypothalamus and permits local synthesis of neuroestradiol. In primates the hypothalamic concentrations of estradiol and estrone during juvenile suppression of GnRH pulse generator are higher than during puberty.[87] Thus, neuroestradiol may be also involved in mechanisms of pubertal onset in humans. Energy balance has a considerable influence on puberty. The link between energy homeostasis and pubertal onset is suggested to be leptin. This hormone is secreted by adipose tissue and plays permissive role in the mechanisms of pubertal onset. After critical mass of adipose tissue is achieved, leptin may participate in hypothalamic pubertal activation, since leptin receptors are found in puberty-related neurons.[88]

Numerous other metabolism-associated and growth axis-associated hormonal factors interact with neuroendocrine hypothalamic network and probably play permissive role in the regulation of pubertal onset (e.g. insulin, ghrelin, growth hormone, insulin-like growth factor-1).[89,90,91] The regulation of the onset of puberty looks like even more complicated after taking into account possible neuronal-glial-endothelial interactions, environmental factors (endocrine disruptors) and the influence of epigenetics.[74,82,92]

The increase in pulsatile secretion of GnRH during pubertal onset is mainly sleep-associated. The pulsatile activity of GnRH neurons is their intrinsic property, which is highly Ca^{2+}-dependent and stimulated by cAMP. After being released at the median eminence into the portal circulation GnRH stimulates the pulsatile secretion of LH and FSH in the hypophysis. The pulsatile activity of GnRH neurones leads to the gradual elevation of pubertal estrogen levels and forming of positive feedback mechanism. In females, it enables GnRH dependent gonadotropin surge and first full ovulation. Thus, the oscillatory GnRH activity plays a crucial regulatory role in the secretion of gonadotropins and, therefore, in pubertal maturation. [93,94]

The pulsatile secretion of gonadotropins (LH, FSH) is detectable before puberty. However, a prominent rise in LH secretion is associated with the onset of puberty and in boys corresponding to testicular size of 3 mL. During early puberty LH levels follow clear diurnal pattern with higher nocturnal secretion. FSH secretion is less dependent on GnRH than LH secretion. The diurnal patterns of testosterone and estradiol levels follow the pulsatile secretory pattern of gonadotropins. Mean LH concentrations reach their maximun at mid-puberty, while FSH secretion increases gradually throughout puberty.[95]

Pubertal growth and maturation of the Leydig cells provide the surge of testosterone, which is important for the start of spermatogenesis, development of the accessory sex glands and external masculinization. The gonadotropin (LH) stimulation is essential for the maturation of adult Leydig cells.[61]

In females, a major source of estrogens is the ovary. In male estrogen production is diffuse. Approximately 20 % of estrogens is produced in the testis by Leydig cells, while the rest is derived from testosterone conversion into estradiol by aromatase in adipose, brain, skin and bone tissues.[96]

Sex hormone binding globulin (SHBG) binds sex hormones (testosterone, dihydrotestosterone, estradiol) in blood, participates in their transportation and determines their bioavailability. SHBG secretion in liver is regulated by sex steroids, insulin and thyroid hormones. SHBG levels increase during infancy, stay stable during childhood and decrease during puberty.[97]

Inhibin B is a glycoprotein hormone produced by the Sertoli cells and considered as a marker of Sertoli cell function. The name of this hormone originates from its ability to inhibit secretion of FSH. The secretion of inhibin B reaches a maximum at 3–6 months of age, then concentrations of inhibin B are even higher than in adults. After that the secretion is declining to a nadir towards 3-6 years of age and increasing again with the onset of puberty.[98]

Anti-Müllerian hormone is another glycoprotein hormone expressed by immature Sertoli cells. Maturation of Sertoli cells is associated with a decline of AMH secretion. A peak level of AMH production is reached at about 3 months of age, then AMH secretion is declining towards 1 year of age. After the onset of puberty, increasing testosterone levels cause a decrease of AMH levels and, finally, they reach low concentrations detectable throughout adulthood. In clinical practice AMH is used as a specific marker of the testis e.g. in bilaterally cryptorchid infants.[99]

The classical screening tool for the detection of central pubertal activation of HPG is GnRH stimulation test. However, the level of LH greater than 0.3 IU/l by ultrasensitive assay is also very specific indicator of pubertal onset.[100]

2.6.2 Physical changes in puberty

The generally accepted tool for the assessment of pubertal development is Tanner classification. It is based on assessment of external genitalia without taking any particular measurements. The classification was developed by regular photographing of the boys and girls during puberty. Below is direct quotation from classical Tanner's publication.[101]

The genital development stages in boys (G):

Stage 1. Pre-adolescent. Testes, scrotum and penis are of about the same size and proportion as in early childhood.

Stage 2. Enlargement of scrotum and of testes. The skin of the scrotum reddens and changes in texture. Little or no enlargement of penis at this stage.

Stage 3. Enlargement of penis, which occurs at first mainly in length. Further growth of testes and scrotum.

Stage 4. Increased size of penis with growth in breadth and development of glans. Further enlargement of testes and scrotum. Increased darkening of scrotal skin.

Stage 5. Genitalia adult in size and shape. No further enlargement takes place after stage 5 is reached.

The pubic hair stages in boy ang girls (P):

Stage 1. Pre-adolescent. The vellus over the pubes is not further developed than that over the abdominal wall, i.e. no pubic hair.

Stage 2. Sparse growth of long, slightly pigmented downy hair, straight or only slightly curled, appearing chiefly at the base of the penis or along labia.

Stage 3. Considerably darker, coarser and more curled. The hair spreads sparsely over the junction of the pubis.

Stage 4. Hair now resembles adult in type, but the area covered by it is still considerably smaller than in the adult. No spread to the medial surface of the thighs.

Stage 5. Adult in quantity and type with distribution of the horizontal (or classically "feminine") pattern. Spread to medial surface of thighs but not up linea alba or elsewhere above the base of the inverse triangle.

Stage 6. Pubic hair spreads further beyond the triangular pattern to the navel.

The breast development stages in girls (B):

Stage 1. Pre-adolescent: elevation of papilla only.

Stage 2. Breast bud stage: elevation of breast and papilla as small mound. Enlargement of areolar diameter.

Stage 3. Further enlargement and elevation of breast and areola, with no separation of their contours.

Stage 4. Projection of areola and papilla to form a secondary mound above the level of the breast.

Stage 5. Mature stage: projection of papilla only, due to recession of the areola to the general contour of the breast.[101]

The pubertal development in boys does not contain any reliable events to detect the onset of puberty taking only a medical history. Testicular growth after juvenile pause is considered as the first and most reliable sign of pubertal onset. Evidence-supported threshold of testicular volume by Prader orchidometer for pubertal onset is more than 3 mL (or $\geq$ 4 mL).[102,103,104,105] The North American longitudinal study showed that further pubertal progression had occurred within 6 months in 82% of boys, who had reached this 3 mL cut-off point.[103] Furthermore, the testicular volume more than 3 mL by Prader orchidometer associated also with an increase in testosterone levels.[106,107] Testicular size could be also assessed by a ruler and ultrasonography. Some endocrine guidelines suggest testicular length of more than 25 mm by a ruler as a threshold point of pubertal onset in boys, obviously,

referencing to the length of Prader orchidometer's 3 mL bead.[108,109] Simultaneously, similar cut-off point for ultrasound measurements is not reliably established.

Pattern of testicular growth is sigmoidal with remarkable acceleration during puberty. Recent Dutch study suggested approximate prepubertal and post-pubertal testicular volumes and cut-off volume for denoting of pubertal onset by ultrasound using ellipsoid equation: 0.6 mL, 13 mL and 1.4 mL respectively.[64] The increase in testicular volume is a result of mitosis of Sertoli cells, growth of seminiferous tubules in diameter and length and beginning of spermatogenesis (increase in the number of spermatogonia, spermatocytes and spermatids). Testicular growth is under hormonal control of FSH, LH and testosterone. FSH stimulates proliferation of Sertoli cells and spermatogonia, whereas LH and testosterone stimulate full spermatogenesis.[110]

The first spermaturia (spermarche) is another considerable pubertal event indicating onset of spermatogenesis. The median age of first spermaturia is 13-14 years.[111]

In girls, the first sign of puberty is the appearance of breast bud. Generally, menarche occurs after the stage of peak height velocity has been passed.[101]

Pubertal growth spurt in girls starts soon after breast growth occurs. In boys, an acceleration of statural growth occurs later than in girls, approximately in one year after pubertal onset.[101]

Penile growth is not the earliest sign of puberty. In a large Bulgarian cross-sectional study published in 2010, the maximum penile growth occurs approximately from 12 to 16 years of age. Until age 10 years, the mean length of penis was less than 5 cm. An enlargement of testes was observed 1 year before the increase in penile length. The penile circumference and penile length increased simultaneously.[112]

Pubic hair occurs soon after the onset of puberty in both sexes. As a first sign of pubertal development, pubic hair is presented only seldom. Axillary hair appears, usually, when person is reaching P4 stage or approximately in 2 years after appearance of pubic hair.[101]

The pubertal growth of the larynx and vocal folds is especially strong in boys. "Breaking" of the voice appears as a late pubertal event. The most significant changes in speaking and singing fundamental frequencies take place between Tanner stages G3 and G4.[113]

The increased production of gonadal and adrenal sex hormones induces appearance of acne, secondary hair growth and adult axillary odour. However, hair on the face, trunk and limbs occurs generally later, in the 20s.[114]

The characteristic swings of the mood and heightened emotionality are also an essential part of pubertal development.[115]

Up to 69 % of pubertal boys experiences enlargement of subareolar mammary gland tissue, pubertal gynecomastia.[116] This phenomenon occurs at about Tanner stage G4 and disappears after 12-18 months period in most cases.[101]

Figure 1 presents pubertal activation of hypothalamic–pituitary–gonadal axis and typical order of pubertal events in both sexes (Tanner stages, PHV and menarche).[82,117,118]

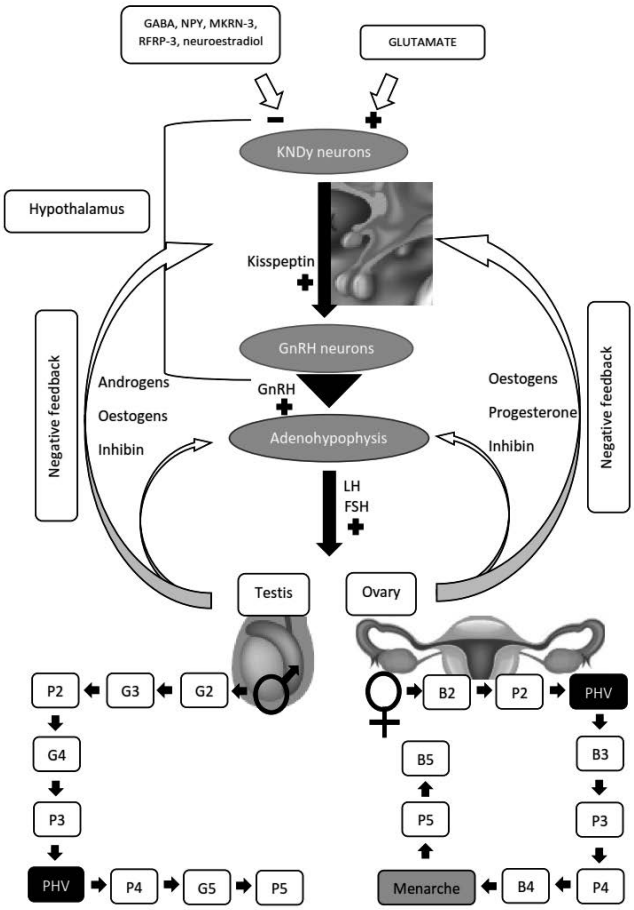

Figure 1. Pubertal activation of hypothalamic–pituitary–gonadal axis and typical order of pubertal events in both sexes (Tanner stages, PHV and menarche).

Modified from Livadas & Chrousos 2016, Wolf & Long 2016 and Barett et al. 2016.[82,117,118] *Anatomical insertions were used with permission (Essentials Collection/Getty Images).*

Hormone-related abbreviations: FSH = follicle-stimulating hormone, GABA = gamma-aminobutyric acid, GnRH = gonadotropin-releasing hormone, KNDy = kisspeptin/neurokinin B/dynorphin A neurons, LH = luteinizing hormone, MKRN3 = makorin RING finger 3, NPY= Neuropeptide Y, RFRP-3 = RFamide-ralated peptide-3. Pubertal events-related abbreviations: PHV = peak height velocity; tanner staging: B = breast development in girls, G = external genitalia in boys, P = pubic hair.

2.6.3 Timing of puberty

Puberty is a sequence of events described in previous section with large individual variation in order and, especially, timing. In the classical British work published in 1970 the mean age at G2 and G5 stage was 11.6 and 14.9 years, respectively.[119] In average, the male genitalia reached the adult stage in 3 years (95% CI, 1.7-4.7) and the peak height velocity reached at a mean age of 14,1 years.[119] According to the same British research group (study published in 1969), in girls, the mean age at stage B2 and B5 was 11.5 and 15.33, respectively. Transition from B2 to B5 took in average 4 years (90% CI, 1.5-9). The peak height velocity reached at a mean age of 12,1 years. The mean age of menarche was 13.5 years.[120]

Current conception about puberty in Finnish boys and girls is based on 30 years old study. According to the longitudinal puberty monitoring performed by Ojajärvi in Helsinki (results were published in 1982), the mean age at G2 stage (years (SD)) was 12.2 (0.85), at G3 13.2 (0.86), at G4 14.2 (0.86), at G5 15.5 (0.70). In girls, mean age at B2 stage was 10.8 (1.27), at B3 12.0 (0.86), at B4 13.5 (1.35), at B5 15.2 (1.10). Development of the pubic hair was also assessed. Mean age at P2 stage was 12.6 (0.80) and 11.6 (1.04), at P3 13.5 (0.76) and 12.4 (0.97), at P4 14.3 (0.84) and 13.5 (1.04), at P5 15.5 (0.70) and 15.1 (0.97) in boys and girls, respectively. In average, the time needed for getting from G2 to G5 stage was 3.35 years (min 2.0 years, max 5.75 years) and from B2 to B5 stage was 4.54 years (min 3 years, max 7 years) in boys and girls, respectively. The mean age at menarche was 13.25 (1.33) years. In this study classical Tanner criteria without any modifications were used. In Finnish adolescents, PHV occurs at mean age of 13.7 (1.09) years and 11.4 (1.25) in boys and girls, respectively.[121]

Publications relating to the timing of puberty may contain a lot of different biases, which could complicate the direct inter-study comparison of the results. The pioneering work of Tanner and Marshall itself has weaknesses. The subjects in this study were not completely representative for the general population of UK, since participants were recruited from a lower socio-economic sector of the population. Furthermore, the photographing is not reliable method for the assessment of pubic hair.[119,120] Traditional Tanner staging is observer dependent and contains the risk of inaccuracy of visual assessment of genitalia. For example, in NHANES III study (highly cited pubertal study from the United States) the observers did not measure testicular volume and quality control allowed a 1-stage variance between the physician's assessment and the quality control standard. That might be especially important for the indication of pubertal onset (evaluation between G1 and G2 stages).[122]

Therefore, the measurement of testicular volume for denoting the onset of puberty is strongly recommended. However, there is no consensus, what testicular volume denotes the onset of puberty in boys: > 3 mL or ≥ 4 mL by Prader's orchidometer.[5] Self-assessment of pubertal stage by the child or parents is also practiced and was found as unreliable.[123] Moreover, some authors use in their studies further modification of Tanner staging e.g. Biro et al. used modified global stages in place of traditional Tanner classification.[124] In overweight and obese girls breast tissue is difficult to distinguish from fat tissue and palpation of breasts by trained examiner is recommended to denote pubertal onset.[125]

The secular trends in pubertal timing is a topical issue in pediatric research. The changes in pubertal timing may have numerous short- and long-term adverse effects on individual health. Currently, there is consensus on trends in female puberty. The age of menarche has been gradually declined from late 1800s to the mid-1900s. Furthermore, the panel of experts consented (using data from the United States), that from 1940 to 1994 the age of menarche has continued to decline and the age of initiation of pubertal breast growth has also declined. The same panel suggested, that the tempo of pubertal development from 1940 to 1994 has declined [126,127] Danish analysis also indicates the decrease of menarcheal age during the time-period from mid-1960s to 1980s. However, the Danish analysis (data from 1964 to 1992) did not confirm secular trends toward earlier breast development.[128] Most recent Danish study on female puberty found significant decline of the mean age at B2 from 10.88 years in 1991 to 9.86 years in 2006.[129]

The data on male puberty is controversial. In the NHANES III (cross-sectional survey from the United States, 1988-1994) median age at G2 among white boys was 10.1 years.[122] In more resent longitudinal study from United States (performed by the NCHID, in 2000-2006) median age at G2 among white boys was 10.4 years.[130] These results would suggest the decline of age at G2 compared to previous studies from United States. However, the expert panel stated, that data are insufficient to conclude about trends in male puberty, mainly, due to the problem of inter-study comparability.[127] Simultaneously, according to European studies (Netherlands, Denmark) from the mid-1960s to the late 1990s the pubertal timing seems to stay almost unchanged. The age at G2 in 1990s was 11.83 and 11.5 years in Denmark and Netherlands, respectively, indicating later pubertal onset in the European male population than in the United States.[128,131] However, the most recent Danish data indicates advancement in male pubertal onset. The mean age at G2 among Danish boys was 11,83 and 11,59 years in 1991-1993 and 2006-2008 cohorts, respectively, suggesting a trend toward earlier onset of puberty.[132]

The data on the tempo of male puberty is also conflicting. In the NHANES III the mean age at G5 was close to the previous studies from the United States, suggesting that the tempo of pubertal development declined, since the age at G2 decreased.[127] Simultaneously, in the Danish study the mean age at G5 in 1991-1993 was 15.4 yr and in 2006-2008 14.3 yr. This statistically significant difference indicates no increase in the duration of male puberty.[132]

The secular trends in timing of pubertal growth also suggest shift toward earlier onset of puberty in both sexes. The large Danish analysis indicates, that between 1935 and 1969 the age at onset of pubertal growth spurt decreased by 0.2 and 0.4 years in girls and boys, respectively, and the age at peak high velocity decreased by 0.5 and 0.3 years in girls and boys, respectively.[133] The decrease in the timing of pubertal growth spurt was also noticed in Finland, when Saari et al. updated Finnish growth curves and compared new growth patterns (1983-2008) to the old growth reference (1959-1971).[134]

The genetics explains approximately 50-80% of variability in pubertal timing. In general, secular shifts in pubertal timing are explained by such environmental factors as improvement in socio-economic conditions, nutrition and hygiene.[135]

The epidemic of obesity in children has been discussed extensively. The connection between obesity and decline of the age of pubertal onset or menarche in girls is well supported by numerous studies.[136] However, in boys, data is still controversial. Most of the studies considering relationships between obesity, nutrition and pubertal timing are cross-sectional. Due to objective reasons, longitudinal interventional studies are not usual. In that context a recent longitudinal intervention study on the effect of weight loss on puberty in overweight German boys and girls should be mentioned. The aim of the one-year lifestyle intervention was BMI-SDS reduction. The intervention was started just before the onset of puberty and at the moment on the onset of intervention all participants were prepubertal. This study demonstrated that the onset of puberty was less frequent in girls and more frequent in boys, if BMI-SDS reduction were achieved: 45.7% vs 75% ($p=0.014$) in girls and 76.9% vs 53.6% ($p=0.024$) in boys, with and without BMI-SDS reduction, respectively. In discussion, the authors of this study hypothesized that the main mechanisms explaining the shifts in pubertal timing in obese children are an action of leptin and action of aromatase in adipose tissue.[137] However, in this study, obese and overweight children were not separated. Simultaneously, some authors speculated, that association between BMI and the age at pubertal onset in boys is nonlinear. Overweight boys have a tendency toward the earlier onset of puberty and obese boys (boys with very high BMI) experience their puberty later.[5]

Numerous other signals, which connect nutritional status, energy balance and hypothalamus may influence timing of puberty in children under specific circumstances (e.g. ghrelin, IGF-1, glucose, insulin and insulin resistance, thyroid hormones). The mechanisms of action and precise role of these factors is still a matter of debate.[138]

The sufficient balance of energy during childhood and puberty is only one aspect how nutrition may influence pubertal development and timing. However, the understanding how qualitative changes in diet could impact on puberty is also important, since modern research data support the idea of very early dietary and lifestyle intervention to prevent i.a. cardiovascular morbidity during adulthood.[139]

The Special Turku Coronary Risk Factor Intervention Project (STRIP) is a large prospective, longitudinal, interventional study launched in 1989. The main aim of this Finnish project was to assess the influence of dietary and lifestyle intervention started at the age of 7 months on development of atherosclerosis. The intervention in the STRIP did not change the total amount of energy in the diet of participants, but it modified quality of fat in diet and restricted the dietary intake of cholesterol. In the STRIP the aim of dietary counselling was to provide 30-35 of daily energy (E%) from fat with saturated to monounsaturated + polyunsaturated fatty acid ratio of 1:2. The aim of cholesterol intake in intervention children was less than 200 mg/d.[140] These dietary restrictions, potentially, may influence sex hormone concentrations and puberty. The alteration of sex hormone metabolism was observed in adults receiving low-fat diet.[14,15]

So far, the intervention STRIP participants demonstrated the effectiveness of low-saturated-fat and low-cholesterol diet during childhood and adolescence. The decrease in saturated fat intake and reduction of serum low-density lipoprotein cholesterol (LDL-C) concentration from infancy until 19 years of age were observed. The serum cholesterol profile improved especially in boys compared to the girls.[8] Furthermore, the dietary counselling was associated with other beneficial effects: the improvement in brachial artery endothelial function[9], the lowering of blood pressure[10], the improvement in insulin sensitivity[11], the increase in ideal cardiovascular health score[12] and the reduction in risk for the metabolic syndrome[13]. Simultaneously, the intervention and control groups showed no difference in growth, BMI, age at menarche or in timing of pubertal development. The median age at menarche was 13.0 and 12.8 years in the intervention and control groups, respectively ($p=0.52$). In the STRIP study, the visual assessment of Tanner staging without assessment of testicular volume was used. Until now, the STRIP data on pubertal hormones was not avaliable.[141]

Previously, similar dietary intervention was done only once in the United States, in the DISC HAS. The DISC is an acronym for the Dietary Intervention Study in Children and the HAS for the Hormone Ancillary Study. The HAS is related to the DISC as an extension, initiated to assess the effect of dietary intervention on sex hormones during puberty in both sexes. The aims of dietary intervention in the DISC HAS were to provide 28% of daily calories from fat, less than 8% of daily calories from saturated fat, up to 9% of daily calories from polyunsaturated fat and at least 11 % of calories from monounsaturated fat. The limit of cholesterol intake was less than 75 mg/4200 kJ (1000 kcal) per day with a maximum recommended intake of 150 mg/d. In the DISC HAS project only children with elevated levels of LDL cholesterol were recruited at the prepubertal stage of development. The follow-up started, when eligible girls were 7.8–10.1 years old and boys 8.6–10.8 years old. During the follow-up hormonal and anthropometric parameters were assessed altogether 5 times (at baseline, after 1, 3 and 5 years and at the last visit).[142,143]

In the intervention group, the total fat, saturated fat and cholesterol intake were lower compared to the control group. Due to dietary intervention, the lowering of serum LDL cholesterol levels was also observed.[144] After low-saturated-fat and low-cholesterol diet, intervention girls had lower concentrations of sex-hormones than controls. However, this difference was observed only in the girls after menarche. The intervention girls had circa 20-30 % lower estradiol, non-sex hormone binding globulin-bound estradiol, estrone, and estrone sulfate levels during the follicular phase of the menstrual cycle, than control girls. They also had 27.2% higher testosterone and 52.9% lower progesterone levels during the luteal phase of the menstrual cycle than girls from the usual care group.[142] The hormonal parameters of intervention and control boys did not differ during entire follow-up period.[143] Both groups and both sexes showed similar growth and progression through puberty.[142,143] Median age at menarche was 12.8 years and 12.9 years in intervention and control girls, respectively (p=0.74).[142]

In addition to the epidemic of obesity, the endocrine-disrupting chemicals are also suggested to be an important environmental factor causing secular trends in pubertal timing. Endocrine disruptors can act as estrogenic, androgenic, antiestrogenic, or antiandrogenic substances. Moreover, some compounds can demonstrate different properties (agonistic or antagonistic) depending on the dose or other specific circumstances. EDCs may influence the production, metabolism and excretion of endogenous hormones, altering the hormonal balance of exposed person. The exposure to EDC may lead to precocious or delayed puberty by central or peripheral mechanisms. The data on EDCs as compounds altering pubertal development is growing continuously.[135] For example, accidental exposure to

polybrominated biphenyls (PBBs) in Michigan (the United States) was associated with earlier age at menarche and earlier pubic hair development in the girls.[145] The other study from the United States showed association between lead exposure and delayed puberty in girls.[146]

Shifts in timing of puberty are considered as events with numerous short- and long-term adverse consequences. In girls, premature adrenarche has been associated with an increased risk of metabolic syndrome and PCOS in adulthood.[147,148] In turn, PCOS is characterized by polycystic ovaries, hyperandrogenism, menstrual disorders, infertility, hirsutism, and obesity.[149] The low age at menarche is also a risk factor for metabolic syndrome and breast cancer in adulthood.[150,151] Pathologically early pubertal development is associated with rapid skeletal maturation and, as a result, loss in adult height.[152] Behavioral and psychiatric disorders are also linked to the changes in pubertal timing. In girls, for example, early maturation is associated with depression, eating, conduct and substance use disorders. Early physical maturation is a risk for being abused sexually, for beginning sexual relationships early and psychosocial problems.[153]

In clinical practice, it is important to define normative references for precocious and delayed puberty. In case of precocious puberty breast development begins before the age of 8 years in girls and enlargement of testis starts before the age of 9 years in boys.[154] Delayed puberty is pubertal onset after the age of 13 years and 14 years in girls and boys, respectively.[155]

2.7 Semen quality

2.7.1. Spermatogenesis

Spermatogenesis is the process where spermatozoa are produced. It takes place in the seminiferous tubules in the testes. In mammals, spermatogenesis is divided into mitotic, meiotic and spermiogenic phases. In humans, spermatogonium needs 74 days to complete its transformation to a mature spermatozoon. After onset of puberty the pool of spermatogonial stem cell (A_{dark} spermatogonia) remains stable. Sperm differentiation begins, when A_{dark} spermatogonia are committed to differentiating A_{pale} spermatogonia which in turn give rise to type B spermatogonia. The niche of spermatogonia cells is localized in basal compartment of the seminiferous epithelium. Further, B spermatogonia divide to produce

preleptotene spermatocytes that enter meiosis, pass through Sertoli cell barrier into adluminal compartment of seminiferous epithelium and develop to leptotene, zygotene, pachytene and finally to diplotene spermatocytes which undergo the first meiotic division that produces secondary spermatocytes. The second meiotic division results in the generation of haploid spermatids. Further differentiation leads to the gradual forming of elongated spermatids. Finally, the mature spermatozoa are released into the lumen of seminiferous tubules.[156]

Spermatogenesis is irreversible recurrent process with a cyclic nature. The seminiferous epithelium has also detectable cyclicity. Different stages of epithelial cycle can be distinguished by associations of cell types. The applying of acrosine immunohistochemical staining in the assessment of acrosomal development enables a detailed analysis of the seminiferous epithelial cycle. Nowadays, 12 stages can be distinguished.[157] Since the duration of the seminiferous epithelial cycle in humans is 16 days, the complete differentiation of spermatozoon includes many of them. In contrast to other species (e.g. rodents or bull), in humans, in the same cross-sectional view of seminiferous tubule 2 to 4 stages can be seen simultaneously.[156,157]

The role of Sertoli cells in spermatogenesis is crucial. These cells provide an essential environment for germ cells at all stages of differentiation. The Sertoli cell barrier (also known as blood-testis barrier) maintains basal compartment for the germ-line stem cells and immune-privileged adluminal compartment to secure meiotic and spermiogenic phases of spermatogenesis. The Sertoli cell can treat only a limited number of germ cells at the same time, which determines the capacity of sperm production in the male gonad. The Sertoli cells play irreplaceable role in endocrine, paracrine and immune regulation of spermatogenesis. The cyclicity in gene expression (GDNF, SCF, DHH) and plasticity are also important features of Sertoli cells.[158,159,160] The stage-dependent cyclicity, in respect to germ cell differentiation, is determined by endocrine regulation, intrinsic properties of the Sertoli cells and interaction between Sertoli and differentiating germ cells. Simultaneously, DMRT1 is considered as a factor essential to maintain the identity of Sertoli cells independently of the spermatogenic cyclicity. In humans, the proliferation of Sertoli cells continues until puberty and then Sertoli cell pool remains stable.[156,161,162]

The main hormonal agents regulating spermatogenesis are LH, testosterone and FSH. LH stimulates production of testosterone by Leydig cells. Testosterone is a constitutional element of regulation of spermatogenesis, especially, for the transition from round to elongated spermatid and spermiation.[163] The level of intratesticular testosterone is higher than

levels of testosterone in circulation. However, the high intratesticular testosterone is not absolutely vital for sperm production.[164] Testosterone exerts its action through androgen receptors, which are expressed mainly in Sertoli cells, Leydig cells and peritubular myoid cells. In humans, the expression of androgen receptor in Sertoli cells increases after the age of 4 years. Thus, Sertoli cells remain insensitive to androgens during fetal and early postnatal period.[165] The androgen receptor expression in Sertoli cells depends on the stage of the seminiferous epithelial cycle with amplification during mid-stages (approximately stage VI in humans). In respect to spermatogenesis, androgen receptor signalling is required for the maintenance of spermatogonia numbers, progression through meiosis, the blood-testis barrier integrity, formation of seminiferous tubule lumen, spermatid adhesion and spermiation.[156,157,166,167]

FSH is less crucial for spermatogenesis. In men inactivating FSH receptor mutations do not cause absolute infertility.[168] The action of this gonadotropin can be compensated by e.g. excessive testosterone. In adult Sertoli cells, FSH participates in the regulation of transcription of many genes depending on the stage of the seminiferous epithelial cycle. The proliferation of immature Sertoli cells occurs under stimulation of FSH.[156,163]

Paracrine, juxtacrine and autocrine signalling in testis is also an integral part of regulation of spermatogenesis. SCF/KIT signaling promotes survival and proliferation of differentiating spermatogonia. SCF production occurs in Sertoli cells under FSH control in the epithelial stage-dependent manner. Retinoic acid is also synthesized in Sertoli cells and acts in an autocrine manner. Simultaneously, retinoic acid induces the differentiation commitment of germ cells towards meiosis.[169] However, the important role of retinoic acid is clear only in rodents, more investigations in humans are needed. Many other paracrine factors participate in the regulation of spermatogenesis e.g. DHH, activin, inhibin, follistatin, estrogen etc.[156]

Major pathways of steroidogenesis in adrenal cortex, testis, and ovary are presented in Figure 2.[170]

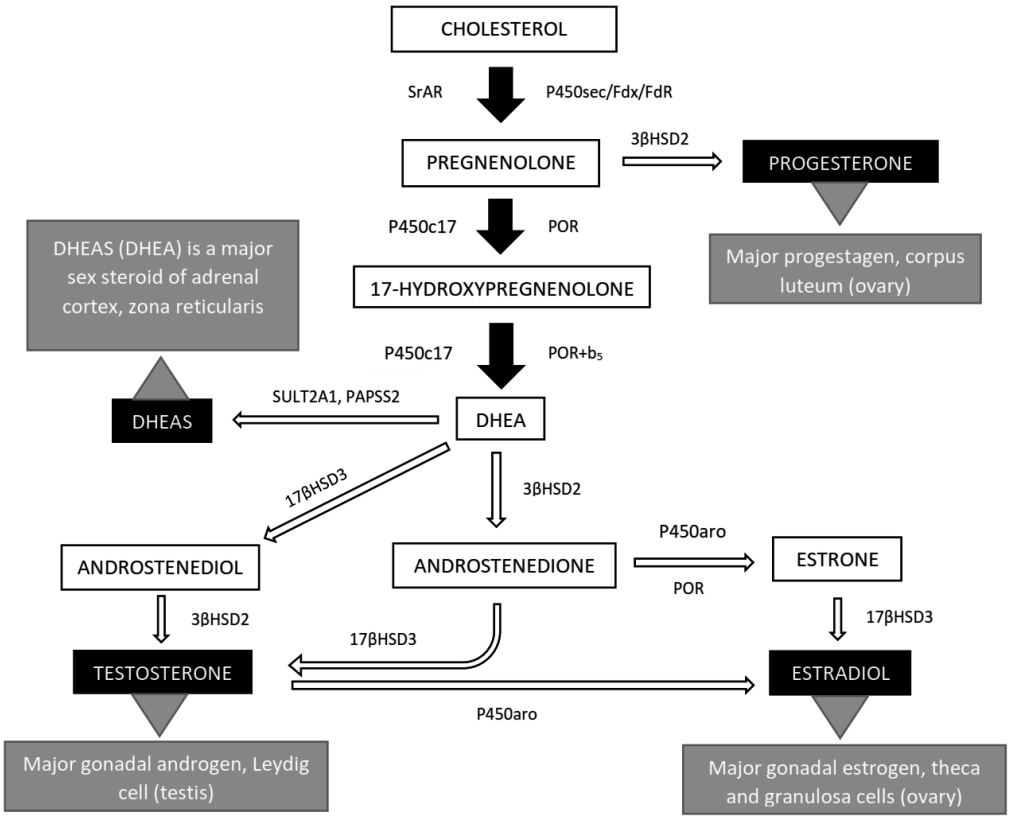

Figure 2.Major pathways of steroidogenesis in adrenal cortex, testis, and ovary.

Modified from Miller & Auchus 2011.[170] *Black boxes indicate the major hormonal end-products. Grey boxes explain the role of hormone and the site of synthesis. Arrows indicate pathway steps and involved enzymes. DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulphate, P450aro = aromatase.*

2.7.2. Epididymis

Anatomically epididymis is divided into a proximal caput, corpus and distal cauda. Furthermore, a functional segmentation of epididymis can also be distinguished. Epididymis is not only a sperm storage, but essential structure for further sperm maturation. Like the

testis itself, the epididymis guarantees immunological protection for spermatozoa. The epididymal transit influences ability of spermatozoa to fertilize, which increases from proximal to distal parts of epididymis. In humans, the epididymal maturation of sperm was studied in infertility patients who were treated by sperm collection from different parts of epididymis or underwent surgical treatment e.g. epididymovasostomy. If procedure was targeted more distally the outcome was preferable in respect to conception.[171] Animal studies suggest that epididymal maturation of spermatozoa includes changes in the sperm membrane, acrosome, other head and tail organelles, which improve the ability of sperm to fertilize the egg. For example, the absence of epididymal-originating surface protein P34H was associated with male infertility, since P34H plays essential role in the binding of the sperm to zona pellucida.[172,173]

Compared to other species, in humans, the length of epididymal tubule is short and caudal part of the epididymis is poorly developed. Thus, in our species the sperm storage capacity of the epididymis is relatively poor. Usually, the human reservoir of sperm is not enough to produce two or three sufficient semen samples in sequence. Transition of the sperm through human epididymis takes only 2-4 days (in domestic and laboratory animals 10-12 days).[172]

2.7.3. Reference values for semen parameters

Current reference values for semen parameters were established by the World Health Organization in 2010 (Table 2). Reference values were generated by analysis of several cross-sectional studies of semen quality among fertile men (TTP $\leq$ 12 months). The mean (+SD) age of the analysed persons was 31$\pm$5 years (range 18–53). According to the WHO manual, eligible sample is a complete, first (if several were collected) semen sample given after 2-7 days of abstinence.[174,175]

Table 2. Lower reference limits (CI 95 %) for main semen parameters.

Modified from WHO Laboratory Manual for the Examination and Processing of Human Semen.[174]

Parameter	Lower reference limit (CI 95%)
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)

PR = Progressive motility, NP = Non-progressive motility.

However, the translation of semen parameters into fertility is not direct. Danish researches reported that TTP begins to increase when sperm concentration declines below 40 x 10^6/ml.[176] Researchers from the United States suggest lower threshold for fertility decline, at 30 x 10^6/ml.[177] According to the large European cross-sectional study the increased TTP was associated with sperm concentration <55 x 10^6/ml.[178]

Composition of human semen in percentages of the volume of ejaculate is presented in Figure 3.[117,179]

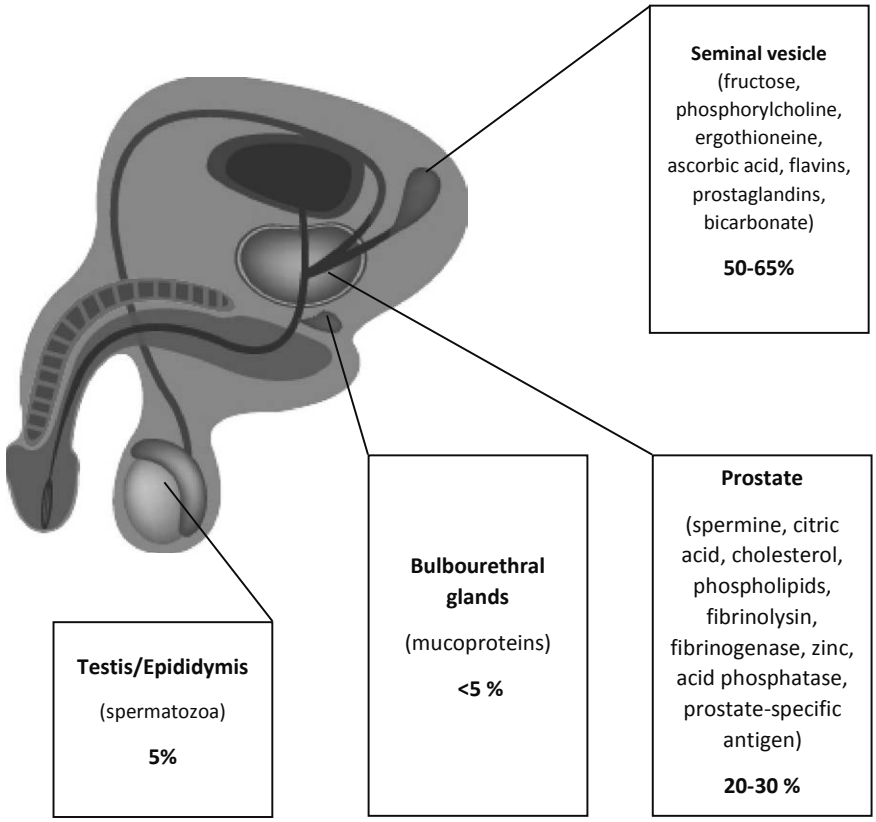

Figure 3. Components of semen in percentages of the volume of ejaculate. Boxes indicate the sites of production.

Anatomical insertion was used with permission (Essentials Collection/Getty Images).

2.7.4. Semen quality and age

Our knowledge about changes in semen parameters with age is based on cross-sectional studies and further meta-analyses. In a recent large meta-analysis, 90 eligible studies (93 839 participants, age range 16.5-80 years) were included. The meta-analysis showed that semen volume, total sperm count, morphology, total motility, progressive motility and DNA fragmentation declined with increasing male age. Surprisingly, the male age did not

influence sperm concentration. However, this meta-analysis did not estimate the age thresholds for such changes in semen traits.[1]

Stone et al. in their retrospective analysis of 5 081 men aged from 16.5 to 72.3 years concluded that decline in sperm quality begins after 34 years of age. Total sperm count and total motile count decline first. Sperm concentration and percentage of sperm with normal morphology decrease after 40 years of age. The percentage of motile sperm falls after 43 years of age and, finally, semen volume decreases after 45 years of age.[180]

In a cross-sectional analysis of 6022 semen samples in relation to the age of participants, Levitas et al. found that the semen volume, total sperm count, and total motility are highest in the age group of men from 30 to 35 years. The most pronounced decline in these parameters were observed in the ≥ 55 years group. Simultaneously, sperm concentration increased gradually with the increasing of age of participants. The top sperm concentration was found in group of men older than 55 years. The highest motility (%) was observed among the youngest participants (< 25 years group) with subsequent gradual decline afterwards.[181]

According to a Chinese cross-sectional study of 998 subjects aged from 20 to 60 years, the age of man correlated negatively with rapid progressive motility and percentage of normal sperm. The decline of these semen parameters was observed after the age of 30 years. Semen volume, sperm concentration and total sperm count showed no correlation with the age.[182]

Schwartz et al. studied semen parameters of 833 fertile men in respect to their age (21-50 years old man). The age-related changes in semen concentration, total sperm count and semen volume were not significant. In the age group of men from 21 to 25 years semen parameters (in particular percentage of normal sperm) were lower, when compared to the age group 26 to 35 years of age. Motility and morphology began to decline in the age group 36 to 40 years of age and 41 to 45 years of age, respectively.[183]

In relatively small cross-sectional study (97 men, aged 22-80 years) performed in the United States, the age-related decline in semen volume and motility was observed. In this analysis the decline was continuous with no evidence of a detectable turning age point.[184]

In the longitudinal Danish study 158 men were followed for up to 4 years. The median age at the entry was 19.1 years. Semen samples were collected quarterly and, in total, 1838

semen samples were analysed. Danish researchers found no change in sperm concentration, total sperm count and morphology. Simultaneously, there was a significant increase in the percentage of rapid progressive motile sperm and semen volume. There also was decrease in the percentage of non-progressive and immotile sperm. However, all these changes were significant only for men up to 23 years of age.[185]

2.7.5. Semen quality trends over time and geographical differences

The deterioration of semen quality in the 20th century was discussed in two large meta-analyses.[1,2] The first was published in 1992 by Carlsen et al.. This analysis included 61 papers published between 1938 and 1991. Carlsen et al. demonstrated a significant decrease of semen volume and mean sperm concentration over the analysed period. Semen volume declined from 3.40 ml in 1940 to 2.75 ml in 1990 ($p=0.027$) and sperm concentration from 113 x 10^6/ml to 66 x 10^6/ml ($p<0.0001$).[2] More recent systematic review and meta-analysis was published by Johnson et al. in 2015. In total, this analysis included 124 papers released between 1938 and 2013. Johnson et al. reported statistically significant decline of sperm concentration by -0.599 x 10^6/ml per year between 1938 and 2013. However, in the separate analysis over period from 1994 to 2013 decline was no longer statistically significant, when other important predictors were taken into consideration: sample source, mean age, abstinence control, gross domestic product of the country.[1] However, Levine et al. in their meta-regression analysis found significant decline in sperm counts between 1973 and 2011. One hundred eighty-five eligible studies and 42 935 men were included in analysis. Over this period sperm concentration and total sperm count declined by 50-60 % in western countries among men unselected by fertility.[186]

Sperm parameters vary evidently between different geographical regions. Jørgensen et al. compared semen quality among young men (approximately 17-20 years of age) representing general population in four Nordic-Baltic countries: Denmark, Norway, Estonia and Finland. Danish and Norwegian men had significantly lower sperm concentration (41 x 10^6/ml) than Estonian and Finnish men (57 x 10^6/ml and 54 x 10^6/ml respectively).[16] Regional differences in semen quality was also reported in studies on fertile men. In comparison between Denmark (Copenhagen), France (Paris), Scotland (Edinburgh) and Finland (Turku) lowest sperm concentrations were found in the Danish population (77 x 10^6/ml)

and highest in Finnish men (105 x 10^6/ml), while sperm concentration in France and Scotland was in between (94 x 10^6/ml and 92 x 10^6/ml respectively). Participants were approximately 30 years of age.[17] Iwamoto et al. extended European comparison and added into analysis 324 fertile Japanese men (from the Kawasaki/Yokohama area). This study showed that semen parameters among Japanese men were close to those in Denmark.[187]

However, not only fertility problems are associated with poor semen quality. The incidence of testicular cancer, cryptorchidism and hypospadias is higher in regions where semen quality is declined, e.g. in Denmark.[6,188,189]

Recent semen quality trends in Denmark were reported by Jørgensen et al. in 2012. This large cross-sectional one-center study comprised 4867 men representing general population with a median age 19 years. Semen characteristics were monitored over 15-years-period (1996 – 2010). Over this time median sperm concentration and total sperm count slightly increased from 41 x 10^6/ml to 48 x 10^6/ml (p=0.02) and from 132 to 151 million (p=0.001), respectively.[190] Another Danish cross-sectional study did not show significant trends in semen quality among young men between 1996 and 2016.[191] While semen quality in Denmark stabilized at low level, Finnish recent trends cause concern. Analysis of 3 consecutive cohorts of young Finnish men born in 1979-81, 1982-83 and 1987 showed a decline of semen concentration, total sperm count and percentage of sperm with normal morphology. Median sperm concentration decreased from 67 x 10^6/ml to 48 x 10^6/ml.[192] Simultaneously, the incidence of testicular cancer in Finland increased annually by 5.1 % over 10 years and in Denmark decreased by 1 % per year.[193,194]

The hypothesis of testicular dysgenesis syndrome was discussed previously in a separate section. Lifestyle and environmental factors associated with deterioration of semen quality will be discussed here. Results of a meta-analysis suggest that tobacco smoking influences negatively semen quality (sperm count, motility and morphology).[195] Prenatal exposure to cigarette smoking is associated with reduction of semen characteristics and smaller testicular size in male offspring.[196] Furthermore, marijuana smokers are also at risk to have a lower semen quality. In a large cross-sectional Danish study, smoking of marijuana was associated with lower sperm concentration, total sperm count, percentage of sperm with normal morphology and percentage of motile sperm, when marijuana was used more than once per week. Decline of semen quality was more pronounced if other recreational drugs were used simultaneously.[197] In a meta-analysis performed by Li et al. they found that alcohol consumption is associated with decreased semen volume.[198] Furthermore, a cross-sectional study published by Jensen et al. showed a negative association between habitual

alcohol consumption and semen quality. Sperm concentration, total sperm count and percentage of sperm with normal morphology declined when alcohol intake was ≥ 5 units per week. Most severe effect was observed when alcohol intake was > 25 units per week. One unit of alcohol contains approximately 12 g of ethanol.[199] Maternal alcohol consumption is also associated with decreased sperm concentration in male offspring, when mother drinks at least 4.5 alcohol unites/week during pregnancy.[200]

Overweight (BMI 25-29.9 kg/m^2) and obese (BMI ≥30 kg/m^2) men are at risk to have abnormal total sperm count (<40 millions) and sperm concentration (<15 x 10^6/ml).[201] This risk grows with increasing of BMI. Underweight persons also have a higher risk for poor semen quality.[201] Psychological stress may also influence negatively semen concentration, total sperm count, semen volume, motility and morphology.[202,198] According to meta-analyses exposure to mobile phone could influence sperm motility and viability negatively.[203,204,205] Exposure to WI-FI could also decrease sperm motility.[206,207]

In humans, the influence of endocrine disrupting chemicals on semen parameter was observed in accidental exposure to persistent organic pollutants. E.g. accidental exposure to polychlorinated biphenyls (PCBs) in Taiwan and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in Italy led to decline of semen quality in prenatally exposed men.[208,209] Perfluorooctanoic acid exposure in utero is also associated with poor semen quality in adult men. Exposure to nonpersistent EDCs (phthalates, bisphenol A) during prenatal and adult life may have a negative effect on semen quality as well.[210]

3. AIMS OF THE STUDY

A. Study I (Testicular growth during puberty in boys with and without a history of congenital cryptorchidism).

We hypothesised that cryptorchidism is a primary testicular damage, which influences pubertal testicular growth and does not alter central mechanisms of puberty. Thus, we aimed to evaluate testicular growth during puberty in boys with and without a history of congenital cryptorchidism. Additionally, our objective was to compare different tools for measurement of testicular size (ultrasound, orchidometer and ruler) during onset and progression of puberty. We also aimed to identify testicular volume based on ultrasound measurement for the definition of pubertal onset. Finally, we intended to validate value of ruler measurement (25 mm testicular length by ruler vs 3 mL by orchidometer) in denoting the onset of puberty.

B. Study II (The influence of low-saturated-fat and low-cholesterol dietary counselling on hormones and physical development during puberty).

In the context of STRIP study we hypothesised that low-saturated-fat and low-cholesterol dietary counselling could affect pubertal hormonal status. Thus, our aim was to assess the influence of low-saturated-fat and low-cholesterol dietary counselling on the level of pubertal hormones and progression of puberty in the subcohort of healthy STRIP participants.

C. Study III (Semen quality during young adulthood).

We also hypothesised that semen quality during young adulthood could improve due to further maturation of spermatogenesis. Thus, our last aim was to study possible changes in semen quality in longitudinal follow-up of young men representing general population from 19 to 29 years of age.

4. SUBJECTS AND METHODS

4.1. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism (Study I)

Population for this project was assembled using a prospective, population-based birth cohort recruited in Turku University Hospital in the period from 1997 to1999 to study the prevalence of congenital cryptorchidism in Finland. From 1494 prenatally recruited boys 35 were cryptorchid.[6] Moreover, 160 cryptorchid and 24 control boys born in 1997-2002 and recruited in Turku University Hospital to study risk factors of congenital cryptorchidism were considered as potential participants in extended pubertal follow-up. Up to 2 control participants were matched on date of birth ±14 d, parity, gestational age ± 7 d, maternal diabetes mellitus [yes/no] and maternal smoking during pregnancy [yes/no] for every cryptorchid boy.[6] Matched controls were selected from both: prospective cohort and boys recruited postnatally. All volunteering participants were examined shortly after birth and at 3 months of age. Furthermore, all boys with a history of congenital cryptorchidism, matched controls and every 10th boy without history of congenital cryptorchidism from the prospective cohort were invited for examination at 18 months of age.[6] Physical examination of the boys included the assessment of testicular position using modified Scorer criteria[6], measurement of testicular volume by ultrasound[211] and the length of penis by ruler[212]. Congenital cryptorchidism was defined as an absence of one or both testes from the normal scrotal position at birth or estimated date of delivery in case of preterm birth. Retractile testis was considered as a normal finding. Levels of reproductive hormones (FSH, LH, testosterone, inhibin B, INSL3, SHBG) were measured at the age of 3 months. The hormonal assay details and related hormonal results were described in previous publications.[213,214]

Our study aimed to continue the follow-up of boys described above throughout their puberty. Due to practical reasons, only candidates who still lived close to the Turku area were selected. Altogether, 165 boys with a history of congenital cryptorchidism and 306 controls were invited to participate in this longitudinal study. Finally, 31.5 % of invited boys with the history of congenital cryptorchidism (52 participants) and 21.2 % of invited controls (65 participants) agreed to take part in pubertal follow-up. Furthermore, 2 additional boys without a history of congenital cryptorchidism from the birth cohort were accepted by their request as controls while their brothers were invited to participate in this study. Thus, initial size of pubertal cohort was 119 boys in total. The first visit was designed at the age of 8.5

years aiming to start the follow-up before the onset of puberty. Seventeen boys had a history of bilateral congenital cryptorchidism and 35 boys were unilaterally cryptorchid at birth. Orchidopexy was needed to treat undescended testis in 15 unilaterally and 9 bilaterally cryptorchid boys. The rest of congenitally cryptorchid boys did not need surgical treatment due to spontaneous permanent descent of the testis. Cryptorchid boys underwent the first orchidopexy at the median age of 1.9 years (range, 0.24–9.3; n=15). Median age at the second orchidopexy was 5.6 years (range, 2.5–12.4; n=4). One participant was treated by orchidopexy four times.

One participant from the control group was ruled out from the final analysis due to a history of acquired cryptorchidism. One boy with a history of congenital cryptorchidism and 1 boy from the control group were excluded from the final analysis due to precocious puberty. Five participants with unilateral cryptorchidism lost their congenitally undescended testis later due to vanishing (1 boy) of the testis or orchidectomy (4 boys). Thus, data presented here contain 46 participants with a history of congenital cryptorchidism (cases), 65 boys without a history of congenital cryptorchidism (controls) and 5 monorchid boys. Altogether 1585 examinations were performed until current analysis. Six cases and seven controls dropped out before their puberty and pubertal testicular growth began. Three boys with a history of congenital cryptorchidism were still not in puberty at the time of the analysis. Thirty-seven cases excluding the five monorchid participants and 58 controls reached puberty. Thirteen cases, two monorchid participants and 33 controls had completed their pubertal follow-up. Five cases and six controls dropped out after onset of puberty. Follow-up of 22 case participants, 19 controls, and three monorchid boys was still uncomplete.

Altogether nine trained medical researchers were involved in examinations of boys. Participants were examined two times annually over the entire period of follow-up. Researchers participated in workshops 1-2 times annually to maintain consistency of examination techniques between observers. At every visit anthropometric measures (height and weight) were obtained. Regular Prader orchidometer (12 wooden beads from 1 to 25 mL)[102], ultrasound machine (Aloka Prosound 6, linear probe, 5–13 MHz and Aloka SSD-500, linear probe, 7.5 MHz; Hitachi Aloka Medical) and a plastic ruler (Hultafors) were used to measure testicular size. When testicular volume was less than 6 mL, results of evaluation by Prader orchidometer were reported to the nearest 0.5 mL, while larger testicular volumes were estimated to the nearest 1 mL. At the time of statistical analysis 147 testicular volumes measured by orchidometer were assessed to be >25 mL. Plastic ruler was used to evaluate testicular length in mm., but actual testicular volume was not calculated. Two equations were implemented to calculate testicular volume by ultrasound: ellipsoid formula ($\pi/6$ x

length x width^2) and Lambert's formula (0.71x length x width x height).[215] Testicular width and length by ultrasound was measured thrice at every examination. The average of three measurements of testicular volume was used in the calculations. Height of testis by ultrasound was measured once.

Testicular volume > 3 mL by Prader orchidometer (one or both testes) at 2 successive visits denoted the onset of puberty since testicular growth is the first physical sign of male puberty.[101]

To assess possible participation biases, we compared infancy hormone levels at 3 months of age and testicular volumes at 3 and 18 months of age between boys who decided to participate in pubertal follow-up after infancy and nonparticipating boys. The age of onset of pubertal testicular growth and anthropometric parameters between participants with and without history of congenital cryptorchidism were also compared at the 8.5 years of age and at the age of onset of puberty. Kolmogorov-Smirnov test was applied to assess the normality of the distributions. In pairwise comparison 2-samples *t* test or Mann-Whitney *U* test was used (in case of violation of the normality assumption).

Due to existence of three prepubertal boys at the time of our analysis, we decided to test their potential influence on the results related to the timing of pubertal onset. The data on the timing of pubertal onset were reanalysed twice assuming that these three participants achieve testicular volume by orchidometer > 3 mL at their next visit or at the age of 20 years. Since contralateral hypertrophy of the testis is common in monorchid boys[216], those five cases were not taken into analysis.

Concordance between orchidometer and ruler thresholds which denote the onset of puberty (> 3 mL and > 25 mm, respectively) was evaluated with cross tabulation method.

We also evaluated correlation between orchidometer and ultrasound in testicular volume measurements. Only boys without a history of congenital cryptorchidism were included in this subanalysis. Altogether 1850 examinations of right and left testes were randomly divided on two subsets (925 and 925 examinations) due to computational reasons. Successive analysis of two subsets using Passing-Bablok regression was performed for the validation of results. Bland-Altman method was applied to calculate the 95% limits of agreement.

Furthermore, in the control group we evaluated mean testicular volume by ultrasound in participants with testicular volume of 3 mL by orchidometer and testicular length of 25 mm by ruler.

Nonlinear mixed-effects model was used to analyze testicular growth pattern and model postpubertal testicular volume. Pubertal growth of the testis follows a sigmoid curve and can be described by four parameters: prepubertal and postpubertal testicular volume, age at mid-puberty and a scale parameter. Age at mid puberty represents the age when half of testicular growth is achieved. The scale parameter describes the duration of most rapid testicular growth (Figure 4).

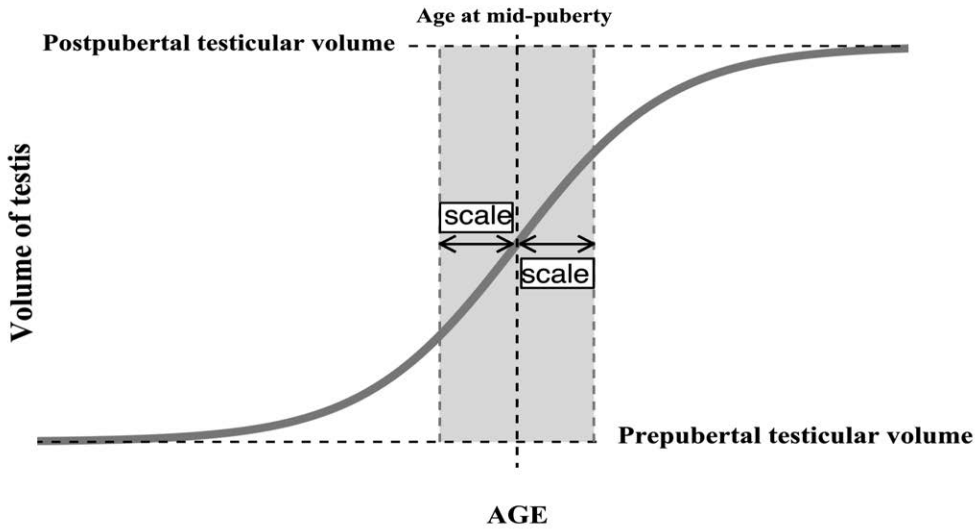

Figure 4. Four parameters describing pubertal growth of testis.

The figure is reproduced from Sergey Sadov, Jaakko J. Koskenniemi, Helena E. Virtanen, Antti Perheentupa, Jørgen H. Petersen, Niels E. Skakkebaek, Katharina M. Main, and Jorma Toppari. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. Journal of Clinical Endocrinology and Metabolism, 2016 Jun;101(6):2570-7 with permission from the Oxford University Press.

The nonlinear mixed-effects model describes both fixed and random effects. Fixed effects estimated means of four parameters mentioned above among boys with and without a history of congenital cryptorchidism, while random effects described variability of these parameters between participants.

Testicular growth patterns of the left and right testis were similar in boys without a history of congenital cryptorchidism and in boys with a history of bilateral congenital cryptorchidism. Simultaneously, among boys with a history of unilateral congenital cryptorchidism

testicular growth patterns of the left and right testis were different. Due to this observation 2 nonlinear mixed-effect models were designed to compare patterns of testicular growth between study groups. For the analysis of testicular growth in controls and boys with a history of bilateral congenital cryptorchidism we used mean volume of the right and left testis. In the first model, mean testicular volumes in controls and bilaterally cryptorchid boys were compared to volumes of the undescended testis in cases with a history of unilateral congenital cryptorchidism. Monorchid boys were excluded from this analysis. In the second model, volumes of the descended testes in the cases with a history of unilateral congenital cryptorchidism and mean testicular volumes in the control boys were compared.

Independent samples *t* test was used to compare modelled postpubertal testicular volumes of the undescended testes among boys with a history of unilateral cryptorchidism between participants who underwent orchidopexy and participants who did not need surgical intervention.

Statistical analysis was performed by the R statistical package, version 3.2.3 (R Development Core Team, R Foundation for Statistical Computing; www.r-project.org). A value of $P<0.05$ was considered statistically significant.

4.2. The influence of low-saturated-fat and low-cholesterol dietary counselling on hormones and physical development during puberty (Study II)

The Special Turku Coronary Risk Factor Intervention Project (STRIP) is open, randomized intervention study performed in the Research Center of Applied and Preventive Cardiovascular Medicine, University of Turku. Participants were enrolled at well-baby clinics in a period from 1990 to 1992 in the city of Turku (Finland). The population of 1062 healthy children were randomised into 540 intervention participants and 522 control participants at the age of 7 months. The intervention included dietary counselling aiming to achieve a fat intake of 30 % to 35 % of daily energy, cholesterol intake < 200 mg/d and an intake ratio of saturated/unsaturated fatty acid of 1:2. The intervention was initiated at 7 months of age. Participants visited research center annually through childhood and adolescence. At every research visit anthropometric measurements (height, weight and BMI) were obtained. Tanner staging (B/G/P) was assessed annually from the 9 years of age onwards. Four-day food record was used to assess food consumption. Food records were examined for accuracy by a nutritionist. Micro Nutrica software (Food and Nutrient Database of the

Social Insurance Institution, Finland) was used to analyse the intake of nutrients. This software was designed to recognize commonly used foods and dishes in Finland and calculate 66 nutrients. The original STRIP cohort was also described in previous publication related to this project in details.[141,217,218]

The subgroup of 200 STRIP participants was randomly selected to evaluate their pubertal hormonal status. Annual blood samples were available for hormonal measurements from 10 to 19 years of age. Tanner staging and anthropometric data were also collected at every visit once a year. In girls, serum levels of pubertal hormones (estradiol, FSH, LH and SHBG) were measured only until menarche since data on menstrual cycle was not collected and standardization of hormonal levels by the phase of menstrual cycle was not possible to perform. Data on the time of menarche were available. In boys, measurements of testosterone, FSH, LH, SHBG, AMH and inhibin B were conducted annually over entire pubertal follow-up period. Finally, data on 89 participants from the intervention group (48 boys and 41 girls) and 104 participants from the control group (52 boys and 52 girls) were analysed. A diagram describing the study flow of STRIP participants is presented in Figure 5.

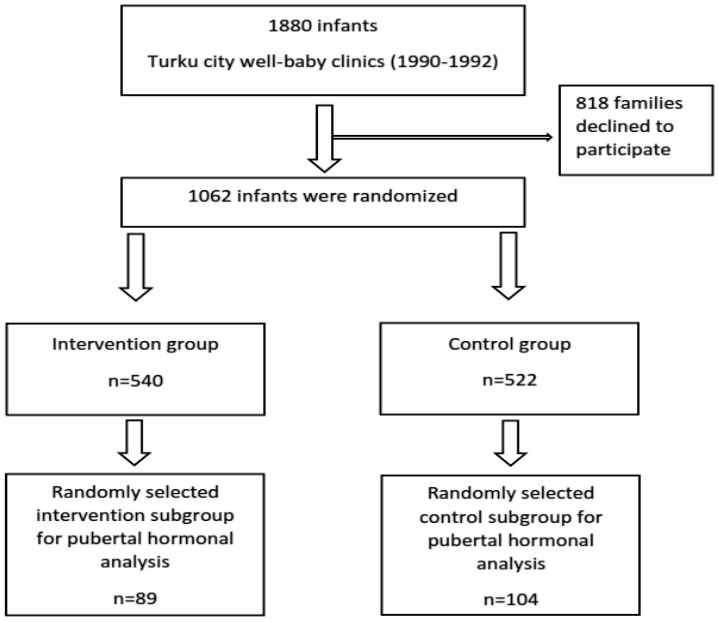

Figure 5. The flow of STRIP participants.

Pubertal blood samples were collected in a period from 2000 to 2011. All blood samples were obtained at standardised morning time and they were fasting samples. After collection samples were clotted and centrifugated. Obtained serum was stored in a refrigerator at 75ºC and, thereafter, used in hormonal analyses. All hormonal measurements were performed blindly at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark. Estradiol concentration was measured with radioimmunoassay (Pantex, USA). Two-sided fluoro-immunometric analysis (AutoDELFIA™ Immunoassay System, Wallac, Finland) was used to determine the concentrations of FSH and LH. Concentrations of SHBG, testosterone and AMH were analysed using chemiluminescent enzyme immunoassays (Access 2, Beckman-Coulter). Specific two-sided enzyme-linked immunoassay (InhibinB genII, Beckman Coulter, USA) was used to analyse concentrations of Inhibin B. Concentration of free testosterone was obtained by the method described by Vermeulen et al. 1999. In calculations total testosterone and SHBG values with a fixed albumin value were used.[219] Table 3. presents limits of detection and inter-assay coefficients of variation for used hormonal assays.

Table 3. Characteristics of hormonal assays.

Hormonal assay	LOD	Inter-assay CV
Estradiol	18 pmol/L	<15%
FSH	0.06 IU/L	<5%
LH	0.05 IU/L	<5%
SHBG	0.33 pmol/L	<6%
Testosterone	0.35 pmol/L	<6%
AMH	0.14 pmol/L	<6%
Inhibin B	3 pg/mL	<11%

LOD = limits of detection, CV = coefficient of variation.

Hormonal, anthropometric, cholesterol and dietary data were analysed using mixed models for repeated measures. Only FSH in the male participants was log-transformed due to violation of the normality assumption. In comparison of the difference in percentages of the control and intervention children who entered puberty at a certain age Fisher's exact test was used. Pairwise comparison was performed by Student's *t* test. In statistical analysis SAS software version 9.4 (SAS Institute, Cary, NC) and R statistical package, version 3.2.3 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org) were used. A value of $P<0.05$ was considered statistically significant.

4.3. Semen quality during young adulthood (Study III)

Compulsory military service in Finland implies a medical examination of all conscripts at the age of 18 years prior to their recruitment. The list of conscripts living in South West of Finland (Turku, Raisio and Kaarina) was provided to us by military authorities. Using this list we recruited our study population, which represented the general population of young men born in Finland. Inclusion to the study also required, that mother of participant was born in Finland. Medical fitness for military service did not influence the process of study recruitment. All listed conscripts were asked for voluntary visit in Turku University Andrology Unit for examination. Recommended abstinence time from ejaculation was at least 48 hours before providing the semen sample for laboratory analysis. The participation rate at 19 years of age (first visit) was 13.4 %.[192] The size of financial reward was 40-50 Euro per participant.

The enrolment to the study was performed between 1998 and 2003. The study population was divided on two cohorts. Cohort A represented by men born 1979-1981 and cohort B by men born in 1983. All participants were invited to Turku University Andrology Unit four times in total (Figure 6). At every visit (at the age of 19, 21, 25 and 29 years) physical examination was performed and semen sample for laboratory analysis was collected. Moreover, every time participants answered a standardized questionnaire. The cohort A was defined as a discovery cohort. Cohort B was designed as a validation cohort for the cohort A.

In the first round of the study (at the age of 19 years), 336 and 197 men participated in the cohort A and B, respectively. Ten years later 111 and 90 men took part in the conclusive fourth visit in the cohort A and B, respectively. Sixty-one men from cohort A and 52 men from the cohort B participated in all four rounds.

The characteristics of the participants at the age of 19 years have also been presented by Jørgensen et al. in 2011 in the publication describing recent trends in semen quality in Finland.[192]

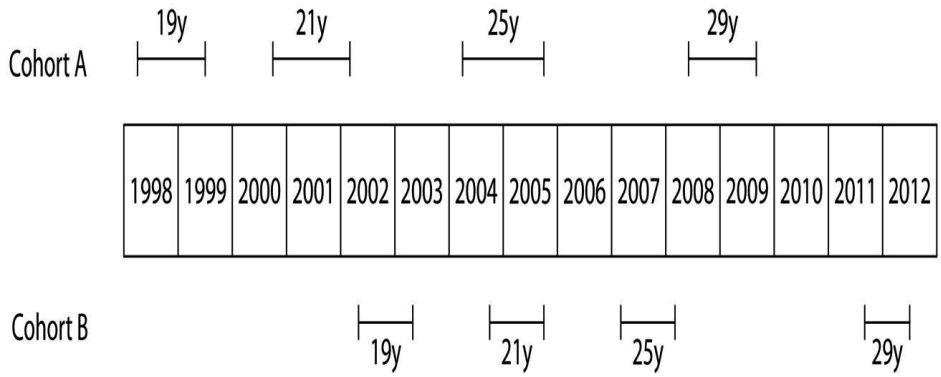

Figure 6. The timeline of study visits for the cohort A and B during the follow-up period.

The figure is reproduced from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 with permission from the Oxford University Press.

Male participants provided semen samples by masturbation in private room next to the laboratory. If the semen sample was produced at home, it was kept close to 37^0C and transferred to the andrological laboratory within 1 hour. The time of previous ejaculation was reported by participants. The abstinence time was calculated as the period between the current and previous ejaculation.

Semen volume was assessed by subtracting the weight of empty collection tube from the weight of tube with semen sample, presuming that 1 ml is equal to 1 g. Sperm motility was estimated by placing of 10 µl of well-mixed semen on a clean glass slide that was covered with 22 × 22 mm cover slip. During laboratory manipulations semen sample was kept at 37^0C and the microscope had a heated stage. The preparation was examined without delay at × 400 magnification. According to WHO manuals published in 1992 and 1999 sperm motility were classified on rapid progressive motility (class A), slow progressive motility (class B), nonprogressive motility (class C) and immotility (class D). Percentage of motile sperm calculated as a sum of classes A, B, and C. Motility was assessed twice in two aliquots of semen and the mean value was calculated. This average was used in further statistical analysis.

A solution of 0.6 mol/l $NaHCO_3$ and 0.4% (v/v) formaldehyde in distilled water was applied for dilution of semen sample, in which sperm concentration was evaluated using an improved Neubauer haemocytometer. Only spermatozoa with tails were accepted into count.

Semen volume, sperm motility and concentration in all semen samples related to this study were assessed by the same technician.

Morphological assessment of the semen was performed using a smear of the sample. It was stained with Papanicolaou and evaluated using strict criteria described by Menkveld et al..[220] Due to longitudinal nature of the project semen morphology was originally assessed at study rounds over a period of over 10 years. Men who participated in all four rounds were combined into two pools according their original cohort: 61 and 52 men from the cohorts A and B, respectively. Thereafter, morphology was assessed in random order during a period of a few weeks by two researchers (M.V. in cohort A and W.R. in cohort B). This protocol was applied to reduce the intra-observer variation in the assessment of semen morphology over time. Only the semen morphology of participants who visited all four times are reported.

To maintain high reliability in assessment of sperm concentration our center participated in an external quality control programme over the whole study period.[16] Five blinded semen samples were sent to the participating centers (including the Turku laboratory) from 8 to 10 times annually. Sperm concentration was evaluated according to the protocol described above. The results of our technician remained stable during the entire study period when compared with other participants in the external quality control programme.

During physical examination the Tanner stage of pubic hair, testicular size by Prader orchidometer and ultrasound machine were evaluated. The presence of vas deference was verified by palpation. The existence and severity of varicocele was assessed by Valsalva testing.

The participants received a standardized questionnaire before every visit at the Andrology Unit. The questionnaire contained information on age, pregnancy, disease and fertility history. Questions related to pregnancy and childhood disease history required collaboration of the participants with their parents.

Semen volume, sperm concentration, total sperm counts, total motile count and percentage of normal sperm had skewed distribution of residuals. Distributions of these variables were

normalized using square root transformation. Mixed model repeated measures analyses were applied to test for mean differences in the studied variables between visits in the cohort A and B. The Tukey–Kramer procedure was used to adjust P-values for pairwise comparisons. Abstinence time and quadratic term for abstinence time were included into statistical models as covariates. The adjustment of motility for the time between ejaculation and start of semen analysis was also conducted. A value of $P<0.05$ was considered statistically significant. Statistical analyses were performed by SAS for Windows version 9.2 (Cary, NC, USA).

4.4. Ethics

All three studies were approved by the Joint Ethics Committee of the University of Turku and Turku University Hospital, Finland. All participants and their parents (puberty follow-up) gave written informed consent for the study. All tree studies were performed according to the Helsinki II declaration.

5. RESULTS

5.1. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism (Study I)

5.1.1. Introduction to the results section (Study I).

Since only part of boys from the original birth cohort continued into pubertal follow-up, we started this section from the results of comparison between participants and nonparticipants to assess potential bias of attendance. Thereafter, the data on the timing of pubertal onset and modelled testicular growth in different subgroups of participants were presented. Finally, we presented the results of comparison of different tools for measurement of testicular size and provided validation and reference data on the use of ruler and ultrasound as a tool for defining the onset of puberty

5.1.2. Participation bias assessment

We compared hormone levels, testicular volumes and penile length between participants and nonparticipants at infancy to assess a potential bias of attendance. Nonparticipants from non-cryptorchid group had slightly larger testicular volumes at birth (0.14 vs 0.11 mL, $P = 0.002$, respectively) and at 3 months of age (0.21 vs 0.17 mL, $P < 0.001$, respectively) than participating non-cryptorchid boys. At the age of 18 months no difference in testicular volumes was observed. Small hormonal differences were discovered at the age of 3 months. Cryptorchid boys, who participated later in pubertal follow-up, had higher FSH levels than non-participant cryptorchid boys (2.0 vs 1.6 IU/L, P=0.009), and non-cryptorchid participants had higher INSL3 concentrations than those who did not participate (0.15 vs 0.10 ng/mL, P=0.003). Other hormonal parameters (LH, testosterone, SHBG, inhibin B, estradiol, FSH-inhibin B-ratio and LH-testosterone-ratio) or length of penis did not differ between participants and nonparticipants (Table 4).

Table 4. Assessment of potential attendance bias (Study I)

The table is modified from Koskenniemi. Clinical studies on testicular growth and descent. University of Turku, 2018 and reproduced with permission from Koskenniemi

	Cryptorchid		p-value	Non-cryptorchid		p-value
	Participant	Nonparticipant		Participant	Nonparticipant	
n	50	143		63	1420	
Volume of testis at birth (mL)[a]	0.09	0.09	0.81	0.11	0.14	**0.002**
Volume of testis at 3 months (mL)[a]	0.18	0.16	0.15	0.17	0.21	**<0.001[c]**
Volume of testis at 18 months (mL)[a]	0.17	0.18	0.42	0.18	0.19	0.28
Length of penis at birth (mm)	32.5	33.0	0.46	33.9	34.6	0.12
Length of penis at 3 moths (mm)	36.7	37.1	0.64	36.3	36.8	0.41
Length of penis at 18 months (mm)	39.9	40.6	0.22	39.4	40.0	0.38
LH IU/l	2.4	2.1	0.31	1.9	1.8	0.84
FSH IU/l	2.0	1.6	**0.009[c]**	1.3	1.4	0.34
Testosterone (nmol/l)	4.3	3.7	0.21	3.6	3.5	0.82
Free testosterone (pmol/l)[b]	25.7	22.1	0.09	20.5	21.2	0.65
SHBG (nmol/l)	149	148	0.90	151	147	0.57
Inhibin B (pg/mL)	436	437	0.97	477	462	0.54
Estradiol pg/mL	18.0	20.4	0.36	24.1	20.4	0.23
INSL3 (ng/mL)	0.11	0.13	0.23	0.10	0.15	**0.003**

[a] Ellipsoid formula was used for calculation. [b] Vermeulen's formula was used for calculation. [c] Pairwise comparison was performed using Mann-Whitney U-test (otherwise with independent samples T-test). Significant P-values (<0.05) shown in bold.

5.1.3. Timing of pubertal onset

The anthropometric parameters and testicular volume did not differ between boys with and without a history of congenital cryptorchidism at the age of 8.5 years and at the onset of

pubertal growth of testis, which could be also considered as a time of pubertal onset (Table 5). Thus, among controls estimated age of pubertal onset (or onset of pubertal testicular growth) was 11.7±1.1 and among cases 11.8±1.0 years (mean ±SD, P=0.56).

Table 5. Characteristics of study population at the age of 8.5 years and at the onset of puberty, defined by the onset of pubertal testicular growth.

The table is reproduced from Sergey Sadov, Jaakko J. Koskenniemi, Helena E. Virtanen, Antti Perheentupa, Jørgen H. Petersen, Niels E. Skakkebaek, Katharina M. Main, and Jorma Toppari. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. Journal of Clinical Endocrinology and Metabolism, 2016 Jun;101(6):2570-7 with permission from the Oxford University Press.

	First visit at age of 8.5 years (Mean ± SD)		At the onset of puberty	
	Case*	Control	Case*	Control
n	46	65	37	58
Age (years)	8.5±0.05	8.5±0.05	11.7±1.1	11.8±1.0
Weight (kg)	29.9±6.0	29.4±4.8	42.2±9.5	41.6±8.8
Height (cm)	132.8±5.5	133.9±6.0	149.3±7.5	150.7±7.9
BMI (kg/m^2)	16.8±2.5	16.3±1.8	18.8±3.2	18.2±2.6
Testicular volume by orchidometer (right, mL)	1.7±0.4	1.7±0.4	4.2±1.0	4.0±0.6
Testicular volume by orchidometer (left, mL)	1.8±0.4	1.7±0.3	4.1±0.9	3.8±0.7

*Monorchid boys (n=5) were excluded from analysis

5.1.4. Modelled testicular growth during puberty assessed by ultrasonography

Among controls, a mean modelled prepubertal and postpubertal volume of testis was 0.22 mL and 10.2 mL, respectively, as judged by ultrasonography. The half of pubertal growth of testis was achieved at 13.4 years of age. Since estimated scale parameter was 0.73 years, approximately 46 % of pubertal testicular growth occurred between 12.7 and 14.1 years, which was a period of the fastest growth of testes.

Modelled growth patterns of testis in different subgroups of participants is presented in Figure 7.

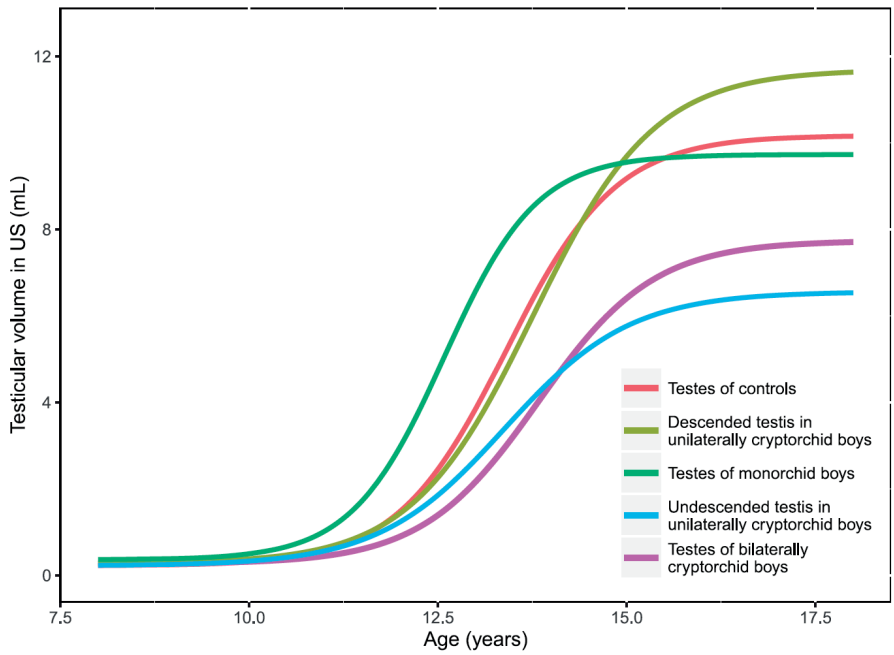

Figure 7. Testicular growth patterns estimated with nonlinear mixed-effects model in different subgroups of participants.

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In comparison of testicular growth patterns between boys with and without a history of congenital cryptorchidism, no differences were observed in the ages at peak testicular growth and scale parameters (Table 6).

The modelled prepubertal volume of descended testis of unilaterally cryptorchid boys was 0.05 mL bigger when compared to the testicular volume of controls (P=0.008). The prepubertal volumes of undescended testes among unilaterally or bilaterally cryptorchid boys did not differ from testicular volumes in controls (Table 6).

Boys without a history of congenital cryptorchidism had bigger modelled postpubertal testicular volumes when compared to undescended testes of boys with a history of unilateral and bilateral congenital cryptorchidism (+3.6 mL, P=0.001 and +2.4 mL, P=0.01, respectively; Table 6).

Monorchid testes were 0.14 mL bigger prepubertally than testes of controls (P=0.002, [0.05, 0.22]). Simultaneously, in subgroup of monorchid children, postubertal testicular volume, age at peak testicular growth, or scale parameter did not differ from controls.

In the comparison of modelled postpubertal volume of spontaneously descended testes and operated testes in participants with a history of unilateral congenital cryptorchidism, no significant difference was observed.

Table 6. Differences in four parameters estimated with nonlinear mixed effect model in boys with a history of congenital cryptorchidism.

Control boys were used as a reference.

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Parameters	Testis of controls	Descended Testis of Unilaterally Cryptorchid Boys Mean Difference (95% CI)*	Undescended Testis of Unilaterally Cryptorchid Boys Mean Difference (95% CI)	Testes of Bilaterally Cryptorchid Boys Mean Difference (95% CI)
Prepubertal volume (mL)	Reference	0.05 (0.01, 0.09)**	0.0003 (-0.04, 0.03)	0.04 (-0.0005, 0.09)
Postpubertal volume (mL)	Reference	1.30 (-0.30, 2.91)	-3.61 (-5.01, -2.22)**	-2.43 (-4.37, -0.50)**
Age at peak testicular growth (y)	Reference	0.32 (-0.25, 0.88)	-0.02 (-0.63, 0.59)	0.42 (-0.36, 1.20)
Scale parameter (y)	Reference	0.07 (-0.03, 0.18)	0.11 (-0.0002, 0.21)	0.04 (-0.09, 0.17)

*Monorchid boys (n=5) were excluded from analysis. ** Statistically significant P-value (<0.05)

5.1.5. Comparison of different tools for measurement of testicular size.

Agreement between puberty onset cut-off values measured by orchidometer (3 mL) and ruler (25mm) is presented in Table 7 (see also Figure 8). The analysis was based on the measurements of 1488 right and 1514 left testes (3002 measurements in a total).

Table 7. Agreement between measurements by orchidometer and ruler for the defining of the onset of puberty.

The table is reproduced from Sergey Sadov, Jaakko J. Koskenniemi, Helena E. Virtanen, Antti Perheentupa, Jørgen H. Petersen, Niels E. Skakkebaek, Katharina M. Main, and Jorma Toppari. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism. Journal of Clinical Endocrinology and Metabolism, 2016 Jun;101(6):2570-7 with permission from the Oxford University Press.

	Length of testis by ruler ≤ 20 mm	Length of testis by ruler 21 – 25 mm	Length of testis by ruler 26–30 mm	Length of testis by ruler > 30 mm
Testicular volume by orchidometer ≤ 3 mL	818 (100%)	516 (91%)	55 (17%)	0 (0%)
Testicular volume by orchidometer > 3 mL	0 (0%)	50 (9%)	263 (83%)	1300 (100%)

Testicular volume by orchidometer was > 3 mL in all 1300 measurements, when length of testis by ruler was > 30 mm. Testicular volume by orchidometer was > 3 mL only in 9% of measurements, when the length of testis by ruler was 21–25 mm, and in 0% of measurements, when length of testis by ruler was ≤ 20 mm. If ruler length was assessed in the range from 26 to 30 mm, 83% of testes measured by orchidometer were >3 mL (see also Figure 8).

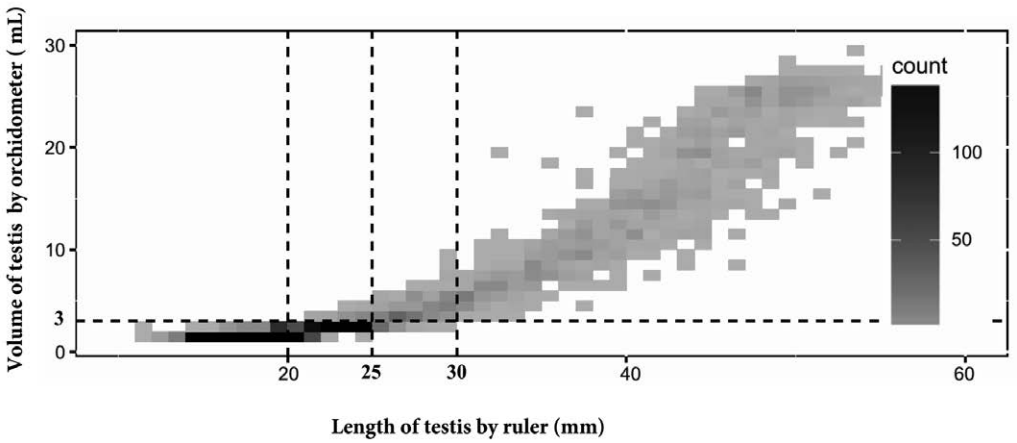

Figure 8. Concordance between measurements of testicular size by orchidometer and ruler.

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When testicular volumes by orchidometer in boys without history of cryptorchidism were equal to 3 mL, volume of testes by ultrasonography (mean ±SD) calculated by ellipsoid and Lambert's formula were 0.75 ±0.35 and 1.58 ±0.39 mL, respectively. The ultrasonography volume equivalent for 25 mm length by ruler was 0.84 ±0.28 and 1.68 ±0.38 mL, respectively.

Agreement between measurements of testicular size by orchidometer and ultrasonography analysed by the Passing-Bablok regression and modified Bland-Altman method is presented in Figure 9. Every milliliter of the testicular volume estimated by ultrasonography amplifies the discordance between orchidometer and ultrasonography by 0.27 and 1.04 mL using Lambert's and ellipsoid formulas, respectively. Moreover, the 95% limits of agreement widen with a growth of testis, more notably, when volume of testes were calculated by ellipsoid formula, e.g. testicular volume of 10 mL calculated by Lambert's formula and ellipsoid has the 95% limits of agreement of 0-7.5 mL and 5-20 mL, respectively.

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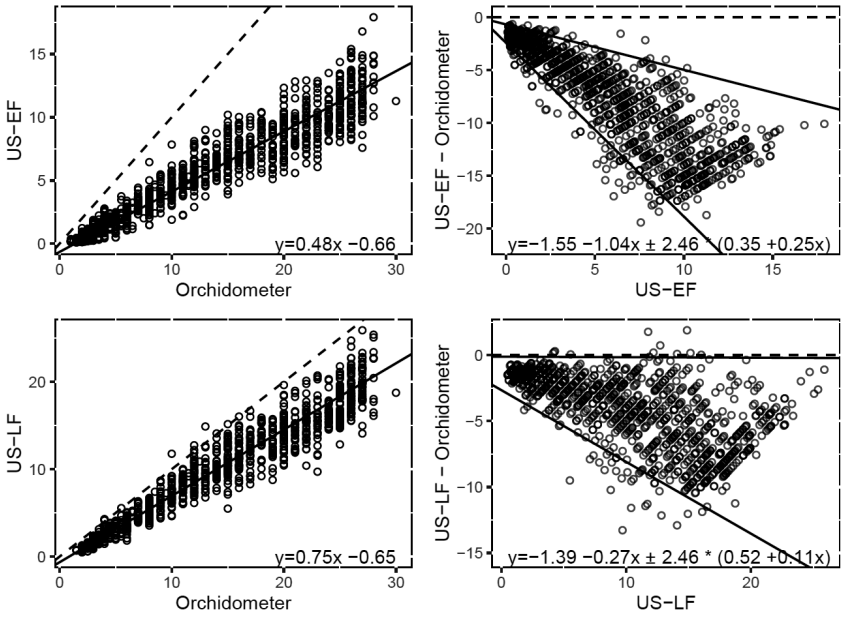

Figure 9. Agreement between measurements of testicular size by orchidometer and ultrasonography.

US-EF = Volume of testis (mL) by ultrasonography calculated with ellipsoid formula, US-LF = volume of testis (mL) by ultrasonography calculated with Lambert's formula, orchidometer = volume of testis (mL) by orchidometer. A perfect agreement between two measurements are indicated by dashed lines. The left-hand panel represents regression analyses, which were performed using Passing-Bablok method. Results of modified Bland-Altman analyses are presented on right-hand panel.

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5.2. The influence of low-saturated-fat and low-cholesterol dietary counselling on hormones and physical development during puberty (Study II)

In boys and girls, pubertal hormone levels did not differ significantly between intervention and control participants over entire observation period. Boys from control group were shorter (P=0.04) than boys from intervention group. Other anthropometric characteristics

(weight and BMI) did not differ between control and intervention boys. In girls, the analysis of anthropometric parameters (height, weight and BMI) did not reveal any statistically significant differences. Hormone and anthropometric results are presented in Tables 8, 9 and Figures 10-13. Data on transition into puberty in boys and girls by certain age are presented in Table 10.

Table 8. Hormonal and anthropometric characteristics of the male participants: mean (SD)[221]

Age (years)	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**p-value**
n, intervention	15-17	40-45	38-43	38-46	39-44	30-39	39-43	28-35	33-36	7-10	
n, control	15-20	38-45	39-45	38-46	45-51	31-41	34-40	31-40	36-41	18-20	
Height, cm, intervention	141.3 (5.9)	148.3 (7.1)	154.3 (7.6)	161.8 (9.3)	169.4 (10.0)	175.1 (8.0)	179.8 (7.4)	181.3 (7.2)	182.3 (7.1)	180.9 (5.1)	**0.04**
Height, cm, control	140.7 (5.6)	145.6 (5.7)	151.1 (6.7)	157.5 (7.4)	166.2 (7.6)	172.3 (7.2)	176.5 (6.7)	178.5 (6.4)	178.9 (6.3)	178.2 (5.4)	
Weight, kg, intervention	34.3 (4.9)	38.7 (7.3)	43.4 (8.53)	49.9 (9.7)	56.0 (9.7)	61.2 (10.1)	67.1 (9.3)	69.2 (9.8)	72.5 (10.2)	75.1 (13.5)	0.37
Weight, kg, control	34.8 (6.1)	37.5 (6.2)	41.5 (8.0)	47.5 (8.7)	54.6 (9.4)	60.3 (10.0)	63.2 (9.4)	68.4 (9.1)	70.2 (9.4)	68.1 (7.7)	
BMI, kg/m^2, intervention	17.2 (2.4)	17.5 (2.5)	18.2 (2.9)	19.0 (2.8)	19.5 (2.8)	20.0 (3.1)	20.8 (2.7)	21.0 (2.8)	21.8 (2.6)	23.0 (4.0)	0.77
BMI, kg/m^2, control	17.5 (2.4)	17.6 (2.3)	18.1 (2.8)	19.1 (2.8)	19.7 (3.0)	20.3 (3.0)	20.3 (2.7)	21.5 (2.7)	22.0 (2.8)	21.5 (2.7)	
Testosterone, nmol/L, intervention	0.04 (0.1)	0.9 (2.2)	4.0 (5.1)	10.2 (6.8)	16.4 (6.3)	19.6 (5.3)	21.8 (5.4)	21.8 (5.6)	22.0 (5.2)	21.7 (7.9)	0.32
Testosterone, nmol/L, control	0.1 (0.1)	0.6 (1.7)	3.1 (4.5)	9.2 (7.4)	15.4 (7.3)	18.6 (5.8)	20.6 (5.9)	21.8 (5.6)	21.3 (5.9)	22.4 (5.4)	
Free testosterone, pmol/L, intervention	0.5 (1.4)	10.5 (28.3)	44.2 (61.7)	135.4 (95.0)	223.4 (98.5)	284.6 (78.6)	336.8 (79.2)	333.3 (87.8)	356.5 (76.2)	350.0 (66.8)	0.41
Free testosterone, pmol/L, control	0.7 (1.7)	5.7 (16.4)	34.6 (52.5)	111.6 (98.8)	211.6 (99.5)	273.0 (86.0)	312.2 (82.9)	336.7 (78.6)	339.8 (81.9)	353.8 (71.2)	
LH, U/L, intervention	0.2 (0.3)	0.8 (0.7)	1.5 (1.1)	2.4 (1.1)	3.0 (1.2)	3.7 (1.4)	4.0 (1.5)	4.2 (1.7)	4.2 (1.4)	5.6 (1.4)	0.08
LH, U/L, control	0.2 (0.3)	0.5 (0.7)	1.4 (1.1)	2.5 (1.5)	2.9 (1.1)	3.4 (1.1)	3.8 (1.4)	3.8 (1.7)	4.2 (1.5)	3.9 (1.1)	
FSH, U/L, intervention	1.0 (0.7)	1.8 (1.1)	2.1 (1.4)	2.5 (1.8)	2.6 (1.6)	3.4 (1.9)	3.8 (2.0)	3.4 (1.6)	3.3 (1.8)	4.0 (1.9)	0.47
FSH, U/L, control	1.0 (0.7)	1.3 (0.9)	2.1 (1.7)	2.7 (1.9)	3.0 (2.2)	3.1 (1.7)	3.8 (2.5)	3.3 (2.3)	3.7 (3.2)	3.8 (4.1)	
SHBG, nmol/L, intervention	94.2 (24.2)	89.8 (30.6)	88.1 (32.4)	65.8 (28.1)	51.0 (22.2)	39.2 (13.4)	35.1 (12.2)	34.0 (9.8)	29.5 (8.7)	35.0 (13.6)	0.93
SHBG, nmol/L, control	92.6 (36.7)	88.5 (30.9)	85.3 (31.2)	70.1 (32.5)	49.7 (21.7)	37.9 (16.7)	36.4 (14.2)	33.8 (13.2)	32.0 (11.9)	30.4 (8.9)	
Inhibin B, pg/mL, intervention	88.8 (43.8)	113.5 (52.7)	154.2 (63.3)	180.6 (67.0)	185.5 (62.3)	192.2 (65.0)	194.8 (66.6)	197.0 (73.3)	210.8 (73.2)	196.1 (71.9)	0.43
Inhibin B, pg/mL, control	91.2 (37.0)	120.1 (67.4)	157.1 (70.2)	181.6 (65.4)	193.8 (69.2)	214.0 (74.9)	205.2 (73.3)	218.7 (81.5)	212.5 (70.5)	221.5 (96.6)	
AMH, pmol/L, intervention	609.1 (281.2)	467.0 (262.5)	325.9 (301.4)	159.4 (209.1)	59.9 (32.0)	57.0 (40.2)	57.9 (25.2)	58.6 (27.5)	66.2 (25.8)	84.0 (35.3)	0.34
AMH, pmol/L, control	514.3 (210.2)	533.5 (285.8)	323.8 (211.7)	170.7 (160.4)	62.5 (33.9)	56.2 (28.7)	58.7 (25.8)	68.6 (33.3)	71.9 (34.0)	78.2 (36.9)	

Table 9.Hormonal and anthropometric characteristics of the premenarcheal female participants: mean (SD)[221]

Age (years)	**10**	**11**	**12**	**13**	**14**	**p-value**
n, intervention/control	18/20	35/43	28/35	19/19	3/7	
Height, cm, intervention	141.2 (5.2)	147.4 (6.3)	153.3 (6.4)	159.6 (6.8)	161.4 (8.6)	0.78
Height, cm, control	143.3 (5.9)	148.5 (6.8)	153.0 (7.1)	155.8 (6.4)	161.0 (6.7)	
Weight, kg, intervention	33.5 (5.4)	37.6 (7.7)	41.1 (6.7)	44.4 (5.0)	46.7 (5.8)	0.23
Weight, kg, control	39.9 (11.1)	41.5 (10.7)	43.4 (10.5)	43.2 (7.8)	46.5 (6.3)	
BMI, kg/m^2, intervention	16.8 (2.1)	17.2 (2.9)	17.4 (2.2)	17.4 (1.3)	17.9 (0.3)	0.20
BMI, kg/m^2, control	19.2 (4.3)	18.7 (3.8)	18.4 (3.5)	17.7 (2.4)	17.9 (1.8)	
Estradiol, pmol/L, intervention	25.9 (22.9)	62.4 (53.6)	104.9 (67.6)	159.1 (60.6)	277.0 (187.6)	0.89
Estradiol, pmol/L, control	32.8 (21.3)	79.1 (66.0)	112.2 (67.2)	156.8 (69.2)	187.4 (45.1)	
LH, U/L, intervention	0.2 (0.3)	0.8 (1.3)	1.8 (1.6)	4.3 (3.0)	6.3 (3.5)	0.53
LH, U/L, control	0.2(0.2)	1.2 (1.5)	2.4 (2.4)	4.0 (2.6)	5.0 (1.6)	
FSH, U/L, intervention	1.9 (1.0)	3.1 (1.8)	4.0 (1.8)	4.7 (1.3)	5.1 (0.6)	0.26
FSH, U/L, control	2.3 (0.9)	3.4 (1.4)	4.4 (1.8)	4.9 (1.4)	4.6 (1.4)	
SHBG, nmol/L, intervention	91.3 (29.9)	81.5 (32.8)	83.3 (27.7)	71.2 (30.0)	93.7(16.6)	0.67
SHBG, nmol/L, control	70.8 (33.0)	74.0 (42.7)	73.0 (39.6)	85.5 (42.3)	89.4 (51.9)	

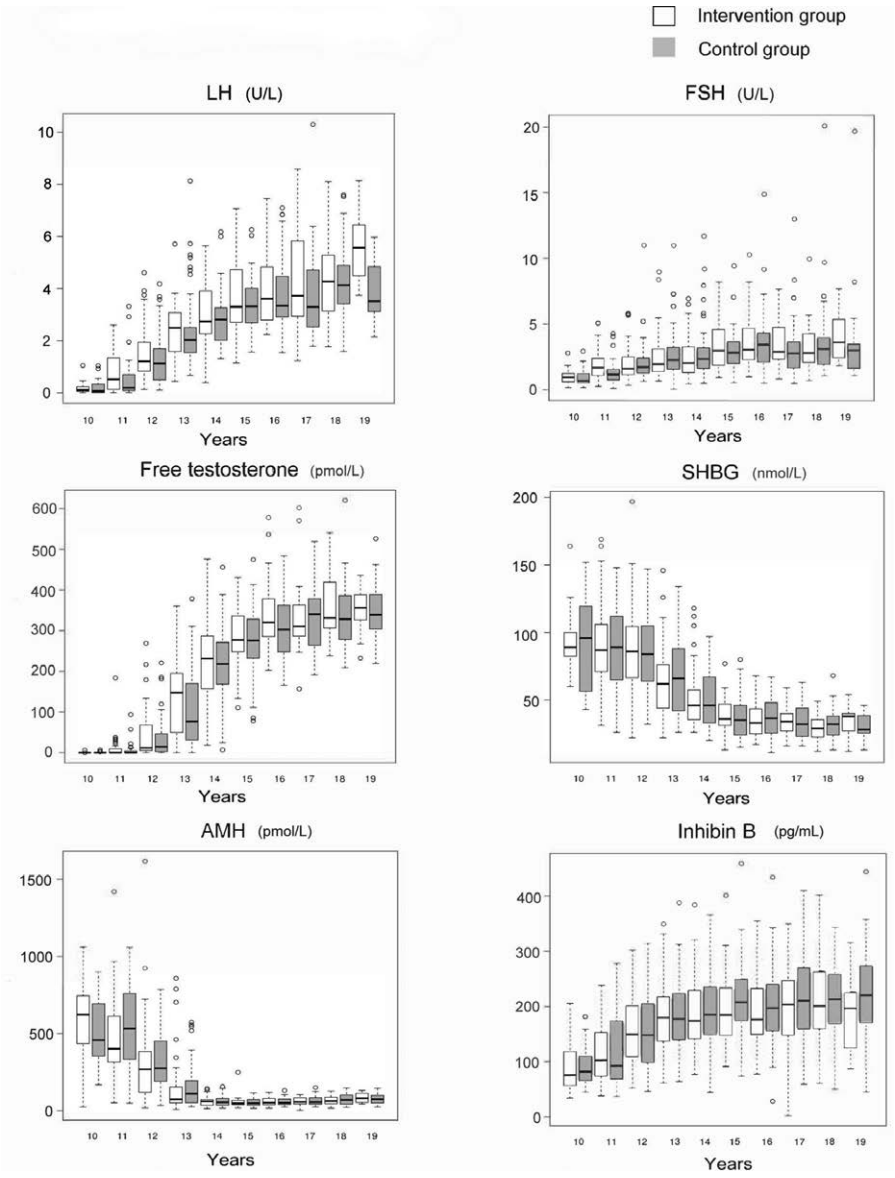

Figure 10. Hormone levels in the boy cohort.[221]

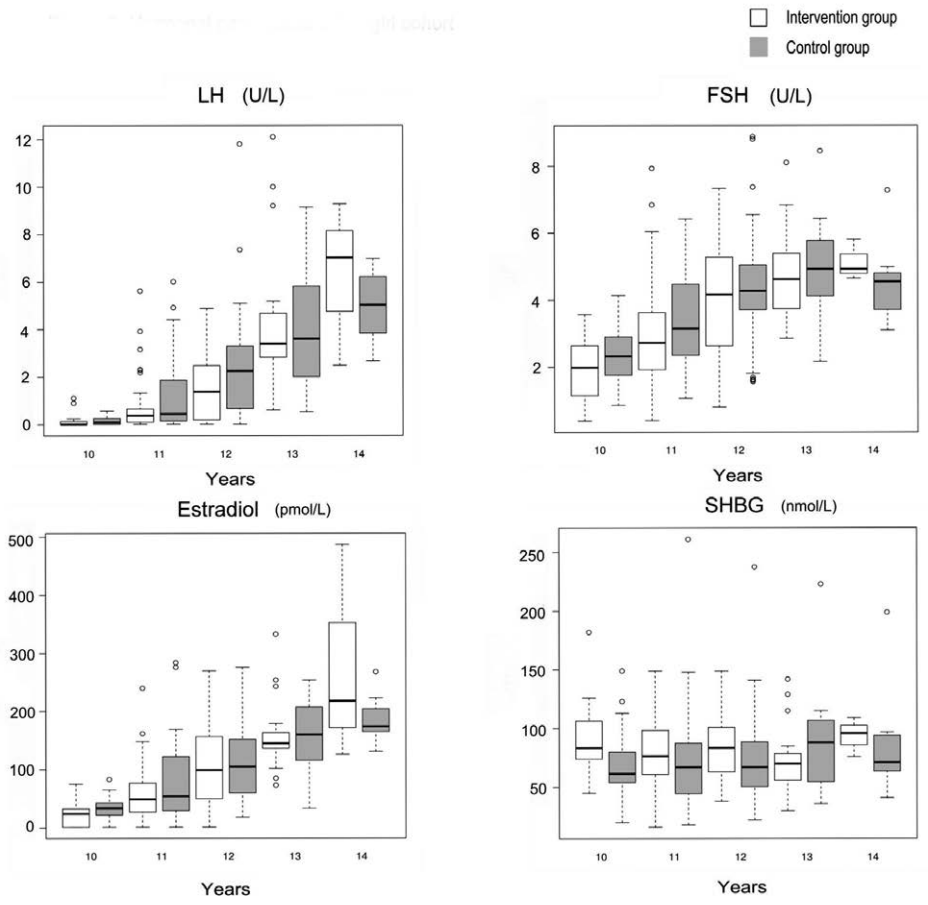

Figure 11. Hormone levels in the girl cohort[221]

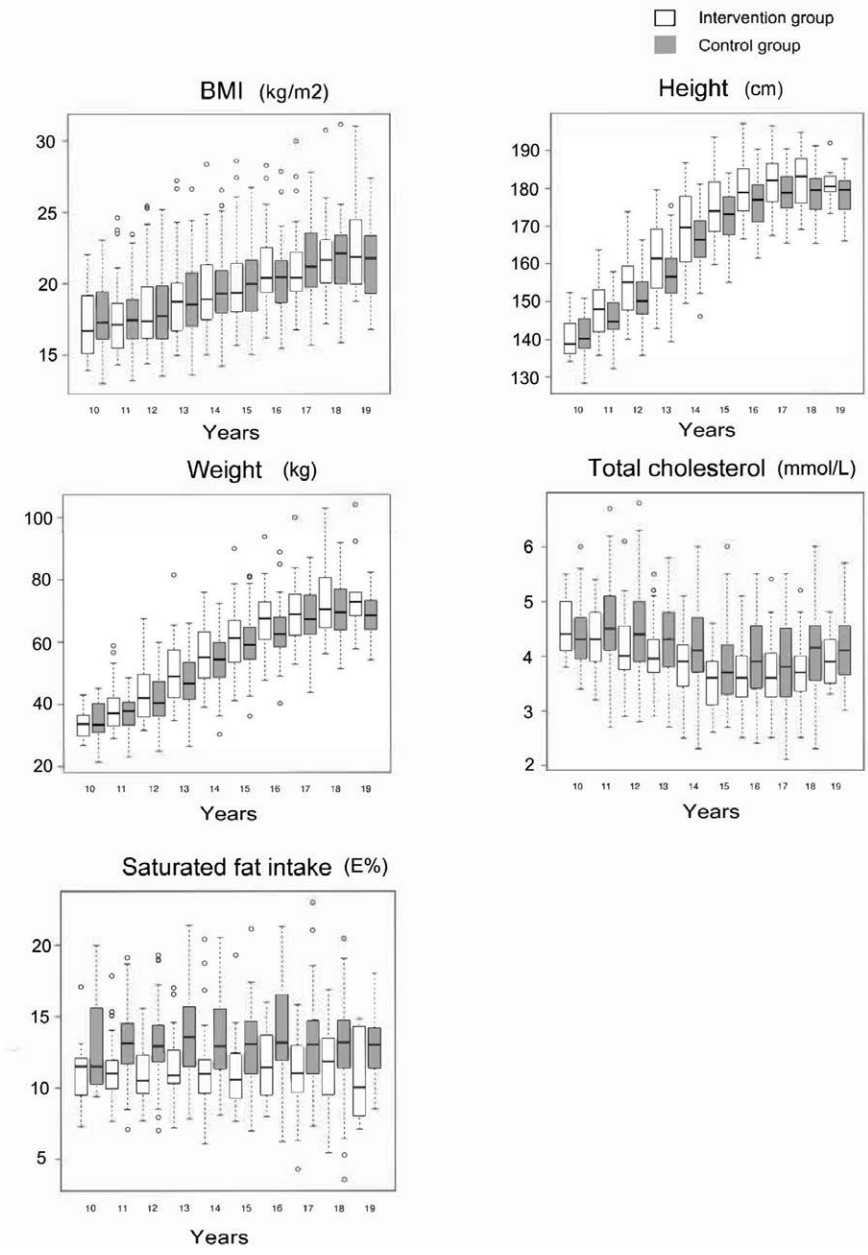

Figure 12. Anthropometric parameters, total cholesterol and saturated fat intake in the boy cohort.[221]

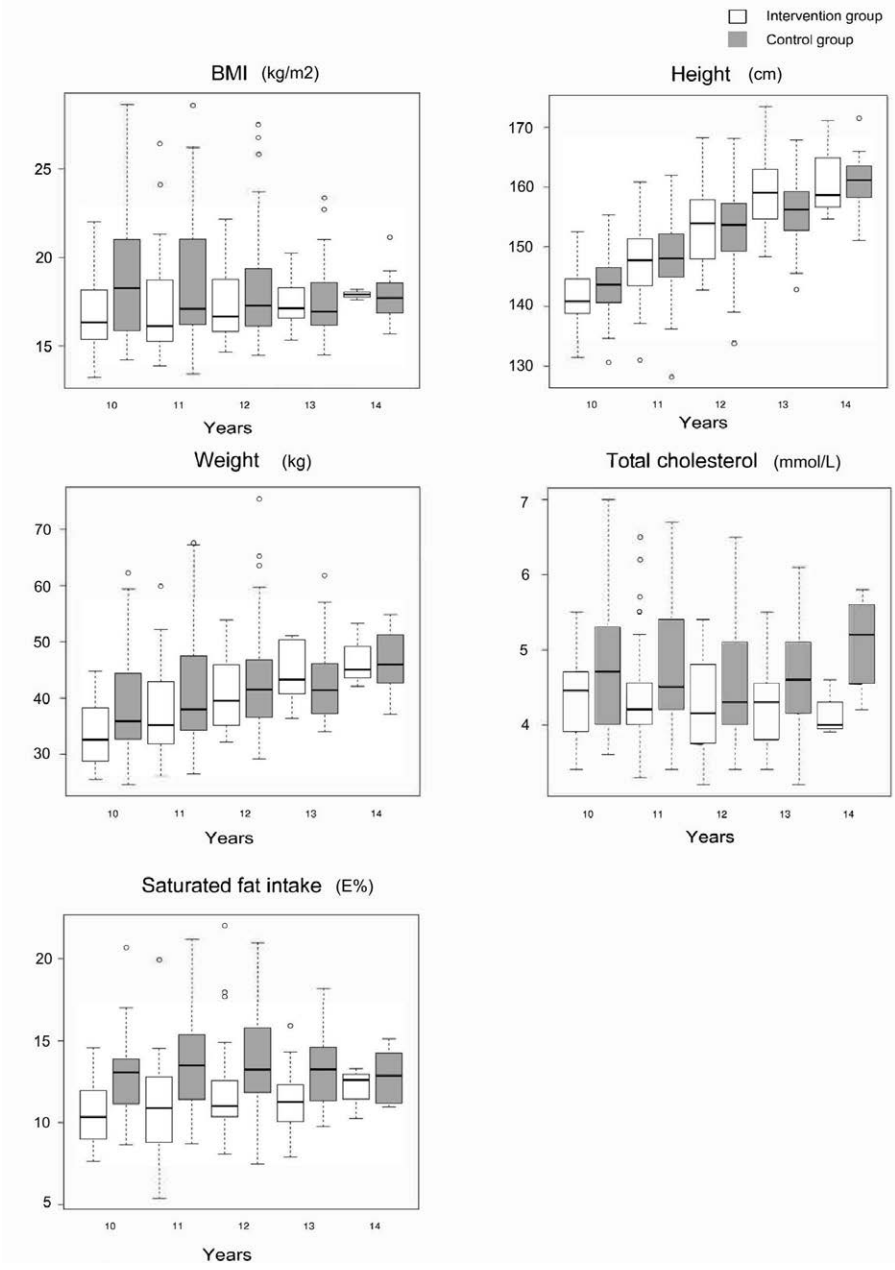

Figure 13. Anthropometric parameters, total cholesterol and saturated fat intake in the girl cohort.[221]

Table 10. Percentage of children who have entered puberty by certain age. (Boys: Tanner > G1P1; Girls: Tanner >B1P1)[221]

	By 10 years,%	By 11 years,%	By 12 years,%	By 13 years,%	By 14 years,%
Boys (p=0.07)					
Control	11	42	82	100	100
Intervention	7	66	88	98	100
Girls (p=0.24)					
Control	15	60	88	100	
Intervention	18	42	85	100	

Pubertal progression described by Tanner stages in boys and girls is shown in Table 11. Control boys reached G4, G5 and P5 stages later than boys from intervention group. Mean age at G4 in control and intervention groups was 14.7 and 14.0 years, respectively (p=0.008). Mean age at G5 in control and intervention groups was 16.4 and 15.8 years, respectively (p=0.008). Mean age at P5 in control and intervention groups was 15.8 and 15.2 years, respectively (p=0.03). In further statistical analysis we did not find interaction between Tanner staging (G) in boys and their hormonal status.

Table 11. Mean age of Tanner stages among study children.[221]

	Control group	**Intervention group**	**P-value**
BOYS, age of G/P-stages in years (mean, CI 95%)			
G2	11.5 (11.3,11.8) n=40	11.4 (11.1,11.7), n=35	0.45
G3	13.1 (12.8,13.4), n=39	12.7 (12.4,13.0), n=38	0.09
G4	14.7 (14.4,15.0), n=42	14.0 (13.6,14.4), n=35	**0.008**
G5	16.4 (16.1,16.7), n=42	15.8 (15.5,16.1), n=42	**0.008**
P2	13.0 (12.7,13.3), n=29	12.6 (12.2,12.9), n=31	0.1
P3	13.8 (13.4,14.3), n=22	13.4 (13.0,13.7), n=26	0.09
P4	14.5 (14.1,14.8), n=31	14.1 (13.7,14.5), n=23	0.22
P5	15.8 (15.4,16.2), n=40	15.2 (14.9,15.6), n=40	**0.03**
GIRLS, age of B/P-stages in years (mean, CI 95%)			
B2	11.4 (11.1,11.6), n=46	11.5 (11.2,11.8), n=32	0.48
B3	12.6 (12.3,12.9), n=44	12.8 (12.5,13.2), n=31	0.25
B4	14.2 (13.9,14.5), n=43	14.2 (13.9,14.6), n=35	0.93
B5	15.9 (15.6,16.3), n=50	16.0 (15.6,16.3), n=39	0.97
P2	12.0 (11.7,12.3), n=30	12.0 (11.6,12.3), n=25	0.86
P3	12.7 (12.4,12.9), n=41	12.6 (12.2,13.0), n=21	0.87
P4	13.9 (13.7,14.2), n=44	13.8 (13.4,14.1), n=35	0.46
P5	15.6 (15.2,15.9), n=51	15.4 (15.0,15.8), n=37	0.46

Statistically significant p-values in bold

In control and intervention girls the mean age at menarche was 12.9 years (P=0.95). In majority of children the first indicator of puberty was G2 and B2 in boys and girls, respectively. The proportion of children, who had P2 before G2/B2, did not differ between control and intervention participants (p=0.23 for boys, p=1.0 for girls).

Serum cholesterol concentrations (P=0.005 and P=0.03 for boys and girls, respectively) and saturated fat intake (P< 0.001 and P= 0.002 for boys and girls, respectively) were lower in the intervention group of children (Figures 12 and 13).

5.3. Semen quality during young adulthood (Study III)

The findings obtained by physical examination and the self-reported information obtained by questionnaire in both cohorts (A and B) at the age of 19 years are presented in Table 12. Semen parameters over entire 10 years follow-up period are presented in Tables 13-16.

P-values were adjusted for the time of abstinence using a multivariate analysis. We found no influence of tobacco smoking on semen quality in further analysis.

Statistically significant improvement of sperm motility was observed in both cohorts. In cohort A, a percentage of motile sperm improved from 66 to 82% ($P < 0.001$) and, in cohort B, from 76 to 81% ($P = 0.03$) between 19 years and 29 years, respectively (Tables 13 and 15). Sperm motility also increased among men from cohort A, who participated in all four rounds (Table 14). Men from cohort B, who participated in all four rounds, showed improvement in total count of motile sperm, simultaneously, the increase in a percentage of motile sperm was significant only between 19 years and 21 years (Table 16).

A percentage of morphologically normal sperm increased during the follow-up of 10 years in both cohorts. However, sperm morphology was assessed only among men, who attended all four rounds (Tables 14 and 16).

Statistically significant improvement of sperm volume between 19 years and 29 years was observed only in cohort A (Tables 13 and 14). In combined analysis of men from cohort A and B together (men participated in all four rounds, n=113), median semen volume increased from 3.5 to 4.0 mL between 19 years and 25 years ($P = 0.03$, no statistically significant changes between other rounds, Figure 14).

During the follow-up period of 10 years slight tendency towards higher total sperm counts and sperm concentrations was observed. In cohort A this slight improvement was not statistically significant (Table 13 and 14). Simultaneously, in cohort B total sperm count improved from 172 millions at 19 years to 225 millions at 29 years ($P=0.009$, Table 15). This finding was not repeatable in men who attended all study visits, when only cohort B or both cohorts together were analysed (Table 16 and Figure 14). In combined analysis of men from both cohorts, who participated in all rounds (n=113), median sperm concentrations increased from 54 million/mL at 19 years to 66 million/mL at 29 years ($P=0.62$) and median total sperm count from 192.2 millions at 19 years to 224.4 millions at 29 years ($P=0.1$), differences between other rounds in pairwise comparison were also not statistically significant (Figure 14).

Nine samples from 6 participants were azoospermic. All these samples were originated from cohort A.

Table 12. The findings obtained by physical examination and the self-reported conditions at the 19 years of age.

The table was modified from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 and reproduced with permission from the Oxford University Press.

	Cohort A; n=336 (%)	Cohort B; n=197 (%)
Findings obtained by physical examination		
Hydrocele	0	1.5
Varicocele	22.3	20
Been diagnosed as having		
Chlamydia	1.2	1.5
Cystitis or pyelonephritis	2.1	2
Diabetes	0	0
Epididymitis	0.6	0
Prostatitis	0.9	0.5
Thyroid disease	0.3	1
Been treated for		
Cryptorchidism	0.9	1.5
Inguinal hernia	6.8	3
Testicular torsion	0.9	1
Testicular cancer	0	0
Varicocele	1.5	0.5
Other diseases of penis, urethra or scrotum	1.2	1
Other history		
Caused a pregnancy	2.1	6.1
Experienced fertility problems	0.9	0
Taken any medication during past 3 months	14.6	20
Subgroup of men not affected by any of the above mention conditions	53.9	55.3

Table 13. Semen parameters of men in the cohort A over entire follow-up period.

The table was modified is from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 and reproduced with permission from the Oxford University Press.

Rounds	**I (19 y)**	**II (21 y)**	**III (25 y)**	**IV (29 y)**	**P-value**
n	336	179	181	111	
Semen volume (mL)	3.0 (3.3) 0.5-8.5	3.4 (3.5) 0.6-9.9	3.5 (3.8) 0.7-9.9	3.5 (3.8) 0.2-8.7	0.06 I vs II **<0.001 I vs III** **<0.001 I vs IV** 0.09 II vs III 0.21 II vs IV 1.00 III vs IV
Semen concen-tration (million/mL)	60 (72) 0-515	56 (65) 0-377	51 (64) 0-225	70 (71) 0-195	0.37 I vs II 0.61 I vs III 0.81 I vs IV 0.98 II vs III 0.20 II vs IV 0.27 III vs IV
Total sperm count (million)	193 (219) 0-1380	184 (217) 0-753	187 (239) 0-1345	219 (251) 0-966	1.00 I vs II 0.46 I vs III 0.06 I vs IV 0.52 II vs III 0.08 II vs IV 0.63 III vs IV
Sperm motility (abc, %)	66 (64) 1-87	72 (70) 15-92	78 (76) 19-95	82 (79) 36-94	**<0.001 I vs II** **<0.001 I vs III** **<0.001 I vs IV** **<0.001 II vs III** **<0.001 II vs IV** 0.21 III vs IV
Total count of motile sperm (million)	124.2(144,5) 0-800.5	128.7(154.6) 0-538.8	138.9(182.1) 0-942.4	184.0(199.3) 0-763.5	0.47 I vs II **0.002 I vs III** **<0.001 I vs IV** 0.13 II vs III **<0.001 II vs IV** 0.23 III vs IV

Significant P-values (<0.05) shown in bold. Results are presented as median (mean) and range. Statistical analysis was performed using mixed model of repeated measures analyses after a square root transformation. The Tukey-Kramer methods was used to adjust p-values for pairwise comparison.

Table 14. Semen parameters of the men, who participated in all the four rounds (cohort A).

The table was modified is from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 and reproduced with permission from the Oxford University Press.

Rounds	**I (19 y)**	**II (21 y)**	**III (25 y)**	**IV (29 y)**	**P value**
n	61	61	61	61	
Semen volume (mL)	3.2 (3.5) 0.9-8.0	3.7 (3.7) 0.8-8.7	3.6 (4.1) 1.0-8.2	3.9 (4.0) 0.2-7.9	0.69 I vs II **0.01 I vs III** **0.02 I vs IV** 0.19 II vs III 0.26 II vs IV 1.00 III vs IV
Sperm concentration (million/mL)	54 (68) 0-309	47 (64) 0-377	56 (67) 0-225	66 (70) 0-195	0.79 I vs II 0.99 I vs III 0.99 I vs IV 0.70 II vs III 0.77 II vs IV 1.00 III vs IV
Total sperm count (million)	177 (222) 0-651	182 (224) 0-753	219 (254) 0- 880	220 (264) 0-966	1.00 I vs II 0.41 I vs III 0.43 I vs IV 0.3 II vs III 0.31 II vs IV 1.00 III vs IV
Sperm motility (abc, %)	67 (65) 30-81	72 (71) 31-87	80 (78) 37-95	82 (79) 41-92	**<0.001 I vs II** **<0.001 I vs III** **<0.001 I vs IV** **<0.001 II vs III** **<0.001 II vs IV** 0.94 III vs IV
Normal sperm morphology (%)	7.5 (8.2) 4-11	7.0 (7.9) 4-11	8.0 (8.7) 4-12	10 (10.6) 6-14	0.57 I vs II 0.99 I vs III **<0.001 I vs IV** 0.44 II vs III **<0.001 II vs IV** **<0.001 III vs IV**
Total count of motile sperm (million)	126.4(145.1) 0-417.6	140.1(162.1) 0-538.8	163.6(198.5) 0-689.3	184(212.4) 0-763.5	0.93 I vs II **0.01 I vs III** **0.008 I vs IV** **0.03 II vs III** **0.02 II vs IV** 0.99 III vs IV

Significant P-values (<0.05) shown in bold. Results are presented as median (mean) and range. Statistical analysis was performed using mixed model of repeated measures analyses after a square root transformation. The Tukey-Kramer methods was used to adjust p-values for pairwise comparison.

Table 15. Semen parameters of men in the cohort B over entire follow-up period.

The table was modified is from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 and reproduced with permission from the Oxford University Press.

Rounds	**I (19 y)**	**II (21 y)**	**III (25 y)**	**IV (29 y)**	**P value**
n	197	110	96	90	
Semen volume (mL)	3.4 (3.5) 0.6-9.0	3.5 (3.6) 0.4-10.9	3.7 (3.9) 1.0-8.9	3.5(3.8) 0.8-8.9	0.51 I vs II 0.09 I vs III 0.59 I vs IV 0.79 II vs III 1.00 II vs IV 0.96 III vs IV
Sperm concen-tration (million/mL)	50 (63) 0-274	57 (63) 0-263	46 (57) 0-195	62 (71) 0-171	0.96 I vs II 0.43 I vs III 0.09 I vs IV 0.76 II vs III **0.03 II vs IV** **0.007 III vs IV**
Total sperm count (million)	172 (199) 0-785	171 (209) 0.-975	168 (217) 0-1361.7	225 (264) 0-1077	1.00 I vs II 1.00 I vs III **0.009 I vs IV** **0.02 II vs IV** 1.00 II vs III **0.02 III vs IV**
Sperm motility (abc,%)	76 (73) 18-91	79 (76) 21-93	82 (77) 21-94	81 (76) 18-96	**0.02 I vs II** **0.002 I vs3** **0.03 I vs IV** 0.85 II vs III 0.90 II vs IV 1.00 III vs IV
Total count of motile sperm (million)	130.9(148.3) 0-573.3	132.6 (162.0) 0-711.5	138.6 (174.9) 0-1034.9	183.0(210.6) 0-717.4	0.88 I vs II 0.73 I vs III **<0.001 I vs IV** 0.99 II vs III **0.01 II vs IV** **0.02 III vs IV**

Significant P-values (<0.05) shown in bold. Results are presented as median (mean) and range. Statistical analysis was performed using mixed model of repeated measures analyses after a square root transformation. The Tukey-Kramer methods was used to adjust p-values for pairwise comparison.

Table 16. Semen parameters of the men, who participated in all the four rounds (cohort B).

The table was modified is from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 and reproduced with permission from the Oxford University Press.

Rounds	**I (19 y)**	**II (21 y)**	**III (25 y)**	**IV (29 y)**	**P value**
n	52	52	52	52	
Semen volume (mL)	3.8 (3.8) 1.1-8.7	3.8 (4.0) 0.4-10.9	4.0 (4.1) 1.6-8.9	3.4 (3.9) 0.8-7.9	0.94 I vs II 0.86 I vs III 0.9992 I vs IV 0.996 II vs III 0.93 II vs IV 0.86 III vs IV
Sperm concen-tration (million/mL)	54 (65) 0-266	59 (61) 0-160	57 (56) 0-153	69 (74) 0-160	0.99 I vs II 0.56 I vs III 0.12 I vs IV 0.53 II vs III **0.03 II vs IV** **0.005 III vs IV**
Total sperm count (million)	202 (233) 0 -701	192 (229) 0-928	193 (222) 0-1362	233 (280) 0-1077	0.9995 I vs II 0.72 I vs III 0.16 I vs IV 0.79 II vs III 0.13 II vs IV **0.02 III vs IV**
Sperm motility (abc, %)	76 (74) 47-88	82 (78) 21-93	83 (79) 44-92	82 (78) 18-94	**0.04 I vs II** 0.05 I vs III 0.2 I vs IV 1.0 II vs III 0.99 II vs IV 0.996 III vs IV
Normal sperm morphology (%)	7.3 (7.6) 0-19 n=51	8.5 (8.4) 0-19 n=49	8.0 (8.5) 1-21 n=49	9.0 (9.7) 0-20.5 n=51	0.22 I vs II 0.59 I vs III **0.001 I vs IV** 0.91 II vs III **0.04 II vs IV** **0.02 III vs IV**
Total count of motile sperm (million)	142.2(173.6) 0-558.6	161.3(183.6) 0-711.5	153.1(180.4) 0-1034.9	193.4(192.9) 0-979.8	1.0 I vs II 0.92 II vs III **0.005 I vs IV** 0.81 II vs III **0.04 II vs IV** **0.007 III vs IV**

Significant P-values (<0.05) shown in bold. Results are presented as median (mean) and range. Statistical analysis was performed using mixed model of repeated measures analyses after a square root transformation. The Tukey-Kramer methods was used to adjust p-values for pairwise comparison.

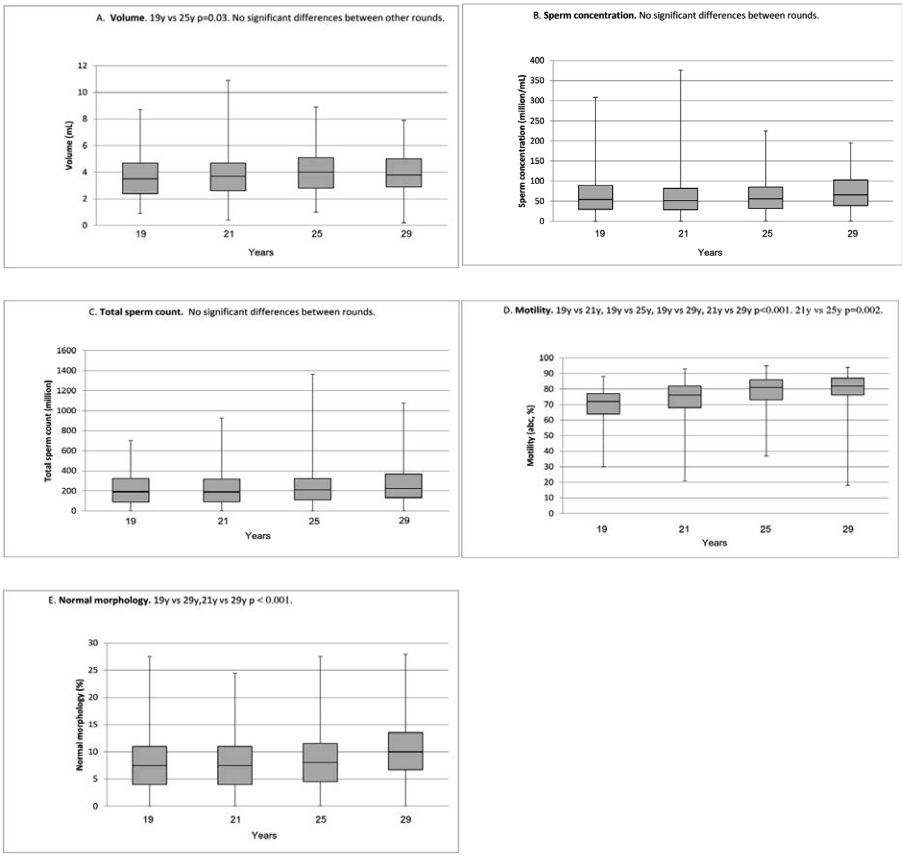

Figure 14. The semen characteristics of all men from cohorts A and B who attended all 4 rounds (n=113).

A) Semen volume B) Sperm concentration C) Total sperm count D) Sperm motility (abc, %) E) Sperm morphology (normal %)

The figure is reproduced from Antti Perheentupa, Sergey Sadov, Riitta Rönkä, Helena E. Virtanen, Wiwat Rodprasert, Matti Vierula, Niels Jørgensen, Niels E. Skakkebæk, Jorma Toppari. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Human Reproduction, 2016 Mar; 31(3): 502–510 with permission from the Oxford University Press.

6. DISCUSSION

6.1. Testicular growth during puberty in boys with and without a history of congenital cryptorchidism (Study I)

This study represents a unique longitudinal project in which boys with congenital cryptorchidism have been followed from birth to the end of puberty. In this work, we used statistical modelling to perform a detailed analysis of testicular growth during puberty among boys with and without a history of congenital cryptorchidism. Furthermore, we compared an orchidometer, ruler and ultrasonography in the assessment of testicular size. We also offered reference data on a ruler and ultrasonography for denoting the onset of puberty.

Within the framework of our longitudinal follow-up, a few important observations were made. In relation to the timing of pubertal onset, no difference between boys with a history of congenital cryptorchidism and those without were found. This indicates that the onset of puberty is driven by central mechanisms, which are substantially independent of the gonads. The analysis of testicular growth showed that the postpubertal volume of congenitally cryptorchid testes was smaller than the size of the descended testis. Simultaneously, the descended testis among boys with a history of unilateral congenital cryptorchidism did not show a statistically significant tendency towards compensatory growth during puberty, while the prepubertal volume of these testes was slightly bigger in comparison to controls. Most likely, poor pubertal testicular growth of congenitally cryptorchid testes is a consequence of testicular damage during prenatal development. Since testicular volume correlates directly with the number of Sertoli cells, the spermatogenic capacity of a congenitally undescended testis would be also reduced.[7] Thus, the reduced postpubertal volume of congenitally undescended testes is in concordance with the hypothesis of testicular dysgenesis syndrome, which connects cryptorchidism, impaired spermatogenesis, hypospadias and testicular cancer via early testicular damage, which occurs during fetal life.[4]

In a previous Finnish study published in 1996, 76 patients treated for cryptorchidism between the ages of 10 months and 13 years were compared to 47 patients without a history of cryptorchidism.[222] This retrospective study showed that among patients treated for cryptorchidism spermarche occurred later. The mean age at spermarche was 13.7 and 13.0 in treated and healthy participants, respectively ($P<0.01$). This finding does not conflict with

our results since spermarche is a relatively late pubertal event and mainly reflects the development of the testis itself. In the same study Taskinen et al. also observed that early spermarche (<13 years of age) is associated with bigger testicular volumes in postpuberty, and vice versa, later spermarche (>14 years of age) was associated with smaller postpubertal testicular volumes.[222]

The modelled postpubertal volume of undescended testes did not differ from participants with a history of unilateral congenital cryptorchidism who were treated surgically and experienced a spontaneous descent. Thus, orchidopexy could secure catch-up growth of the testis in more severe cases of cryptorchidism. Previously, Kollin et al. showed beneficial effect of orchidopexy performed at 9 months of age compared to orchidopexy at 3 years of age in relation to testicular growth.[223]

Nonlinear mixed-effect analysis fits the sigmoidal pattern of testicular growth during puberty. At the time of our analysis, the follow-up with the pubertal cohort was not finished and it is still ongoing. Thus, the chosen statistical method enabled the modelling of postpubertal testicular size on an individual level. This approach could be useful not only from an academic point of view but it is also practical since testicular volume is associated with the spermatogenic potential of the testis and, moreover, can be easily obtained through physical examination. Calculation of postpubertal testicular volume in advance is a potential clinical tool that could be used to predict of poor spermatogenic capacity in adulthood. Consequently, the modelled postpubertal testicular volume could potentially guide clinical work-up and follow-up protocols as one of the risk factors for impaired fertility.

The denoting of pubertal onset in boys is a challenging academic and clinical problem, due to the absence of self-evident signs that could manifest pubertal development. The widely used Tanner classification denotes the onset of puberty in boys as a transition to G2. The assessment of Tanner staging is based on the external appearance of the genitalia and is thus highly dependent on the skills and experience of the examiner.

In Finland all children are screened for the onset and progression of puberty by school healthcare physicians. If abnormality in pubertal development is detected, the child must be referred to a paediatrician. Throughout primary school, the physician examines the students three times altogether. The first health check occurs during the first year of school when the child is approximately 7 years of age. In relation to pubertal development, the purpose of this examination is the detection of precocious puberty. The second check occurs during the fifth school year when the child is approximately 12 years of age. This

examination is the most important with respect to the detection of pubertal onset. The majority of boys are in puberty at this age. However, some children could be prepubertal without a delay in pubertal development. The screening rules for clinicians are presented in Table 17. The last health check occurs during the eighth year of school, or around 15 years of age, when a delay in pubertal development is obvious if the child remains at G1.[224,225]

Table 17. Puberty screening rules, which are used in Finnish school healthcare for boys.

Modified from Raivio T. Puberteettikehitys ja sen häiriöt. Lääkärin käsikirja.[225]

	Not before (years)	At the latest (years)
G2*	9	13.5
P2	9	13.5
Growth spurt	10	15

*Length of the testis by ruler ≥ 25 mm

For decades in Finnish primary healthcare testicular length by ruler was used as an additional criterion for G2. However, rulers and callipers have been poorly studied as a tool for denoting pubertal onset in boys. In fact, we did not find original validation studies for the ruler or calliper. The ruler length cut-off of 25 mm refers to the length of the 3 mL bead of the Prader orchidometer. Since the pubertal acceleration of testicular growth and activation of the HPG axis is associated with a testicular volume of 3 mL by the Prader orchidometer, this volume is considered a cut-off level for denoting pubertal onset.[124,107] Our study provides evidence for the use of the ruler in clinical practice. If the length of the testis by ruler was 26-30 mm, the majority of testes were >3 mL. However, 100% confidence was reached when testicular length by ruler was >30 mm. This finding should be acknowledged in primary healthcare for more credible screenings of children for puberty at 12 years of age by school healthcare physicians, particularly when the individual definition of G2 is based exclusively on the ruler criterion. In uncertain clinical situations the pubertal status of the child could be assessed repeatedly before the examination at 15 years of age.

The orchidometer was used and studied extensively for decades after it was introduced by Professor Andrea Prader in 1966.[102] It seems that in modern clinical practice, use of the Prader orchidometer is limited to a narrow group of specialists (e.g., andrologists). In Finland, primary healthcare physicians do not routinely use the orchidometer to evaluate pubertal status or to examine infertile men. Years of experience and our study showed that

the ruler could successfully compensate for the orchidometer in assessing the onset of puberty. Previous studies and our study demonstrate a correlation between the orchidometer and ultrasonography.[226,227,228,229] However, further Bland-Altman analysis revealed a large disagreement between the two methods. Testicular volumes measured by the Prader orchidometer were much larger than those obtained by ultrasonography, and this disagreement increased with growth of the testis, especially when an ellipsoid formula was used. A weak reproducibility of measures obtained by the Prader orchidometer further corrupts the application of this tool in academic and clinical practice.[230]

The availability of ultrasonographic technologies is continuously increasing. Thus, reference data on ultrasonographic measurements is required. In our study, ultrasound volumes of the testis that correspond to the 3 mL volume by the Prader orchidometer were calculated. We also found that a >3 mL testicular volume by the orchidometer corresponds to the ultrasonographic volumes of >1.3 mL and >2.4 mL with a likelihood of 97.5 % by ellipsoid and Lambert's formula, respectively. Our results are in line with other recent studies on the same issue. Joustra et al. stated that a testicular volume of 4 mL by orchidometer corresponds to a 1.4 mL ultrasonographic volume, which was calculated by ellipsoid formula.[64]

6.2. The influence of low-saturated-fat and low-cholesterol dietary counselling on hormones and physical development during puberty (Study II)

The quantitative and qualitative properties of nutrition may affect pubertal timing and hormonal status.[138,142,231] In most human studies, the assessment of nutritional status is based on anthropometric measurements.[138] Interventional prospective longitudinal studies on nutrient intake are not common, and STRIP is one of these important trials.

Due to the absence of significant differences between intervention and control groups, our findings support the safety of a low-saturated-fat and low-cholesterol dietary intervention during childhood and adolescence in respect to pubertal development in boys and girls. Importantly, the aim of the dietary intervention was to achieve qualitative changes in fat intake without attempting to reduce the total intake of energy. Since we know from previous studies on adult participants that changes in fat intake can influence hormonal status[14,15]

and that alteration of the timing of puberty could have numerous short- and long-term adverse consequences[153] (see chapter 2.6.3.), a "null result" in this study along with previously reported beneficial effects of such dietary intervention encourages the implementation of this interventional strategy in the general population.

The main disadvantages of our study are a relatively small number of participants and the absence of hormonal data after menarche in girls.

However, our study also has some strengths. Since the STRIP cohort was designed to represent the general population, selection biases are minimised. In a previous study on the same issue (DISC HAS, see chapter 2.6.3.), the elevated level of LDL-cholesterol was one criterion for inclusion in the study.[142,143] Thus, the DISC HAS cohort represents a selected population. Dietary intervention in the STRIP was initiated at infancy (7 months of age), and regular follow-up was continued throughout infancy, childhood and adolescence. Pubertal follow-up visits were carried out annually. A long intervention period with regular controlling ensures the higher reliability of results. Intervention in the DISC HAS started much later in prepuberty than in the STRIP study, and the follow-up included only 5 visits altogether (see chapter 2.6.3.).[142,143]

Due to the similarity of results in both the puberty follow-up subcohort and the main STRIP cohort, the hormonal results obtained in our analysis are likely to be generalisable to the whole STRIP population. In particular, anthropometric characteristics, the timing of progression throughout Tanner stages and the age at menarche did not differ much between our subpopulation and the general STRIP population. Furthermore, in intervention children from the pubertal follow-up, we observed a decrease in levels of serum total cholesterol and an intake of saturated fat of a similar magnitude, when our results were compared to findings in the general STRIP.[141]

Our analysis discovered slight statistically significant differences between intervention and control boys in anthropometric parameters and progression of puberty assessed by Tanner staging. We did not find any statistically significant differences between intervention and control groups of children in respect to pubertal hormones. However, some slight statistically non-significant differences can be noticed. Among intervention boys there was a tendency to the earlier induction of pubertal growth and pubertal activation of the HPG axis (Figures 10 and 12; Table 8). In girls, the effect of intervention seemed to be opposite and even more subtle compared to the boys (Figures 11 and 13; Table 9). The direction of these

changes is similar to the shifts observed by Reinehr et al. in their recent longitudinal interventional study. However, in this German study, life-style and nutritional interventions were performed in a group of overweight children and shifts in the timing of pubertal activation of HPG were associated with the reduction in BMI-SDS.[137] Thus, our result is not directly comparable. Nevertheless, underlying mechanisms that explain these shifts could be similar. We hypothesise that changes in body composition and consequent hormonal shifts (action of aromatase, leptin etc) could explain the slight difference in the timing of puberty between intervention and control participants in our study. A precise understanding of these underlying mechanisms is important in the context of the earlier onset of puberty in the general population of many countries.

In girls, postmenarcheal hormones were excluded from the analysis due to the lack of a systematic standardisation of blood samples by the day of menstrual cycle. Removing postmenarcheal hormones from the analysis was also done due to the use of hormonal contraceptives by some participants. In the DISC HAS girls, hormonal differences between intervention and control groups were observed after the first menstrual bleeding (see chapter 2.6.3.).[142] The association between diet and postmenarcheal hormonal changes were observed in other studies also. Persky et al. demonstrated that a vegetarian diet among postmenarcheal adolescents was associated with higher levels of estradiol at the follicular phase and DHEAS at the luteal phase.[232] Thus, the results of these two studies are conflicting since follicular estradiol in the DISC HAS intervention group was lower than in the control group, and levels of DHEAS did not differ. In another study on postmenarcheal girls, no differences were found in hormone levels between vegetarian and nonvegetarian participants.[233]

All above-mentioned studies represent selected populations, and the aims of dietary intervention in the DISC HAS were more strict. Thus, we still need data on the influence of a low-saturated-fat and low-cholesterol diet on the hormonal status of postmenarcheal girls from the general population.

6.3. Semen quality during young adulthood (Study III)

Over the entire follow-up period in a group of young men from 19 to 29 years of age, we observed a slight statistically significant improvement of sperm motility and morphology. However, changes in sperm production were minor. This finding indicates that production of sperm at 19 years of age represents capacity, which is close to the best on an individual level. Results in discovery and validation cohorts were similar as well as results of a combined analysis of both cohorts.

Most studies on age-related changes in semen quality are cross-sectional. Moreover, these studies were often based on men who visited infertility or andrology clinics, which could cause potential selection-related biases. Age-related semen quality studies based on the general population are less common.[180,181,182,183,184]

Data on changes in semen quality during young adulthood (20-30 years of age) are not abundant. Only one longitudinal study, carried out by Danish researchers, has been done on this topic. As described in the review of the literature (see chapter 2.7.4.), in that study, a slight but significant increase in semen volume and sperm motility was observed. In contrast to our study, the morphology of sperm remained unchanged, which, could probably be explained by a shorter follow-up period in the Danish cohort (only 4 years). The size of the Danish cohort was also smaller than ours.[185]

Thus, in addition to the longitudinal design of our research, our study has a few strengths compared to previous studies. Since our participants represent the general population, selection biases are minimised and our results are easier to generalise. It is unlikely that fertility problems caused any participation biases at 19 years of age since at this age, fertility status is usually unknown. The follow-up period covered the entire young adulthood from 19 to 29 years of age, and thus, a complete pattern of physiological changes in semen quality during this period was discovered. Furthermore, the relatively large size of our longitudinal cohort (533 participants and 1299 observations in total) adds to the reliability of our results.

The main limitation of our study is the low participation rate (< 20 %), which is a common problem for studies on semen quality. In the Danish study, which had a similarly designed recruitment process, levels of reproductive hormones between the group of young men who participated in the semen quality study and those who did not were compared. Hormone levels (FSH, LH, inhibin B and testosterone) did not differ between these two groups,

which suggests a similar capacity of spermatogenesis.[234] Thus, the low participation rate in our study did not likely cause a significant selection bias.

Sertoli cells create a niche for spermatogenesis. Only a limited number of germ cells can be treated by Sertoli cells at once. Since the proliferation of Sertoli cells stops after the onset of puberty, sperm production should not increase after pubertal development is completed.[156] Our sperm count-related findings support this view. Importantly, at 19 years of age, the men born in 1979-1981 had adult levels of testosterone and developed Tanner stage 6 pubic hair and adult-size testes.[16] This indicates mature testicular function and adult hormonal status at this age.

If sperm production and hormonal testicular function are at the adult level in 19-year-old men, we can hypothesise that an observed slight improvement in sperm motility is a result of further functional maturation of accessory sex glands (seminal vesicle, prostate, bulbourethral and urethral glands) and the epididymis. Further maturation of these organs may lead to an increase in seminal plasma and better functional support of the spermatozoa. This view is supported by the increase in semen volume among participants in cohort A since we know that the main role in the production of semen plasma belongs to the accessory sex glands. Data on the impact of post-testicular mechanisms on sperm motility are limited. In humans, these studies are based on measurements of post-testicular seminal markers, which characterise the functional activity of the epididymis and accessory sex glands. The association between these markers and motility of the sperm was shown in such studies. For example, Elzanaty et al. demonstrated a significant positive correlation between the seminal neutral α-glucosidase (marker of epididymal function), prostate-specific antigen (marker of prostatic function) and percentage of motile sperm.[235] The testicular or epididymal “check-up” mechanisms that secure the normal structure of the spermatozoa could also undergo functional maturation during young adulthood, which could consequently lead to a slight improvement in sperm morphology.

Recent European studies on semen quality compared sperm variables between countries and generations using men from 19 to 30 years of age.[16,17,190,192] Since we found no significant change in sperm counts over the entire follow-up period, results of the above-mentioned comparisons were not biased by changes in sperm production. Thus, the decrease in sperm counts during previous decades cannot be explained by age dependent physiological changes in semen quality during young adulthood.[192] Furthermore, Finnish studies on se-

men quality demonstrated that fertile men at 30 years of age have better sperm concentrations than 19-year-old men from the general population, which is not a result of an increase in sperm count after 19 years of age.[3]

In humans, relations between semen characteristics and fertility were usually assessed by the analysis of the correlation between TTP and sperm concentration. As reviewed previously, different levels of sperm concentrations were proposed as thresholds for normal fertility: >30 x 10^6/ml, >40 x 10^6/ml and >55 x 10^6/ml.[176,177,178] According to data collected in our research center in 2008-2011, median sperm concentrations among 19-year-old men were 49 x 10^6/ml.[236] Fertility of these men is a cause for concern since at this age their full spermatogenic capacity is already reached.

The influence of the increase in sperm motility and morphology on subsequent fertility during young adulthood is obviously favourable. However, the precise effect of such improvement is difficult to estimate. This change would not likely secure male fecundity if sperm production is reduced significantly.

7. CONCLUSIONS

Study I.

The postpubertal size of congenitally cryptorchid testes are smaller than postpuberal size of normally descendent testes. This finding could be explained by early testicular damage during fetal life and anticipates impaired spermatogenic potential in adulthood. Since timing of pubertal onset did not differ between boys with and without a history of congenital cryptorchidism, the central regulation of puberty is functioning normally in both groups.

We discovered that the length of the testis by ruler >30 mm corresponds to Prader orchidometer size >3 mL in 100 % of measurement and testicular length by ruler 26-30 mm corresponds to orchidometer size >3 mL in only 83 % of measurements. This finding has been acknowledged, when ruler is used in the denoting of pubertal onset in clinical practice. Since availability and popularity of ultrasonography grows continuously, more reference data on ultrasonography in respect to the assessment of testicular volume at different ages is needed.

Study II

We found no significant influence of a low-saturated-fat and low-cholesterol dietary intervention on hormonal status during puberty. Our results confirm the safety of such intervention in children. However, due to limitation of our study hormonal changes in postmenarcheal adolescents were not ruled out.

Study III.

At the age of 19 years spermatogenic potential of the man is almost reached. However, over follow-up period from 19 to 29 years of age slight improvement in percentage of motile sperm and percentage of morphologically normal sperm was observed. This improvement may, however, not compensate for low sperm numbers in respect to fertility.

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