DELAYED PUBERTY
ETIOLOGY, OUTCOME AND INTERACTIONS WITH GROWTH
TERO VARIMO
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DELAYED PUBERTY

ETIOLOGY, OUTCOME AND INTERACTIONS WITH GROWTH

Tero Varimo

ACADEMIC DISSERTATION

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Helsinki 2016
To Eveliina and Joonatan
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This thesis is based on the following articles which are referred to in the text by Roman numerals I-V:


ABBREVIATIONS

AI  aromatase inhibitor
AMH  anti-müllerian hormone
ANOS1  anosmin 1
ARC  arcuate nucleus
BDI  beck’s depression index
BMD  bone mineral density
BMI  body mass index
CAH  congenital adrenal hyperplasia
CDGP  constitutional delay of growth and puberty
CHD7  chromodomain helicase DNA-binding protein 7
CHH  congenital hypogonadotropic hypogonadism
CI  confidence interval
CNS  central nervous system
CPP  central precocious puberty
DP  delayed puberty
DYN  dynorphin
ELISA  enzyme-linked immunosorbent assay
FGFR1  fibroblast growth factor receptor 1
FHH  functional hypogonadotropic hypogonadism
FSH  follicle-stimulating hormone
GABA  gamma-amino butyric acid
GH  growth hormone
GnRH  gonadotropin-releasing hormone
GNRHR  gonadotropin-releasing hormone receptor
GPR54  g protein-coupled receptor 54
hCG  human chorionic gonadotropin
HPG axis  hypothalamic-pituitary-gonadal axis
Hyper H  hypergonadotropic hypogonadism
HRQoL  health-related quality of life
ICD  international classification of diseases
IGF  insulin-like growth factor
IGSF10  immunoglobulin superfamily member 10
ISO-BMI  age- and sex-adjusted body mass index
ISS  idiopathic short stature
KISS1R  kisspeptin 1 receptor
KNDy  kisspeptin/neurokinin B/dynorphin
KS  Kallmann syndrome
LH  luteinizing hormone
Lz  letrozole
MKRN3  makorin ring finger protein 3
MPH  mid-parental target height
<table>
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<th>Full Form</th>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NKB</td>
<td>neurokinin B</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>PHH</td>
<td>permanent hypogonadotropic hypogonadism</td>
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<tr>
<td>PROK2</td>
<td>prokineticin 2</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>standard deviation score</td>
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<tr>
<td>TAC3</td>
<td>tachykinin 3</td>
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<tr>
<td>TAC3R</td>
<td>tachykinin 3 receptor</td>
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<tr>
<td>TS</td>
<td>Turner syndrome</td>
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<td>VNN</td>
<td>vomero-nasal nerve</td>
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ABSTRACT

During puberty, adolescents achieve reproductive capability and attain adult height. An intact hypothalamic-pituitary-gonadal (HPG) axis is crucial for the normal onset of puberty. Pubertal timing is influenced by genetic and environmental factors such as nutrition and stress. However, the exact mechanisms that trigger puberty remain elusive. A recent finding of a paternally-inherited loss-of-function mutation in \textit{MKRN3} gene in patients who were diagnosed with central precocious puberty suggests that key genes regulate the central restrain on the HPG axis during childhood, and that the loosening of the restrain precedes the onset of puberty. At the moment, the methods to measure the amount of the central restrain are limited to only observing its influence further down the HPG axis. Thus, a marker which reflects the strength of the central restrain directly could aid in the differential diagnosis and the follow-up of pubertal disorders. A variety of diseases can delay puberty, but it is particularly important to early identify the patients who suffer from acquired or congenital hypogonadotropic hypogonadism (CHH) which results from the absent or impaired functions of gonadotropin-releasing hormone. This congenital sex steroid deficiency predisposes to long-term consequences, but very little is known about its role in the modulation of growth during early infancy and its impact on health-related quality of life (HRQoL) in adulthood.

In the first study, circulating MKRN3 levels were measured in boys with idiopathic short stature (ISS) (n=30) before and after the onset of puberty. Sixteen boys were treated with an aromatase inhibitor letrozole (Lz) for two years in an attempt to increase their adult height. In boys, serum levels of MKRN3 declined faster before than after the clinical onset of puberty. The boys who were treated with Lz had a slower decrease in MKRN3 levels than placebo-treated boys, and the decline in MKRN3 had a reciprocal relationship with the clinical and the biochemical markers (\textit{i.e.} testis size, luteinizing hormone, inhibin B, testosterone) of puberty onset.

In the second study, the diagnoses that underlie delayed puberty (DP) and its outcome predictors were investigated in a large (n=244) series of patients who were examined for delayed puberty at the Helsinki University Central Hospital between 2004 and 2014. A multitude of different diagnoses were found to cause DP, albeit constitutional delay of growth puberty (CDGP) was the most common cause in both sexes. Further, annual growth velocity, a history of prior cryptorchidism, and a positive family history of DP were useful for the differential diagnosis of DP. Additionally, measuring testicular size with a ruler emerged as an effective parameter which could aid in differentiating the prepubertal boys with CHH from those with CDGP.

The third and fourth study consisted of growth charts of 42 patients (36 boys and 6 girls) with CHH, and evaluated the influence of congenital sex steroid deficiency on growth from birth length to adult height. In girls with CHH and \textit{FGFR1} mutations, the growth rate decreased from mid-childhood. Conversely, the boys with CHH experienced a length deflection during the first six months of life (minipuberty), which was likely to result from deficient testosterone surge during
the same period. In both sexes, the height standard deviation score nadir was reached at the age of pubertal induction.

In the fifth study, the HRQoL was measured in men with CHH (n=30) with the generic 15D questionnaire. The men with CHH experienced an impaired HRQoL, especially in the dimensions of depression and distress. The finding that the age of diagnosis correlated negatively with HRQoL scores supports the view that timely diagnosis of CHH is an important goal when evaluating a patient with late puberty.

In summary, sex steroids modulate growth from early infancy to adulthood. In boys, circulating MKRN3 levels decline before the clinical onset of puberty which supports the current view that MKRN3 is a key regulator of pubertal onset. Our findings suggest a relationship between estrogen and MKRN3 secretion. Additionally, the results pave the way for MKRN3 as a novel biochemical marker that may turn out to be useful in the evaluation of pubertal disorders in a research setting. Many diseases can delay pubertal maturation, but CDGP is the most common cause in Finland. In men, the timely diagnosis of CHH is crucial to avoid the long-term adverse effects of sex steroid deficiency on HRQoL. Finally, the results not only emphasize the importance of thorough review of medical history and a careful physical examination when evaluating an adolescent with DP, but also bring new tools in clinical decision-making.
FINNISH SUMMARY (TIIVISTELMÄ)


Tutkimuksen ensimmäisessä osakokonaisuudessa määrättiin seerumin MKRN3-tasot lyhytkasvuisilla pojilla puberteetin aikana. Osaa pojista hoidettiin kahden vuoden ajan aromataasin estäjä letrotsolilla aikuispituuden lisäämiseksi. Tulosten mukaan seerumin MKRN3-tasot laskevat voimakkaasti ennen murrosiän alkua sopien keskushermoston murrosiän estovaikutuksen määrän vähenemiseen. MKRN3-tasojen laskuun liittyi myös murrosiän biokemiallisten merkkiaineiden (luteinisoiva hormoni, testosteroni, inhibiini B) nousu sekä puberteetin eteneminen. Letrotsoli-hoidetuilla todettiin ennen murrosiän käynnistymistä hitaampi seerumin MKRN3-tasojen lasku kuin lumelääkettä saaneilla pojilla, viitaten siihen, että estrogeeni osallistuu murrosiän käynnistymisen säätelöyn.


Kolmas osakokonaisuus selvitti synynäisen HH:n vaikutuksia imeväisiän ja lapsuusiän kasvuun. Tutkimuksessa kuvattiin FGFR1-geenivirhettä kantavien synynäisistä HH:ta sairastavien tyttöjen lapsuusiän kasvuun piirteet. Heidän
pituuskasvunsa oli keskimäärin ikätovereita hitaampaa 4–7 vuoden iästä alkaen jatkuen aina sukupuolihormonihoidon aloitukseen asti. Vastaavasti HH-pojilla sukupuolihormonivaje associoitui kasvun hidastumiseen jo minipuberteetin eli ensimmäisen puolen vuoden aikana, sekä johtaa myös murrosiän kasvupyhädyksen puuttumiseen.

Tutkimuksen neljäs osakokonaisuus käsitteli synnynnäisen sukupuolihormonivajeen vaikutusta terveyteen liittyvään elämänlaatuun aikuisiällä. HH-miehillä todettiin 15D-elämänlaatumittarilla heikentyneet elämänlaatu aikuisena. HH-potilaiden varhaista tunnistamista puoltaa tutkimuksen havainto, että HH-miehillä alentunut terveyteen liittyvä elämänlaatu associoitui korkeampaan diagnoosi-ikään.

INTRODUCTION

In 1977, the Nobel Prize in physiology was awarded to the scientists who discovered a small peptide, gonadotropin-releasing hormone (GnRH), secreted in brain (Burgus et al. 1971, Schally et al. 1971). The discovery of GnRH inspired further research which eventually led to the understanding of the basic functions and the genetic regulation of the hypothalamic-pituitary-gonadal (HPG) axis (Bennett et al. 1975, Blake et al. 1980, Mason et al. 1986, Franco et al. 1991, Lei & Rao 1994). Subsequently, gene mutations found in patients with congenital hypogonadotropic hypogonadism (CHH) and pubertal disorders have increased our understanding of the complex genetic control of puberty (Elks et al. 2010, Bianco & Kaiser 2009, Howard et al. 2016, Abreu et al. 2013). This was highlighted by the recent finding of a paternally inherited loss-of-function mutation in makorin ring finger protein 3 (MKRN3) gene in patients with central precocious puberty (CPP), which suggested that MKRN3 has a pivotal role in the inhibition of GnRH secretion (Abreu et al. 2013). Measuring circulating GnRH levels has been difficult, and observing the activity of GnRH neurons has been limited to measuring its effects further downstream on the HPG axis (i.e. gonadotropin and sex steroid levels). However, in girls circulating MKRN3 levels have shown promising results for serving as a direct marker of central restrain on GnRH neurons, whereas similar studies in boys have not been published (Hagen et al. 2015).

Before puberty, the HPG axis faces two prolonged activation periods. The first occurs during fetal life and the second activation right after birth. The levels of sex steroids start to increase after the first week of life, and reach adult-like levels at 1 to 3 months of age, and then gradually decline by the age of 6 months (Forest et al. 1974, Winter et al. 1976, Bolton et al. 1989, Andersson et al. 1998, Kuiri-Hanninen et al. 2011, Bergada et al. 2006). This minipuberty has been known for over 40 years (Forest et al. 1974, Winter et al. 1976), but its importance in human development and influence on growth remains unclear. After infancy, the HPG axis remains quiescent and under central restrain until it is reactivated at the onset of puberty.

During puberty, adolescents achieve secondary sexual characteristics, experience growth spurt, and attain adult height. Thus, delayed pubertal maturation can cause low self-esteem and significant stress for adolescents and even limit their activities (Gross & Duke 1980). Delayed puberty (DP) is most commonly caused by constitutional delay of growth and puberty (CDGP) which is an idiopathic condition – an extreme end of the normal spectrum (Sedlmeyer & Palmert 2002). However, DP can also result from a variety of functional and chronic diseases. Patients with CHH have delayed or absent pubertal development, and CHH also serves as a practical model to investigate the long-term impact of sex steroid deficiency (Sedlmeyer & Palmert 2002). The differential diagnosis of DP, especially between CDGP and CHH, can be challenging (Harrington & Palmert 2012). Therefore, physicians who evaluate patients with DP in school health care or at the pediatric outpatient clinic have a crucial role in the early identification of the patients with a pathological underlying cause of DP. Consequently, understanding the diagnoses that underlie DP and the predictors of its clinical course could lead to better patient care.
INTRODUCTION

The aim of this study was to evaluate if circulating MKRN3 levels could serve as a read-out of the central restrain on the HPG axis in peripubertal boys, characterize the diagnoses that underlie DP, describe the childhood growth of CHH patients, especially length growth during minipuberty, and to evaluate the long-term impact of sex steroid deficiency on health-related quality of life (HRQoL).
REVIEW OF THE LITERATURE

1. HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS AND PUBERTY

1.1. Current concepts on the development and function of GnRH neurons

Normally functioning HPG axis has a critical role in the reproductive development. This neuroendocrinological cascade is initiated by GnRH neurons located in the hypothalamus. However, GnRH neurons rise from the olfactory placodes where they migrate towards the forebrain and the hypothalamus (Kim et al. 1999, Wray 2010, Wierman, Kiseljak-Vassiliades & Tobet 2011). The sensitivity of this period is underlined by the fact that an impaired migration process results in congenital hypogonadism which clinically manifests in disorders of puberty in later life (Wray 2010, Wierman, Kiseljak-Vassiliades & Tobet 2011, Boehm et al. 2015, Dwyer et al. 2015, Young 2012, Kallmann, Schonfeld & Barrera 1944, Valdes-Socin et al. 2014, Legouis et al. 1991).

1.1.1. The ontogeny and migration of GnRH neurons

There is substantial evidence that GnRH neurons originate from the progenitor cells of the olfactory placodes of the developing embryo, whereas some have suggested that GnRH neurons arise from the neural crest (Kim et al. 1999, Wray 2010, Schwanzel-Fukuda & Pfaff 1989, Wray, Grant & Gainer 1989, Forni et al. 2011, Katoh et al. 2011). Nevertheless, these neurons are unique as they origin outside the central nervous system (CNS) and migrate towards the forebrain (Kim et al. 1999, Wray 2010, Schwanzel-Fukuda & Pfaff 1989). Based on studies in rodents, the migration pathway of GnRH neurons is marked by a variety of adhesion molecules (Bless et al. 2006, Garcia-Gonzalez et al. 2016), neurotransmitters (Casoni et al. 2012, Vastagh et al. 2015), and growth factors (Sabado et al. 2012, Chung & Tsai 2010). Initially, GnRH neurons attach to the axons of the vomeronasal-nerves (VNN) and climb towards the cribriform plate located at the nasal-forebrain junction. At the cribriform plate, GnRH neurons have to find their way caudally to the forebrain. Importantly, this phase reflects the close developmental relationship between the olfactory system and GnRH neurons, since an impaired GnRH migration at this stage results in a clinical syndrome of hypogonadotropic hypogonadism and anosmia (Kallmann syndrome, KS) (Boehm et al. 2015, Kallmann, Schonfeld & Barrera 1944, Laitinen et al. 2011, Hutchins et al. 2016, Lewkowitz-Shpuntoff et al. 2012). After the cribriform plate, GnRH neurons continue their pathway to the forebrain until the migration ends at the hypothalamic area. At this point, GnRH neurons detach from the guidance of the VNN (Kim et al. 1999, Wray 2010, Schwanzel-Fukuda & Pfaff 1989). Subsequently, the neurons form extensions towards the anterior pituitary which enable the secretion of GnRH to the hypophyseal portal blood (Wierman, Kiseljak-Vassiliades & Tobet 2011). At the anterior pituitary, GnRH regulates the secretion of gonadotropins which are critical for the normal development of gonads.
1.1.2. The composition and function of the GnRH pulse generator

Anatomically, the GnRH pulse generator consists of a complex neuronal network which regulates the pulsatile secretion of GnRH and gonadotropins (Herbison 2016, Piet et al. 2015). The hypothalamus receives a vast amount of intrinsic and environmental impulses. Subsequently, the information is filtered, processed, and transmitted onwards to the GnRH neurons through the adjacent neurons (Figure 1A). These neurons secrete neuropeptides such as kisspeptin (Herbison 2016, Ward et al. 2009, Millar & Newton 2013). In humans, kisspeptin secreting neurons are located in the preoptic area and the arcuate nucleus of the hypothalamus (ARC). Kisspeptin activates the G protein-coupled receptor (GPR54)/kisspeptin-1 receptor (KISS1R), and GnRH neurons are sensitive to kisspeptin and express KISS1R (Pinilla et al. 2012). In mice, the amount of kisspeptin sensitive GnRH neurons increase during peripuberty (Kallo et al. 2012). Kisspeptin stimulates GnRH secretion, and the administration of a kisspeptin analog to prepubertal mice induces the progression of puberty (Decourt et al. 2016). Conversely, an inactivating mutation in Kiss1 results in hypogonadism marked by the absence of puberty (Seminara et al. 2003). In humans, a loss-of-function mutation in KISS1 is reported to cause CHH (Seminara et al. 2003), whereas activating mutations in KISS1 have been found in patients with CPP (Silveira et al. 2010). Most of the kisspeptin neurons secrete two co-transmitters: dynorphin (Dyn) and neurokinin B (NKB) (Skrapits et al. 2015). NKB and its receptor are encoded by tachykinin-3 (TAC3) and its receptor (TACR3), respectively. In mice, an NKB analog induces GnRH activity and LH pulses through kisspeptin/gpr54 signaling (Grachev et al. 2012). The administration of NKB alone does not appear to provoke gonadotropin secretion in humans, whereas administering it with kisspeptin increases LH secretion (Narayanaswamy et al. 2016). In support of the view that NKB stimulates GnRH secretion, a loss-of-function mutation in TAC3 or TACR3 results in congenital hypogonadotropism in humans (Topaloglu et al. 2012). Dynorphin synchronize and modulate the secretion of kisspeptin and transmit inhibitory signals to the GnRH neurons, thus in Dyn knockout mice gonadotropin secretion is compromised (Navarro et al. 2009). A subpopulation of kisspeptin neurons co-secrete kisspeptin, NKB, and DYN, and are located in the ARC (Skrapits et al. 2015). These neurons are called KNDy neurons, and they regulate the GnRH neurons (Figure 1A) (Pinilla et al. 2012). Gamma-amino butyric acid (GABA) influences on GnRH neurons through GABA_A receptors (Watanabe, Fukuda & Nabekura 2014). Despite being an inhibitory neurotransmitter in the adult brain, in vivo studies suggest that GABA mediates excitatory impulses to GnRH neurons (Watanabe, Fukuda & Nabekura 2014). All in all, the intensity of transmitted signals to GnRH neurons determines whether the GnRH secretion is maintained at the basal amplitude or if the information is transmitted onwards in a form of altered GnRH secretion.

GnRH is released into the pituitary portal blood with a diurnal pattern, particularly during the puberty onset (Rosenfield, Bordini & Yu 2013, Rosenfield, Bordini & Yu 2012, Albertsson-Wikland et al. 1997). An increase in GnRH secretion stimulates the anterior pituitary to secrete gonadotropins (i.e. luteinizing hormone (LH) and follicle-stimulating hormone (FSH)). In women, LH is required in the induction of ovulation, and FSH for the maturation of the follicles in the ovaries (Simonneaux & Bahougne 2015). In men, FSH acts directly on the Sertoli cells, whereas LH stimulates androgen production in Leydig cells to promote spermatogenesis (O’Shaughnessy 2014, Huhtaniemi 2015). Additionally, the
testes secrete inhibin B which inhibits FSH secretion at the pituitary level (de Kretser et al. 2000, Andersson et al. 2004, Boepple et al. 2008, Sehested et al. 2000), and anti-müllerian hormone (AMH) which is crucial for the development of sexual dimorphism as it induces the atrophy of the Müllerian ducts in male fetuses (Josso, Picard & Tran 1980, Josso, Rey & Picard 2013) (Figure 1A).

Figure 1. Panel A, The negative-feedback loop of the hypothalamic-pituitary-gonadal (HPG) axis after puberty. Gonadotropin-releasing hormone (GnRH) neurons secrete GnRH into the portal blood of anterior pituitary, which induces the secretion of gonadotropins. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) induce sex steroid production in the gonads. Sex steroids, especially estrogen, inhibit GnRH and gonadotropin secretion. At least a part of the feedback on GnRH neurons is mediated through kisspeptin, neurokinin B, and dynorphin. Panel B, the activity of the HPG axis from fetal life to adolescence (Pinilla et al. 2012, de Kretser et al. 2000, Kuiri-Hanninen, Sankilampi & Dunkel 2014, Jin & Yang 2014).
1.1.3. The activation phases of the HPG axis

Before puberty, the HPG axis faces two activation periods (Figure 1B). The first takes place during fetal life when the levels of LH and FSH peak at the mid-gestation (Kaplan & Grumbach 1976, Debieve et al. 2000). This hormonal surge is more pronounced in the female than in the male fetuses (Reyes, Winter & Faiman 1973, Clements et al. 1976). Thereafter, the levels of LH and FSH decline reaching only low levels at birth (Debieve et al. 2000). The second activation period occurs after birth. During the first 24 hours of life, there is a transient peak in LH secretion which resolves quickly (Corbier et al. 1990). Then, the HPG axis is reactivated at the age of one week and the active phase lasts for the next 6 to 9 months (Forest et al. 1974, Winter et al. 1976, Kuiri-Hanninen, Sankilampi & Dunkel 2014). During this minipuberty, gonadotropin and sex steroid levels increase and reach adult-like levels at 1 to 3 months of age (Winter et al. 1976, Tapanainen et al. 1981, Andersson et al. 1998, Bergada et al. 2006, Kuiri-Hanninen, Sankilampi & Dunkel 2014), and serum inhibin B and AMH levels surge concomitantly (Andersson et al. 1998, Aksglæde et al. 2010). However, the postnatal surge in hormonal levels differs between sexes. In boys, the gonadotropin response is LH dominant, whereas in girls FSH is more profoundly elevated (Bergada et al. 2006, Ibanez et al. 2002). Testosterone levels decline by 6 months of age, but girls may still show elevated estradiol levels at 3-4 yrs of age (Kuiri-Hanninen et al. 2011).

Estradiol levels fluctuate, whereas testosterone levels peak more sharply. Besides hormonal changes, maturing follicles can be detected in girls during minipuberty (Kuiri-Hanninen et al. 2013). In primates testis, the number of Sertoli cells increase from birth to infancy concomitantly with the elevated gonadotropin levels (Simorangkir, Marshall & Plant 2003), which suggests that minipuberty is important for the proliferation of Sertoli cells (Simorangkir, Marshall & Plant 2003). In support of this, boys with CHH may show normal genitalia at birth, but a progressive involution of the scrotum during minipuberty (Main, Schmidt & Skakkebaek 2000). At the same time, the number of germ cells, Sertoli cells and Leydig cells in healthy boys increase, and urinary prostate specific antigen levels are transiently elevated (Kuiri-Hanninen et al. 2011, Codesal et al. 1990, Muller & Skakkebaek 1984, Cortes, Muller & Skakkebaek 1987). However, the initiation of spermatogenesis is prevented by the absence of androgen receptors in Sertoli cells (Chemes et al. 2008). During the first six months, testicular and penis size increase (Kuiri-Hanninen et al. 2011, Boas et al. 2006). The hormonal minipuberty has been known for 40 years, but only recently some of its importance in human development has been understood. The early hormonal surge, especially testosterone, is associated with male-type behavior in both boys and girls (Lamminmaki et al. 2012). Low testosterone levels are associated with early phonological development and language organization in both sexes (Friederici et al. 2008, Quast et al. 2016).

During childhood, the HPG axis remains quiescent under the central restrain with the exception of some overnight LH secretion (Mitamura et al. 2000, Mitamura et al. 1999, Apter et al. 1993, Wu et al. 1996). Despite of the gonadostat, the HPG axis remains functional since it can be evoked with a GnRH test which in prepubertal patients shows a small but a significant increase in LH levels and a considerable FSH response (Dickerman, Prager-Lewin & Laron 1979). The onset of puberty is preceded by the removal of the gonadostat which results in an increased amplitude and frequency of GnRH secretion (Herbison 2016, Oerter et
Subsequently, the levels of gonadotropins increase and stimulate sex steroids secretion which ultimately induce pubertal maturation (Oerter et al. 1990, Andersson et al. 1997).

1.1.4. The regulation of the HPG axis

After the onset of puberty the HPG axis is under the regulation of a negative-feedback loop (Figure 1A). The role of estrogen in regulating GnRH secretion appears to be bipartite. Estrogen inhibits Kiss1 expression and gonadotropin secretion in mice, but there is evidence of a positive estrogen-kisspeptin feedback circuit as well (Dorling et al. 2003, Clarkson et al. 2010, Dubois et al. 2015, Dubois et al. 2016). In pubertal primates, the removal of circulating estrogen increases the secretion of kisspeptin, and the administration of estrogen restores the kisspeptin activity suggesting that estrogen is a crucial inhibitor of GnRH secretion (Kenealy et al. 2016, Kenealy et al. 2013). Testosterone also appear to regulate GnRH secretion, since a treatment with testosterone in castrated male monkeys suppresses LH and FSH secretion and the levels of kisspeptin mRNA in mediobasal hypothalamus (Shibata et al. 2007). Due to the aromatization of androgens into estrogens, the inhibitory effects of testosterone on the HPG axis are believed to be estrogen-mediated. The administration of estrogen decreases the LH secretion in both healthy men and men with isolated GnRH deficiency (Finkelstein et al. 1991), whereas the removal of the estrogen inhibition with an aromatase inhibitor (AI), which is a blocker of estrogen biosynthesis, results in increased levels of gonadotropins (Finkelstein et al. 1991, Hayes et al. 2000, Wickman & Dunkel 2001). Moreover, in late maturing boys who were treated simultaneously with an AI and testosterone, the levels of baseline gonadotropins and GnRH induced LH-response increased significantly, which further supports the key role of estrogen in the regulation of human puberty (Wickman & Dunkel 2001).

1.2. Normal puberty

1.2.1. General concepts

During puberty, the adolescent achieves secondary sexual characteristics and reproductive capability, and eventually attains adult height. During the last century, the timing of puberty has declined rapidly until the 1950s, and, thereafter, the decrease has shown a steadier phase (Euling et al. 2008). The onset of puberty is marked by the development of glandular breast tissue in girls and an increase in testicular volume in boys (Marshall & Tanner 1969, Marshall & Tanner 1970). Timing of puberty shows large individual variation. Puberty may begin too early or it can be delayed. In Finland, all adolescents are screened for abnormal pubertal maturation at the age of 13 to 14 yrs in the school health care.

1.2.2. Normal variation in the timing of puberty and secular trends

Puberty onset normally occurs in boys at the average age of 11 to 12 yrs, but can vary from 9 to 14 yrs (Marshall & Tanner 1970, Ojajärvi 1982), whereas in girls, puberty begins at an average age of 10 to 11 yrs, but is still considered normal if glandular breast tissue (Tanner B2 starge) is found between 8 to 13 yrs of age (Marshall & Tanner 1969, Ojajärvi 1982). Another difference between the sexes
is observed in bone maturation. The onset of puberty occurs at a mean bone age of 11.1 years in girls and 11.7 years in boys (Marshall 1974).

The decline in the age of pubertal timing has been biphasic. The age of menarche decreased gradually until the 1950s, and then the decline has plateaued as a result of better nutrition, hygiene, and diet (Sorensen et al. 2012). During the last two decades, several studies have investigated pubertal timing, and a trend towards an earlier puberty in girls has been reported both in the US and Europe (Sun et al. 2002, Aksglaede et al. 2009, Biro et al. 2013). However, the results of the studies are inconsistent. Studies over longer period of time failed to show a decline in the age of puberty onset during the last 20 yrs, which suggested a difference in the timing of puberty between the US and Europe (Juul et al. 2006, Mul et al. 2001). The Copenhagen puberty study reported a decline in the age of thelarche from 10.9 to 9.9 yrs during a period of 15 yrs, but the decrease in the age of thelarche was not as significant as reported in the US (Sun et al. 2002, Aksglaede et al. 2009, Wu, Mendola & Buck 2002). Further, the decline in the timing of puberty was not supported by a change in the biochemical markers of puberty (Aksglaede et al. 2009). The levels of gonadotropins and estrogen did not increase in parallel to the decrease in the age of thelarche, which pointed towards the earlier appearance of glandular breast tissues independent of gonadotropin secretion (Aksglaede et al. 2009). The age at menarche appears to decline. A recent study reported a trend towards an earlier age of thelarche, and also an earlier menarche in daughters than their mothers (Wohlfahrt-Veje et al. 2016). The age at menarche in mothers correlate with the age at menarche in daughters, and similarly the pubertal timing of mothers and fathers influence on the age of puberty onset of their sons and daughters (Wohlfahrt-Veje et al. 2016, Ersoy et al. 2005). Thus, the timing of puberty appears to be parentally inherited.

1.2.3. Clinical and biochemical markers of puberty

Pubertal progress is categorized by using the Tanner stage classification which divides the development of the testes, breasts, and pubic hair into five categories (Marshall & Tanner 1969, Marshall & Tanner 1970). In boys, prepubertal genitals are categorized into genital stage 1 (G1), and when puberty is complete, the genital stage is 5 (G5) (Marshall & Tanner 1970). Similarly, in girls, the pubertal progression is classified from breast stage 1 to 5 (B1-B5) (Marshall & Tanner 1969).

In boys, a testis size more than 3 mL is widely used to indicate the clinical onset of puberty, whereas some studies have suggested that testicular volume of 4 mL marks the activation of the HPG axis (Sadov et al. 2016, Joustra et al. 2015, Ankarberg-Lindgren & Norjavaara 2004). Correspondingly, the appearance of glandular breast tissue (thelarche) (B2) indicates the clinical onset of puberty in girls (Marshall & Tanner 1969, Ojajärvi 1982).

Tanner genital stage is a simple and the most widely used clinical assessment of the clinical onset and progression of puberty. However, it relies solely on the visual estimate of the breast, the genital, and the pubic hair stage. In boys, measuring the length and the width of the testes is an accurate method to estimate testicular volume. Several formulas have been used to estimate testis size, one of which is the Hansen formula: (mL) is length (cm) x width² (cm) x 0.52 (Behre, Nashan & Nieschlag 1989). Another method used widely in clinical practice to determine testis size is the Prader orchidometry (Prader 1966). Testicular size can
be also measured with ultrasound (Diamond et al. 2000), but its usefulness is limited by costs and availability. Additionally, none of the three measuring methods is superior to the others in accuracy (Taskinen, Taavitsainen & Wikstrom 1996). In girls, evaluating the presence of breast tissue can be very difficult, especially in an obese subject. In unclear cases, breast tissues can be determined by using the ultrasound (Garcia et al. 2000).

Biochemical markers of puberty illustrate the activity of the HPG axis at the pituitary and at the gonadal level. A single measurement of LH or FSH represent indirectly the GnRH secretion. Additionally, the activity of the HPG axis can be assessed with two stress-tests. In a human chorionic gonadotropin (hCG) test, gonadal testosterone secretion is stimulated with hCG (Segal et al. 2009, Osuna, Arata-Bellabarba & Tortolero 2001), whereas in a GnRH test, LH and FSH releases are augmented by administering a GnRH analog (Rosenfield, Bordini & Yu 2013, Rosenfield, Bordini & Yu 2012, Dunkel et al. 1985). At gonadal level, the Sertoli cells inside the seminiferous tubules of the testes secrete inhibin B which can be used in the biochemical evaluation of puberty. However, the measurement of low inhibin B levels is subject to a high variation (Kalra et al. 2010). Another Sertoli cell origin peptide, AMH, has been reported in studies addressing pubertal disorders. Serum AMH levels declined during puberty which reflected the gonadotropin influence on the Sertoli cell maturation (Hero et al. 2012). Moreover, inhibin B and AMH levels have been suggested to discriminate between the boys with idiopathic pubertal delay and CHH (Coutant et al. 2010, Adan et al. 2010).

1.2.4. Molecular genetic basis of puberty

During the last 30 yrs, the discovery of novel genes in patients with pubertal disorders, especially CHH, has revealed the genetic complexity of human puberty (Elks et al. 2010, Bianco & Kaiser 2009, Boehm et al. 2015). In the past, the genes associated with CHH were discovered by identifying single candidate CHH genes and linking them to the phenotypic features of CHH (Pitteloud et al. 2006, Dode et al. 2003, Dode et al. 2006). Additionally, depicting the pedigrees of CHH patients has been fruitful in identifying new candidate genes and revealing the low penetrance for most of the CHH genes (Gurbuz et al. 2012). By using these methods approximately 30 to 35% of genes that underlie CHH have been discovered (Crowley 2011), which suggests that many patients with CHH are currently without a genetic diagnosis. Recently, new methods such as next-generation sequencing, proteomics, and genomics have yielded additional precision in finding novel CHH genes. The problem in identifying causative CHH genes lies in the absence of consensus of validation standards (MacArthur et al. 2014). However, whole-genome sequencing and its implications are likely to reveal the remaining portion of unknown CHH genes in the near future (Stamou, Cox & Crowley 2015).

Very recent discoveries of genes that underlie delayed and precocious puberty have increased our knowledge on the genetic control of human puberty. A loss-of-function mutation in MKRN3 was discovered in patients with CPP (Abreu et al. 2013). Subsequently, circulating MKRN3 levels were measured in girls before and after the onset of puberty (Hagen et al. 2015). These findings suggested that a normally functioning MKRN3 is critical for maintaining the central inhibition on the GnRH neurons. MKRN3 is expressed widely in human tissues, and is a putative E3 ubiquitin ligase, but its exact mechanisms are unclear (reviewed in Abreu et
al. 2015). Very recently, a pathologic mutation in immunoglobulin superfamily member 10 (IGSF10) gene was found in six unrelated Finnish families with late puberty (Howard et al. 2016). Apparently, these genes control the timing of puberty, but, at the same time, they represent only a fraction of potential puberty genes that are yet to be found.

The knowledge of genetic regulation of puberty stems mainly from the mutations found in patients with CHH. The most common genes that are found mutated in CHH patients, especially the ones reported in Finnish patients, are listed in Table 1 (Table 1). In brief, CHH genes regulate GnRH neuron migration and development or GnRH secretion and action (Table 1). The inheritance pattern differs between CHH genes, for example, anosmin 1 (ANOS1) is located in chromosome X, thus mutations in ANOS1 manifest commonly in male CHH patients (Boehm et al. 2015). Remarkably, only less than 10% of CHH cases have a molecular genetic verification of CHH (Boehm et al. 2015). In Finland, the most common mutated CHH gene is fibroblast growth factor receptor 1 (FGFR1). In fact, one third of the Finnish patients with CHH carry a mutation in FGFR1 (Laitinen et al. 2011). Further, FGFR1 mutations manifest with skeletal anomalies and, at a population level, are suggested to be associated with childhood obesity (Jiao et al. 2011, Costa-Barbosa et al. 2013), whereas ANOS1 associates with a more severe reproductive phenotype of CHH (Costa-Barbosa et al. 2013).
Table 1. Phenotypes and non-reproductive features of the most common genes found mutated in patients with Kallmann syndrome (KS), congenital hypogonadotropic hypogonadism (CHH), constitutional delay of growth and puberty (CDGP) and central precocious puberty (CPP).

<table>
<thead>
<tr>
<th>Gene</th>
<th>HGNC ID</th>
<th>Phenotypes</th>
<th>Reported in Finnish patients</th>
<th>Mode of inheritance</th>
<th>Distinctive non-reproductive features</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>KS</td>
<td>CHH</td>
<td>CHH reversal</td>
<td>CDGP</td>
<td>CPP</td>
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<td>Development and migration of GnRH neurons</td>
<td></td>
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<td></td>
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<tr>
<td>ANOS1</td>
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<td>x</td>
<td>x</td>
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<td></td>
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<tr>
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<td>x</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>TAC3</td>
<td>HGNC:11521</td>
<td>x</td>
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<tr>
<td>TACR3</td>
<td>HGNC:11528</td>
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<tr>
<td>KISS1</td>
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<td>x</td>
<td></td>
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<tr>
<td>KISS1R</td>
<td>HGNC:4510</td>
<td>x</td>
<td></td>
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<tr>
<td>Inhibition of GnRH secretion</td>
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<tr>
<td>MKRN3</td>
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<td>x</td>
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</table>
1.3. Delayed puberty (DP)

The majority of adolescents enter puberty within the normal age range. However, sometimes pubertal maturation occurs later than expected. Delayed puberty is defined as the absence of clinical signs of puberty at the age of 2 to 2.5 standard deviation (SD) above the mean age of the general population, and it affects approximately 2% of adolescents (Marshall & Tanner 1969, Marshall & Tanner 1970, Lee 1980). Delayed puberty is caused by a variety of conditions (Sedlmeyer & Palmert 2002, Reindollar & McDonough 1981, Lawaetz et al. 2015). The most common cause is constitutional delay of growth and puberty (CDGP) which constitutes up to 65% of cases with DP at a single pediatric endocrinology outpatient clinic (Sedlmeyer & Palmert 2002). On the other hand, DP can be caused by chronic diseases and pathological entities, such as craniopharyngioma and Crohn’s disease, which need to be diagnosed and treated as early as possible. This emphasizes the key role of first-line evaluation in identifying these patients early in school health care and referring them onwards to the pediatric outpatient clinic.

1.3.1. Diagnostic criteria in Finland

Due to the secular trend in the timing of puberty, the diagnostic criteria of DP show variation between countries. Since two different testicular sizes have used to describe the clinical onset of puberty, the diagnostic criteria are not uniform either. In Finland, the age limits for precocious and delayed puberty was recently updated by an expert consensus (Raivio 2013). Consequently, a boy is considered to have DP if he is prepubertal (i.e. the pubertal stage G1 and/or testis volume less than 3 mL) at the age of 13.5–14 yrs (Ojajärvi 1982), and in a girl if she is at the B1 stage at the age of 13.0 or more (Ojajärvi 1982).

1.3.2. Underlying causes

The most frequent cause of DP is CDGP which is an extreme end of normal variation (Sedlmeyer & Palmert 2002, Clayton et al. 1988). Thus, CDGP is an idiopathic condition which is often inherited, since up to 65% of the first degree relatives of CDGP patients report a history of DP (Sedlmeyer & Palmert 2002). However, a family history of DP is not pathognomonic to CDGP as late maturation is also found among the close relatives of patients with CHH (Sedlmeyer & Palmert 2002). The diagnosis of CDGP is set after the pathological causes of DP are ruled out. The other diagnoses can be classified into three categories. Patients with permanent hypogonadotropic hypogonadism (PHH) have low gonadotropin and sex steroid levels that result from a pathology in the CNS or a permanent disease that delays puberty. Importantly, CHH constitutes the majority of cases with PHH (Sedlmeyer & Palmert 2002). Similarly, functional hypogonadotropic hypogonadism (FHH) category consists of patients who have low gonadotropin and sex steroid levels but do not have any biochemical and clinical signs that would suggest the presence of a permanent cause of DP and an identified disease, such as celiac disease that is likely to delay maturation (Sedlmeyer & Palmert 2002). Finally, high basal gonadotropin levels and gonadal failure is characteristic to patients with hypergonadotropic hypogonadism (Hyper H). The frequency and distribution of the four diagnostic categories of DP at a pediatric outpatient clinic of a single tertiary center is presented in Figure 4 (Figure 2).
1.3.3. Evaluation of patient with DP

The evaluation of DP includes a careful clinical examination and a review of the medical history. A history of undescended testes (i.e. cryptorchidism), micropenis, anosmia/hyposmia, and prior infertility treatments in close relatives are clinical cues that increase the risk for permanent hypogonadism (reviewed in Bouvattier et al. 2011). History of headache and other neuro-ophthalmic symptoms should be screened as well (Taylor et al. 2012). Subsequently, adolescents who present with the above medical history or signs and symptoms that raise a suspicion of pathologic cause of DP should be referred to the pediatric outpatient clinic for further investigation.

The diagnostic process of DP is categorized into the first and second-line investigations (Palmert & Dunkel 2012). The first-line investigations include the evaluation of genital and pubic hair stage, determining the annual growth velocity and bone age, measuring baseline sex steroid and gonadotropin levels, and biochemical analyses (e.g. thyrotropin, free thyroxine, and creatine) to rule out chronic diseases such as hypothyroidism or celiac disease (Palmert & Dunkel 2012). High basal LH and FSH levels easily identify patients with Hyper H (Abitbol, Zborovski & Palmert 2016). Sex steroid levels can be used in predicting the progress of puberty. In a previous study, 75% of boys with testosterone level above 0.7 nmol/L entered puberty within the next 12 months, and everyone within the next 15 months (Wu et al. 1993). Gonadotropin, estradiol, and testosterone levels show diurnal variation during puberty. After the onset of puberty, LH secretion increases during the day, whereas the diurnal secretion of FSH is less prominent (Albertsson-Wikland et al. 1997, Mitamura et al. 1999, Goji & Tanikaze 1993). Testosterone secretion is induced during the night before the clinical onset of puberty, and thereafter the levels of testosterone increase both during day and night (Albertsson-Wikland et al. 1997, Mitamura et al. 1999, Goji & Tanikaze 1993). The circadian rhythm of the estradiol is similar to that of testosterone (Mitamura et al. 2000).
During puberty, the gradual increase in sex steroid levels, especially estrogen, induces skeletal maturation. A delay in the onset of puberty results in a deviation also in the bone age. Thus, a patient with DP has a bone age which is approximately 2 to 3 yrs behind the calendar age (Lawaetz et al. 2015). Additionally, the absence of sex steroid-induced growth spurt can be visualized in the growth curve (Figure 3). The adolescents with DP continue to grow with the childhood growth velocity, whereas the height velocity of their peers rapidly increases. This results in a “dip” seen in the growth curve (Figure 3). Importantly, being shorter than their peers may be one of the reasons why patients with DP, especially boys, suffer from psychosocial burden (Gross & Duke 1980, Palmert & Dunkel 2012). Half of the boys with CDGP experience a decrease in height velocity already during childhood (Wehkalampi et al. 2007). In adolescence, a very slow height velocity (< 2 cm/yr), especially in a combination with neuro-ophthalmic symptoms, increases the risk for chronic diseases and panhypopituitarism (Taylor et al. 2012). However, only limited evidence of height velocity’s discrimination between the four diagnostic categories of DP exists (i.e. CDGP, PHH, FHH, and Hyper H) (Palmert & Dunkel 2012).

In some cases, the first-line investigations of DP cannot provide sufficient information to rule out the pathological causes of DP. Thus, the second-line investigations offer more precise information about the HPG axis. The studies include GnRH and hCG stress testing, a brain MRI, and the markers of Sertoli cell function, and genetic testing (reviewed in Palmert & Dunkel 2012). The performance of inhibin B and AMH together with GnRH and hCG tests in the differential diagnosis of DP (i.e. CDGP vs CHH) is discussed in detail below. A brain MRI scan is performed in cases where the clinician suspects that pubertal delay might be caused by CHH, or a lesion in the CNS, or requires the visualization of the olfactory or inner ear structures (i.e. suspicion of CHH) (Lewkowitz-Shpuntoff et al. 2012).

Figure 3. A typical growth curve, mid-parental target height (MPH) (asterisk), and predicted adult height (vertical line) of a boy with constitutional delay of growth and puberty (CDGP) who received testosterone treatment (1 mg/kg) (arrow) for 6 months.
1.3.4. Treatment

Before hormonal induction of puberty, the pathologic causes of DP must be excluded with a sufficient probability (Palmert & Dunkel 2012). The patients with CDGP have two treatment options: sex steroid treatment or watchful waiting (Dunkel & Quinton 2014). The onset of puberty can be waited with a careful follow-up (Dunkel & Quinton 2014). However, the absence of puberty can cause significant psychosocial stress for the adolescent (Gross & Duke 1980), which supports the induction of puberty (Palmert & Dunkel 2012). The treatment of DP (girls with estrogen gel and boys with low-dose intramuscular testosterone in Finland) intends to induce sexual maturation and an increased growth velocity, and appears to affect positively on self-esteem (Figure 3) (Soliman, Khadir & Asfour 1995, Bergada & Bergada 1995, Kaplowitz 1998).

Usually, estrogen gel is administered with gradually increasing amounts, and the response is evaluated at the follow-up visits (Wehkalampi et al. 2011, Ankarberg-Lindgren, Kristrom & Norjavaara 2014). The concept of starting the treatment with low estrogen doses stems from the idea of mimicking the normal puberty. Indeed, at early puberty low estrogen levels promote growth, whereas a higher level that occurs late in puberty is required to complete the physical development (Albin et al. 2012). Low-dose testosterone treatment is well-tolerated and has relatively few adverse effects, but the dosage and the treatment period vary within and between countries (Palmert & Dunkel 2012, Ambler 2009). Ideally, the hormonal treatment of DP should result in the activation of the HPG axis. Currently, this may not be the case with boys who are treated with intramuscular testosterone, because low-dose testosterone treatment suppresses gonadotropin and testosterone secretion (Soliman, Khadir & Asfour 1995, Dunkel & Wickman 2001). Testosterone is aromatized into estrogens which mediate the negative feedback to the hypothalamic and the pituitary levels of the HPG axis (Figure 1A) (Dunkel & Wickman 2001). Removing the negative feedback of estrogen is a potential novel treatment modality for DP (reviewed in Palmert & Dunkel 2012). Aromatase inhibitor letrozole increases gonadotropin and testosterone levels during peripuberty (Hero, Norjavaara & Dunkel 2005), which suggests that it also augments the progression of puberty. On the other hand, the impact of AIs on bone health remains unclear (Hero et al. 2010, Hero 2016). A well-designed randomized controlled study on the use of AI for the treatment of DP is definitely required (Palmert & Dunkel 2012). The treatment of DP should always include a follow-up to ensure the normal progression of puberty, and the absence or deceleration of the tempo of puberty and growth during the follow-up should raise the suspicion of a pathologic cause of DP, even though the initial diagnostic work-up resulted in the diagnosis of CDGP.

1.4. Congenital hypogonadotropic hypogonadism (CHH)

Congenital hypogonadotropic hypogonadism is a rare reproductive disorder which is caused by an impaired secretion, production, or action of GnRH (Boehm et al. 2015, de Roux et al. 1997, Bouligand et al. 2009). When hypogonadotropism is accompanied by anosmia or hyposmia, it is called Kallmann syndrome (KS). KS affects approximately 1:48000 live births in Finland (Laitinen et al. 2011). The patients with most severe CHH suffer from sex steroid deficiency throughout their life, starting from fetal life when the deficiency may manifest in cryptorchidism or micropenis, and result in an absent or incomplete puberty in adolescence (Main,
Schmidt & Skakkebaek 2000, Grumbach 2005, Dwyer, Jayasena & Quinton 2016). In some cases, patients with CHH may experience a reversal of hypogonadism later in life. In fact, the reversal variants constitute up to 18% of CHH cases, and the reversal of hypogonadism occurs despite of partial or absent puberty (Raivio et al. 2007, Sidhoum et al. 2014). The objectives in treating a CHH patient are to achieve secondary sexual characteristics and fertility, and induce linear growth and bone maturation (Boehm et al. 2015). The long-term health effects of CHH include an increased risk for osteoporosis (Laitinen et al. 2012a, Behre et al. 1997), metabolic diseases (Zarotsky et al. 2014), and impaired health-related quality of life (Aydogan et al. 2012, Shiraishi, Oka & Matsuyama 2014). Consequently, this underpins the importance of the appropriate timing of diagnosis and early treatment initiation.

1.4.1. Diagnostic criteria

The classical diagnostic criteria of CHH include an absent or incomplete puberty at the age of 18 years, low sex steroid levels with normal or subnormal gonadotropin levels, and otherwise normal anterior pituitary function, and no organic cause for the condition (Raivio et al. 2009, Villanueva et al. 2015, Shaw et al. 2011). Thus, excluding other causes of permanent hypogonadism, such as craniopharyngioma, is essential for the diagnosis of CHH. Although these diagnostic criteria are widely used, there is a lack of international diagnostic criteria for CHH (Boehm et al. 2015).

1.4.2. Clinical and biochemical evaluation

The diagnosis of CHH is typically made during late adolescence or early adulthood. To discriminate patients with CHH from those with CDGP before puberty may be extremely difficult, and diagnosing CHH without genital anomalies during childhood or infancy is even more challenging (Dwyer, Jayasena & Quinton 2016). The same clinical and biochemical markers as in the evaluation of patients with DP are used in the diagnostic process of CHH. Additionally, a brain MRI is used to exclude lesions in the CNS and to visualize the olfactory bulbs and the inner ear (Della Valle et al. 2013). Ultrasonography of abdomen and pelvis can reveal renal agenesis and visualize the reproductive organs (ovaries, uterus, and testis). Since sex steroids are critical for normal pubertal growth spurt and the accumulation of bone mineral content, patients with CHH have an increased risk for osteoporosis (Laitinen et al. 2012a). The bone density is typically assessed with the dual-energy X-ray absorptiometry (DXA).

The recent advances in molecular genetic testing have implemented the genetic testing of CHH into the diagnostic process of CHH. Molecular genetic testing is performed in order to find the inheritance pattern and the penetrance of the disease in the family (reviewed in Boehm et al. 2015, Pitteloud et al. 2010). Non-reproductive phenotype may be used in selecting patients to be screened for CHH genes. In fact, bony anomalies and dental agenesia are enriched in CHH patients with *FGFR1* mutations (Costa-Barbosa et al. 2013, Zenaty et al. 2006), whereas mutations on *ANOS1* are associated with a more severe reproductive phenotype and an increased prevalence of synkinesia (*i.e.* mirror movements) of the upper extremities (Salenave et al. 2008). In Finnish CHH patients, an algorithm which uses the non-reproductive phenotype can be used to guide the molecular genetic testing (Laitinen 2012). Genetic testing provides additional
information to the clinical practice, but it should be focused on the selected patients with CHH (Boehm et al. 2015).

The reversal CHH variants manifest in a broad spectrum of pubertal progress and non-reproductive phenotypes (Raivio et al. 2007). Testicular growth during follow-up may reveal the partial gonadotropin secretion seen in the reversal phenotype, but, again, differentiating these subjects from patients with CDGP is challenging (Raivio et al. 2007). Recently, a study of Chan et al. suggested that administration of kisspeptin could aid in identifying patients with a reversal form of CHH (Chan et al. 2014). Patients with CHH, except reversal phenotypes, did not present an LH response to administered kisspeptin (Chan et al. 2014). Consequently, an experiment of treatment pause in hormone replacement therapy can reveal subjects with reversal phenotype later in life.

1.4.3. Clinical features

CHH is associated with a variable non-reproductive phenotype which includes, for example, the lack of smell (anosmia) or reduced capability to smell (hyposmia), cleft lip or palate, renal agenesis, mirror movements of hands (synkinesia), dental defects, micropenis, and cryptorchidism (Table 1) (Laitinen et al. 2011, Costa-Barbosa et al. 2013, Krams et al. 1999, Bailliec-Forster et al. 2010, Molsted et al. 1997). The presence of these clinical cues can aid in the evaluation of an adolescent with suspected hypogonadism (Costa-Barbosa et al. 2013). Additionally, the non-reproductive features emphasize the need for a careful physical examination with special attention to the genitalia, teeth, and the neurological examination, when evaluating a patient with late puberty. Patients with CHH appear to grow relatively normally during childhood, but the impaired pubertal development results in the absence of growth spurt (Raboch & Reisenauer 1976, Kaushanski & Laron 1979)

1.4.4. The differential diagnosis between CHH and constitutional delay of growth and puberty

The early diagnosis of CHH remains as a dilemma (Boehm et al. 2015). Distinguishing prepubertal patients with CHH from those with CDGP, especially boys, is the most challenging process (Harrington & Palmert 2012). The only definite method to separate these two groups is to wait for the CDGP patients to show a complete and spontaneous progression of puberty. The difficult differential diagnosis between CDGP and CHH can result in an unnecessary delay in the diagnosis of CHH (Boehm et al. 2015). Several clinical and biochemical markers with varying success are used in this diagnostic process (reviewed in Harrington & Palmert 2012, Palmert & Dunkel 2012) (Table 2). These include GnRH and hCG stimulation tests, sex hormone levels, and the levels of Sertoli cell markers (Table 2). However, none of the tests show a complete diagnostic accuracy (Table 2) (Segal et al. 2009, Dunkel et al. 1985, Coutant et al. 2010, Adan et al. 2010, Grinspon et al. 2010), and most of the studies have been retrospective (Segal et al. 2009, Coutant et al. 2010, Sequera et al. 2002). Additionally, the absence of clinical signs used in the differential diagnostics in these studies is striking. The effectiveness of clinical cues in predicting the progression of puberty, such as cryptorchidism, has not been studied before. Thus, clinicians who evaluate patients with DP would benefit from such clinical predictors of pubertal outcome.
Table 2. The suggested cutoff levels, sensitivities, and specificities of clinical and biochemical markers used to differentiate between congenital hypogonadotropic hypogonadism and constitutional delay of growth and puberty.

<table>
<thead>
<tr>
<th>Marker(s)</th>
<th>Suggested cutoff levels</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline LH, IU/L</td>
<td>0.3-0.7</td>
<td>85-100</td>
<td>88-90</td>
<td>(Grinspon et al. 2010, Binder et al. 2015)</td>
</tr>
<tr>
<td>Baseline FSH, IU/L</td>
<td>0.9-1.2</td>
<td>88-100</td>
<td>55-88</td>
<td>(Segal et al. 2009, Grinspon et al. 2010)</td>
</tr>
<tr>
<td>GnRH induced LH response, IU/L</td>
<td>2.8-5.8</td>
<td>90-100</td>
<td>84</td>
<td>(Segal et al. 2009)</td>
</tr>
<tr>
<td>GnRH induced FSH response, IU/L</td>
<td>3.7-4.6</td>
<td>90</td>
<td>52-80</td>
<td>(Grinspon et al. 2010, Sequera et al. 2002, Binder et al. 2015)</td>
</tr>
<tr>
<td>Testosterone, nM</td>
<td>0.5</td>
<td>93</td>
<td>100</td>
<td>(Segal et al. 2009, Grinspon et al. 2010)</td>
</tr>
<tr>
<td>Inhibin B, pg/mL</td>
<td>28-35</td>
<td>80-100</td>
<td>75-100</td>
<td>(Segal et al. 2009)</td>
</tr>
<tr>
<td>hCG test, nM</td>
<td>3.6</td>
<td>92</td>
<td>92</td>
<td>(Coutant et al. 2010, Rohayem et al. 2015)</td>
</tr>
<tr>
<td>AMH, pmol/L</td>
<td>110</td>
<td>80</td>
<td>100</td>
<td>(Coutant et al. 2010)</td>
</tr>
<tr>
<td>Testicular volume (mL)</td>
<td>3</td>
<td>93</td>
<td>45</td>
<td>(Segal et al. 2009)</td>
</tr>
<tr>
<td>Combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibin B and LH (pg/mL, IU/L)</td>
<td>35 and 0.5</td>
<td>100</td>
<td>100</td>
<td>(Coutant et al. 2010)</td>
</tr>
</tbody>
</table>

Values lower than the cutoff levels increase the probability of CHH

1.4.5. Treatment

The treatment options for CHH depend on the aim of the treatment. Similarly to the treatment of CDGP, sex hormones are used in CHH to induce secondary sexual characteristics and height growth (reviewed in Boehm et al. 2015). In infants, cryptorchidism should be corrected during the first year of life as the untreated undescended testes are associated with impaired future fertility potential (Lee et al. 1996, van Brakel et al. 2013, van Brakel et al. 2014). Similarly, short term low-dose testosterone treatment in early infancy induces sufficient penile growth in patients with micropenis (Hatipoglu & Kurtoglu 2013, Landier, Chaussain & Job 1984). The adolescent boys are treated with testosterone and girls with estrogen to induce virilization and pubertal growth. The treatment reduces the risk for psychosocial disturbances caused by sexual infantilism (Dwyer et al. 2014). The dosage of sex steroids is gradually increased, mimicking the physiological rise in sex steroid levels that occurs during puberty. Also, the secondary sexual characteristics can be achieved by using pulsatile GnRH or hCG alone or in combination with recombinant FSH (Raivio, Wikstrom & Dunkel 2007, Zacharin et al. 2012). These treatment modalities induce testicular growth, spermatogenesis, and are associated with improved HRQoL scores (Boehm et al. 2015, Shiraishi, Oka & Matsuyama 2014). However, the previous patient series have included only a limited number of subjects, and the results need to be repeated in a large sample of CHH patients. After the induction of puberty, the
adequate sex hormone dosage is titrated by using the baseline levels of testosterone or estradiol.

1.4.6. Long-term impact of sex steroid deficiency on bone-health and health-related quality of life

Pubertal development is crucial for a normal acquisition of bone mineral content and bone density. Findings in menopausal women have shown a direct negative relationship between estrogen levels and bone density (Ahlborg et al. 2003). Similarly, hypogonadal men have a lower bone mineral density than those with normal testosterone levels (Canale et al. 2000). Patients with CHH and insufficient hormone replacement therapy have an increased risk for osteoporosis (Laitinen et al. 2012a, Finkelstein et al. 1989). Hormone replacement therapy restores bone health and emphasizes the life-long need of hormonal replacement therapy in patients with CHH (Laitinen et al. 2012a, Canale et al. 2000).

Hypogonadism has adverse effects on general well-being and psychosocial long-term outcomes. HRQoL scores in men with CHH suggest that CHH is associated with an impaired quality of life (Aydogan et al. 2012, Shiraishi, Oka & Matsuyama 2014, Dwyer et al. 2014, Lasaite et al. 2013). These studies included mixed study populations and an unclear characterization of CHH (Aydogan et al. 2012, Shiraishi, Oka & Matsuyama 2014, Lasaite et al. 2013). Aydogan et al. reported lower HRQoL scores in men with CHH, particularly in general health and emotional domains of HRQoL (Aydogan et al. 2012). These men had higher depression scores when evaluated with Beck Depression Inventory (BDI). After the beginning of testosterone therapy, both the HRQoL and BDI scores improved (Aydogan et al. 2012). The studies, however, did not evaluate the impact of the non-reproductive phenotype of CHH or the age of diagnosis on HRQoL.

2. THE HORMONAL REGULATION OF GROWTH

Human growth has been categorized into three different components (infancy, childhood, and puberty) by using the Karlberg’s growth model (Karlberg 1989). Each phase is controlled by a distinct hormonal and biological mechanisms: for example, sex steroids are the major regulators of growth during puberty (Karlberg 1989). During the last decade, several studies in both developing and developed countries have reported an increase in mean height (Saari et al. 2011, Cole 2000, Roelants, Hauspie & Hoppenbrouwers 2009). This trend is probably due to the changes in environmental factors such as nutrition that regulate growth. Due to this secular trend observed also in Finland, national growth references should be updated from time to time (Saari et al. 2011, Sorva et al. 1990, Sorva, Tolppanen & Perheentupa 1990).

2.1. Fetal life and infancy

Fetal growth is dependent on materno-placental environment, and the main hormonal contributors to growth are insulin, IGF-1, and IGF-2 (Murray & Clayton 2013). In rodents, the expression of Igf2 is widespread in fetal tissues during the last trimester, and deleting Igf1 or Igf2 results in fetal growth retardation (Fowden 2003). Elevated transcription of IGF-2 results in overgrowth (Sparago et al. 2004), whereas a down-regulated IGF-2 expression is associated with perinatal growth...
retardation (Gicquel et al. 2005). Altogether, linear growth in early gestation is more rapid in boys (Pedersen 1982). Growth velocity increases during the first two trimesters and peaks at mid-gestation (2.5 cm/week) (Underwood & Van Wyk 1992). The role of androgens in this process is unclear. Infants with congenital adrenal hyperplasia (CAH), which is a condition of fetal androgen excess, have a higher birth length than healthy controls. Conversely, the newborns with complete androgen insensitivity syndrome (CAIS) show similar birth length to the general population (Balsamo et al. 2006, Miles et al. 2010). These findings suggest that androgens may contribute to fetal growth.

Very little is known about the contribution of sex steroids on the growth during infancy as well. The transient postnatal activation of the HPG axis appears to interact with an early infancy growth. During minipuberty, serum testosterone levels, but not estradiol, correlates with penile growth and growth velocity (Boas et al. 2006, Kiviranta et al. 2016). Boys experienced a length velocity up to 4 cm/yr faster than the girls during minipuberty. The peak growth velocity occurs simultaneously with the average age of the peak gonadal activation (at 1 month of age) (Kiviranta et al. 2016). Interestingly, the levels of IGF-1 remain similar in boys and girls during the first six month of life, which suggests that the GH/IGF-1 axis is not stimulated despite the elevated sex steroid levels (Kiviranta et al. 2016). These findings suggest that androgens modulate growth in early infancy, and the influence appears to be mediated directly on target tissues.

During the first two years of life, infants experience catch-up or catch-down growth which suggests that early infancy growth compensates the growth during fetal life. In detail, birth size correlates poorly with parental size, whereas after the first two years the correlation improves significantly (Murray & Clayton 2013). The phenomenon has been explained with a central mechanism that detects and balances the difference between the expected and the actual size, or that it is a result of compensatory mechanisms of growth plate stem cells to growth delay (Murray & Clayton 2013). The growth during infancy is controlled by the combined effects of thyroid hormone, GH/IGF-1 axis, and nutrition. Diet induces IGF-1 production in the liver (Socha et al. 2011, Ong et al. 2009, Low et al. 2001). Besides liver, IGFs are produced in the target tissues. Locally produced IGF-1 is a more potent growth contributor than the liver produced molecule, since in mice, removing the hepatic igf1 does not inflict a significant growth retardation (Liu, Yakar & LeRoith 2000). In humans, the phenotype of genetic IGF-1 deficiency includes deafness, intellectual disability, and pre- and postnatal growth retardation (Netchine et al. 2011). The most important target tissue of IGFs is the epiphyseal growth plate. In the growth plate, linear bone growth is achieved by the endochondral ossification. Chondrocytes not only express receptors for IGF-1, but also produce IGF-1 locally. In mice, the chondrocyte-derived IGF-1 and IGF-2 contributes significantly to the linear bone growth (Smink et al. 2002). Consequently, the infancy growth is first a continuation of the rapid fetal growth. Length velocity is at the highest level during the first 3 months (20 cm/year), and then it gradually declines to 9 cm/yr at the average age of 3 yrs (Thiering et al. 2012, Molinari, Gasser & Largo 2013).

2.2. Childhood

After infancy, the primary role of nutritional component in the regulation of growth is replaced by GH stimulation on IGF-1 synthesis in liver and in target tissues
At the same time, the importance of thyroid hormone in the growth regulation increases. Hypothyroidism results in the reduction of GH and IGF-1 levels, and also an impaired chondrocyte proliferation and hypertrophy in the growth plate (Schmid et al. 2006, Kindblom et al. 2001, Stevens et al. 2000). The influence of sex steroids on childhood growth is unclear. Supraphysiological levels of androgens, if aromatized to estrogens, promote growth since children with excessive androgen production (i.e. CAH) have higher growth velocity and BMI values and a higher risk of childhood obesity than their peers (Volkl et al. 2006) (Merke et al. 2000). This suggests a programming contribution of androgens to the body composition during childhood. Furthermore, obese children show increased levels of dehydroepiandrosterone, a more advanced bone age throughout childhood, and an increased height standard deviation score (SDS) at the onset of puberty (Denzer et al. 2007, Johnson et al. 2012, Lass et al. 2011). This has been thought to result from an increased aromatase activity in the adipose tissue (Polari et al. 2015). In contrast, hypogonadism has been considered to contribute little to childhood growth. Importantly, male patients with androgen insensitivity syndrome have a tendency towards increased height growth during late-childhood and adolescence (Hellmann et al. 2012). Children with very rapid infant weight gain have higher levels of adrenal androgens during childhood (Ong et al. 2004). An excessive weight gain associates with an earlier pubertal development (Lee et al. 2016), albeit a certain amount of weight is critical for the normal onset of puberty (Frisch 1994). All in all, childhood growth is mainly GH dependent, which results in an average annual height velocity of 6 cm in mid-childhood which then declines to 5 cm/yr before the onset of puberty (Kelly et al. 2014). Further, the height velocity continues to decline steadily in a case of an absent pubertal growth spurt (Rikken & Wit 1992).

### 2.3. Puberty

During puberty, the constant childhood growth is gradually changed to the rapid pubertal growth. During the transition, childhood growth slows to its nadir right before the pubertal growth acceleration, which results in a period of very slow height velocity (i.e. peripubertal growth dip) (Rogol, Roemmich & Clark 2002). Pubertal growth has been traditionally categorized into three phases: the time of growth acceleration (i.e. take-off velocity) right before the actual growth spurt, the time of most rapid growth (i.e. peak height velocity), and the phase of decreased growth velocity and the fusion of the epiphyseal cartilage (Tanner et al. 1976). The peak pubertal height velocity is reached at 1 to 2 yrs after the onset of puberty (Wehkalampi et al. 2011, Abbassi 1998), and girls reach it approximately 2 yrs before boys, which is likely due to the more rapid rise in estradiol levels reported in early pubertal girls (Marshall & Tanner 1970, Janfaza et al. 2006). During the growth spurt, the average height velocity is 8 cm/yr in girls, whereas boys exhibit even faster growth (9 cm/yr) (Abbassi 1998). Then the height velocity steadily decreases as the epiphyseal plates fuse. In both sexes, estrogen has a pivotal role in the induction of the growth spurt (Juul 2001, Morishima et al. 1995, Carani et al. 1997). Estrogen levels stimulate pituitary GH release and IGF-1 production in the liver and in target tissues (Venken et al. 2005, Mauras et al. 1987). More evidence supporting the role of estrogen in the stimulation of GH/IGF-I axis has accumulated from studies in boys treated with AIs. Adolescent boys treated with AI (anastrozole or letrozole) showed a decrease in IGF-1 concentrations during
follow-up (Mauras et al. 2000, Neely et al. 2014), whereas one study found no change in IGF-1 levels during anastrozole-treatment (Mauras et al. 2008). Treating CPP patients with a GnRH analog decreases not only the levels of gonadotropins and sex steroids but also the levels of IGF-1 and IGF-binding protein 3 (Muller et al. 2000, Juul et al. 1995). Additionally, estrogen induces growth plate senescence and maintains bone health by stimulating chondrocytes and osteoblast invasion in the epiphyseal plate (Shim 2015, Kusec et al. 1998, Yin et al. 2015). In boys, estradiol levels correlate positively with bone mineral density (BMD) (Doneray & Orbak 2010). The highest bone mineral content is achieved earlier in girls, whereas boys accumulate higher bone mass and more pronounced bone expansion (i.e. periosteal apposition) mainly due to longer period of bone acquisition (reviewed in Bonjour & Chevalley 2014). During puberty, testosterone induces protein synthesis and lipolysis, and increases muscle mass and BMD (Doneray & Orbak 2010, Mauras et al. 2003, Hero et al. 2006). Thus, adolescent boys accumulate significantly more fat free mass in contrast to girls who gain more fat mass during the same period (Fintini et al. 2011, Garnett et al. 2004). Altogether, the absence of puberty results in low bone mineral content, osteoporosis, and the absence of pubertal growth spurt which are all observed in patients with CHH (Laitinen et al. 2012a, Van Dop et al. 1987).

2.4. Adult height

Adult height is regulated by genetic factors. Interestingly, a genome-wide association study found 423 loci, but could not focus single genes, that influenced on adult height (Wood et al. 2014). Evidence suggests that the majority of these genes do not participate in the GH/IGF-1 axis, but are associated with for example fibroblast growth factor signaling (Wood et al. 2014, Lui et al. 2012). After the growth spurt, the adolescents continue to grow until the epiphyseal plates fuse. Thus, the deficiency of sex steroids and the absence of puberty are likely to result in a longer period of growth and a taller adult height. In fact, this is best demonstrated in the patients with aromatase deficiency or with CHH as they typically exceed their genetic growth potential and their adult height is above the height of the general population (Morishima et al. 1995, Raboch & Reisenauer 1976, Dickerman, Cohen & Laron 1992, Uriarte et al. 1992). Conversely, a premature closure of the epiphyseal plates reduces the adult height. In patients with CPP, a short childhood growth phase together with an early timing of the growth spurt and bone maturation result in a height loss and shorter adult height in contrast to the general population (Carel & Leger 2008, Bertelloni & Mul 2008).

2.5. Evaluation of growth

Adult height estimates are used by clinicians to predict the height reached after puberty. Genetic growth potential can be estimated with the mid-parental target height (MPH). In Finland, the MPH is calculated by using the formula: 0.886 x ([father’s height + mother’s height] / 2 + 6.8 – 178.9066) / 6.6784 – 0.071 (Saari et al. 2011). However, MPH is only a rough estimate of the growth potential regardless of the individual variation in pubertal timing and bone maturation. Statural growth continues until the epiphyseal cartilages fuse. Thus, a more precise estimate of the individual growth potential at a certain calendar age is achieved by determining the current bone age from the wrist and hand x-ray. Bone age is visually estimated by using the radiographic atlases of skeletal maturation
REVIEW OF THE LITERATURE

(Tanner et al. 1983, Greulich & Pyle 1959). Subsequently, the individual timing of puberty (precocious, normal, and delayed) and the determined bone age are subjected to an adult height prediction model which produces a prediction of adult height (Bayley & Pinneau 1952). As the bone age estimate depends solely on the view of the assessor, it is susceptible to variation. Recently, the discovery of computerized assessment of bone age has made the evaluation of skeletal maturation easier and the adult height predictions more precise (Thodberg et al. 2009). This automated bone age calculation is widely implemented to the clinical practice.

Growth velocity may provide cues of chronic illnesses, albeit a number of conditions may manifest in impaired growth (Tuchman et al. 2008, Taylor et al. 2012, Sankilampi et al. 2013). The evidence that support growth velocity to be used in the screening chronic diseases is inadequate. A recent review collected data from 69 articles which reported growth monitoring algorithms to detect chronic growth inhibiting disorders such as GH deficiency, Turner syndrome (TS), celiac disease, and cystic fibrosis (Scherdel et al. 2016). The meta-analysis found out that only the clinical decisions rules to detect patients with TS, coeliac disease and cystic fibrosis had sufficient specificities (Scherdel et al. 2016). In accordance, the evidence on the diagnostic performance of growth velocity to detect subjects with an abnormal pubertal development is scarce and limited to delayed puberty (Palmert & Dunkel 2012). It has been suggested that the annual growth velocity less than 3 cm could aid in identifying patients with delayed puberty caused by a chronic disease (Palmert & Dunkel 2012). Conversely, a steep growth curve together with advanced bone age in subjects with clinical signs of CPP supports the premature activation of the HPG axis (Fuqua 2013).
AIMS OF THE STUDY

This study was conducted to evaluate the interactions between the HPG axis and growth from early infancy to adult height, and to describe the etiology and outcome of delayed puberty. The specific aims were:

1. to investigate if circulating MKRN3 levels could serve as a read-out of the diminishing central restrain on the HPG axis (I)

2. to characterize the diagnoses that underlie delayed puberty and predictors of its clinical course (II)

3. to depict the growth patterns of patients with congenital hypogonadotropic hypogonadism from birth to adult height (III-IV)

4. to evaluate the health-related quality of life in men with CHH (V)
PATIENTS AND METHODS

3. STUDY SUBJECTS

3.1. Boys with idiopathic short stature (ISS) (I)

The boys with idiopathic short stature (ISS) participated in a randomized placebo-controlled double-blind trial, in which 16 boys were treated with letrozole (Lz) (2.5 mg/d) and 14 received placebo (Pl) for two years (Hero, Norjavaara & Dunkel 2005). At the beginning of the study, the boys had a mean age of 11 years (range: 9.1 - 14.2 yrs). The chronic diseases that can cause short stature were excluded by using growth curve analysis, serum IGF-I level, and in subject with a suspicion of GH deficiency, a GH stimulation test. The boys were investigated every 6 months for 2 years, and the follow-up protocol included a final visit at 36 months after the beginning of the study. At the visits, testis size was calculated by using the formula: length x width$^2$ x 0.52, and pubertal stage was recorded (Marshall & Tanner 1970, Behre, Nashan & Nieschlag 1989). At the beginning of the study, 28 boys were prepubertal (G1), one boy was at G2 (testicular volume above 3 mL), and one at G3 (Hero, Norjavaara & Dunkel 2005). During the two years of the treatment, eight boys who were treated with Lz and eight who received Pl showed signs of puberty progression, whereas at 36 the months visit seven out of 10 with Lz and six out of 11 boys with Pl were pubertal (Hero, Norjavaara & Dunkel 2005). Additionally, six boys fulfilled the criteria of delayed puberty (i.e. G1 at the age of 14 yrs or older). Letrozole which is a potent third-generation AI, effectively inhibited the estrogen biosynthesis as indicated by low serum estradiol levels in the treated boys despite stimulated testosterone secretion (Hero, Norjavaara & Dunkel 2005, Wit, Hero & Nunez 2011).

3.1.1. Collection and analyses of the serum samples

The serum samples of boys with ISS were used (Hero, Norjavaara & Dunkel 2005). The obtained serum was stored at -20 or -80 C until the hormonal analyses were performed. Gonadotropin levels were measured with an ultrasensitive immunofluorometric assays (Wallac, Turku, Finland), inhibin B levels with immunoenzymometric assay (Serotec, Oxford, UK), and testosterone levels with a modified RIA (Hero, Norjavaara & Dunkel 2005). Serum MKRN3 levels were quantified by using the commercially available Human MKRN3 enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA) with the sensitivity of 7.8 pg/mL, the within assay CV of 6%, and the between assay CV of 9.2%. Before the analyses, the samples were diluted (1:8) according to the instructions in order to lower the MKRN3 levels to the measuring range of the kit. The final MKRN3 level was achieved by multiplying the measured level with 8.

3.2. Patients with DP (II)

The patients (408 boys and 181 girls) had been investigated for DP at the Pediatric Endocrine Outpatient Clinic of Helsinki University Hospital between 2004 and 2014 and were identified with an ICD-10 code-based inquiry to the electronic patient information system (Figure 4). The used ICD-10 codes were E28, E28.3, E28.8, E28.9, E29, E29.0, E29.1, E29.8, E29.9, E30.00, E30.09, E30.8, and E30.9.
Of the 589 patients, 244 (174 boys and 70 girls) fulfilled the criteria of DP (Figure 4). The medical records included information about Tanner stages in 244 (100%), bone age in 227 (93%), baseline LH 216 (88%) and FSH levels in 216 (88%) and 218 (89%), sex steroid levels in 216 (89%) and 218 (89%). Of the second line investigations of DP, a GnRH test was performed in 104 (43%) and inhibin B levels in 126 (72%) boys. A growth hormone (GH) stimulation test was performed in 15 patients (8 boys and 7 girls), and two of them (panhypopituitarism and juvenile rheumatoid arthritis) had a result which suggested a GH deficiency. A ruler-measured testicular volume was available in 120 (69%) subjects. Height measurements, which were taken at least every six months, were available in 230 (94%) boys, which enabled the calculation of the annual height velocity. The timing of puberty in the first degree relatives of the patients was recorded in all patients, and puberty was considered late if a sister or the mother had reached menarche after 14 yrs, or a brother or the father had experienced late puberty or delayed growth spurt.

![Figure 4. A flowchart describing the patient recruitment for study II.](Modified from II)
PATIENTS AND METHODS

3.2.1. Classification of different DP types

The diagnoses of DP were classified into the four main diagnostic categories: FHH, PHH, Hyper H, and CDGP (Sedlmeyer & Palmert 2002). The diagnosis of CDGP was confirmed when the clinical, the hormonal, and the radiologic data ruled out pathological causes of DP. Further, in most of the cases with CDGP (86%), the progression of puberty was confirmed in the hospital outpatient clinic, whereas the remaining cases were followed up in the school health care. However, none of the patients were referred back to the tertiary center for an evaluation of pubertal progress.

The PHH group consisted of patients with a confirmed pathology in the CNS, or an organic cause for the hypogonadism, and/or low LH and FSH and sex steroid levels, and without any spontaneous progression of puberty until the age of 18 yrs. Of the 19 patients with PHH, 16 (11 boys and five girls) had CHH as the cause of the permanent hypogonadism. In the remaining three patients, PHH was caused by Prader-Willi syndrome, panhypopituitarism, and craniopharyngioma.

The patients with FHH had an identified disease that likely delayed pubertal onset, but the patients showed a spontaneous progression of puberty during the follow-up. Interestingly, a spontaneous progression of puberty was confirmed in 14 girls and 15 boys (97%) with FHH within the next three yrs after the initial evaluation of puberty. The patients with FHH had no hormonal or clinical signs which suggested the presence of permanent hypogonadism. Additionally, the FHH group included patients with anorexia nervosa and those with severe underweight. These patients had age- and sex-matched BMI (ISO-BMI) values below 16 kg/m² prior to the initial evaluation (Saari et al. 2011). Finally, the diagnosis of Hyper H was confirmed with gonadal failure and high baseline LH and FSH levels. Remarkably, a history of prior cryptorchidism was reported in the three boys with CDGP (unilateral) and in four boys with CHH (two unilateral and two bilateral), whereas none of the patients had a history of micropenis. The methods used to determine the levels of gonadotropins, sex steroids and inhibin B between 2004 and 2014 are described in Table 3 (Table 3).

Table 3. The methods used to determine gonadotropins, sex steroids and inhibin B levels in studies II-V.

<table>
<thead>
<tr>
<th>Hormone(s)</th>
<th>Method</th>
<th>Period</th>
<th>Detection limit</th>
<th>Manufacturer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH and FSH</td>
<td>Immunofluorometric assay</td>
<td>2004 - 2011</td>
<td>0.1 IU/L</td>
<td>AutoDELFIA, Wallac, Turku, Finland</td>
<td>II, III, IV, V</td>
</tr>
<tr>
<td></td>
<td>Electrochemiluminescence immunoassay</td>
<td>2011 -</td>
<td>0.1 IU/L</td>
<td>Roche Diagnostics, USA</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Radioimmunoassay</td>
<td>2009 - 2014</td>
<td>30 pmol/L</td>
<td>AutoDELFIA, Perkin-Elmer, Turku, Finland</td>
<td>II, IV</td>
</tr>
<tr>
<td></td>
<td>Immunoluminometric assay or mass spectrometry</td>
<td>2014 -</td>
<td>10 pmol/L</td>
<td>Siemens Healthcare Diagnostics, Germany</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Packard-Becker, Groningen, The Netherlands</td>
<td>II</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Radioimmunoassay</td>
<td>2004 - 2005</td>
<td>&lt; 1.0 nmol/L</td>
<td>AE Sciex, Foster City, California, USA</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Mass spectrometry</td>
<td>2005 -</td>
<td>0.05 nmol/L</td>
<td>Oxford Brooks Innovation Diagnostics Systems</td>
<td>II, III, V</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Laboratories, UK</td>
<td></td>
</tr>
<tr>
<td>Inhibin B</td>
<td>ELISA</td>
<td>2004 - 2010</td>
<td>7 pg/mL</td>
<td>Beckman Coulter, Inc, USA</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>2010 -</td>
<td>2.6 pg/mL</td>
<td></td>
<td>II</td>
</tr>
</tbody>
</table>
3.3. Patients with CHH (III-V)

The third study cohort included 36 patients (30 men and 6 females) with CHH who participated in the nationwide study of KS in Finland (Laitinen et al. 2011). In addition to the Finnish boys with CHH, the growth data of nine Danish patients were included (Tommiska et al. 2014). The diagnosis of CHH was based on i) absent or incomplete puberty at the age of 18 years and ii) low sex steroid levels accompanied by normal or subnormal gonadotropin levels and iii) otherwise normal anterior pituitary function and iv) no organic cause for their condition. Seven boys (six Finns and one Dane) did not meet the criteria since they were less than 16 yrs old at the time of diagnosis. Their diagnosis, however, was confirmed since the Finns had a history of prior cryptorchidism and/or micropenis, and no testicular growth during the clinical follow-up, whereas the Dane had a micropenis and very low minipubertal LH (< 0.05 IU/L) and testosterone (< 0.23 nmol/L) levels. The molecular genetic diagnosis of CHH was confirmed in 22 patients (Laitinen et al. 2011, Laitinen et al. 2012b, Tommiska et al. 2014). The girls all had a mutation in FGFR1 (n=6), whereas in boys with CHH, the gene mutations were categorized into three groups: the boys with mutations in ANOS1 (n=7), FGFR1 (n=5), and into those with mutations in gonadotropin-releasing hormone receptor (GNRHR), chromodomain helicase DNA-binding protein 7 (CHD7), or prokineticin 2 (PROK2) (n=4). Clinical signs of profound GnRH deficiency (history of micropenis or/and cryptorchidism) were reported in 23 (53%) (14 Finns and 9 Danes) subjects, whereas one girl had a cleft lip and none had bony anomalies. Hormonal markers were determined in the laboratory of Helsinki University Central Hospital (HUCH). The used methods are described in detail in Table 3 (Table 3).

3.3.1. Acquisition of growth data

The growth data of six CHH girls with an FGFR1 mutation, and 36 boys with CHH (27 from Finland and 9 from Denmark) were reviewed retrospectively (Laitinen et al. 2011, Laitinen et al. 2012b, Tommiska et al. 2014). Height/length and weight were measured by trained nurses, and bone age was determined by the physician according to the Greulich and Pyle atlas. National reference data were used to calculate the height/length SDSs. We were especially interested in the linear growth during minipuberty. Thus, growth charts were carefully searched to obtain the length measurements at birth, and three and six months of age. When an exact measurement was not present at three or six months of age, we selected the nearest available measurement (at 3 months, range: 2-4 months, and at 6 months, range: 5-7 months). Fifteen (42%) of the boys with CHH had representative length measurements (i.e. birth length and a length measurement at three or six months of age) during minipuberty, which allowed us to calculate the decline in length SDS from birth to three and six months of age. Subsequently, the length SDSs were compared with MPH SDSs, available in 11 (73%) boys with representative length measurements during minipuberty. Adult heights were available in 31 boys (23 Finns and 8 Danes) and in three girls. The MPH SDS (n=22) was calculated in Danes with the mean height SDS of mother and father, and in Finnish boys and girls by using the equations: 0.886 x ([father’s height + mother’s height] / 2 + 6.8 – 178.9066) / 6.6784 – 0.071, and 0.0611 x (father’s height + 0.0703 x mother’s height - 22.37), respectively (Saari et al. 2011).
In the boys with CHH, the mean age of pubertal induction was 16.1 yrs (range: 12–21), whereas the corresponding age in girls was 15.7 yrs (range: 14.8–16.8). In 14 boys, the data permitted us to calculate the dosage of testosterone used during the first year of pubertal induction (mg/kg/yr).

Before the age of 2 yrs, weight gain was characterized by using the weight-for-length which is the percentage deviation of weight from the median weight for length and sex (%DW) (Sorva, Tolppanen & Perheentupa 1990). Thereafter, ISO-BMI or BMI was used (Saari et al. 2011). The ISO-BMI values were categorized into severe underweight (ISO-BMI less than 16 kg/m²), underweight (16-17 kg/m²), normal weight (17-25 kg/m²), overweight (25-30 kg/m²), and obesity (more than 30 kg/m²) (Saari et al. 2011).

3.3.2. Health-related quality of life

Health-related quality of life was evaluated in 30 men with CHH by using the 15D instrument (Sintonen 2001). At the evaluation visit, the mean age of the patients was 38.1 yrs (range: 16–61), and four patients had a reversal phenotype of CHH. The 15D is a descriptive questionnaire which includes questions about the 15 different dimensions of health (mobility, vision, hearing, breathing, sleeping, eating, speech, excretion, usual activities, mental function, discomfort and symptoms, depression, distress, vitality, and sexual activity). The answer to each question is classified into 5 levels (the best level=1; the worst level=5), and the alternative that best describes the current state is chosen. Thus, the questionnaire produces a single index score (15D score, scale from 0-1, 1=full health, 0=being dead) which represents the overall HRQoL, and a similar score for each dimension. Subsequently, population-based preference weights are implemented to the 15D scores which result in final comparable HRQoL scores (range 0–1). In this study, we compared the mean 15D score and the mean dimension values in patients with CHH to those of a general male population (n=1227). A clinically relevant difference in the 15D score is 0.03 (Alanne et al. 2015).

4. STATISTICS

Statistical analyses were conducted with SPSS statistical software, release 22.0 (SPSS, Chicago, IL, USA). The data is presented with mean (SD) unless otherwise mentioned. The analyses between two groups were carried out with the t-test (I-II, V) or the Mann-Whitney U-test (I-II), whereas when comparing the height SDS to general population, one-sample t-test was used. Longitudinal length/height and weight measurements were analysed with the paired samples t-test. Correlations were determined with Spearman’s rank correlation (r_s) (I-III, V). The correlations were two-sided, except for the correlation between the age of diagnosis and HRQoL (V). However, this was accepted since delayed diagnosis has been shown to associate with long-term psychological consequences (Dwyer et al. 2014).

Categorical variables were analysed with the Fisher’s exact test. Odds ratios (OR) with 95% confidence intervals (CI) were calculated (II). The diagnostic accuracy of the clinical and hormonal markers of puberty were modelled with the receiver operating characteristic (ROC) curves with area under the ROC curve (AUC) and 95% CI. The ROC curves were interpreted and cutoff values which
PATIENTS AND METHODS

maximized the sensitivity and the specificity were selected. For the risk prediction model of CHH in study II, the quality control (QC) measures of inhibin B were obtained between 2004 and 2014, and fitted into a linear model that predicted the dispersion for each mean. Based on mean ± 2 standard error of the QC measurements, three inhibin B categories were created (10-49, 49-111, and 111-212 ng/L). Subsequently, inhibin B levels of the patients were divided into the three categories. Finally, the combination of inhibin B category and testis size was subjected to a logistic regression model which predicted the disease risk.

The longitudinal measurements of MKRN3, gonadotropins, testosterone, and inhibin B were investigated by implementing the method of summary measures (Matthews et al. 1990). Thus, a regression line was fitted to each patient’s measurements, with age as an independent variable and circulating MKRN3, testosterone, LH, FSH, or inhibin B levels as dependent variables. The obtained regression coefficients were analyzed as raw data with the t-test. The statistically significant level was set to $P$ value less than 0.05.

5. ETHICS

The study protocol was approved by the Ethics Committee of the HUCH and Danish regional ethical committee, and the national agency of medicines (FIMEA) (I, III-V). A written informed consent was obtained from all subjects and their guardians. The study on the etiology of delayed puberty contained only data from the medical records without any contact to the patients. Thus, according to the Finnish medical research act, no ethical permission was required (II).
RESULTS

1. Serum MKRN3 levels in boys before the clinical onset of puberty

During the three year follow-up, circulating MKRN3 declined significantly (-669 ± 713 pg/mL/yr, \( P < 0.001 \)) (Figure 5). The overall decrease was similar between the Lz-treated boys (n=16) and those who received Pl (n=14) (-657 ± 750 vs -683 ± 690 pg/mL/yr, \( P = \) NS). Subsequently, we investigated how MKRN3 levels changed during the progression of puberty, and analyzed the longitudinal changes in MKRN3 levels in 13 boys with available measurements before and after the clinical onset of puberty (defined as genital stage 2). In these boys, the decline in MKRN3 levels was more profound before G2 (2931 ± 2750 pg/mL/yr), than after the onset of puberty (560 ± 1510 pg/mL/yr, \( P < 0.05 \)). Interestingly, before pubertal onset, the boys who were treated with Lz had a slower decline in MKRN3 levels (-782 ± 3190 pg/mL/yr) than those who received Pl (-2030 ± 1821 pg/mL/yr, \( P < 0.05 \)).

![Figure 5. Panel A, the mean MKRN3 levels in boys with idiopathic short stature during the follow-up. Panel B, the mean MKRN3 levels adjusted for the clinical onset of puberty (genital stage 2, G2). Lines represent mean (blue) ±1 standard deviation (dashed line).](image)

Since MKRN3 is suggested to be a key inhibitor of GnRH neurons, we examined if circulating MKRN3 levels would associate with the clinical and biochemical markers of puberty. Indeed, the decline in MKRN3 levels correlated negatively with testis sizes (\( r_s = -0.3, P < 0.001 \)), and the decline appeared to be faster at small testis sizes (Figure 6). Additionally, we examined the relationship between testicular growth and MKRN3 by determining the testicular volume for each patient at the time of the highest and the lowest measured MKRN3 level. At the highest MKRN3 level, the mean testicular volume was small (2.0 ± 2.9 mL), whereas larger testes sizes were observed at the lowest MKRN3 level (5.8 ± 4.5 mL, \( P < 0.001 \)). After excluding the two boys who showed an increasing longitudinal change in MKRN3 levels during the study period, we found that the rate of decline in MKRN3 levels (n=26) correlated negatively with the corresponding change in testosterone (\( r_s = -0.6, P < 0.01 \)), LH (\( r_s = -0.5, P < 0.01 \)).
RESULTS

n=26), and inhibin B levels \( (r_s = -0.44, P < 0.05) \). The reciprocal association between serum MKRN3 levels and the levels of inhibin B in four individuals are described in Figure 8 (Figure 7).

Figure 6. The association between testis size and serum MKRN3 levels (160 measurements) in 30 boys with idiopathic short stature (ISS) followed for 3 yrs \( (r_s = -0.3, P < 0.001) \). The line represents the relationship between testis size and MKRN3 in 14 boys with ISS who received placebo \( (r_s = -0.4, P < 0.001) \) (Modified from I).

Figure 7. The reciprocal changes in circulating MKRN3 and inhibin B levels in four boys with ISS before and after the onset of puberty.

Prepubertal boys treated with Lz and those with Pl had similar levels of testosterone, gonadotropin and inhibin B levels, testis sizes, and no difference in
the age of puberty onset (Hero, Norjavaara & Dunkel 2005). The six boys who were diagnosed with DP, however, experienced a significantly lower decline in MKRN3 levels than those with normal pubertal timing (-81 ± 612 vs -812 ± 671 pg/mL/yr, \( P < 0.05 \)), albeit we found no association between circulating MKRN3 and parameters of pubertal timing: age at G2, time from the initial evaluation to G2, or bone age at G2 (\( r_s = 0.05 – 0.3, P = NS \)).

2. The underlying causes of DP

We found 30 different diagnoses that underlie DP (Table 4). In both sexes, the most common cause was CDGP, and it was more frequent in the boys than in the girls (82% vs 56%, \( P < 0.001 \)). Additionally, 63% of the boys and 69% of the girls with CDGP had a positive family history of DP (\( P = NS \)). The absolute number of girls with familial CDGP (n=27) was lower than the number of boys (n=90). In consequence of the lower number of girls with CDGP, the relative frequency of pathological conditions that underlie DP (i.e. FHH, PHH or Hyper H) was higher in girls (44% vs 18%, \( P < 0.05 \)). The second most frequent cause of DP in both sexes was FHH. Further, a difference between the sexes was apparent in the FHH and Hyper H categories, since they both affected the girls more frequently than the boys (\( P < 0.001 \)).

Table 4. The number of different diagnoses that underlie delayed puberty at the pediatric outpatient clinic of Helsinki University Central Hospital between 2004 and 2014 (n=244) (Modified from II).

<table>
<thead>
<tr>
<th>Diagnoses</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDGP</td>
<td>142 (82%)</td>
<td>39 (56%)</td>
</tr>
<tr>
<td>PHH</td>
<td>13 (7%)</td>
<td>6 (9%)</td>
</tr>
<tr>
<td>Congenital hypogonadotropic hypogonadism</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Hypopituitarism</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Craniopharyngioma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Prader-Willi syndrome</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Severe underweight</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Anorexia</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Severe developmental delay</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Clinical CHARGE syndrome without CHD7 mutation</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VATER association</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cornelia de Lange syndrome</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cartilage-hair hypoplasia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intense exercise</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Jacobsen syndrome and hypothyroidism</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hydrocephalus</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Juvenile idiopathic arthritis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Radiation therapy for malignancy</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CNS disorders (Small Rathke’s cleft cyst or empty cella)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Hyper H</td>
<td>3 (2%)</td>
<td>11 (15%)</td>
</tr>
<tr>
<td>Anorchia</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Idiopathic testicular failure</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Idiopathic ovarian failure</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Chemotherapy for malignancy (Askin tumor)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Turner syndrome</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>FSH receptor gene mutation</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SRY-positive XX male</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

CDGP, constitutional delay of growth and puberty; PHH, permanent hypogonadotropic hypogonadism; FHH, functional hypogonadotropic hypogonadism; Hyper H, hypergonadotropic hypogonadism; CHD7, chromodomain helicase binding protein 7.
RESULTS

Subsequently, we compared the distribution of the four diagnostic categories in our series to the frequencies reported in the most comprehensive study published so far (Sedlmeyer & Palmert 2002). In our series, the frequency of CDGP was significantly higher (in girls 56% and in boys 82%) than reported before (32% and 64%, P < 0.001, respectively) (Figure 8) (Sedlmeyer & Palmert 2002). By contrast, the frequency of FHH (9%) and Hyper H (2%) in boys, and PHH (15%) in girls, was significantly lower than proportion in the previous series (20%, 7%, and 25%, P < 0.05, respectively) (Figure 8).

![Figure 8. The distributions of the four main diagnostic groups that underlie late puberty in the two largest tertiary center patient series (Study II and Sedlmeyer & Palmert 2002). CDGP, constitutional delayed of growth and puberty; PHH, permanent hypogonadotropic hypogonadism; FHH, functional hypogonadotropic hypogonadism; Hyper H, hypergonadotropic hypogonadism.](image)

3. The differential diagnosis of DP

A history of prior cryptorchidism was more frequent in those with PHH (30%) than in those with CDGP (2%) (P < 0.05). Similarly, patients with CHH (a subgroup of PHH) had undescended testes (36%) more commonly than those with CDGP (2%, P < 0.05). Thus, OR for a boy with DP and a history of cryptorchidism to have CHH was 26.5 (95% CI; 4.9-142, P < 0.001). By contrast, in late maturing boys without a history of cryptorchidism the risk of CHH was low (OR 0.04, 95% CI; 0.01-0.2, P < 0.001), and the probability of CHH decreased even more if the boy had also a positive family history of DP (OR 0.01, 95% CI; 0.001-0.1, P < 0.01).

Testicular volume, measured with a ruler, inhibin B and GnRH-induced LH levels were the most effective markers to discriminate between the prepubertal (genital stage 1 or testis size < 3mL) boys with CHH (n=10) and those with CDGP.
RESULTS

(n=76), whereas equally effective markers could not be found in girls. Additionally, when testis size was combined with inhibin B or GnRH-induced LH levels, the diagnostic efficacy was improved even more. The performance of clinical and biochemical predictors of DP, and their combinations, are described in Table 5 (Table 5) and Table 6 (Table 6).

Table 5. The clinical and biochemical markers to discriminate between prepubertal boys with congenital hypogonadotropic hypogonadism (n=10) and those with constitutional delay of growth and puberty (n=76).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>AUC (95% CI)</th>
<th>Cutoff level</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline LH, IU/L</td>
<td>0.86 (0.73-0.98)</td>
<td>0.5</td>
<td>100</td>
<td>68</td>
</tr>
<tr>
<td>Baseline FSH, IU/L</td>
<td>0.89 (0.75-1.00)</td>
<td>1.2</td>
<td>86</td>
<td>88</td>
</tr>
<tr>
<td>GnRH-induced LH, IU/L</td>
<td>0.92 (0.83-1.00)</td>
<td>4.3</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>GnRH-induced FSH, IU/L</td>
<td>0.81 (0.6-1.00)</td>
<td>3.3</td>
<td>86</td>
<td>72</td>
</tr>
<tr>
<td>Testosterone, nM</td>
<td>0.74 (0.59-0.88)</td>
<td>0.6</td>
<td>91</td>
<td>42</td>
</tr>
<tr>
<td>Inhibin B, ng/L</td>
<td>0.88 (0.74-1.00)</td>
<td>60</td>
<td>90</td>
<td>83</td>
</tr>
<tr>
<td>Testicular volume, mL</td>
<td>0.95 (0.87-1.00)</td>
<td>1.1</td>
<td>100</td>
<td>91</td>
</tr>
</tbody>
</table>

**Combinations**

- Inhibin B and testicular volume: 0.95 (0.87-1.00)
- GnRH-induced LH and testicular volume: 0.98 (0.94-1.00)

AUC, area under the curve; CI, confidence interval. Values lower level than the cutoff levels increase the probability of CHH.

Table 6. The biochemical markers used to discriminate between prepubertal girls with congenital hypogonadotropic hypogonadism (n=5) and those with constitutional delay of growth and puberty (n=36).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>AUC (95% CI)</th>
<th>Cutoff level</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline LH, IU/L</td>
<td>0.53 (0.26-0.80)</td>
<td>0.3</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Baseline FSH, IU/L</td>
<td>0.82 (0.53-1.00)</td>
<td>1.2</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>GnRH-induced LH, IU/L</td>
<td>0.78 (0.43-1.00)</td>
<td>1.7</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>GnRH-induced FSH, IU/L</td>
<td>0.80 (0.45-1.00)</td>
<td>4.2</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>Estradiol, nM</td>
<td>0.58 (0.34-0.80)</td>
<td>0.05</td>
<td>80</td>
<td>45</td>
</tr>
</tbody>
</table>

AUC, area under the curve; CI, confidence interval. * Estradiol level 0.05 nmol/l is the detection limit of the assay. Values lower level than the cutoff levels increase the probability of CHH.

While in a search for new methods that would aid in the challenging differential diagnosis between CHH and CDGP, we developed a clinical risk prediction model for CHH which takes advantage of the performance of different inhibin B analyte concentrations and testicular volume. In brief, testicular volumes and inhibin B levels were combined in a logistic regression analysis which estimated the probability of CHH in a given inhibin B level and testicular volume (Table 7). The
RESULTS

risk for CHH was the highest at small testicular volumes (< 1mL) and low inhibin B levels (< 50 ng/L).

Table 7. The mean probability of CHH according to testicular volume and inhibin B level. The inhibin B categories were calculated from the quality control measurements of inhibin B (mean ± 2 standard deviation) between 2004 and 2014.

<table>
<thead>
<tr>
<th>Inhibin B (ng/L)</th>
<th>Testis size</th>
<th>&lt;1.0 mL</th>
<th>1.1-2 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-49</td>
<td>90 (50-100)*</td>
<td>20 (0-80)</td>
<td></td>
</tr>
<tr>
<td>49-111</td>
<td>60 (10-100)</td>
<td>10 (0-40)</td>
<td></td>
</tr>
<tr>
<td>111-212</td>
<td>20 (0-90)</td>
<td>0 (0-10)</td>
<td></td>
</tr>
</tbody>
</table>

*Probability is presented with mean percentage (range)

Subsequently, the performance of the growth velocity to differentiate between the four diagnostic categories of DP was evaluated. We found that boys with FHH (n=18) had a lower mean height velocity than those with CDGP or CHH (n=142) (3.2 ± 1.2 vs 4.1 ± 1.7 cm/yr, P < 0.05, respectively). Since it has been suggested that height velocity below 3 cm/yr could identify patients with FHH from those with CDGP or CHH (Palmert & Dunkel 2012), we categorized the boys and the girls into those whose height velocity was above 3 cm/yr and into those who grew slowly (< 3 cm/yr). In girls, the frequencies of the four diagnostic categories (i.e. CDGP, PHH, FHH, and Hyper H) did not differ between the two growth categories. Conversely, in boys, the number of cases with FHH was significantly higher in boys who grew slowly than in those with height velocity more than 3 cm/yr (19% vs 4%, P < 0.05). However, the ROC analysis of growth velocity revealed that the previously suggested growth velocity cutoff level of 3 cm/yr discriminated the boys with FHH from those with CDGP or CHH only with a sensitivity of 50% and a specificity of 80% (Palmert and Dunkel, 2012). Next we evaluated the efficacy of annual growth velocity to identify the patients with an abnormal brain MRI scan. Brain MRI was performed in 39 patients (24 boys and 15 girls), and it was considered abnormal in 6 cases: one girl with a craniopharyngioma, two boys with a Rathke’s cleft cyst, two with a hypothalamic hamartoma, and one with empty sella. The girl with the craniopharyngioma grew very slowly (2 cm/yr), while the mean growth velocity of the boys with an abnormal MRI scan was similar to those with a normal MRI scan (n=18) (3.5±0.9 vs 4.2±2.4 cm/yr, P = NS).

4. The growth of patients with CHH from birth to adult height

The boys with CHH (III) showed a significant decrease in the mean length SDS from birth (0.2 ± 1.6 SDS) to 3 months (-0.9 ± 1.2 SDS, P < 0.01) and to 6 months of age (-0.7 ± 1.3 SDS, P < 0.05). The mean length SDSs at 3 and 6 months of age were lower than their MPH SDSs (-0.02 ± 0.9 SDS, P < 0.05) and, at 3 months of age, lower than the length SDSs of the general population (-0.9 ± 1.2 SDS, P < 0.05). The presence of minipubertal growth deflection was further evaluated by excluding four patients with birth length above the normal range (±2SD). Despite excluding the four patients, the length deflected from birth (-0.3 ± 1.3) to 3 months
of age (-1.0 ± 1.3, \( P < 0.01 \)). The boys with CHH experienced a reduction in weight-for-length from birth (2 ± 10%) to 6 months of age (-4 ± 10%, \( P < 0.05 \)).

Subsequently, we evaluated separately the growth rate of patients with signs of a severe GnRH deficiency (i.e. a history of cryptorchidism and/or micropenis). These males (n=8) experienced a profound length SDS deflection from birth (0.8 ± 1.8 SDS) to 3 (-1.0 ± 1.4 SDS, \( P < 0.01 \)) and to 6 months of age (-0.5 ± 1.2 SDS, \( P < 0.05 \)) (Figure 9).

![Figure 9](image.png)

Figure 9. The length standard deviation scores (SDS) of CHH patients with (dark grey) and without (light grey) a history of micropenis and/or cryptorchidism.* \( P < 0.05 \), ** \( P < 0.01 \).

The length SDS of CHH patients with verified gene mutations decreased significantly from birth (0.1 ± 1.1 SDS) to 3 months of age (-1.0 ± 0.9 SDS, \( P < 0.01 \)). At 3 months of age, the boys had lower height SDS than the general population (-1.0 ± 0.9 SDS, \( P < 0.05 \)), and the boys with ANOS1 (n=7) had a lower birth length than those with FGFR1 (n=4) (-0.7 ± 0.8 vs 0.8 ± 0.8 SDS, \( P < 0.05 \), respectively).

The boys with CHH grew relatively normally during childhood, but at the average age of 7 years (n=31), their mean height SDS (-0.8 ± 1.3 SDS) was lower than their mean MPH SDS (0.07 ± 0.7 SDS, \( P < 0.05 \)) and the height SDSs of the general population (\( P < 0.01 \)). During adolescence, the height SDS reached its nadir (-1.8 ± 1.4 SDS) at a mean age of 15.8 yrs, and thereafter the boys experienced an increased growth velocity as a result of the induction of puberty. The dosage of testosterone used during the first year of puberty induction (mean dosage 30, range 5 – 114 mg/kg/yr) in 14 patients correlated negatively with the adult height (\( r_s = -0.7 \), \( P < 0.05 \)) (Figure 10A), but no association was found between the age at puberty induction and the adult height (\( r_s = 0.1 \), \( P = \text{NS} \)). Further, the boys with CHH had a mean adult height of -0.4 ± 1.3 SDS which was similar to the adult heights of the general population, their MPH SDSs, and did not differ between gene mutations (\( P = \text{NS} \)). We found a decreasing trend in the age at induction of puberty from the 1970s onwards (\( r_s = -0.4 \), \( P < 0.05 \)) (Figure 10B).
RESULTS

The girls with CHH (IV) and FGFR1 mutations experience an unremarkable growth during early infancy, since length SDS did not deviate with the exception of one girl whose height SDS decreased from birth (-0.8 SDS) to 6 months of age (-1.5 SDS). Thus, the transient height SDS deflection seen in boys with CHH boys was not evident in the growth curves of the girls with CHH. Similar to the boys with CHH, the height SDSs of the girls decreased during childhood. The deflection in the height curve began at the age of 4 to 7 yrs, and the mean height SDS of the girls reached its nadir (-1.8 SDS) at the average age of 14.5 yrs, which occurred, as in boys with CHH, right before the induction of puberty.

The late sexual maturation was also apparent in the bone maturation of the girls, since they had a mean delay of 2.4 yrs (range: -3.2 to (-1.9) yrs) in the bone age. In the three girls, with available adult heights, the adult height SDSs minus MPH SDSs ranged from -0.2 to 1.2 SDS. All subjects had normal weight development, as their BMI trajectories were within the normal limits.

5. Health-related quality of life in males with CHH

The male patients with CHH had a lower mean 15D score than the age-matched general male population (0.918 ± 0.074 vs 0.948 ± 0.067, \(P < 0.05\)). In particular, the dimensions most affected were depression and distress (Figure 11). Although a continuous sex steroid treatment is essential to prevent the long-term consequences of CHH, we found no association between the testosterone levels, or the total duration of hormone replacement therapy pauses and the 15D scores (\(P = \text{NS}\)). Subjects with a reversal phenotype (n=4) experienced HRQoL similar to the general population, whereas the males with a history of prior cryptorchidism and/or micropenis (n=14) reported significantly lower 15D scores in the dimension of sexual activity (\(P < 0.01\)). Interestingly, the age at diagnosis correlated negatively with the 15D score (\(r_s = -0.3, P < 0.05\)) and the dimension of depression (\(r_s = -0.3, P < 0.05\)).
Eight patients with CHH had been diagnosed with depression, bipolar disease or anxiety disorder, and all of them were on antidepressants or anxiety medication. The subjects who received antidepressants or anxiety medication had lower 15D score and the scores in the dimensions of depression, distress, and usual activities than those without medication ($P < 0.05$). Co-morbidities appeared not to influence on HRQoL, since the patients with or without co-morbidities reported similar 15D scores ($P = NS$).

The main results of the five studies (I-V) are summarized in Table 8 (Table 8).
Table 8. Summary of materials, methods, and main results of the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Material</th>
<th>Methods used</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>30 peripubertal boys with ISS. 16 were treated with Lz, and 14 received Pl</td>
<td>Serum MKRN3 ELISA, testosterone, inhibin B, gonadotropins, and testicular volume</td>
<td>MKRN3 levels declined during prepuberty, and the decline was slower in boys who were treated with Lz suggesting that estrogen influences on the loosening of the central restrain on the HPG axis. The rate of decline in MKRN3 levels associated inversely with the clinical and biochemical markers of puberty onset.</td>
</tr>
<tr>
<td>II</td>
<td>244 patients (174 boys and 70 girls) with DP</td>
<td>Medical history, gonadotropins, testosterone, estradiol, inhibin B, testicular volume, growth velocity, brain MRI</td>
<td>A variety of different diagnoses underlie DP. In boys with DP, prior cryptorchidism, small testicular size, and low inhibin B levels increased the risk for CHH.</td>
</tr>
<tr>
<td>III</td>
<td>Growth charts of 36 (27 Finnish and 9 Danish) patients with CHH</td>
<td>Length and weight measurements</td>
<td>Sex steroids appear have an effect on growth during early infancy, since the boys with CHH experienced a reduced growth rate during the first six months of life.</td>
</tr>
<tr>
<td>IV</td>
<td>Growth charts of 6 girls with CHH and FGFR1 mutations</td>
<td>Length and weight measurements</td>
<td>In girls with CHH, no significant length deflection was found during the first six months of life, but the girls showed a decreased height SDS in mid-childhood.</td>
</tr>
<tr>
<td>V</td>
<td>30 men with CHH</td>
<td>15D questionnaire</td>
<td>Adult men with CHH experienced an impaired HRQoL, particularly in the dimensions of depression and distress. A history of genital anomalies associated with a reduced HRQoL in the dimension of sexual activity, and the age at diagnosis correlated negatively with the HRQoL.</td>
</tr>
</tbody>
</table>

ISS, idiopathic short stature; Lz, letrozole; Pl, placebo; ELISA, enzyme-linked immunosorbent assay; DP, delayed puberty; CHH, congenital hypogonadotropic hypogonadism; FGFR1, fibroblast growth factor receptor 1; SDS, standard deviation score; HRQoL, health-related quality of life.
DISCUSSION

The exact mechanisms that trigger the onset of puberty are inadequately understood. Similarly, the influence of sex steroids on growth during infancy is unknown. This study addressed these issues by showing for the first time that pubertal activation of the HPG axis is preceded by a decline in serum MKRN3 levels, and that the boys with congenital sex steroid deficiency experience a reduced growth velocity during the first six months of life. Additionally, the reduced growth velocity may serve as a non-reproductive cue to detect boys with CHH. The timely diagnosis of CHH is emphasized by the finding that adult males with CHH experience an impaired HRQoL which associated with the later age of diagnosis. The results also show that a careful review of medical history and physical examination are effective clinical tools in the differential diagnosis of DP, particularly in distinguishing patients with CHH from those with CDGP. The importance of these results is discussed in detail in the subsequent section.

1. Circulating MKRN3 levels in boys before the clinical onset puberty

In boys, circulating MKRN3 levels declined before the onset of puberty, and the decrease was more rapid during prepuberty than after the onset of puberty. Further, the rate of decline in MKRN3 levels associated negatively with the clinical and the biochemical markers of HPG axis activity. Interestingly, after our results were published, a Danish group reported serum MKRN3 levels in healthy peripubertal boys (Busch et al. 2016). The study confirmed our findings that in boys MKRN3 levels decline during peripuberty (Busch et al. 2016). Conversely, Busch et al. reported no correlation between serum MKRN3 levels and the levels of LH, inhibin B or testosterone (Busch et al. 2016). This difference may be explained by the fact that circulating MKRN3 exhibited a large inter-individual variation which could have diluted the possible correlations between MKRN3 and biomarkers of puberty (I, (Busch et al. 2016) ). On the other hand, 16 of our boys with ISS were treated with an AI, which enhances the secretion of gonadotropins and testosterone by removing the negative feedback of estrogen on the HPG axis (Figure 1A). This may have resulted in the strengthening of the reciprocal changes between the MKRN3 levels and the biochemical markers of puberty. Altogether, the decline in MKRN3 levels appear to precede the onset of puberty.

The exact mechanisms of MKRN3 in the control of puberty are unclear, albeit it is expressed ubiquitously in human tissue, especially in the testis, (Uhlen et al. 2015). MKRN3 is located at the Prader-Willi and Angelman syndrome critical region in chromosome 15 (Jong et al. 1999). MKRN3 is suggested to participate in protein ubiquitination, in which proteins are tagged with an ubiquitin and then send to proteasome and degraded into small peptides (Rock et al. 1994). In this process, MKRN3 and other ring zinc-finger proteins tag the target proteins by mediating the transfer on ubiquitin (Schwabe & Klug 1994, Deshaies & Joazeiro 2009). In mice, Mkrn3 is expressed in the ARC which regulates the activity of GnRH neurons, and the decrease in hypothalamic Mkrn3 occurs right before the onset of puberty (Abreu et al. 2013). In support of this, the zinc-finger protein family appears to control the epigenetic repression of puberty in primates, and the
DISCUSSION

expression of several zinc-finger protein genes is down-regulated at the time of
the HPG reactivation (Lomniczi et al. 2015). The levels of MKRN3 in adolescents
decline months and yrs before the onset of puberty, which suggests that MKRN3
serves as a transmitter of inhibitory signals to the GnRH neurons (I, (Abreu et al.
2013, Hagen et al. 2015, Busch et al. 2016) ). The temporal correlation between
the decline in circulating MKRN3, together with the activation of the HPG axis, and
the result that MKRN3 levels of boys decline faster before than after the onset of
puberty, pinpoints the loosening of the MKRN3 brake to the prepubertal time. The
observed inter-individual variation in MKRN3 levels, and the finding that the
decine in MKRN3 levels continues with a slower pace throughout the puberty (I,
(Busch et al. 2016)), suggest the presence of individual MKRN3 threshold for the
release of the MKRN3 brake. However, it is noteworthy that MKRN3 is likely not
the only regulator of puberty onset, and its functions may be compensated by
other factors. Interestingly, circulating MKRN3 levels appear to differ between
sexes (Hagen et al. 2015, Busch et al. 2016), since MKRN3 levels are higher in
girls and decline steadier as compared to the biphasic change observed in boys
(I, (Hagen et al. 2015, Busch et al. 2016)). Similarly, CPP is more common in girls
than boys ((Abreu et al. 2013, Macedo et al. 2014), and the boys with CPP and a
paternally inherited MKRN3 mutation experience a smaller decline in the age of
puberty onset than the corresponding girls (reviewed in Simon et al. 2015). Thus,
the role of MKRN3 in the inhibition of GnRH secretion appear to be more profound
in girls.

The absence of evidence describing the relationship between estrogen and
MKRN3 is striking. Hagen et al. reported longitudinal MKRN3 changes in girls, but
they did not find correlation between circulating MKRN3 and estradiol levels
(Hagen et al. 2015). In contrast, we found that the boys treated with an AI had a
slower rate of decline in MKRN3 levels, which suggests that estrogen influences
MKRN3 secretion and supports a role of estrogen as a factor that promotes the
loosening of the central restrain on the HPG axis (Flor-Cisneros et al. 2004, Cui
et al. 2015). To this end, there appears to be a negative association between a
measure of childhood estrogen exposure – bone age – and the age at the onset
of puberty (Flor-Cisneros et al. 2004). Boys with chronic diseases that have
delayed bone maturation, such as malnutrition or GH deficiency, present delayed
reactivation of the HPG axis, whereas patients with condition that accelerate bone
development, such as CAH, also enter puberty at an earlier age (Flor-Cisneros et
al. 2004). These findings beg the question if circulating MKRN3 could be used in
the differential diagnosis of DP. We found that the boys with late puberty
experienced a smaller decline in MKRN3 levels than those with normal timing of
puberty, and Hagen et al. reported lower levels of MKRN3 in early maturing girls
(Hagen et al. 2015). However, serum MKRN3 levels in boys are similar to those
reported in healthy men and men with CHH (Varimo et al. 2016). The effectiveness
of serum MKRN3 in detecting disorders of pubertal development should be
investigated in a prospective study including adolescents with CDGP, CPP, and
CHH.

The studies on circulating MKRN3 levels in humans published so far have
shown a large inter-individual variation I, (Hagen et al. 2015, Busch et al. 2016).
The levels of MKRN3 differ between laboratories (Hagen et al. 2015, Busch et al.
2016, Varimo et al. 2016), albeit all the studies in humans have used the same
commercial ELISA kit. Consequently, the use of MKRN3 ELISA kit needs to be
DISCUSSION

validated and replicated in larger cohorts. Additionally, international age- and sex-specific reference standards for circulating MKRN3 are required.

2. The diagnoses and predictors of DP

A wide spectrum of different diagnoses were found to cause DP, of which CDGP was the most common cause in both sexes. The results are largely consistent with the findings of previous studies on DP (Sedlmeyer & Palmert 2002, Reindollar & McDonough 1981, Lawaetz et al. 2015, Toublanc, Roger & Chaussain 1991). In our series, however, the frequency of CDGP was higher, whereas the proportions of PHH and FHH were lower than reported in the most comprehensive series of DP published to date (Sedlmeyer & Palmert 2002). The latter difference may be explained by the fact that the study of Sedlmeyer et al., as well as most of the previous studies on DP, have been conducted in tertiary centers which may have increased the frequency of rare conditions that underlie DP such as PHH (Sedlmeyer & Palmert 2002, Reindollar & McDonough 1981, Lawaetz et al. 2015). This may have resulted in a higher proportion of patients with PHH (Sedlmeyer & Palmert 2002). A recent review, which included a small retrospective evaluation of patients investigated for DP during a two-year period in an academic center, reported a frequency of CDGP (70%) very similar to ours (Abitbol, Zborovski & Palmert 2016). Moreover, the authors of the review speculate that the true number of CDGP patients is probably even higher due to the fact that previous studies have included patients evaluated in a specialized tertiary center (Abitbol, Zborovski & Palmert 2016). Indeed, the high proportion of CDGP in our series might be explained by the patient referral. Our tertiary center outpatient clinic receives direct referrals from general practitioners who evaluate children for pubertal disorders in the Helsinki area school health care. Thus, our series probably resembles the general pediatric population well.

In our series, the proportion of Hyper H was half of the frequency reported before (Sedlmeyer & Palmert 2002). Interestingly, TS was found in 27% of girls with Hyper H, which is a low proportion since TS is reported to affect 1:2500 live births (Stochholm et al. 2006), but a frequency that is consistent with the study of Sedlmeyer et al. (Sedlmeyer & Palmert 2002). Hence, this suggests that patients with TS are diagnosed earlier than in adolescence with clinical features other than DP, such as reduced growth velocity and short stature. Surprisingly, the proportion of girls with idiopathic ovarian dysfunction was high in our center. The girls tested negative for the Finnish-type FSH receptor gene mutation which causes 29% of cases with primary ovarian failure in Finland (Aittomaki et al. 1995, Aittomaki 1994). Since these girls had normal anatomy of the reproductive organs and no ovarian auto-antibodies, this suggests that they may harbor a novel gene defect which results in ovarian dysfunction that should be further studied.

Physicians in school healthcare, pediatricians, and pediatric subspecialists evaluate patients with DP. Performing a wide range of diagnostic tests at the initial evaluation may not always be necessary (Palmert & Dunkel 2012, Abitbol, Zborovski & Palmert 2016). Our results on the outcome predictors of DP emphasize the importance of a careful review of medical history. A history of cryptorchidism was a risk factor for CHH, whereas normally descended testes decreased the risk of permanent hypogonadism. The OR of normally descended testes decreased when it was combined with the knowledge of pubertal timing in
DISCUSSION

the patient’s family. Thus, a boy with normally descended testes who reported DP in a close relative, was likely to have CDGP or FHH as the cause of DP. Further, if these boys do not report any neurological or visual symptoms or findings, the second-line investigations of DP, if needed at all, can be focused on excluding chronic diseases that delay puberty (i.e. FHH) and to those fail to show spontaneous pubertal development during follow-up.

Although the annual growth velocity less than 3 cm was associated with the increased frequency of FHH in boys, the further analyses of growth velocity failed to reveal any effective threshold for identifying patients with a pathological underlying cause for DP. However, this may result from the fact that our series included only a few patients with a hypothalamic or pituitary lesion. In girls with DP, the previously reported growth velocity cutoff level (3 cm/yr) performs inadequately (Palmert & Dunkel 2012). Growth velocity did not prove to be useful in pinpointing the patients who require a brain MRI either. This is important since the patients with lesions in the hypothalamic or pituitary area of CNS show reduced growth velocity long before any neuro-ophthalmic symptoms or signs of delayed puberty are present (Taylor et al. 2012). The results of the clinical and the biochemical predictors of DP are summarized in Table 9 (Table 9).

Table 9. Clinical and hormonal markers which suggest the diagnostic category of delayed puberty (II).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CHH/PHH</th>
<th>CDGP</th>
<th>FHH</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of cryptorchidism</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height velocity &lt; 3 cm/yr</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Small testicles (&lt; 1.1 mL)</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>LH &lt; 0.5 IU/L</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FSH &lt; 1.2 IU/L</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Inhibin B &lt; 60 ng/L</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>GnRH-induced LH &lt; 4.3 IU/L</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TV &lt; 1 mL and inhibin B &lt; 50 ng/L</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

PHH, permanent hypogonadotropic hypogonadism; CHH, congenital hypogonadotropic hypogonadism; CDGP, constitutional delay of growth and puberty; FHH, functional hypogonadotropic hypogonadism; TV, testicular volume.

The differential diagnosis between CHH and CDGP is extremely challenging, and new evidence-based clinical parameters that could aid in this process are warranted (Harrington & Palmert 2012). Testis size, measured with a ruler, inhibin B and GnRH-induced LH levels emerged as the most effective markers to discriminate between the prepubertal boys with CHH and those with CDGP. After testis size was combined with inhibin B or GnRH-induced LH levels, a slightly improved discriminatory power was reached. Sertoli cell markers, GnRH and hCG stimulation tests, and their combinations have been investigated before in order to find an effective diagnostic parameter that would identify patients with CHH. Unfortunately, none of the tests show a complete accuracy (Harrington & Palmert 2012, Segal et al. 2009, Dunkel et al. 1985, Coutant et al. 2010, Adan et al. 2010,
Grinspon et al. 2010). We addressed the dilemma from a new viewpoint, and developed a testis size and inhibin B-based clinical prediction tool to estimate the risk of CHH in a prepubertal boy with DP. In the model, subject’s inhibin B value is classified in one of the three inhibin B categories obtained from QC measurements instead of using a single inhibin B cutoff level (Coutant et al. 2010, Rohayem et al. 2015). This is crucial since we know that the performance of inhibin B assay is not optimal at low analyte levels (Kalra et al. 2010). The prediction model may serve as an additional support for the clinician pondering between CHH and CDGP.

3. The influence of sex steroid deficiency on growth during minipuberty, childhood and adolescence

We depicted the growth charts of patients with CHH from birth to adult height. During minipuberty, the boys with CHH experienced a transient deflection in the length SDS, whereas a similar decrease was not evident in girls. The findings suggest that the minipubertal length deflection is a new non-reproductive feature of CHH, and that androgens modulate growth during the first six months of life. Importantly, we found that the decline in length SDS was more profound in boys with signs of a severe GnRH deficiency (i.e. a history of cryptorchidism or micropenis). In line with our finding, minipubertal testosterone levels in healthy boys correlate positively with penile growth and length velocity (Boas et al. 2006, Kiviranta et al. 2016). Boys have a more rapid growth velocity than girls during the first six months of life (Kiviranta et al. 2016), which may be explained by a sex-difference in hormonal milieu. During minipuberty, testosterone levels rise rapidly in boys, whereas in girls estradiol levels increase, but fluctuate and decline more slowly (reviewed in Kuiri-Hanninen, Sankilampi & Dunkel 2014). Thus, it would be tempting to postulate that, in boys, the growth during early infancy is more sensitive to the influence of sex-steroids. Nevertheless, the importance of nutrition in growth during early infancy cannot be excluded. During the first year of life, liver IGF-1 secretion is nutrition-dependent and carbohydrate-containing diet associates positively with growth rate (Socha et al. 2011, Chellakooty et al. 2006). The information on the nutrition during minipuberty was not available in our patients due to the retrospective setting. The study included the growth measurements of only six girls with CHH, thus, some changes in growth may have escaped detection. However, the findings in healthy boys treated with testosterone for micropenis, strongly support the role of androgens in the modulation of growth during early infancy (Landier, Chaussain & Job 1984, Guthrie, Smith & Graham 1973).

Both the boys and the girls with CHH experienced a relatively normal childhood growth, which is consistent with the sparse growth data of CHH patients published before (Van Dop et al. 1987, Raboch & Reisenauer 1976, Dickerman, Cohen & Laron 1992, Kaushanski & Laron 1979). The decreased growth rate observed in patients with CHH at the age of 4 to 7 yrs supports the view that estrogen regulates bone and linear growth already during prepuberty (Cutler 1997, Janner, Fluck & Mullis 2012). In both sexes, the height SDS reached its nadir right before the induction of puberty.

The initiation of sex steroid treatment recovered the linear growth, since the adult heights were similar to MPHs. In contrast, previous studies have reported
that patients with CHH usually exceed their MPHs and adult heights of the general population due to the delayed fusion of the epiphyseal plates (Raboch & Reisenauer 1976, Dickerman, Cohen & Laron 1992, Uriarte et al. 1992). This difference may be explained by the findings that the average age of puberty induction was lower in our study than reported before (Raboch & Reisenauer 1976, Dickerman, Cohen & Laron 1992, Uriarte et al. 1992), and the secular trend in the age of puberty induction in Finland had declined from the 1970s onwards. Since the cohort extended as far as the 1970s, there were patients who received very high doses of testosterone to induce pubertal maturation at the expense of losing some of their growth potential. Indeed, we found that the cumulative dosage of testosterone during the first treatment year correlated negatively with the adult height. Altogether, these results underline the importance of timely diagnosis and treatment of CHH.

The influence of different genotypes to linear growth remain speculative. We found no association between molecular genetic diagnosis and growth during infancy, childhood or adult height. The patients with CHH and a verified gene mutation experienced weight gain during infancy, childhood, and adolescence, which was similar to those without gene mutations. In fact, the only difference found was that the boys with ANOS1 were shorter than those with FGFR1 mutations at birth. The further investigation of this genotype-growth correlation is hindered by the absence of anos1 mouse model. However, since an excess androgen milieu during fetal life results in a higher birth length (Balsamo et al. 2006) and ANOS1 usually relates to a more severe gonadotropin and sex steroid deficiency (Costa-Barbosa et al. 2013), it could be speculated that the lower birth length in patients with ANOS1 stems from the altered intrauterine sex steroid milieu.

4. Health-related quality of life in males with CHH

Congenital sex steroid deficiency associated with an impaired HRQoL in adulthood. Specifically, the males with CHH reported lower HRQoL scores than the age- and sex-matched sample of general population, and the dimensions mostly affected were depression and distress. This is in agreement with the two previous studies on HRQoL in patients with CHH (Aydogan et al. 2012, Shiraishi, Oka & Matsuyama 2014), and it supports the view that congenital sex steroid deficiency causes long-term consequences on general well-being in adulthood. Remarkably, the HRQoL dimensions affected in our cohort were similar to those reported in the study by Aydogan et al. (Aydogan et al. 2012). In the previous studies, hormone replacement therapy improved significantly several domains of HRQoL, especially the dimensions related to psychological well-being (Aydogan et al. 2012, Shiraishi, Oka & Matsuyama 2014). This supports a causative relationship between long-term testosterone deficiency and impaired HRQoL. In contrast, we found no association between the adequate testosterone replacement therapy during adulthood and HRQoL scores. The results may be explained by the cross-sectional design of our study, or by the fact that the primary effects of delayed physical changes of puberty occur already during adolescence and early adulthood, before the onset of testosterone therapy, and paves the way for the long-lasting psychosocial consequences. In support of this, we found a negative correlation between the age at diagnosis and the HRQoL scores, and that CHH men with a history genital anomalies (micropenis and/or cryptorchidism)
have experienced a lower HRQoL in the dimension of sexual activity. A recent survey, which included males with CHH, identified negative psychological effects attributed to late diagnosis of CHH (Dwyer et al. 2014). In particular, the patients expressed anxiety and depression, body image concerns, low self-esteem, and a sensation of being left-behind as their peers developed into adult bodies (Dwyer et al. 2014). Although the data are limited, these findings support the concept that the timely diagnosis of males with CHH is vital for the prevention of long-term psychological consequences.

5. Future perspectives

The mechanisms that trigger the onset of puberty remain elusive. The discovery of *MKRN3* as one of the gatekeepers of pubertal onset, has focused the research on finding novel genes that contribute to the timing of puberty. Similarly, the exact role of extrogen in this process is unknown. At the same time, clinicians who evaluate adolescents with DP face not only the problem of identifying patients with pathological underlying cause, but also selecting right patients for the second-line investigations. Thus, the need for new evidence-based clinical tools to aid in this process is evident.

Late puberty can cause significant psychosocial burden to adolescents, especially to boys, and even limit their social integration with peers (Gross & Duke 1980). Currently, the boys with CDGP have two options for the management of late puberty in Finland: watchful waiting and expediting pubertal changes with a low-dose intramuscular testosterone. The dose and the treatment period of low-dose testosterone vary (Ambler 2009). Moreover, intra-muscular testosterone appears to suppress rather than activate the HPG axis, which questions its role as a standard of care for CDGP (Palmert & Dunkel 2012, Ambler 2009). Interestingly, the studies in boys with ISS have shown that AIs stimulate endogenous gonadotropin and testosterone production, and augment the progression of puberty (Dunkel & Wickman 2001, Hero, Norjavaara & Dunkel 2005). In an attempt to compare AIs to the current standard of care in the DP, this needs to be evaluated in a randomized-controlled setting, such as the one currently ongoing in five Finnish hospitals.
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