Testosterone, 17ß-estradiol and pubertal growth

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To be conscious that you are ignorant of the facts is a great step to knowledge

Att inse att man är okunnig är ett bra steg mot kunskap

Benjamin Disraeli 1804–1881

ABSTRACT

Background and aims: It is well established that the interaction of sex steroids with the growth hormone (GH)/ insulin-like growth factor 1 (IGF-1) axis is of major importance in children for normal pubertal growth. However, detailed understanding is still lacking. The overall aims of this thesis were to study the association between testosterone, estradiol and pubertal growth in healthy girls (Paper I), in boys (Paper II), and in GH-treated short boys without deficient GH secretion (Paper III), and to study the impact of GH treatment on pubertal development (Paper IV).

Patients and Methods: In the first two papers, 35+37 profiles of 24-hour serum 17ß-estradiol and 41 profiles of serum testosterone were analyzed in relation to pubertal height velocity in 27 girls and 26 boys. The children were referred to the endocrine unit for short or tall stature, or were recruited as healthy volunteers at the Göteborg Pediatric Growth Research Center. The short children without deficient GH secretion in Paper III and IV were enrolled in a randomized, controlled, multicenter dose-response study performed in Sweden and were randomized into three groups: untreated controls, GH 33 µg/kg/day, or GH 67 µg/kg/day. Paper III studied 65 boys and Paper IV studied 124 children (33 girls). Serum testosterone was measured by a modified radioimmunoassay (RIA), detection limit 0.03 nmol/L. Serum 17ß-estradiol was determined using an ultrasensitive extraction RIA, detection limit 4 pmol/L. To calculate height velocity, a sixth-degree polynomial was fitted to each child's individual height measurements and its derivatives were used to estimate height velocity with accelerations and decelerations.

Results: Using a dose–response model, the EC_{50} for serum estradiol and testosterone was calculated as the concentration at a 50% gain in height velocity from prepuberty up to peak height velocity (PHV) in puberty. The EC_{50} for estradiol in Paper I and II was 20 pmol/L (95% confidence interval 13–31) for girls and 6.5 pmol/L (3.2–13) for boys. The EC_{50} for testosterone in boys was 3.1 nmol/L (2.4–4.2). Serum estradiol levels >51 pmol/L were found in girls close to PHV. In boys close to PHV, serum levels of estradiol and testosterone were >9 pmol/L and >10 nmol/L, respectively. GH-treated boys in Paper III showed lower testosterone levels in relation to pubertal height velocity in a GH dose-dependent manner compared to untreated controls. However, it was apparent that the calculated PHV did not accurately represent pubertal PHV, as this calculation could not discriminate pubertal PHV from catch-up growth stimulated by the GH treatment. Boys with longer duration from GH start to PHV or from puberty onset to PHV, where most of the catch-up growth finished before pubertal growth started, had similar EC_{50} values to the untreated boys. GH treatment in the boys and girls in Paper IV had no effect on age at onset of puberty or final maturation compared to controls. GH-treated boys had significantly greater maximum mean testicular volumes without differences in testosterone levels, and GH-treated girls showed a significantly longer pubertal duration compared to their controls.

Conclusions: Serum estradiol levels seen in early puberty in girls and serum testosterone in early transition to midpuberty in boys are associated with accelerated height velocity. There was no indication of negative impact of GH treatment on pubertal onset or progression in short children without deficient GH secretion.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Könshormoner tillsammans med tillväxthormon är viktiga för längdtillväxt under puberteten. Tidigare ansågs östrogen vara det viktigaste könshormonet för tillväxt hos flickor och testosteron hos pojkar. Både hos flickor och pojkar ses dock ökande nivåer av testosteron och östrogen under puberteten. Studier av patienter med olika endokrina störningar har visat att östrogen har stor betydelse för normal pubertets-tillväxt och framför allt slutning av tillväxtzonerna och avslutande av tillväxt hos både flickor och pojkar. Störningar i pubertetsutveckling och pubertetstillväxt är vanliga orsaker till att barn remitteras till barnendokrinolog. Med ökad kunskap kring relationen mellan tillväxttakt och könshormonnivåer kan vi på ett bättre sätt bedöma dessa barns tillväxtpotential och optimera en eventuell pubertetsstödjande behandling. Huvudsyftet med denna avhandling är att fördjupa kunskapen om sambandet mellan könshormonnivåer och tillväxttakt. Detta har undersökts hos normalt växande friska barn samt hos friska korta barn som behandlats med tillväxthormon (GH).

Studiens resultat visade att de låga nivåer av östrogen som vi ser i tidig pubertet hos flickor samt testosteron och östrogen som vi finner hos pojkar tidigt till i mitten av puberteten var associerade med ökande tillväxttakt. Flickornas östrogennivåer låg kring 20 pmol/L (95% konfidensintervall 13–31) då de nått upp till halva sin maximala tillväxthastighet under puberteten. Motsvarande värde för pojkarnas testosteronnivåer var 3.1 nmol/L (2.4–4.2) och östrogennivåer 6.5 pmol/L (3.2–13). Det fanns en stor variation i känsligheten för östrogen hos flickor under pubertet avseende tillväxttakt men alla flickor med östrogennivåer över 51 pmol/L hade mindre än 25 % kvar att växa upp till sin maxhastighet. Pojkarna som växte nära sin maxhastighet hade testosteronnivåer över 10 nmol/L och östrogennivåer över 9 pmol/L. Hos pojkar har det mesta av östrogenet omvandlats från testosteron via ett enzym, aromatas, som finns ute i kroppens olika vävnader. Förmågan att omvandla testosteron till östrogen är inte fullt utvecklad och varierar mellan pojkar i tidig pubertet. Därför är det svårt att i serum spegla de östrogennivåer som vi tror påverkar tillväxten ute i vävnaderna. Hos pojkar under pubertet visade därför testosteron i serum ett stabilare samband med ökande tillväxttakt. För att säkerställa sambandet mellan könshormoner och tillväxttakt analyserades även gruppen med korta GH behandlade barn. En del tidigare studier har observerat att GH behandling kan påverka tid för pubertetsstart och även accelerera tempot för utveckling av sekundära könskarakteristika, även om inte resultaten har varit samstämmiga. I denna studiepopulation framkom ingen indikation på detta och flickorna som behandlats med den högre dosen av GH hade till och med en något längre duration av puberteten jämfört med obehandlade flickor. Detta resultat tillsammans med kunskapen om att GH behandling ger störst längdvinst innan pubertet genererade hypotesen: Att samma nivåer av testosteron är relaterade till ökande tillväxttakt hos GH-behandlade och obehandlade korta pojkar som hos normalt växande pojkar. Studiens resultat visade att så också var fallet när start av GH- behandlingen var väl skiljd från pubertetsstart.

Sammanfattningsvis har resultaten i denna avhandling påvisat de nivåer av östrogen och testosteron som är associerade med ökad tillväxttakt hos pojkar och flickor i tidig pubertet. Detta har tidigare inte beskrivits i detalj och innebär ny information som är viktig och till nytta vid bedömning och behandling av barn med pubertets och tillväxtstörningar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Albin AK**, Niklasson A, Westgren U, Norjavaara E.
Estradiol and pubertal growth in girls.
Horm Res Paediatr. **2012**;78(4):218-25

II. **Albin AK**, Norjavaara E.
Pubertal growth and serum testosterone and estradiol levels in boys.
Horm Res Paediatr, **2013**;80(2):100-10.

III. **Albin AK**, Ankarberg-Lindgren C, Nilsson S, Niklasson A, Norjavaara E, Albertsson-Wikland K;
Growth and serum testosterone during puberty in growth-hormone-treated short boys without growth hormone deficiency.
In manuscript

IV. **Albin AK**, Ankarberg-Lindgren C, Tuvemo T, Jonsson B, Albertsson-Wikland K, Ritzén EM; on behalf of the study group.
Does growth hormone treatment influence pubertal development in short children?
Horm Res Paediatr. **2011**;76(4):262-72.

ABBREVIATIONS

ACTH	Adrenocortical hormone
AGA	Appropriate for gestational age
ALS	Acid-labile subunit
AR	Androgen receptor
BMI	Body mass index
DHEA	Dihydroepiandrosterone
DHEAS	Dihydroepiandrosterone sulfate
EC_{50}	Half maximal effective concentration
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERT	Estrogen replacement therapy
FSH	Follicular stimulating hormone
GH	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GnRH	Gonadotropin releasing hormone
GP-GRC	Göteborg Pediatric Growth Research Center
GPR30	G protein-coupled receptor 30
HPG	Hypothalamus pituitary gonad
17β-HSD	17β-hydroxysteroid dehydrogenase
ICP	Infancy–childhood–puberty
IGF-1	Insuline-like growth factor 1
IGF-2	Insuline-like growth factor 2
IGFBP	Insulin-like growth factor binding protein
IGFBP-3	Insulin-like growth factor binding protein 3
ISS	Idiopathic short stature
ITT	Intention to treat
LH	Luteinizing hormone
PHV	Peak height velocity
PP	Per protocol
RIA	Radioimmunoassay
SD	Standard deviation
SDS	Standard deviation score
SGA	Small for gestational age
SHBG	Sex hormone-binding globulin

CONTENTS

1 INTRODUCTION

1.1 Background

Human growth is a complex process regulated by several hormones, genetic factors, nutrition, and environment. A pubertal disorder is a common reason for referral to a pediatric endocrinologist. We know that sex steroids are of great importance in normal growth, especially during puberty, when they control initiation, maintenance and cessation of the pubertal growth spurt. The sex steroids act both locally in the growth plate and systemically via the growth hormone (GH)/ insulin-like growth factor 1 (IGF-1) axis. It is now accepted that estrogen is the sex steroid of crucial importance in both sexes regarding growth acceleration and eventually fusion of the growth plates. It is known that there is a diurnal variation in the levels of testosterone and estradiol and that the levels increase through pubertal stages. However, the detailed association between serum levels of testosterone and estradiol and pubertal height velocity is not known. It is important to understand the relationship between sex steroid levels and growth in children in order to be able to evaluate their growth potential and optimize potential treatment of children with growth and puberty disorders.

1.2 The growth pattern of the growing child

The child's growth pattern varies through different time periods from birth to adult height. Somatic growth and maturation are influenced by several factors, which can broadly be defined as genetic, nutritional, environmental and hormonal. The trend in adult height and timing of adolescent development over the last century is evidence for the influence of environmental factors on the individual's genetic growth potential (1). Nutrition is a major determinant of growth and is the most frequent cause of growth retardation worldwide, although malnutrition in developed countries is more often due to systemic disease or self-induced restriction of food intake than as a result of poverty (1-3).

The important observation by James Tanner in 1987 (4), "Growth is a mirror of health", provides the background for the importance of describing human growth. Mathematics plays an important role in auxology and several mathematical models of human growth have been developed. In modern auxology, the current mathematical description of child and adolescent growth favors solutions that can describe individual growth patterns. Some attempts at developing these growth-descriptive nonlinear models have failed to describe the entire growth pattern from birth to adult height; without

biological concomitants of the mathematical functions, they have shown little clinical relevance. Some models were summarized by Ledford and Cole in 1998 (5). However, new advanced models are being developed and detailed computer modeling of human growth is now a reality using, for example, the SiTAR model (superimposition by translation and rotation) (6, 7) and the QEPS model with four distinct functions: quadratic, exponential, puberty, stopping (8, 9). This will improve the evaluation of the timing, duration and intensity of the pubertal part of growth during adolescence.

The previous way of describing the child's growth biologically is through the infancy–childhood–puberty (ICP) model (10, 11), which describes human growth phases from the latter part of the intrauterine life to adult height. This is a mathematical model that divides the growth process into three additive and partly superimposed components: infancy, childhood, and puberty. The exponential infancy component, the quadratic childhood component and the sigmoid puberty component together describe the total combined growth as shown in figure 1. The model can also be extended with the juvenility growth phase between childhood and puberty (12). The components of the human growth curve from birth to adulthood strongly reflect the different hormonal phases of the growth process. The growth of a child expressed as height velocity over age is shown in figure 2, together with important growth factors of each growth phase.

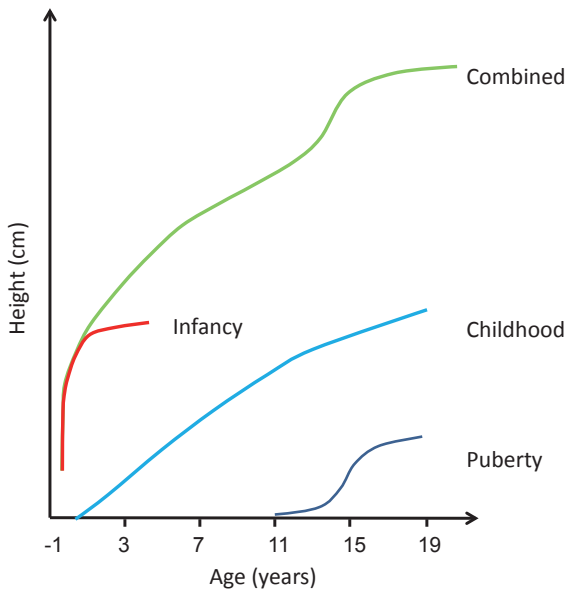


Figure 1. The infancy–childhood–puberty (ICP) model, with its different components. Modified by permission of Acta Paediatr Scand; Karlberg, J., Acta Paediatr Scand Suppl, 1989 (13).

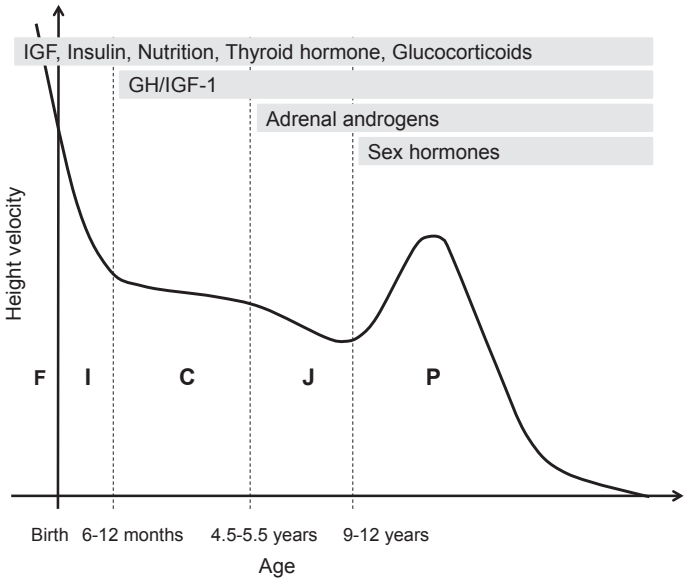


Figure 2. Height velocity chart with markers for the different growth phases and concomitant important growth factors. (F=fetal, I=infancy, C=childhood, J=juvenility, P=puberty. IGF=insulin-like growth factor, GH=growth hormone)

Fetal growth is the fastest growth of any phases of life; here we can find crown–rump velocity of 62 cm per year during the second trimester and 48 cm per year in the third trimester (14). The size at birth is determined more by maternal nutrition and placental function than by genetic makeup. Birth length and adult height have a correlation coefficient of only 0.25, whereas height at the age of two years and adult height show a correlation of 0.80 (15). Growth factors such as insulin, IGF-1, and IGF-2 are important in fetal life for growth and metabolism (16, 17) and for development of the brain (18-20). Thus, children born to diabetic mothers with hyperglycemia are often large for gestational age (21) and there is a correlation between serum IGF-1 and IGF-2 levels and size at birth (22). Boys are heavier and taller than girls at term birth (23-25) and the difference is thought to be generated by androgen action in utero (23, 24). In children with congenital adrenal hyperplasia with an excess of androgens, both birth weight and height over normal reference values have been reported. This supports that increased androgen levels could increase fetal growth (26).

The onset of the *infancy growth phase* occurs around mid-gestation and lasts until three or four years of age with a decelerating influence, and represents the postnatal contribution of fetal growth (10).The infancy component is an

extension of fetal growth, with diminishing height velocity at the end of pregnancy and continuing to decline after birth. The average gain in height during infancy is about 25 cm with boys slightly taller at the age of one year (25).
Nutrition plays a major role during infancy, as well as thyroid hormone, insulin and IGF-1 whereas GH is of metabolic importance but not crucial for normal growth within this period (10, 11, 27).

During the first 12–18 months, children usually shift centiles in their growth charts. According to the ICP model, this is due to a decline in influence of the infancy component during the transition into the *childhood component* of growth. The onset of the childhood component is around 6–12 months of age. During the third year of life, growth is more stable as a result of the childhood component being the main contributor to growth. In this phase, GH gains more importance in the regulation of growth. In children without deficient GH secretion, there is an increase in growth at the start of the childhood component. This is not seen in children with deficient GH secretion until they are treated with GH (10, 28). In addition to GH, other important growth factors during the childhood growth phase are thyroid hormones, nutrition, and psychosocial factors. There is a fairly stable growth rate of about 4–8 cm per year during the childhood phase in both sexes, with a mild mid-growth spurt around the age of six to seven years that coincides with adrenarche. This mid-childhood spurt is not always seen in height velocity charts. The growth rate decelerates gradually during the *juvenility phase* of growth, as described by the quadratic childhood function, usually reaching the lowest height velocity just before puberty starts (3, 12, 29).

The onset of *puberty* can be described in various ways: as physical signs of puberty, increased height velocity, or increase in gonadotropins or sex steroid levels. Attainment of breast stage 2 in girls and testicular volume of 4 ml in boys are generally the definitions of onset of puberty. (29-31). However, in girls there is ovarian enlargement and increased estradiol levels during the two years ahead of breast development and increased height velocity is seen about six months before breast buds form (29, 30, 32). During puberty there is a substantial increase in height velocity up to peak height velocity (PHV). Thereafter, height velocity declines until the epiphyseal growth plates are fused, whereupon longitudinal growth is no longer possible and adult height is reached. Pubertal growth contributes to up to 15–20% of adult height. The amplitude of PHV correlates negatively with the age of pubertal onset, and the pubertal growth spurt correlates with the clinical pubertal development, although there is a different timing between genders (30, 31, 33): girls enter puberty approximately two years ahead of boys and the age at puberty onset corresponds to a skeletal (biological) age of approximately 11 years in girls

and 13 years in boys (34). On average, girls enter and complete each stage of puberty earlier than boys, but there is significant inter-individual variation in the timing and tempo of puberty (2). The PHV usually occurs at Tanner breast stage 2–3 in girls at an average age of 12 years (30, 33). Their mean PHV is 9 cm per year with a total height gain of 25 cm during puberty (30). Boys reach a higher PHV of 10.3 cm per year two years later than girls, resulting in a larger total height gain, 28 cm, during puberty (31). The longer duration of prepubertal growth, together with the greater PHV, give the adult height difference of about 13 cm between women and men (14) that existed in all populations over millennia (35).

With the onset of puberty, the hormonal regulation of growth becomes more complex. Thyroid hormones and nutrition are still important. However, during the pubertal growth phase, GH and IGF-1 together with the sex steroids, estradiol, and testosterone play an important role as growth regulators (10, 11, 36). Patients with gonadal disorders and dysfunctioning GH/IGF-1 axis illustrate the importance of the interaction between these hormonal systems in pubertal growth. Hypogonadal children lack the pubertal growth spurt, and their growth during adolescence will correspond to the sum of the infancy and childhood growth components (10); in contrast, untreated children with impaired GH secretion do have a pubertal growth spurt but at a rate below normal (10, 37).

1.3 Short Stature

Short stature is one common reason for children to visit the pediatric endocrine unit. Most of these children, up to 80%, do not belong to a well-defined group and will be given the diagnosis idiopathic short stature (ISS) (38). ISS is defined as a condition characterized by height below two standard deviations of the corresponding mean height for a given age, sex, and population group, without evidence of disease or chromosomal abnormalities in children born appropriate for gestational age (39, 40). This definition covers different degrees of GH secretion and responsiveness, and is a part of the continuum extending from complete GH deficiency to normality. According to the definition above, this heterogeneous ISS group includes normal variants of growth, such as familial short stature and constitutional delay of growth and puberty, characterized by achievement of adult height within the target range (41).
GH treatment of children with ISS has been approved by the Food and Drug Administration in the United States since 2003. There are few studies that report on the efficacy and safety of long-term GH treatment of children with ISS and it is not approved in Europe. GH therapy is effective in increasing

height velocity in most children with ISS in the first year of treatment but there are controversies about the long-term effects due to the broad individual variation in responsiveness, and few randomized controlled studies including ISS children have been conducted. This topic has been extensively reviewed in the literature (39, 42-45). The current conclusion that can be drawn from these reviews is that long-term GH therapy can partially reduce the height deficit of children with ISS, but with a large inter-individual variation in growth response. Mathematic prediction models for GH response to GH treatment have shown that the observed variability in growth response can be reduced with GH dose given according to estimated GH responsiveness (46). However, the variability in adult height in response to GH treatment in short children without deficient GH secretion could also be a result of the possible effect of GH treatment on pubertal onset and tempo.

1.4 Regulation of longitudinal bone growth

1.4.1 The growth plate

Longitudinal bone growth occurs at the epiphyseal plate, which is a thin layer of cartilage between the epiphyseal and metaphyseal bone at the distal ends of the long bones.
Bone growth is the result of maturation, growth of chondrocytes, their production of bone matrix, and finally calcification (47). The growth plate is a complex structure consisting of different layers of cells, as shown in figure 3. The most immature cells, the stem cells, are found towards the epiphyseal end of the growth plate in the stem cell zone, or resting zone; the proliferating zone contains more mature chondrocytes and the hypertrophic zone contains the larger chondrocytes. The resting stem cells in the resting zone are recruited, whereupon proliferation and differentiation are initiated, followed by apoptosis and mineralization. Maturation of the growth plate occurs during the child's growth and its width decreases until it finally fuses at the end of puberty, replaced by bone (47-49).

There appears to be an intrinsic mechanism within the growth plate controlling the termination of cell division. The term *senescence* is used to describe this process of decline in function and cellularity of the growth plate. The stem-cell-like cells in the growth plate have a finite proliferative capacity that is gradually exhausted, and this is believed to trigger the process of epiphyseal fusion when the growth plate is replaced by bone (50, 51). This phenomenon of senescence could also explain the catch-up growth seen in children recovering from impaired growth resulting from severe illness or malnutrition. The hypothesis is that the growth-inhibiting period conserves the

proliferative capacity of the chondrocytes and thus slows down the senescence (52).

Although this is an intrinsic mechanism of the growth plate, it is affected by several hormones and growth factors that act both systemically and locally. In addition to GH/IGF-1, thyroid hormone, glucocorticoids, and sex steroids exert effects on the growth plate (47, 48).

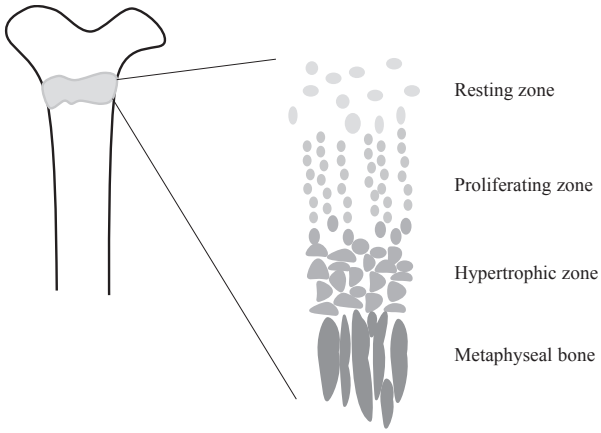


Figure 3. Schematic illustration of the growth plate, showing its cell layers.

The following subchapters will describe the secretion of GH/IGF-1, gonadal sex steroids, thyroid hormone and glucocorticoids, and their importance in growth regulation.

1.4.2 Growth hormone and IGF-1

GH is one of the most important hormones involved in human growth. Excess of GH due to, for example, pituitary adenomas during childhood leads to gigantism, whereas GH deficiency and GH insensitivity (from GH receptor (GHR) defects and IGF-1 depletion) impair postnatal growth and result in severe short stature (53). Although birth length of babies with congenital GH deficiency is just slightly below normal (54), congenital IGF deficiency results in severely diminished birth size (19), which leads us to conclude that IGF has the key role of regulating intrauterine growth independent of GH. The importance of IGF-1 and IGF-2 in fetal growth has been shown in knockout mouse models, where mice lacking the IGF-1 or IGF-2 gene were small at birth, and mice without functioning IGF-1 receptor are even smaller with high mortality (16).

1.4.2.1 Secretion of GH and production of IGF-1

GH is secreted from the pituitary in a pulsatile manner influenced by the stimulating GH-releasing hormone (GHRH) and the inhibiting somatostatin from the hypothalamus. GH has direct effects but also stimulates the production of IGF-1 in the liver and locally at target tissues to mediate its effects on metabolism, body composition and bone growth. There is a negative feedback loop on GH secretion caused by IGF-1 produced mainly by the liver. GH is also capable of inhibiting its own release through a short feedback loop, by inhibiting GHRH secretion and stimulating somatostatin secretion (55, 56). GH secretion is stimulated by stress, hypoglycemia, sleep, nutrition and certain amino acids such as arginine but is inhibited by metabolic signals such as insulin, glucose and nonesterified fatty acids (56). The peptide ghrelin, produced in the stomach as an appetite stimulator, can also stimulate GH secretion; the levels of ghrelin are highest prior to eating and are suppressed after food intake (57). The regulation of GH secretion is illustrated in figure 4.

About 45% of the GH circulating in the blood is bound to GH-binding protein (GHBP) (58). GHBP prolongs GH half-life in serum and thus GH bioavailability. The liver is the main source of GHBP and it is derived from proteolytic cleavage from the extracellular domain of the GHR (58).

Most of the circulating IGF is bound to IGF binding proteins (IGFBP), of which IGFBP-3 is the most abundant protein. The IGF–IGFBP-3 compound forms a ternary complex with acid-labile subunit (ALS), prolonging the half-life of IGF in serum and leading to relatively stable 24-hour plasma concentrations in contrast to the pulsatile secretion pattern of GH (59). ALS is produced exclusively by the liver, whereas IGFBP-3 is produced in many peripheral tissues (59, 60). The production of IGFBP-3 and ALS is also GH dependent (61).

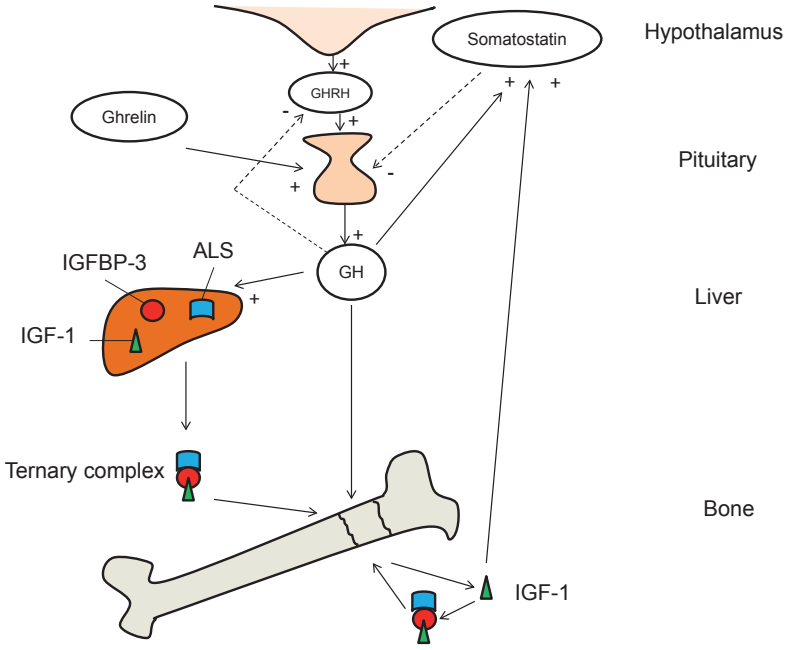


Figure 4. Regulation of the GH/IGF-1 axis. GH secretion is stimulated by GHRH and ghrelin, and inhibited by somatostatin. GH inhibits its own release by stimulating somatostatin and inhibiting GHRH. GH exerts direct effects on the growth plate and stimulates local production of IGF-1 in the growth plate and from the liver. Circulating IGF-1 is bound to IGFBP-3 and forms a ternary complex with ALS. (GH = growth hormone; GHRH = GH releasing hormone; IGF-1 = insulin-like growth factor 1; IGFBP-3 = IGF binding protein 3; ALS = acid-labile subunit.)

1.4.2.2 Patterns of GH and IGF-1 secretion

GH

Patterns of GH secretion are similar in boys and girls during childhood, with a marked night-day rhythm. The secretion of GH is at its maximum during the night, and there are bursts of GH secretion in the daytime at lower amplitudes; in between the pulses of GH secretion the trough levels are very low (62-64). However, in puberty there is a dramatic change in GH secretion. Gonadal hormones stimulate GH secretion and thereafter the secretion pattern differs between genders. In girls, this is an early event in puberty whereas in boys it is a late event, and it parallels the timing of the height velocity curves for both genders in puberty. The marked increase in GH secretion during puberty is due to higher pulse amplitudes, higher for girls than for boys, both during the day and night without change in pulse frequency. After reaching pubertal stage 5, GH secretion changes to gender-specific adult patterns in both sexes (63-67).

IGF-1

Throughout childhood the IGF-1 levels increase slowly. In puberty, when the pulsatile secretion of GH increases up to threefold, there is a more than threefold increase in serum IGF-1 levels. The peak of IGF-1 is seen in pubertal stage 3–4 at 14.5 years of age in girls and in pubertal stage 4 about a year later in boys (68). IGF-1 levels correlate with spontaneous GH secretion in most studies (69) and there could be an increased sensitivity to GH during puberty, as evidenced by the steeper regression lines of GH secretion vs. IGF-1 levels found in pubertal children compared to prepubertal children (70). The levels of IGFBP-3 also exhibit changes during puberty but these are less pronounced compared to IGF-1, resulting in increased levels of free IGF-1 during puberty (69).

1.4.2.3 Interaction between sex steroids and the GH/IGF-1 axis

There is a parallel increase in gonadal steroids and GH at onset of puberty, which suggests regulatory interactions in the secretion of these hormones. During puberty, the change in GH secretion is sex specific and parallels the change in height velocity as mentioned above. The difference in timing of pubertal growth is partly explained by the sex difference in the age of onset of estrogen synthesis. It is further known that GH levels are higher in adult women than in men (71) and highest in the periovulatory phase, when estrogen concentration is at its maximum (72). The difference seen in GH levels between men and women disappears after menopause, when estrogen levels decrease (71). This indicates the importance of estrogen in increasing GH secretion.

There are several clinical observations to verify the importance and relationship between GH and sex steroids during puberty. Girls with Turner syndrome, with no or low levels of endogenous estrogen, will show an increase in GH after supplementation with estrogen (73). An effect of estrogen via the GH/IGF-1 axis is also supported by the fact that estrogen receptor (ER) blockade down-regulates the GH/IGF-1 axis (74). Further proof of the importance of estrogen regarding GH secretion is the finding that testosterone, but not the non-aromatizable androgen dihydrotestosterone (DHT), stimulates GH secretion from the pituitary in boys with constitutionally delayed puberty (75, 76). In addition, 46,XY individuals with androgen insensitivity and female phenotype do have a normal pubertal growth spurt, demonstrating that estrogen in the absence of androgen action could increase growth during puberty and achieve pubertal levels of GH and IGF-1 (77). In children with central precocious puberty, treatment with

gonadotropin releasing hormone (GnRH) analogues suppresses the hypothalamic–pituitary–gonadal (HPG) axis, resulting in a decline in GH secretion, IGF-1 levels and height velocity (78, 79). Furthermore, the enhanced response of GH to pharmacological stimuli in both sexes is used clinically when priming with gonadal steroids.

GH secretion could be increased in two ways: by factors activating the central drive that enhances the GH/IGF-1 axis or by reduction of inhibitory feedback signals such as IGF-1. This is also how sex steroids modulate GH secretion.
Studies on the effects of exogenous estrogen on the GH/IGF-1 axis have shown an attenuating effect on GH action by inhibiting hepatic IGF-1 production. This seems to be route-dependent and is due to hepatic first-pass metabolism. When estrogen is administered orally it is mostly metabolized in the liver and high oral doses are needed to increase the level of estrogen in the systemic circulation; thus, there will be supraphysiological levels of estrogen in the liver resulting in reduced hepatic IGF-1 production and increased GH secretion. This is not seen with transdermal administration of estrogens, because first-pass metabolism is avoided and the liver is exposed to similar levels as in the systemic circulation. However, when women were treated with high doses of transdermal estrogen, circulating levels of IGF-1 fell, suggesting a dose-dependent effect regardless of whether it is achieved from the portal circulation (oral administration) or systemic circulation (transdermal administration) (56, 60, 80). GHBP concentrations rise with oral estrogen treatment, probably due to the hepatic first-pass mechanism. This inhibits GH binding to the GHR through competitive binding to the GHR and GH sequestering, which results in lower IGF-1 levels and thus increased GH secretion (58, 60). The effects of estrogen on ALS and IGFBP-3 parallels the changes in IGF-1, and estrogen seems to exert inhibitory effects on all the three compounds of the IGF-1 ternary complex in a route- and dose-dependent manner (60). At the cellular level, estrogens affect GH action in different ways. It is known that estrogen can regulate GHR expression, which seems to be tissue specific with upregulation in, for example, osteoblasts and hepatocytes (81, 82) but not in the uterus or fallopian tube (83, 84). It has also been shown that estrogen can exert inhibiting effects on GHR signalling (85) and also direct effects on the IGF-1 promoter gene (86).

However, exogenous testosterone does not regulate circulating IGF-1 levels but requires GH to exert a stimulatory effect on IGF-1(56). Thus there are divergent effects of estrogen and testosterone on the production of circulating IGF-1. The neurosecretory effects of testosterone on GH secretion seem to be dependent on prior aromatization to estrogen but the effect of GH responsiveness is more likely mediated through the androgen receptor. Higher levels of GH are found in adult premenopausal women than in men, although

no difference is seen in IGF-1 levels between genders (69, 87). It is also seen that women with deficient GH secretion require a higher replacement dose than the men, suggesting partial GH resistance associated with the presence of estrogen (88).

The interaction between estrogen and the GH/IGF-1 system influences bone growth on different levels and is schematically shown in figure 5. Estrogen exerts its effect on growth centrally by enhancing the GH secretion from the pituitary, both by decreased inhibition from IGF-1 and by activating secretion through estrogen receptors found in both the hypothalamus and the pituitary (60, 89, 90). In addition to this, estrogen also exerts direct action at the growth plate through the ER.

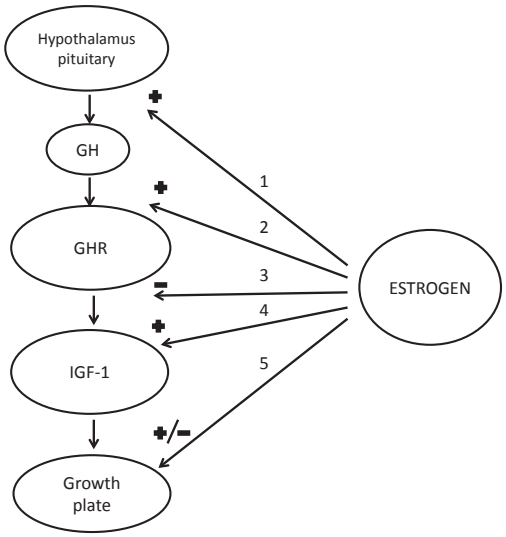


Figure 5. Levels of action of estrogen: 1) stimulation of GH secretion at the hypothalamic–pituitary level, 2) increased GHR expression and enhanced GH binding to GHR, 3) inhibitory effect of GH signaling, 4) stimulatory direct effect at IGF-1 promoter gene, 5) direct action at the growth plate through the estrogen receptor. (GH = growth hormone, GHR = growth hormone receptor, IGF-1 = insulin-like growth factor 1).Modified by permission of Pediatr Endocrinol Rev; Simm, P.J., et al., Pediatr Endocrinol Rev, 2008 (90).

Taken together, these studies and clinical observations indicate that both GH/IGF-1 and sex steroids are needed for normal pubertal growth, and that there is a clear synergism between the hormones. It is also known that the action of estrogen on the GH/IGF-1 axis is complicated and occurs in different ways at different levels: in the hypothalamus and the pituitary, as well as through the production of both liver-derived and locally produced IGF-1. Furthermore, there are gender differences in these actions.

1.4.2.4 Effects of GH/IGF-1 on the growth plate

It has been shown that GH exerts its effect on the growth plate both through circulating IGF-1 and by increasing local production of IGF-1 in the growth

plate, which then acts in a paracrine or autocrine manner to increase bone growth. It is believed that locally produced IGF-1 is of greater importance in longitudinal growth than circulating IGF-1; this is supported by animal studies with mice, in which selective hepatic IGF-1 deletion did not cause impaired growth despite substantially reduced levels of circulating IGF-1 (91). The triple liver deletion of IGF, IGFBP-3 and ALS in mice causes a reduction in circulating IGF-1 levels of almost 98% but only a 6% reduction in body length (92); in contrast, a child with a deletion in the IGF-1 gene (in all tissues), who will have severely reduced height (19). It has also been suggested that GH has an IGF-1-independent action in the growth plate to recruit the chondrocytes in the resting zone into the proliferative zone (93).
In summary, GH is known to act in different pathways, both directly and indirectly through circulating and locally produced IGF-1, to stimulate longitudinal bone growth (91-95).

1.4.2.5 Effects of sex steroids on the growth plate

At puberty onset, the increase in height velocity and the increase of GH secretion and IGF-1 levels in serum have traditionally been associated with the rise in testicular androgens in boys and estrogens in girls. However, experiences from patients with endocrine disorders show that this is not the case. In 1994 a male patient was described with an inactivating mutation in the estrogen receptor alpha (ERα) gene; he had no pubertal growth spurt, tall stature, and osteoporosis, which proves that estrogen is an important factor in epiphyseal fusion (96). A similar phenotype has been found in male patients with an aromatase p450 deficiency, who cannot convert testosterone into estrogen. Treatment with estrogen in men with aromatase deficiency led to growth plate fusion and cessation of longitudinal growth (97, 98). These case reports have taught us that estrogen is the crucial sex steroid for pubertal bone growth and maturation.

Estrogen receptors, ERα and ERβ, and the androgen receptor (AR) are found throughout all cell zones in the human growth plate throughout puberty regardless of gender. The expression of ERα and AR is similar throughout puberty, whereas the expression of ERβ slightly decreases (99). It has been suggested that ERβ acts as a negative regulator of ERα-mediated transcription, which leads to the speculation that there could be an enhanced ERα signaling in the growth plate caused by the decrease in ERβ during puberty (99). A lack of functional ERα is shown to be associated with a phenotype of no pubertal growth spurt and no possibility to fuse the epiphyses (96). Evidence for direct effects of estrogen on bone growth comes from the occurrence of the pubertal growth spurt (although impaired) and epiphyseal

closure in children with Laron syndrome, despite having a defect in the GHR (100). This suggests that ERα is important in normal human pubertal growth. However, it was recently shown in a mouse model that ERα is not important for longitudinal bone growth in early puberty. In contrast, ERα is essential for high dose treatment of estrogen to reduce the growth plate height in adult mice. The authors propose that the growth-stimulating low level of estrogen seen in early puberty exerts its effect on the growth plate through GH/IGF-1 although higher levels of estrogen in latter part of puberty reduce growth by ERα in the growth plate (101). In mice and rats, the growth plates do not fuse after sexual maturation but do fuse after treatment with supraphysiological levels of estrogen (102). If female rodents are ovariectomized there is an increase in longitudinal bone growth (103) that can be reversed with estrogen treatment, which suggests that estrogen has an inhibiting effect on growth in rodents.

There is also a third estrogen receptor, the membrane G protein-coupled receptor (GPR30), which has been localized to the resting and hypertrophic zones of the growth plate in both girls and boys (104). The expression declines with pubertal progress, which indicates that the receptor could be involved in modulation of pubertal growth. GPR30 has been found to be important for the normal inhibitory effect of estrogen on bone growth in ovariectomized mice (105).

A study of growth rate in rabbits before epiphyseal fusion occurs suggested that fusion is the result of growth cessation (51). Estrogen has been reported to accelerate the normal process of growth plate senescence, leading to an earlier exhaustion of the growth plate and earlier fusion (51, 106). This concept would explain why estrogen exposure does not induce fusion rapidly, but must often act for years before fusion occurs, particularly in young children, in whom the growth plates are less senescent. For example, in young children with untreated precocious puberty, it is possible that the epiphyses do not fuse for many years despite being exposed to high levels of estradiol. In contrast, older men with aromatase deficiency show fusion of the epiphyses within less than a year after start of estrogen treatment (51).

Some of the effect of androgens on growth is mediated by estrogen due to aromatization from androgens into estrogens by aromatase in peripheral tissues, for example in adipose tissue. Aromatase is also present in both the rat and the human growth plate, which indicates that sex steroid metabolism occurs in the growth plate (107, 108). The local production of estrogens in the growth plate could provide an additional and important mechanism for modulating local estrogen levels. However, androgens can also act directly on the growth plate without conversion into estrogen. AR is present in all layers

of the human growth plate, which suggests direct action of androgens (99). Testosterone stimulates growth in the absence of GH in hypophysectomized and castrated rats (109). Further evidence of the direct effect of androgen on growth is that treatment with non-aromatizable androgens, DHT, and oxandrolone increases growth without any detectable increase in GH or IGF-1 levels (75, 110-112). When girls with Turner syndrome are treated with GH in combination with oxandrolone there is an increase in adult height (113-115). Another example of the growth-enhancing effects of androgens is the androgen insensitivity syndrome, where 46,XY children with a female phenotype do not reach the normal adult height of 46,XY individuals with a normal male phenotype due to loss of the androgen effect on growth (77). Furthermore, in patients with aromatase deficiency or estrogen resistance, androgens succeed in keeping growth rate stable without the presence of estrogen (96, 97).

1.4.3 Sex steroids

A schematic description of the variation of sex steroid levels in different phases of child growth is shown in figure 6 for girls and boys. This is further described in the following subchapters.

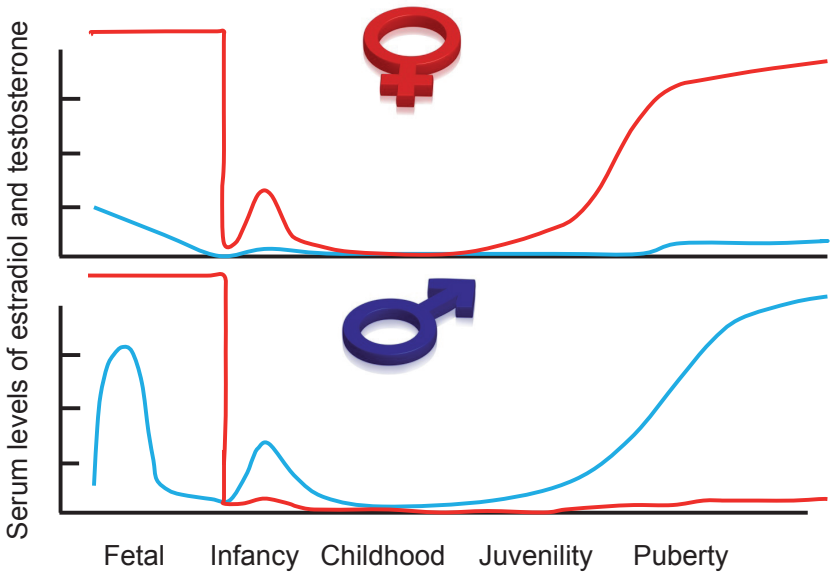


Figure 6. Schematic description of testosterone and estradiol levels in girls and boys in different phases of growth. (Blue line=testosterone, red line=estradiol) Modifed by permission of Best Practice & Research Clinical Endocrinology & Metabolism: Alonso, L.C. and R.L. Rosenfield, Best Pract Res Clin Endocrinol Metab, 2002 (116).

1.4.3.1 Fetal life and infancy

The HPG axis is active in utero and during the first years of life, becoming quiescent until reactivation leads to the onset of puberty. The neurons of the hypothalamic GnRH pulse generator originate in the primary olfactory placode and migrate during early fetal life to the medial hypothalamus. GnRH stimulates the pituitary to pulsatile secretion of LH and FSH, which in turn stimulates the gonads to produce testosterone and estrogen (3, 117, 118). In the fetus, gonadotropin secretion reaches its peak during mid-gestation and then decreases until birth, probably due to increasing sensitivity to the negative feedback of steroids.

In the male fetus, initial testosterone production and sexual differentiation occur in response to the fetal levels of human chorionic gonadotropin. Further testosterone production and masculine differentiation are maintained by the fetal pituitary gonadotropins. Decreased testosterone levels in late gestation reflect the decrease in gonadotropin levels. There are lower circulating FSH and LH levels in male fetuses. This is due to testicular testosterone and inhibin production, given that there is no gender difference in levels of circulating estrogens during intrauterine life (117, 118). After birth, there is an immediate feedback interruption of sex steroids on gonadotropin secretion from the pituitary. Thus, in the newborn child an activation of the HPG axis is seen, with raised levels of gonadotropins and increased sex steroid secretion. In the newborn boy, FSH and LH levels start to rise within a week and peak at two to three months of age, followed by decreasing levels down to prepubertal levels at six to nine months of age. There is a parallel pattern of testosterone with a peak level at one to three months (117-119).

Compared to boys, girls have a slightly lower level of LH but their FSH levels are several times higher, which can last for a longer time period, up to two or three years, when gonadotropin levels fall to prepubertal levels. This period of activity in the HPG axis is often called *minipuberty* (117-120). For unknown reasons the HPG axis then becomes less active until the onset of puberty, when it is reactivated at gonadarche.

1.4.3.2 Childhood and prepuberty

Sensitive assays now allow the measurement of serum levels of FSH, LH, testosterone, and estradiol even in prepuberty, and they have revealed a diurnal pattern of FSH and LH in both girls and boys several years before the onset of puberty. There is a diurnal rhythm in gonadotropin secretion and, after a lag time, a rise in testosterone and estradiol levels is registered early in the morning (121-124). As the onset of puberty approaches, the serum concentration of gonadotropins increases, LH to a greater extent than FSH,

due to an increase of pulse amplitude during the night and not to a change in pulse frequency (121, 122). During prepuberty, higher levels of estradiol are found in girls than in boys (125-127). This is in line with the earlier pubertal growth spurt and skeletal maturation seen in girls, as well as the onset of the growth spurt preceding breast development (30).

1.4.3.3 Juvenility and adrenarche

In both sexes, an increase in androgen secretion from the adrenals, adrenarche, usually occurs at an age of six to eight years. It is a separate process from the gonadarche. The androgens secreted from the adrenals include androstenedione, dihydroepiandrosterone (DHEA), and DHEA sulfate (DHEAS), producing adult body odor, pubic hair, and acne (128, 129). Some children have a mild growth spurt, "mid-childhood-spurt", usually more apparent in girls compared to boys, although it is not a consistent finding (130). This mid-childhood spurt coincides with adrenarche, although there are inconsistent findings regarding the direct relation between these events (131).

1.4.3.4 Puberty

Throughout puberty there is a progression in daytime pulsatility of gonadotropins with increasing concentrations of testosterone and estradiol (124, 132). The exact trigger of pubertal onset, an increase in pulses of GnRH from the hypothalamus, is still something of a mystery, but it is thought to be influenced by a complex interplay between genetics, nutrition, neuro-transmitters, hormones, and the psychosocial environment. The signal to start puberty might be coupled to loss of inhibitory signals including opioid peptides, GABA, and additional excitatory signals such as the neuropeptide kisspeptide. The adipocyte-derived hormone leptin could signal that energy reserves in the body are sufficient to maintain and complete puberty (3, 118, 133, 134). GnRH stimulates the pituitary to release LH and FSH that prompts gonadal growth and further production of sex steroids. In boys, LH stimulates the Leydig cells to produce testosterone and FSH stimulates Sertoli cells and initiates spermatogenesis. In girls, LH stimulates the theca cells in the ovary to produce androstenedione and testosterone and FSH stimulates the granulosa cells to produce estrogens through aromatization from androgens (3, 118).

Androgens and estrogens by gender

The serum levels of testosterone and estradiol increase with pubertal maturity and vary over 24 hours, as will be described in the following section, but first a short description of steroid synthesis is presented.

The synthesis of the steroid hormones starts in the ovaries, testes, and adrenal glands with cleavage of the side chain of cholesterol to form pregnenolone. This and the ensuing enzymatic steps are shown in figure 7. Androstenedione and testosterone are converted into estrogens by 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase in the ovary or testes but also in peripheral tissues (116, 135).

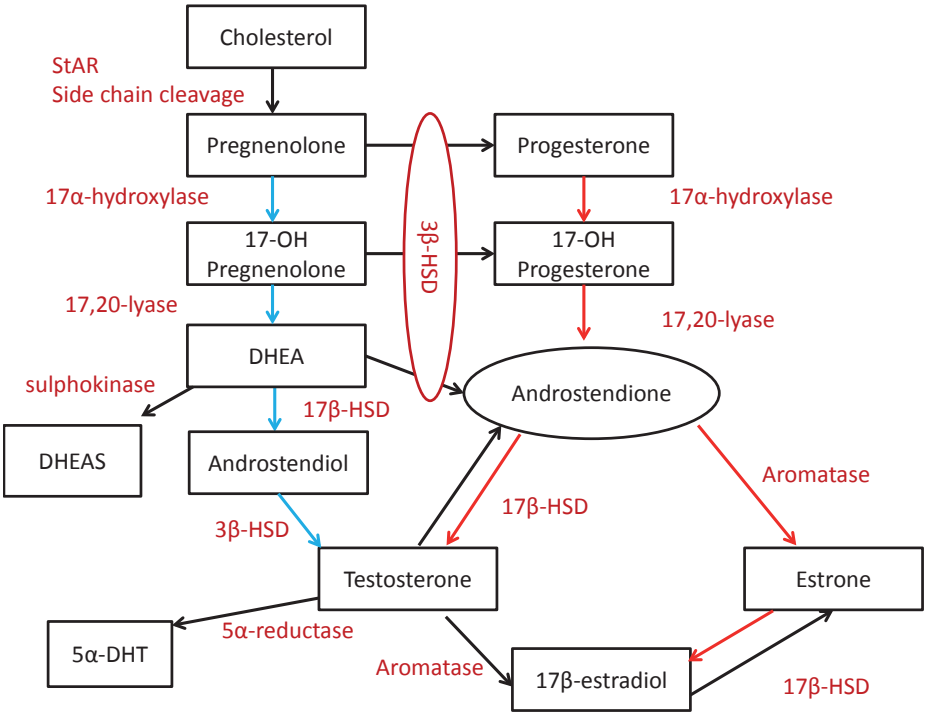


Figure 7. Steroid synthesis. Blue arrows indicate the dominant pathway in the testis and red arrows indicate the dominant pathway in the ovary. (StAR=steroidogenic acute regulatory protein, 3β-HSD= 3β-hydroxysteroid dehydrogenase, 17β-HSD = 17β-hydroxysteroid dehydrogenase, DHEA = dehydroepiandrosterone, DHEAS = DHEA sulfate, 5α-DHT = 5α-dihydrotestosterone.)

Androgens in girls

Androgens are important in girls even though they are considered predominantly male hormones. The androgens are responsible for the growth of pubic and axillary hair and the development of apocrine glands, and also influence the development of the adolescent brain during puberty (136). The regulation of androgen production includes LH (ovary) and ACTH (adrenals), together with the peripheral conversion through paracrine and autocrine mechanisms. Testosterone, DHT, DHEA, and androstenedione are produced both in the ovary and the adrenal cortex. The most potent androgens are testosterone and DHT, while the other androgens act more like precursors and require conversion to testosterone or DHT to interact with the AR. DHEA is the major product of androgens from the adrenals and follows the secretion pattern of ACTH. In the circulation it is more frequently found as DHEAS, with a longer half-life compared to DHEA. Testosterone can be irreversibly converted to DHT in peripheral tissues by 5α-reductase (135, 137).

The prepubertal androgens are derived mainly from the adrenals and the testosterone and DHEAS concentrations are similar in boys and girls (32). After gonadarche there is a correlation between estrogen and testosterone in girls. This suggests that testosterone during puberty derives to a greater extent from the ovaries. During early puberty there is a relative hyperandrogenism with higher androgen levels than estrogen levels in girls. During puberty, estrogens increase more than androgens (138).

At start of puberty there is a gradual increase in serum testosterone level up to midpuberty in girls premenarche. There is already a diurnal variation in prepuberty, with the highest level in the morning (06:00–10:00) and the lowest around midnight, which reflects the diurnal secretion patterns of ACTH and gonadotropins. This day–night variation remains until two years after menarche, probably due to predominantly anovulatory cycles directly after menarche in combination with adrenal androgen production (122, 138). In adult women, the gonads produce about 25% of the testosterone, the adrenals 25%, and the rest is produced by peripheral conversion (137).

Androgens in boys

Ninety percent of the androgens in adult men are produced by the testes, and the remainder is produced by the adrenals and by conversion in peripheral tissues. Before puberty, androgens in boys as well as in girls are produced mainly by the adrenals and follow the rhythm of cortisol secretion. At gonadarche there is a change in the secretion pattern of testosterone, with nocturnal levels increasing earlier in the night as a result of gonadotropin

secretion. The diurnal variation of testosterone shows the highest levels in early morning and the lowest levels in the evening. This variation is more distinct at the start of puberty and diminishes during the latter part of puberty. Serum testosterone levels increase through puberty, with the largest step from early puberty to midpuberty; boys in late puberty have 15 times higher testosterone levels compared to girls (121, 123, 124, 139).

Estrogens in girls

There are three naturally occurring estrogens in women: estrone, estradiol, and estriol. The most important estrogen during puberty and the reproductive years is estradiol, which is also the most potent estrogen with the highest affinity to the estrogen receptor. In pregnancy, estriol is the most important estrogen, and is produced by the placenta. Estrone becomes the predominant circulating estrogen after menopause (48, 116).

The sources of estrogens are direct secretion from the ovaries or conversion of precursor steroids in peripheral tissues. In the ovary, the synthesis of androstenedione from cholesterol starts in the theca cells. Androstenedione is converted in the surrounding granulosa cells, either immediately into estrone or into testosterone and then into estradiol in an additional step. The conversion of androstenedione to testosterone is catalyzed by 17β-HSD, and the conversion of androstenedione and testosterone into estrone and estradiol, respectively, is catalyzed by aromatase; these enzymes are expressed in granulosa cells. Hence, both granulosa and theca cells are essential for the production of estrogen in the ovaries, which can be referred to as the *two-cell theory* (48, 116, 140). In addition there is the local production of estrogens in the peripheral tissues and target organs, as mentioned above (107, 108).

The levels of estradiol increase progressively during puberty and girls with still anovulatory cycles at the end of puberty have estradiol levels three to four times higher than the levels in boys. Estrogen is secreted in a pulsatile pattern reflecting the diurnal variations of GnRH and gonadotropins. The highest levels occur in the morning and the lowest levels in the late evening. In midpuberty, before menarche, the diurnal pattern is most pronounced and disappears one year after menarche, when estradiol starts to vary with the menstrual cycle (122, 127, 141).

Estrogens in boys

In adult men, only 20% of the 17ß-estradiol is synthesized by the testes and 80% is produced by peripheral aromatization of testosterone and androgen

precursors. The effects of local paracrine or intracrine estrogen production could be more important than those of circulating estrogen in boys (142, 143). Aromatase activity is not well expressed in the testes before the start of puberty, when LH increases (144), and it is also known that aromatase activity in extraglandular tissues, including adipose tissues, is low in childhood (145).

Serum 17β-estradiol in prepubertal boys is low and increases gradually from prepuberty until midpuberty; thereafter there is a marked rise in estradiol level into the latter part of puberty. The diurnal rhythm, with lowest levels at night and highest levels in early morning, is not distinct until mid-to-late puberty (123, 127, 146).

The variation of testosterone and estradiol over 24 hours throughout puberty is shown for boys and girls in figure 8.

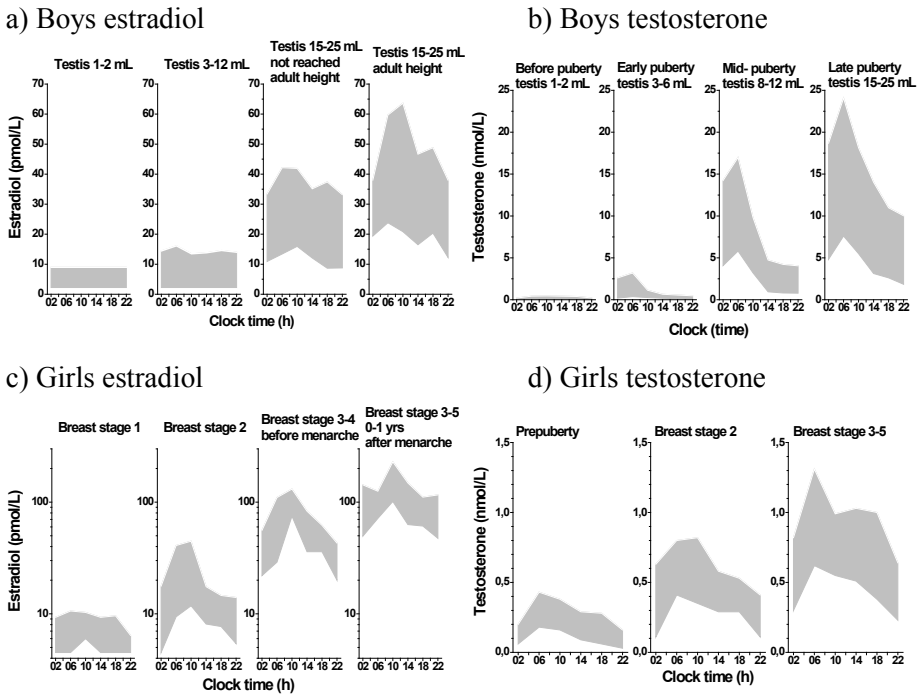


Figure 8. 24-hour reference intervals for serum 17β-estradiol and testosterone in (a,b) boys and (c,d) girls in different pubertal stages. By permission of: a) Carina Ankarberg-Lindgren; C. Ankarberg-Lindgren and E. Norjavaara, BMC Endocr Disord, 2008. (146). b) Eur J Endocrinol; Ankarberg-Lindgren, C. and E. Norjavaara, Eur J Endocrinol, 2004. (139). c) Am J Clin Pathol; Ankarberg-Lindgren, C. and E. Norjavaara, Am J Clin Pathol, 2009. (147). d) J Clin Endocrinol Metab; Ankarberg, C. and E. Norjavaara, J Clin Endocrinol Metab, 1999. (138).

Sex hormone-binding globulin (SHBG)

SHBG is produced in the liver with high affinity for testosterone and estradiol and acts as a carrier of these sex steroids and also regulates their bio-availability. The biological activity of sex steroids is correlated with the free fraction rather than the SHBG-bound fraction. In adult men, only about 1–2% of the testosterone is free whereas the remainder is bound mainly to albumin (~50%) and SHBG (~45%). SHBG binds to testosterone with high affinity compared to albumin and the bioavailability of testosterone in men equals the free and albumin-bound fraction of testosterone together. In adult women, 1–2% of the testosterone is free and the remainder is bound to albumin (~30%) and to SHBG (~70%) (148, 149). The corresponding numbers for estradiol bound to SHBG are 20% in men and 40% in women (148). The levels of SHBG are increased by estrogen and decreased by androgens. SHBG levels are higher in prepubertal children compared to adults and start to decline with the rise in androgens. In adults, higher levels of SHBG are seen in women than in men due to their higher estradiol levels. SHBG levels are also affected by obesity, where levels decrease with high insulin and IGF-1 levels. In contrast, thyroid hormones increase SHBG levels (150). Children with deficient GH secretion show higher levels of SHBG compared to children with normal GH secretion, and GH treatment is associated with a decrease in SHBG levels. How GH affects SHBG levels is still unclear, although it is known to be secreted by the liver where the GHR is also present (151).

1.4.4 Thyroid hormone

It is well known that congenital hypothyroidism is associated with severe postnatal growth retardation. Excess of thyroid hormones has been shown to cause increased height velocity and leads to premature growth plate fusion, resulting in short stature (152, 153). The thyroid hormones act on the growth plate directly through chondrocytes expressing thyroid hormone receptors; they also act indirectly by modulating GH secretion and local GH or IGF-1 actions (47, 48).

1.4.5 Glucocorticoids

Glucocorticoid excess, whether exogenous or endogenous, often leads to impaired longitudinal growth. Thus, children with familial glucocorticoid deficiency present with tall stature (154). Glucocorticoids exert their inhibitory effect on bone growth partly through a direct effect on the growth plate, as evidenced by glucocorticoid receptors found in the chondrocytes in the growth plate. Glucocorticoids have also been found to inhibit bone growth through the GH/IGF-1 pathway; some studies have shown decreased secretion

of GH from the pituitary, and effects on the growth plate from changes in the local IGF-1 system. Furthermore, glucocorticoids can regulate local T3 levels in the growth plate (47, 48). Taken together, these findings indicate that glucocorticoids can cause growth retardation, not only directly via the glucocorticoid receptors but also via interference with other growth-modulating pathways.

2 AIMS AND HYPOTHESES

The overall aim of this thesis is to increase current understanding of the association between serum testosterone, estradiol and pubertal height velocity in boys and girls.

2.1 Specific aims

- To determine the relationship between serum levels of 17β-estradiol and pubertal height velocity up to PHV in girls.
- To determine the relationship between serum levels of 17β-estradiol, serum levels of testosterone, and pubertal height velocity up to PHV in boys.
- To investigate whether GH treatment in short children without deficient GH secretion influences onset or progression of the development of secondary sex characteristics.
- If GH treatment does not influence the development of secondary sex characteristics in short boys without deficient GH secretion; to find further support for the association between serum levels of testosterone and pubertal height velocity up to PHV seen in another population of boys.

2.2 Hypotheses

- Girls with morning 17β-estradiol levels above prepubertal levels (>10 pmol/L) but within the lower range for early puberty have an increased height velocity.
- Levels of serum testosterone in boys during early and initial midpuberty are associated with increased height velocity.
- GH treatment of short children without deficient GH secretion does not affect onset or progression of the development of secondary sex characteristics.
- GH treatment of short boys without deficient GH secretion does not affect the association between serum levels of testosterone and height velocity up to PHV.

3 PATIENTS AND METHODS

3.1 Study design and study subjects

This thesis embraces two main study groups: the healthy children in Paper I and II and the short children without deficient GH secretion, with or without GH treatment, in Paper III and IV.

3.1.1 Healthy children

Paper I and II are retrospective investigations of sex steroids in relation to pubertal growth. The participants were children who were referred to the endocrine unit at the Queen Silvia Children's hospital in Gothenburg, Sweden, for short or tall stature, or who were recruited as healthy volunteers. The children were all born at between 37 and 42 weeks of gestation. Children born SGA were excluded, as well as children diagnosed with a significant severe or chronic disease, endocrine disorders, inflammatory disease, skeletal dysplasia or chromosome aberrations. GH deficiency was excluded, based on 24-hour GH measurement or results of an arginine–insulin tolerance test. Children were also excluded if their prepubertal growth was continuously outside the range of ±3 SDS in height or weight according to Swedish growth reference values for healthy children (25). Inclusion and exclusion criteria are summarized in table 1. Growth data were collected from child health centers, school health services and the endocrine unit where the children had been followed. Breast and pubic hair development were assessed according to Tanner (29) and testicular volumes were determined by orchidometer according to Prader (155). The 24-hour 17ß-estradiol profile and/or 24-hour testosterone profile was assessed at least once in each child. Bone age was measured in some children and was evaluated by the same radiologist at the Queen Silvia Children's Hospital, according to Tanner–Whitehouse method II (156).

Table 1. Inclusion and exclusion criteria for study subjects in Paper I and II.

Inclusion criteria
Prepubertal height within -3 to + 3 SDS[a]
Prepubertal weight within -3 to + 3 SDS[a]
Gestational age within 37–42 weeks
One 24-hour sex steroid profile

Exclusion criteria
Prepubertal height beyond -3 to +3 SDS[a]
Prepubertal weight beyond -3 to +3 SDS[a]
Small for gestational age[b]
Growth hormone deficiency
Severe or chronic disease

[a] *reference population (Albertsson-Wikland 2002) (25)*
[b] *reference population (Niklasson 1991) (157)*
SDS = standard deviation score.

The study groups in Paper I consisted of 27 girls and in Paper II there were 26 boys, as shown in table 2. Thirty-seven 24-hour profiles of 17ß-estradiol were available among the girls; among the boys there were forty-one 24-hour profiles of testosterone and thirty-seven of 17ß-estradiol.

Six girls and six boys were followed longitudinally with multiple profiles of estradiol, and eight boys were followed with multiple profiles of testosterone. Table 2 shows the number of girls and boys who were referred to the endocrine clinic for short or tall stature and the number of recruited volunteers.

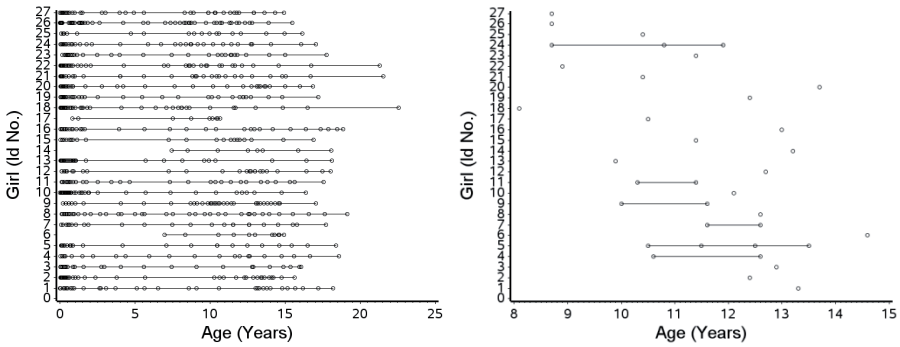
Table 2. Study subjects in Paper I and II

Paper	Gender	N	short	Tall	volunteers	24-h E2 profiles	24-h T profiles	Subjects with multiple 24-h E2 profiles	Subjects with multiple 24-h T profiles
I	Girls	27	17	3	7	37	-	6	-
II	Boys	26	15	3	8	37	41	6	8

(E2=17β-estradiol, T=testosterone.)

Figure 9 shows the number of height measurements obtained in each child by age and the number of assessed sex steroid profiles by age.

a) Height measurements (girls) b) Sex steroid profiles (girls)



c) Height measurements (boys) d) Sex steroid profiles (boys)

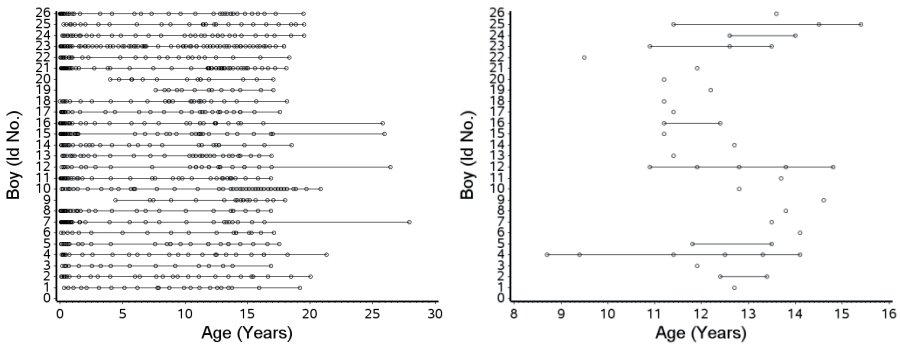


Figure 9a–d. Distribution of height measurements and sex steroid profiles by age in girls (a, b) and boys (c, d).

3.1.2 Short children with and without GH treatment

The children included in Paper III and IV were participants in a randomized multicenter dose–response study of GH treatment in short children without deficient GH secretion (158). The primary aim of this original study was to investigate the effects of GH treatment on adult height in short children without deficient GH secretion compared with the randomized untreated control group. The children were all short, -2 SDS, according to the Swedish population-based reference values (25), and a total of 177 children were enrolled into this original study between 1988 to 1999. Table 3 shows the inclusion and exclusion criteria. When the study was initiated, short children born SGA were not excluded from the ISS group of children as they are today, and thus it is important to bear in mind that these data included SGA

children. After a prestudy year of evaluation, the children who were still prepubertal were randomized into either of three groups: no treatment (control group), GH treatment with a standard dose of 33 µg/kg/day (GH_{33}) or a double dose of 67 µg/kg/day (GH_{67}). The children who had entered puberty after the first prestudy year were randomized into either the control group without treatment or the GH_{67} group. The children were followed once yearly at a university hospital and every three months at their local hospital until attaining adult height. The yearly follow-up included safety and efficacy measurements such as laboratory tests, bone age determination, auxology, and pubertal staging.

Table 3. Inclusion and exclusion criteria for study subjects in Paper III and IV.

Inclusion criteria
Height SDS below -2 SDS[a]
Chronological age 8–13 years (girls) Chronological age 10–15 years (boys)
Bone age under 11 years (girls) [b] Bone age under 13 years (boys) [b]

Exclusion criteria
Growth hormone deficiency
Bone age retardation more than 3 years [b]
Chronic disease
Premature at birth, under 35 weeks
Extreme intrauterine growth retardation

[a] reference population (Albertsson-Wikland 2002) (25)
[b] reference (Tanner 1983) (156)
SDS = standard deviation score

Paper III examines pubertal height velocity related to serum levels of testosterone in the boys in the original study above. Serum testosterone measurements and the possibility to calculate PHV from available height measurements were required, which resulted in further exclusion of 25 boys. The final number in this analysis was 65 boys, including two who were not randomized in the original study but followed as voluntary controls according to the protocol. Figure 10 shows the distribution of the boys in the study.

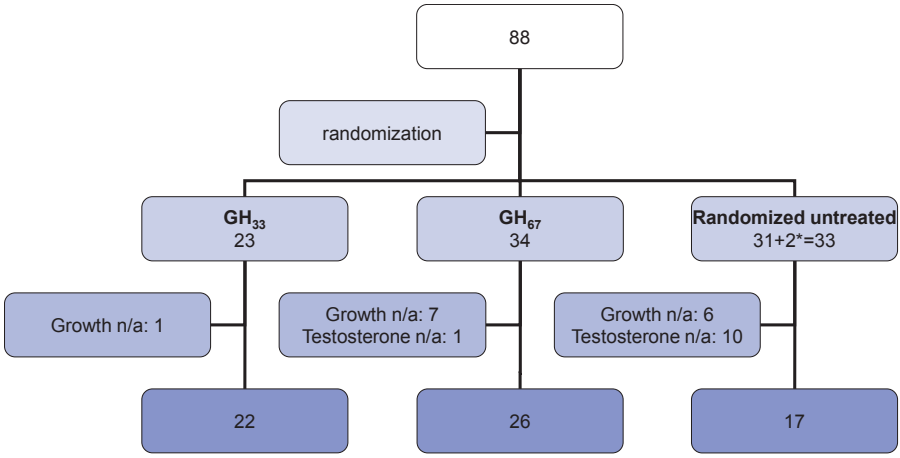


*Figure 10. Flowchart of the distribution of the boys in Paper III. Excluded children: growth n/a = not possible to calculate peak height velocity; Testosterone n/a = no testosterone measurements available. *Two boys who did not participate in the study were included in the randomized untreated group because they were followed according to the protocol as voluntary controls. (GH_{33}=treatment with GH 33 µg/kg/day, GH_{67}=treatment with GH 67 µg/kg/day.)*

Paper IV explored the possible effect of GH on the pubertal development of secondary sex characteristics among the children from the original study. Children were excluded if they had missing data on final pubertal maturity, defined as menarche in girls and maximum mean testicular volume in boys, leaving 124 children (33 girls) for the analysis, see figure 11 and figure 12.

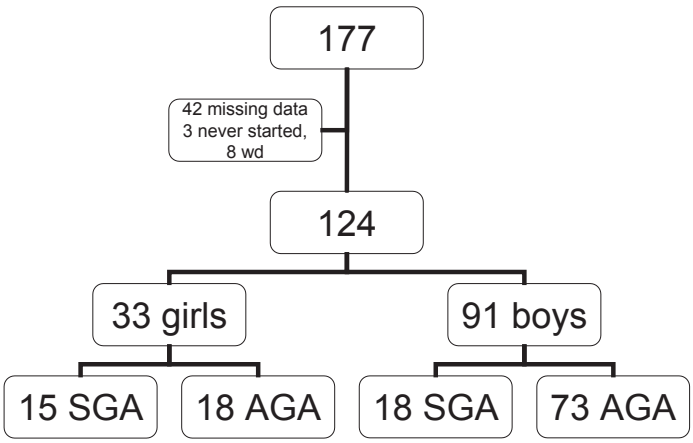


Figure 11. Flowchart of the children included in Paper IV showing the number of girls and boys after exclusions and the distribution of children born small for gestational age (SGA) and appropriate for gestational age (AGA). Wd=withdrawn.

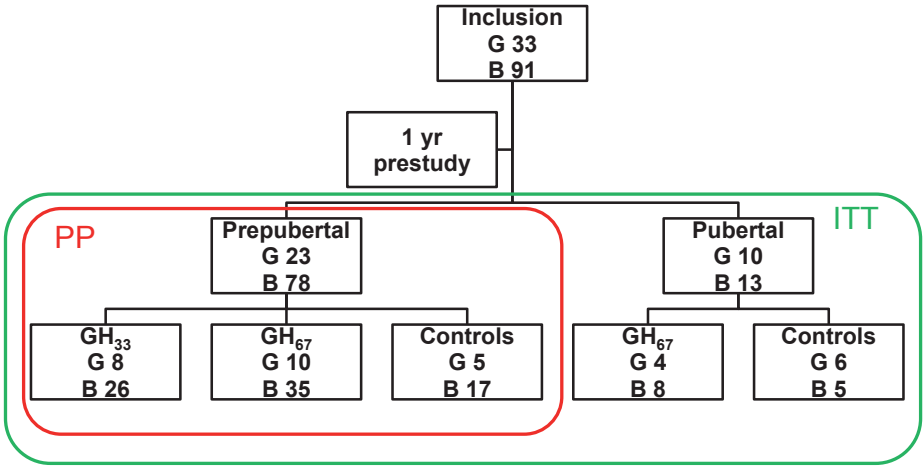


Figure 12. Flowchart of the randomization of children into different treatment groups after the first prestudy year. (B=boys, G=girls.) The per-protocol (PP) population includes the children who were prepubertal at randomization and the intention-to-treat (ITT) population also includes children who were pubertal at randomization.

3.2 Ethical considerations

Healthy children

Assent was obtained from the children and informed written consent was obtained from their parents for future analysis of these data. The regional ethics committee at the University of Gothenburg approved the research protocol.

Short children

The study was approved by the Ethical Committees of Sweden at Gothenburg, Lund, Linköping, Uppsala, Huddinge, Umeå, and the Karolinska Institute. Informed consent was obtained from all the children and their parents.

3.3 Methods

3.3.1 Auxology

Height was measured at the Queen Silvia Children's Hospital in Gothenburg, the other university hospitals and local hospitals involved in the study of GH treatment of short children without deficient GH secretion. Height data were also collected from child health centers and schools. At the hospitals, the mean of three measurements was used and converted into SDS using the Swedish growth reference values for healthy children (25).

3.3.2 Pubertal staging

Pubertal staging was assessed according to Tanner method (29) and testicular volume according to Prader using an orchidometer (155). Pubertal staging and testicular volume measurements were performed by a trained physician at the local hospital or at the university hospital.

3.3.3 Height velocity

Height velocity was calculated by fitting a 6th degree polynomial to each child's individual height measurements. The first derivative of the polynomial equals the child's height velocity. The second derivative mirrors the acceleration and deceleration of the height velocity curve and enables us to find the point where height velocity is at its maximum, in other words, the time point of the child reaching PHV. This corresponds to when the second derivative of the fitted polynomial passes from positive to negative values through zero, as shown in figure 13. The growth charts were checked manually for plausibility of PHV and age at PHV to exclude overfitting.

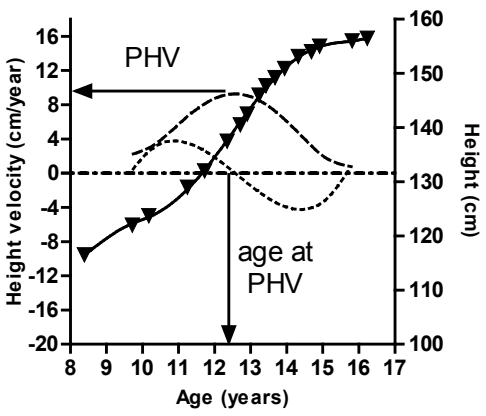


Figure 13. Principles for calculation of height velocity and timing of PHV. The child's height measurements ▼ are shown with the fitted 6th degree polynomial (solid line). The height velocity (dashed line) equals the first derivative of the 6th degree polynomial. The second derivative (dotted line) passes through zero at the time point of age at PHV.

3.3.4 Pubertal growth ratio

To be able to compare the pubertal height velocity between children, a pubertal growth ratio was designed. This consists of current height velocity as a ratio of the PHV corrected for prepubertal height velocity. This means that the pubertal growth ratio equals zero in prepuberty and 1 at its maximal height velocity, PHV. The ratio is calculated as follows:

$$\frac{\text{Current height velocity - prepubertal height velocity}}{\text{PHV - prepubertal height velocity}}$$

Prepubertal height velocity was calculated at a time point 2.5 years before age of PHV in order to cover prepubertal height velocity.

3.3.5 Definitions

In Paper III and partly in Paper IV age of onset of puberty and age at reaching maturity were defined to allow comparisons of time points between different treatment groups. The definitions were not meant to describe the true age of pubertal events. The onset of puberty was defined as the age at the first visit following attainment of Tanner breast stage 2 in girls and testicular volume of over 3 ml in boys. The timing of pubertal maturity in girls was measured as the age at the first three-monthly visit after menarche; in boys this was measured as the age when the maximum mean volume of the two testes (= the maximum mean testicular volume) was first recorded. The duration of puberty was defined as the elapsed time between pubertal onset and pubertal maturity (as defined above) in both genders.
Near-adult height was defined as the height when height velocity was less than 1 cm per year.

3.3.6 Blood sampling

The children stayed in hospital for 24 hours to provide serum profiles of testosterone and estradiol. A heparinized needle was inserted and blood for sex steroid measurement was taken at: 10:00, 14:00, 18:00, 22:00, 02:00, 04:00, 06:00, and again at 10:00 the next morning. In Paper III and IV, single samples were used in addition to the 24-hour profiles. The serum samples were centrifuged and stored in the freezer until assayed. Serum samples obtained after 1995 have been stored at –80°C from the beginning, whereas serum samples obtained earlier were at first stored at –20°C but after 1995 at –80°C.

3.3.7 Laboratory measurements

3.3.7.1 17ß-estradiol

Serum 17ß-estradiol concentrations were determined in duplicate with a modified sensitive commercial immunoassay, (Spectria® Estradiol RIA, Orion Diagnostica; Espoo, Finland), including a diethyl ether extraction step prior to quantification. The method has been described and published previously (32, 159).

The analytical detection limit for the modified extraction RIA was 4 pmol/L with a functional sensitivity of 6 pmol/L (146, 159). The functional sensitivity was defined as the lowest concentration of 17ß-estradiol to be measured with an interassay coefficient of variation of 20%. Total imprecision was 25% at 10 pmol/L and 9% at 250 pmol/L. The stability of the samples has been estimated regarding long duration storage and repeated freeze–thaw cycles by yearly determination of estradiol concentrations in three pooled samples at different serum estradiol levels. The samples were not affected by either long time storage in the freezer nor repeated freeze–thaw cycles (32, 146).

The extraction RIA is an accredited assay by SWEDAC in Sweden, SS-EN ISO 15189 (No. 1899). The method was developed and all analyses were performed at at the Göteborg Pediatric Growth Research Center (GP-GRC) laboratory, Gothenburg, Sweden.

3.3.7.2 Testosterone

Serum testosterone concentrations were determined in duplicate with a modified sensitive commercial immunoassay, (Spectria® Testosterone RIA, Orion Diagnostica; Espoo, Finland), designed for hormone measurements in human serum. To increase the sensitivity, twice the serum volume was used but in all other respects the RIA was conducted according to the manufacturer's instructions. The detection limit was 0.03 nmol/L (32, 138, 139). Total imprecision was 26% at 0,3 nmol/L and 11% at 15 nmol/L. The effect of long-term storage in the freezer and repeated freeze–thaw cycles was determined by analyzing testosterone in serum pools at three different testosterone levels yearly for over a decade. The serum concentrations of testosterone were not affected by long duration storage or repeated freeze–thaw cycles (32, 139).

The RIA for testosterone is an accredited assay by SWEDAC quality control agency in Sweden, SS-EN ISO 15189 (no 1899). The analyses were performed at the GP-GRC laboratory, Gothenburg, Sweden.

3.3.8 Statistical procedures

Results are expressed as mean (SD) or median (range), as appropriate. A Bland–Altman analysis (160) was used to compare the measured and calculated height values. Testosterone and estradiol values were not normally distributed and linear and nonlinear regression analyses were made on log transformed data. Correlation analyses were conducted using Spearman's rank correlation coefficient (rho) or the Pearson's product-moment correlation coefficient, and comparison between groups were conducted using independent sample t-tests or Mann–Whitney tests as appropriate. Multiple linear regression analyses were also used. A p-value of < 0.05 was considered significant. In girls and boys with repeated measurements of estradiol and/or testosterone, the last measurement was included in the regression and correlation analyses. In order to include all data of all boys in Paper III a mixed effect model was used when analyzing the association between testosterone and pubertal growth ratio as described below.

Software

To perform the statistical analyses the standard statistical package SPSS, version 15.0, 17.0 and 22.0 (SPSS, Inc., Chicago, Ill., USA), GraphPad Prism, version 5.03 (GraphPad Software, Inc., San Diego, CA, USA; http://www.graphpad.com), R version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) and WinNonlin 4.1 (Pharsight Corporation, CA, USA) were used. The statistical program SAS, version 8.1 and 9.3 (SAS Institute Inc.; Cary, NC, USA) was used to find the individual 6^{th} degree polynomial fitting the growth data for each child. Calculations of the age at PHV by solving the second derivative of the 6^{th} degree polynomial were performed using Deadline 2.3 (http://deadline.3x.ro/).

3.3.8.1 Morning values of sex steroids

A diurnal variation of sex steroids is seen in boys and girls during puberty. Where 24-hour profiles were available, a morning value was chosen for the analysis because the highest levels are found in the morning. In the boys in Paper II, the value of testosterone and estradiol at 06:00 (or 04:00 if 06:00 was missing) was chosen as a morning value. In girls, the peak of estradiol is seen slightly later and the value at 10:00 (or 06:00 if 10:00 was missing) was chosen instead in Paper I. In the statistical analyses and figures, values of estradiol below the detection limits were set to the expected value of 2 pmol/L (the detection limit divided by 2).

The single samples of testosterone in Paper III were obtained at different time points during the day, but for the analysis they were adjusted to the expected values at 06:00 at the current testicular volume using an extrapolation model.

In this model, a reference distribution for log testosterone during the day at even hours (02:00 to 24:00) and for testicular volume was created from the measurements of 87 boys (161) by calculating means and SD using an order restriction with respect to testicular volume. For study measurements not taken at 06:00 a z-score at time *t* was calculated as a distance weighted mean of the two z-scores at even hours surrounding time *t* using the reference distribution; the testosterone value corresponding to that z-score at 06:00 for the subject's testicular volume was used as an estimate.

In Paper IV, the testosterone values at final maximum testicular volume were measured and compared between treatment groups. A cubic linear function was fitted to reference data for testosterone levels in boys close to adult height and close to reaching maximum mean testicular volume (139). Testosterone levels expected at certain time points and the residual SD could then be estimated, making it possible to calculate SDS for comparisons between the groups.

3.3.8.2 Calculation of EC_{50}

The main goal of fitting a dose–response curve to the data was to determine the best-fit value of the EC_{50}, which is the concentration that provokes a response halfway between the top and bottom plateaus. If those plateaus are not well defined, the EC_{50} will be very uncertain. In the model we have used, we constrained the bottom of the dose–response curve to 0 (the prepubertal value of the pubertal growth ratio) and the top to 1 (the value of the pubertal growth ratio at PHV). This model does not assume a standard slope but rather fits the Hill slope from the data. The calculations with the dose–response model were performed using the software Graphpad prism on log transformed estradiol and testosterone values and the results were expressed as the EC_{50} and its 95% confidence interval. Using the software WinNonlin to confirm the results from the dose–response model, the calculations were made on non log-transformed data in a simple Emax model and the results are presented as the EC_{50}±SD. Figure 14a shows the dose–response curve equation with an example and 14b shows the Emax model equation with an example.

a) b)

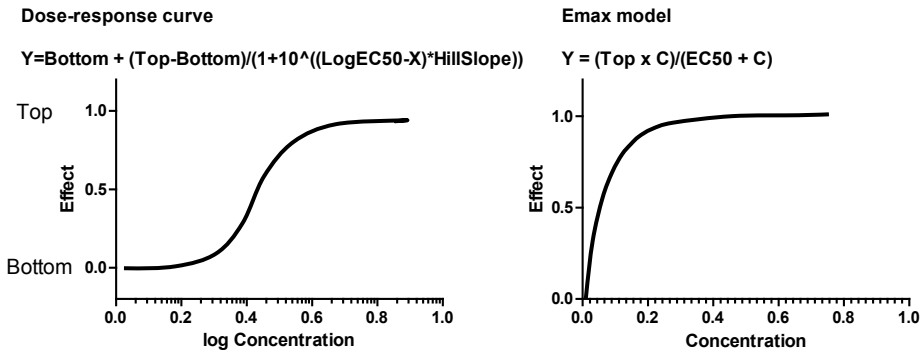


Figure 14a–b. The dose–response curve (a) and the Emax curve (b) and their equations.

In Paper III, a mixed effect model was used to include all available data for all individuals in the analysis of the relationship between testosterone and pubertal growth ratio. In this analysis, log10 testosterone was used as the dependent variable, with subjects as random intercepts. The fixed independent variables were treatment groups, pubertal growth ratio, and their interaction.

4 RESULTS

4.1 Paper I and II: Estradiol, testosterone, and height velocity during puberty in healthy girls and boys

4.1.1 Height measurements and calculated heights

As the accuracy of the polynomial fit is important for the following analyses, height measurements were compared with the calculated values of height from the polynomial fit using a Bland–Altman plot. The measured and calculated heights were similar: the bias (difference between the means ±SD) was 0.0019±0.40 cm among the girls, figure 15, and 0.0088±0.54 cm for the boys.

a) b)

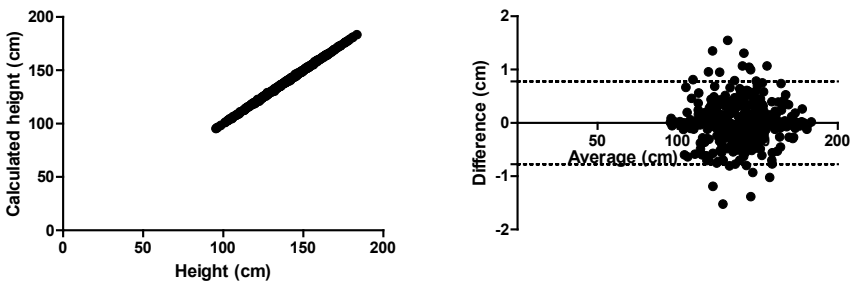


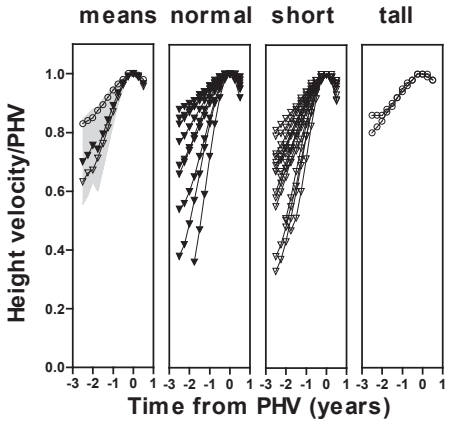
Figure 15a–b. (a) Calculated and measured height in girls in Paper I and (b) the Bland Altman plot, including the 95% confidence interval of the difference between measured and calculated height values.

4.1.2 Pubertal growth patterns

The healthy children showed a variety of growth patterns and their prepubertal height ranged from -3 SDS to +3 SDS according to the Swedish reference population (25). During prepuberty and puberty, tall children grow at a greater height velocity than short children. To describe the children's growth patterns in Paper I and II, their height velocity was expressed as a ratio of their PHV. This ratio was calculated every three months from prepuberty until six months after PHV and was plotted for short stature (prepubertal height <-2 SDS but >-3 SDS), tall stature (prepubertal height >2 SDS but <3 SDS) and normal stature (prepubertal height >-2 SD but <2 SD), as shown in

figure 16. There was no difference in the height velocity/PHV ratio between the tall, short and normal-stature children.
The figures also show the difference in pubertal growth between girls and boys. The boys' prepubertal height velocity/PHV ratio is lower compared to the girls and shows a steeper slope up to PHV, which confirms that boys reach a higher PHV.

a) Girls



c) Boys

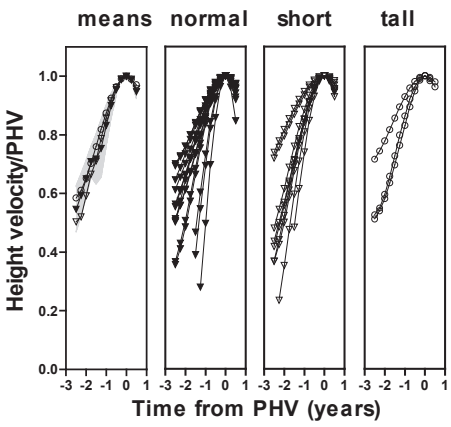


Figure 16a–b. Height velocity/PHV ratio at different time points from prepuberty up to six months after PHV in (a) normally growing, short, and tall girls and (b) boys in Paper I and II. The shaded area shows the 95% confidence interval of the mean of the normally growing children, which includes the means of the tall and short children. (▼=normal ▽=short ○=tall; PHV=peak height velocity.)

4.1.3 Anthropometrics

The calculated mean PHV and age at PHV are shown in table 4 for girls (Paper I) and boys (Paper II) together with values from Swedish reference populations (162, 163). There was a high percentage of short girls and boys: 15 of the 27 girls (55%) and 9 of the 26 boys (35%). The late occurrence of PHV in the study populations compared to the reference population could partly be explained by the higher age at PHV that is typical for short children. Likewise there was a later age at menarche among the girls, 13.9±1.4 years, compared to 12.8 years in a Swedish reference population of girls born in 1971–1975 (164). The table also shows the mean bone age in children with observations close to PHV, together with the corresponding chronological age.

Table 4. Mean (±SD) PHV, age at PHV, and age at menarche, with corresponding values from a Swedish reference population. Mean chronological age and mean bone age are shown for children with observations within ±3 months from PHV.

Study population	Mean PHV (cm/year)	Mean age at PHV (years)	Mean bone age close to PHV (years)	Mean chronological age at bone age close to PHV (years)	Mean Age at menarche (years)
Girls (Paper I)	7.2±1.32 (n=27)	12.8±1.32 (n=27)	11.9±1.16 (n=6)	11.8±1.25 (n=6)	13.9±1.40 (n=21)
Short girls (Paper I)	7.2±1.26 (n=15)	13.0±1.28 (n=15)			
Ref girls	8.6±1.15[a]	11.6±1.0[b]			12.8[c]
Boys (Paper II)	9.0±1.23 (n=26)	14.2±1.01 (n=26)	13.0±0.40 (n=5)	13.9±0.44 (n=5)	
Short boys (Paper II)	9.1±1.07 (n=9)	14.6±0.91 (n=9)			
Ref boys	9.9±1.14[a]	13.5±1.0[b]			

[a]=*Taranger et al. 1976 (162),*
[b]=*Liu et al. 2000 (163),*
[c]=*Lindgren et al. 1991 (164).*

4.1.4 Height velocity, estradiol, testosterone, and time to PHV

To describe the pattern of sex steroid levels and growth during puberty, the height velocity/PHV ratio and concomitant morning estradiol and testosterone levels are plotted in figure 17a (girls) and 17b (boys). Even values beyond PHV (not included in Paper I and II) are shown in order to outline the period covering accelerated and decelerated growth during puberty. To visualize the course of growth and sex steroids over time, the parameters are shown with smoothed superimposed lines. Table 5 shows the values of morning estradiol and testosterone close to PHV (±3 months) and before onset of puberty.

a) Girls b) Boys

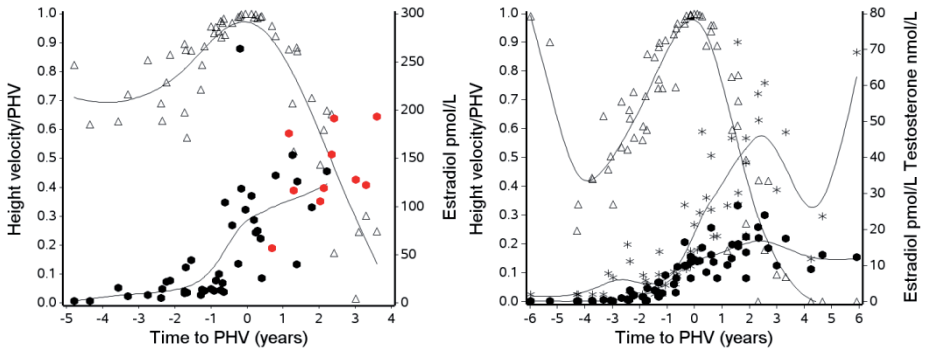


*Figure 17a–b. The height velocity/PHV ratio is plotted over time to PHV with (a) concomitant serum levels of morning estradiol in girls, and (b) morning estradiol and testosterone in boys. The parameters are shown with a smoothed superimposed line, smoothing factor 0.5. (Black dots=estradiol, *=testosterone, triangles=height velocity/PHV ratio, red dots= estradiol levels after menarche.)*

Table 5. Median (range) morning sex steroid values close to PHV (±3 months), and before onset of puberty.

	Close to PHV		Prepuberty	
Study population	**Median morning 17ß-estradiol (pmol/L)**	**Median morning testosterone (nmol/L)**	**Median morning 17ß-estradiol (pmol/L)**	**Median morning testosterone (nmol/L)**
Girls Paper I	97 (41–264) (n=7)		7.4 (2–15.9) (n=5)	
Boys Paper II	14.2 (9.9–24.8) (n=6)	11.4 (6.5–12.6) (n=6)	2 (2–7.9) (n=7)	0.3 (0.1–1.1) (n=10)

4.1.5 Pubertal stage, height velocity and sex steroid levels

The girls' height velocity/PHV ratio and concomitant morning 17β-estradiol levels in breast stage 1–5 are shown in figure 18a and 18b, showing a gradual rise in estradiol levels during puberty and PHV found early in puberty. Growth and estradiol values even after PHV are shown (not included in Paper I)

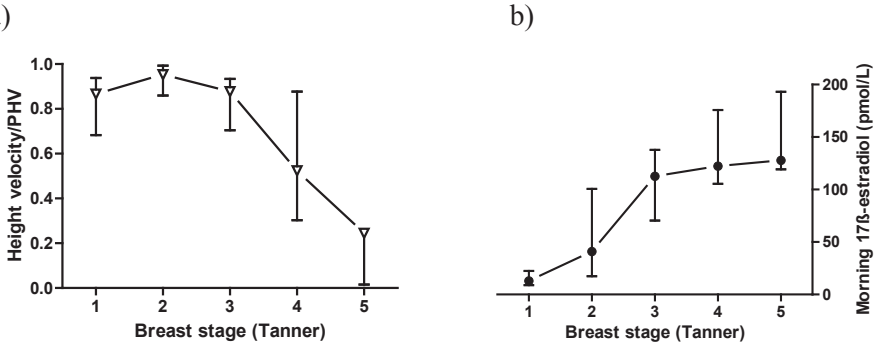


Figure 18a–b. (a) The median height velocity/PHV ratio with its interquartile range and (b) the median of morning 17ß-estradiol with interquartile range in each breast stage for girls. Values of height velocity and estradiol even after PHV are included in the figures (not included in Paper I).

The boys' results are shown in figure 19: (a) height velocity/PHV ratio, (b) morning 17β-estradiol, and (c) morning testosterone; figures show values even after PHV (not included in Paper II). The PHV was seen at testicular volumes of 8–12 ml, and testosterone and estradiol increased gradually through pubertal development; the largest increase in testosterone occurred from early to midpuberty, whereas the largest increase in 17β-estradiol occurred later in puberty.

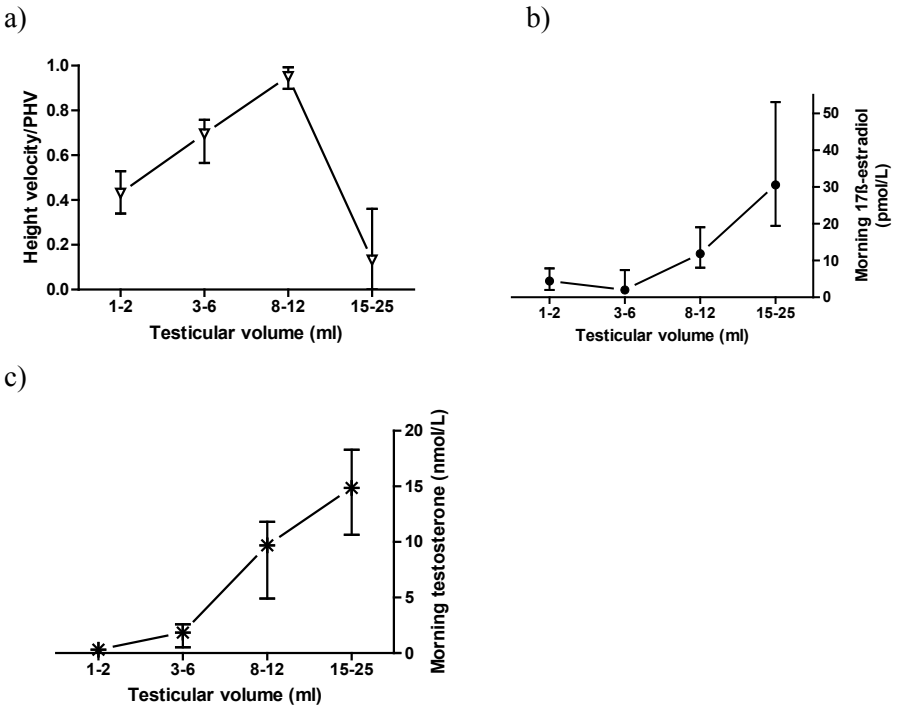


Figure 19a–c. The median and interquartile range for (a) height velocity/PHV ratio, (b) morning 17ß-estradiol, and (c) morning testosterone, by testicular volume in boys. Values of height velocity, 17ß-estradiol and testosterone even after PHV are included in the figures (not included in Paper II).

4.1.6 Correlations: sex steroids and height velocity

There was a positive correlation between morning sex steroids and height velocity in both girls and boys in Paper I and II during the pubertal growth spurt up to PHV.

In girls, morning 17ß-estradiol and height velocity showed a slightly stronger correlation at the time the samples were obtained than three months after the investigation: Spearman's rho=0.66, $p<0.001$ and rho=0.59, $p<0.01$, respectively (Paper I). When growth was expressed as the height velocity/PHV ratio, the correlation with morning 17ß-estradiol was even stronger at the time of investigation, rho=0.80, $p<0.001$.

The same pattern was seen in the boys in Paper II, where the correlation between morning testosterone (log-transformed) and height velocity at the

time the samples were obtained was slightly stronger than at three months after the investigation: Pearson bivariate correlation $r=0.88$, $p<0.001$ and $r=0.82$ $p<0.001$ respectively. A weaker correlation was found between morning estradiol and height velocity: $r=0.54$, $p<0.01$ at investigation and $r=0.44$, $p<0.05$ three months after the investigation.

Recalculating the correlations in Paper II with Spearman's rank correlation coefficient, the corresponding values regarding morning testosterone and height velocity at investigation versus three months later were $rho=0.80$ $p<0.001$ and $rho=0.69$ $p<0.001$, respectively. The correlation between morning estradiol and height velocity at investigation was $rho=0.49$, $p<0.05$, but there was no significant correlation three months after the investigation. The height velocity/PHV ratio was strongly correlated with morning testosterone, $rho=0.90$, $p<0.001$ and to a lesser extent with morning estradiol, $rho=0.65$, $p<0.001$ at time of investigation.

Results when the boys were stratified according to their testicular volumes are presented in table 6.

Table 6. Spearman correlation for selected variables during the accelerating part of the pubertal growth spurt.

Variable	Girls	Boys	
	Morning 17ß-estradiol	**Morning Testosterone**	**Morning 17ß-estradiol**
Height velocity at investigation, all children	0.66**	0.80**	0.49*
Height velocity 3 months after investigation, all children	0.59**	0.69**	NS
Height velocity/PHV ratio at investigation, all children	0.80**	0.90**	0.65**
Height velocity at investigation, < 3 ml testicular volume		NS	NS
Height velocity at investigation, 3–6 ml testicular volume		0.88**	NS
Height velocity at investigation, >6 ml testicular volume		NS	NS
Height velocity/PHV ratio at investigation, <3 ml testicular volume		0.79*	NS
Height velocity/PHV ratio at investigation, 3–6 ml testicular volume		0.85**	NS
Height velocity/PHV ratio at investigation, >6 ml testicular volume		0.68**	0.55*

*p<0.05, **p<0.01

4.1.7 EC_{50}

The pubertal growth ratio, as described in the Methods section, was analyzed in relation to morning sex steroid levels searching for a dose–response relationship between sex steroids and height velocity during the pubertal growth spurt from prepuberty up to PHV. Using this ratio we have created a variable that mirrors the accelerating part of the pubertal growth spurt and

makes it possible to compare individuals despite their different growth patterns. The EC_{50} was calculated in three different ways: nonlinear regression in a dose–response model, linear regression and a simple Emax model (as described in the Methods section). The differences in results are negligible, as shown in table 7, regardless of which of the three methods is used. In four boys and four girls it was not possible to calculate the prepubertal height velocity correctly through the fitted polynomial because the fitting process excluded the height measurements close to prepuberty in favor of the best fit around the time when sex steroids were obtained. These eight children were therefore excluded from this part of the analysis.

Table 7. Results from three different methods of calculating EC_{50} of morning testosterone and estradiol in girls and boys in Paper I and II.

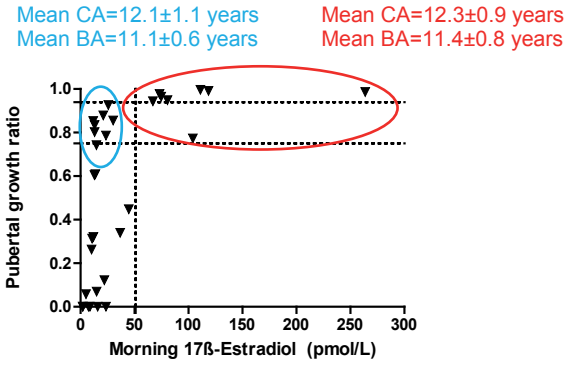
Method	Girls; 17ß-estradiol (pmol/L)	Boys; Testosterone (nmol/L)	Boys; 17ß-estradiol (pmol/L)
Linear regression: EC_{50}(95%CI)	19 (10–30)	2.5 (1.8–3.5)	6.4 (2.6–13.3)
Dose–response model: EC_{50}(95%CI)	20 (13–31)	3.1 (2.4–4.2)	6.5 (3.2–13)
Simple Emax model: EC_{50}(±SD)	18 (±10)	3.1 (±0.9)	6.4 (±5.3)

Figure 20a–c shows the pubertal growth ratio in relation to the non-log level of morning sex steroid. All girls with estradiol levels above 51 pmol/L had less than 25 % of their growth left up to PHV. However, there were girls with estradiol below 51 pmol/L whose pubertal growth had progressed as far as the girls with higher estradiol. Figure 20a shows the mean chronological age of the girls with observations close to PHV with levels of estradiol below and above 51 pmol/L, respectively. (51 pmol/L is the upper range of what is seen in early puberty (141)). The mean bone age of the girls with the higher estradiol levels did not differ significantly from the girls with lower levels of estradiol.

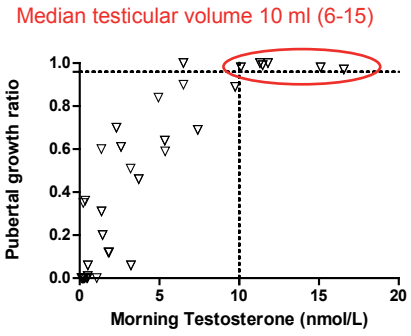
From figure 20b we see that boys with testosterone levels above 10 nmol/L grew very close to PHV (less than 5% remaining to PHV) and their median testicular volume was 10 ml (6–15). Figure 20c shows that all the boys with height velocity this close to PHV had estradiol levels over 9 pmol/L and their median testicular volumes was 12 ml (6–15). However, some boys with

similar estradiol levels have not progressed this far regarding their pubertal growth. Estradiol levels in serum show a more scattered pattern in relation to the pubertal growth ratio compared to testosterone levels in the boys.

a) Girls



b) Boys



c) Boys

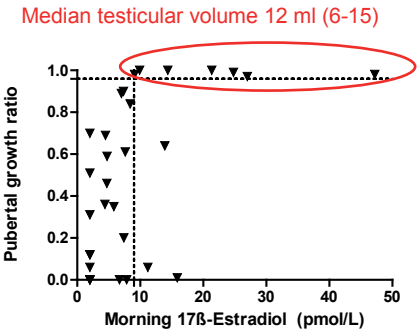


Figure 20a–c. Scatterplots of pubertal growth ratio and (a) morning 17β-estradiol in girls, (b) morning testosterone in boys, and (c) morning 17β-estradiol in boys. The mean (±SD) chronological age (CA) and bone age (BA) are shown for girls with height velocity close to PHV and estradiol levels above and below 51 pmol/L (marked red and blue respectively). The median (range) testicular volume is shown for the boys with testosterone levels above 10 nmol/L and height velocity close to PHV and for the boys with estradiol levels above 9 pmol/L and height velocity close to PHV (marked in red).

Paper I

In Paper I it was shown that low levels of estradiol seen in early puberty in girls are associated with increased height velocity, while higher levels are needed for the development of secondary sex characteristics.

Paper II

Paper II demonstrated the levels of testosterone and estradiol that are associated with increased height velocity up to PHV in boys. The results suggest that testosterone is the preferred sex steroid for the assessment of increased height velocity during puberty in boys.

Clinical implications of paper I and II

The information above is important when treating children without spontaneous puberty with pubertal hormone replacement therapy. At start of treatment the goal should be to mimic the physiological levels of estradiol and testosterone in early puberty in order to obtain normal longitudinal bone growth and pubertal maturation.

4.2 Paper III: Testosterone and height velocity during puberty in GH-treated short boys

4.2.1 Height measurements and calculated heights

Height measurements were compared to the calculated height values from the polynomial fit using a Bland–Altman plot. The measured and calculated heights were similar and the bias (difference between the means ±SD) was 0.019±0.60 cm.

4.2.2 Anthropometrics

Table 9 shows the mean calculated PHV and age at PHV in the three randomized groups. All groups reached PHV at a later age than the reference population (163), reflecting the fact that the boys are short, and short stature is known to be related to later pubertal onset.

Table 9. Mean (±SD) PHV and age at PHV in boys in Paper III, with corresponding values from different Swedish reference populations. The mean bone age close to PHV (±3 months from PHV) is also shown.

Randomization group Boys Paper III	Mean PHV (cm/year)	Mean age at PHV (years)	Mean bone age close to PHV (years)
Untreated (n=17)	8.4±1.19	14.5±1.15	14.1±1.19 (n=4)
GH_{33} (n=22)	8.7±1.13	14.2±1.13	14.1±1.10 (n=11)
GH_{67} (n=26)	9.3±1.35	13.9±1.25	14.1±1.30 (n=13)
Ref boys	9.9±1.14[a]	13.5±1.0[b]	

[a]=*Taranger et al. 1976 (162)*
[b]=*Liu et al. 2000 (163)*
(GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

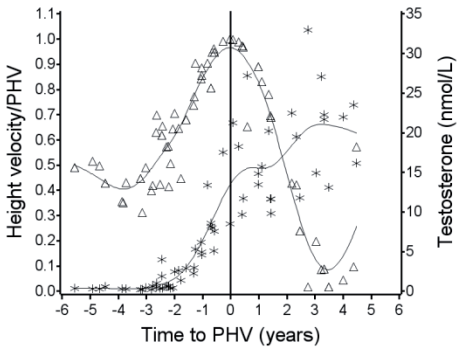
4.2.3 Height velocity, testosterone and time to PHV

Figure 21a–c shows the height velocity/PHV ratio and concomitant morning testosterone levels over time to PHV. In GH-treated boys, the testosterone levels appear to decrease with increasing GH dose at the time of PHV, shown as a vertical line in the figures, compared to the untreated control group. The median morning testosterone levels close to PHV (±0.5 years) and in the year

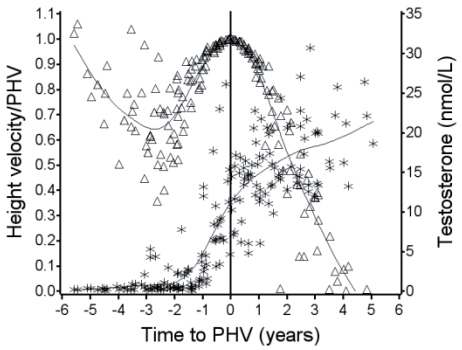
prior to PHV are shown in table 10. The median morning testosterone levels were significantly lower in GH_{67} boys compared to both the untreated control group and to GH_{33} boys close to PHV, p=0.009 and p=0.014, respectively. The table also shows that median morning testosterone levels during the year prior to PHV were significantly lower in GH_{67} boys compared to the untreated control group, and in GH_{67} boys compared to GH_{33} boys, p=0.014 and p=0.041, respectively.

To find the most extreme outliers regarding single samples of testosterone, the predicted values at 06:00 from the extrapolation model with a z score beyond ±3 were identified and were excluded, n=11.

a) Untreated



b) GH_{33}



c) GH_{67}

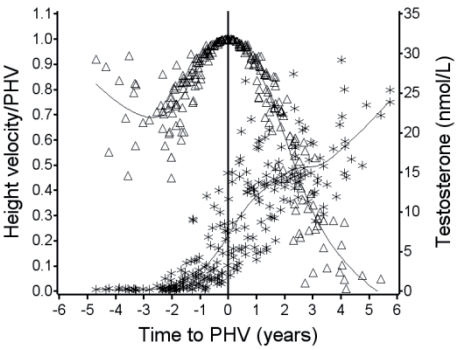


Figure 21a–c. Height velocity/PHV ratio and serum morning testosterone levels over time to PHV in the three randomized groups: (a) untreated (b) GH treatment with 33 µg/kg/day, and (c) GH treatment with 67 µg/kg/day. The figures are shown with smoothed superimposed lines with a smoothing factor of 0.5.

Table 10. Median morning testosterone levels with range at PHV (±0.5 years) and during the year prior to PHV for boys in the different randomized groups (Paper III).

	Median morning testosterone (nmol/L)		
Time period for Median morning testosterone	**Randomization groups**		
	Untreated	**GH_{33}**	**GH_{67}**
Close to PHV (±0.5 years)	17.6 (8.5–21.3) (n=5)	11.5 (4.7–17.2)[a] (n=17)	7.1 (0.9–16.3)[b] (n=24)
In the year prior to PHV	8.3 (5.1–17.6) (n=7)	6.6 (1.0–17.7)[c] (n=16)	2.9 (0.9–12.8)[d] (n=23)

[a] *GH_{33} vs. GH_{67}: p=0.014*
[b] *untreated vs. GH_{67}: p=0.009*
[c] *GH_{33} vs. GH_{67}: p=0.041*
[d] *untreated vs. GH_{67}: p=0.014*
(GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

4.2.4 Pubertal stage, height velocity, and serum testosterone

The morning testosterone levels associated with accelerated growth during puberty appear to be lower for GH-treated boys in a dose dependent manner compared to the untreated boys. It was investigated whether the GH-treated boys also differed from the untreated boys regarding their testicular size. The median testicular volume close to PHV and in the year prior to PHV are shown in table 11. There were no significant differences between the groups, although the range in testicular volume extended down to 3 ml in the GH-treated groups but not less than 8 ml in the untreated group in the year prior to PHV. Notably, 31% of the GH_{33} boys in the year prior to PHV had testicular volume below 8 ml and the corresponding value for GH_{67} boys was 65%.

Table 11. Median testicular volume with range at PHV (±0.5 years) and during the year prior to PHV for boys in the different randomized groups (Paper III).

	Median morning testicular volume (ml)		
Time period for Median testicular volume	**Randomization groups**		
	Untreated	**GH_{33}**	**GH_{67}**
Close to PHV (±0.5 years)	12 (8–20) (n=5)	12 (6–15) (n=17)	8 (4–15) (n=24)
In the year prior to PHV	8 (8–10) (n=7)	8 (3–12) (n=16)	6 (3–15) (n=23)

(GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

Figure 22a–c shows the morning testosterone levels and the height velocity/PHV ratio at the different testicular volume intervals. These figures include all data from all individuals. The pattern of increasing testosterone levels with increasing testicular volume is similar in all three randomized groups. However, the pattern of the height velocity/PHV ratio differs between groups, with higher values in early puberty in GH_{33} boys and even higher in GH_{67} boys compared to the untreated control group.

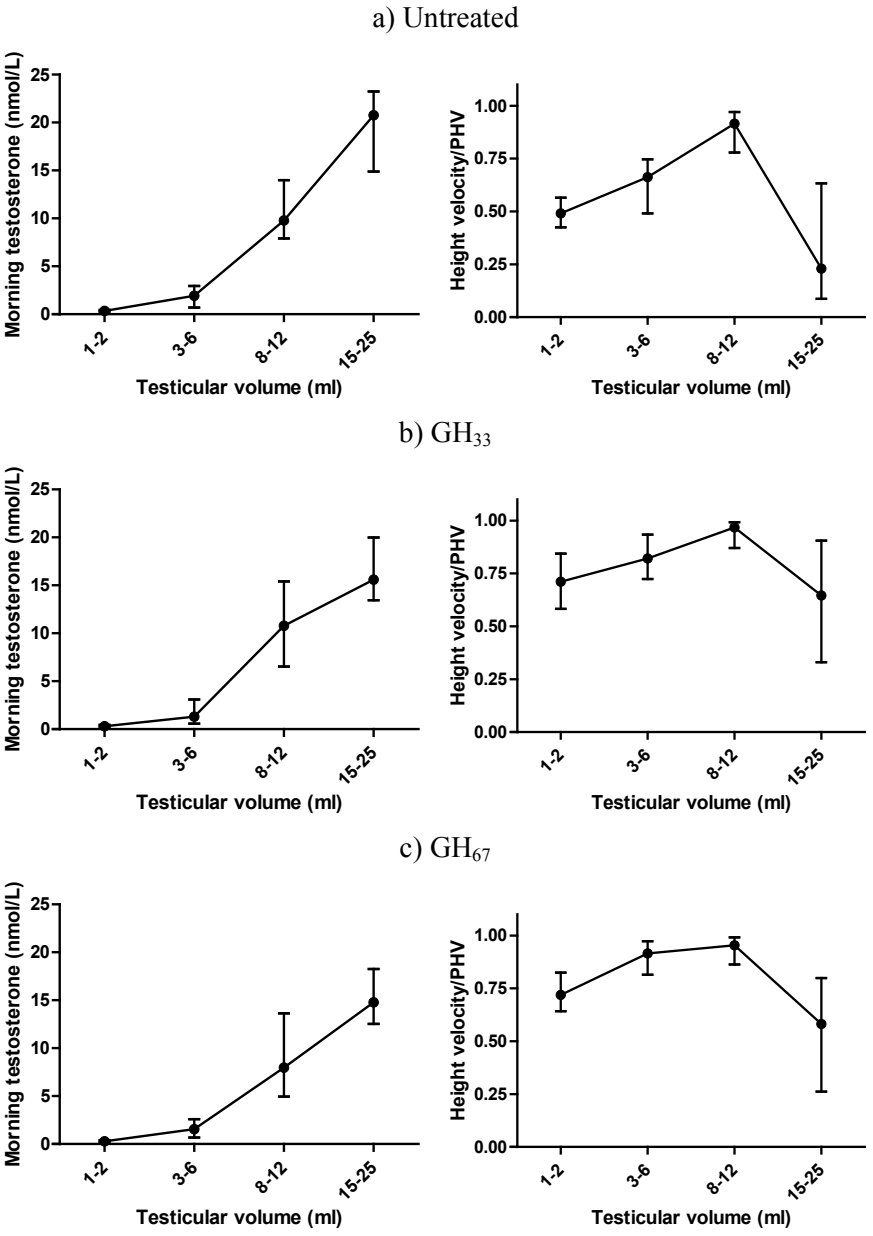


Figure 22a–c. Morning testosterone and height velocity/PHV ratio at different groups of testicular volume intervals in (a) untreated boys (b) boys treated with GH 33 µg/kg/day, and (c) boys treated with GH 67 µg/kg/day. The figures show the median and the interquartile range. Note that these figures concern descriptive data including all data for all individuals, which means that one individual can have more than one observation in each testicular volume interval.

In figure 9 in Paper III, the testicular volumes are plotted by chronological age with comparison to a reference population (162), with age at PHV adjusted down by six months to compensate for the secular trend of pubertal timing in the population born in 1974 (163). The analysis showed that GH-treated boys have larger testicles from the age of 13 years than untreated boys, although all boys in the study group had smaller testicles than the reference population from the age of 13 years.

4.2.5 EC_{50}

Figure 23 shows all boys' serum morning testosterone data and the calculated pubertal growth ratios during puberty up to PHV. There was a significant difference in the testosterone levels related to height velocity up to PHV in a dose–dependent manner. When height velocity had reached halfway from prepubertal height velocity up to PHV (pubertal growth ratio 0.5), the corresponding morning testosterone levels (geometric mean (95% CI)) were: 2.6 (1.63–4.22) nmol/L for the untreated control group, 1.3 (0.91–1.92) nmol/L for GH_{33} boys and 1.0 (0.69–1.32) nmol/L for GH_{67} boys. The morning testosterone levels were significantly lower in GH_{33} boys than in the untreated control group, p=0.03, and even more so in GH_{67} boys compared to the untreated control group, p=0.001.

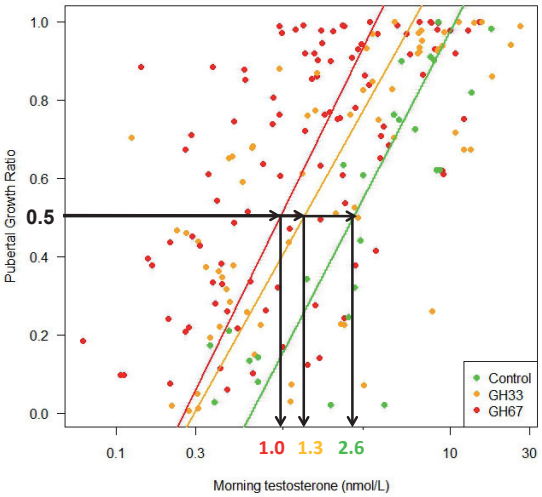


Figure 23. Pubertal growth ratio and serum morning testosterone levels in the three randomized groups, with associated regression lines derived from the linear mixed effect model: green=untreated, orange=boys treated with GH 33 µg/kg/day, and red=boys treated with GH 67 µg/kg/day.

The same pattern could be seen in the individual EC_{50} values available for 20 boys, although the groups are too small to enable statistical calculations. The individual EC_{50} values are shown in table 12 and figure 24.

Table 12. Mean (±SD) and median (range) of the individual EC_{50} values for the three randomized groups.

Treatment group		EC_{50} testosterone nmol/L mean±SD	EC_{50} testosterone nmol/L median (range)
Untreated	N=2	4.0±0.95	4.0 (3.3–4.7)
GH_{33}	N=9	1.6±1.22	1.1 (0.5–4.5)
GH_{67}	N=9	1.0±1.03	0.7 (0.2–3.6)

(GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

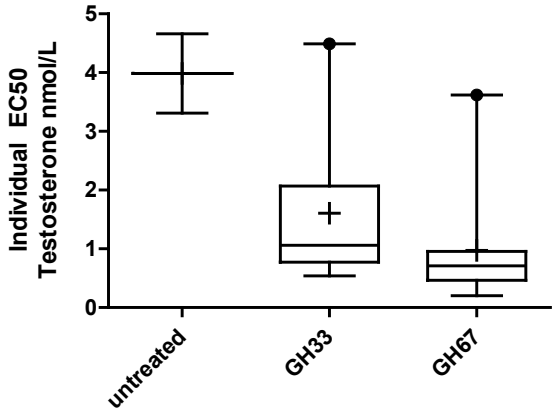


Figure 24. Median of the individual EC_{50} values in each randomized group. The figure shows the interquartile range, where the whiskers represent 10th to 90th percentiles; means are shown with +, medians with horizontal lines. (GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

The individual EC_{50} values of the GH-treated boys were plotted against the elapsed time from GH start to puberty start, and from puberty start to PHV in figure 12 in Paper III. The figure shows that boys with longer duration between GH start, puberty start and PHV have higher EC_{50} values and more similarity to the untreated boys. The treatment groups differ significantly regarding the elapsed time between puberty start and PHV, as shown in table 13.

Table 13. Mean time difference between puberty start and PHV in the randomized groups.

Treatment group		Time from Puberty start to PHV (years) mean±SD
Untreated	n=19	1.9±1.00
GH-treated	n=49	1.2±0.72[a]
GH_{33}	n=22	1.4±0.59
GH_{67}	n=27	1.1±0.78[b]

[a] *GH-treated vs. untreated p=0.008*
[b] *GH_{67} vs. untreated p=0.007*
(GH_{33}= treatment with GH 33µg/kg/day, GH_{67}= treatment with GH 67 µg/kg/day)

Boys with more than two years between GH start and puberty start (n=8) had a significantly longer duration between puberty start and PHV, 1.9±0.66 years, compared to boys with less than two years between GH start and puberty start (n=40), 1.1±0.66 years, p=0.006, and are similar to the untreated control group, 1.9±1.00 years.

In summary, the more time that elapsed between GH start, puberty onset and PHV, the more similar the GH-treated boys were to the untreated control group regarding the association between serum levels of testosterone and height velocity up to PHV.

Paper III

Paper III found no indication that GH treatment influenced the association between testosterone levels and pubertal height velocity up to PHV in short boys with normal GH secretion with or without GH treatment when GH treatment start was well before the onset of puberty.

In boys treated with GH closer to puberty, a different tool for calculating height velocity should be used to enable analyses of testosterone levels and pubertal height velocity in these boys.

4.3 Paper IV: Development of secondary sex characteristics and GH treatment in short children

Paper IV focused on the possible influence of GH treatment on pubertal development regarding secondary sex characteristics in short children without deficient GH secretion. Certain time points were chosen, as described in the methods section, to allow comparisons between children in the different randomized groups regarding their development of secondary sex characteristics during puberty. In Paper IV, separate analyses were conducted on the ITT population and PP population, as well as comparisons of boys and girls born AGA or SGA. It is important to bear in mind the small number of study subjects in some of the groups when interpreting the results.

The results regarding significant differences between groups are summarized in table 1 (ITT population) and table 2 (PP population) in Paper IV.

No differences were found between the randomized groups regarding age at onset of puberty or at final maturation, although onset of puberty and age at menarche were delayed in the girls compared to reference populations. The mean age at onset of puberty in the girls was 11.9 years in the control group, 12.1 years in GH_{33} girls and 11.7 years in GH_{67} girls. In a Danish study where girls were examined in 1991–1993, breast stage 2 was attained at a mean age of 10.88 years, and in a cohort examined in 2006–2008, breast development was significantly earlier, at a mean age of 9.86 years (165). The mean age at menarche in Paper IV was 14.1±1.35 years, compared with 12.8 years for girls in the Swedish reference population (164).
GH treatment did not have any negative impact on testicular growth as previously speculated (166). Testicular size was even significantly larger in GH-treated boys compared to the untreated boys in the ITT population although the boys in all groups, treated as well as untreated, generally had smaller testes in adolescence and onwards compared to the reference population, as shown in figure 1 in Paper IV. Testicular function, measured as testosterone production at final maturation (defined as mean maximum testicular volume) did not differ between treatment groups. There were no signs of acceleration of pubertal pace among the children studied. However, there was a slightly longer duration of puberty in GH-treated girls in the ITT population compared to the control group, both among the girls born AGA and those born SGA.

Paper IV

In this study, GH treatment relatively close to puberty did not have a negative impact on the onset or progression of the development of secondary sex characteristics in short children without deficient GH secretion.

5 DISCUSSION

This chapter begins with an overall discussion about the essential elements of a study on height velocity during puberty in relation to serum levels of testosterone and estradiol. This is followed by a discussion of the outcome of the papers included in this thesis and a general discussion.

5.1 Essential requirements

5.1.1 Accurate height measurements

To assess height velocity correctly, the accuracy of the height measurements is crucial. In the best of worlds we would have had the same examiner measuring all the children, on all test occasions and at the same time of day, because we know this is of importance for the accuracy of the measurements (167, 168). For all the children, the growth data were collected from child health centers, schools, local hospitals, and university hospitals. The mean of three height measurements was used. In another study at Queen Silvia Children's hospital with the same measuring technique (169) the level of technical error was within ±0.23 cm. The measurements that were not performed at Queen Silvia Children's hospital or a hospital endocrine unit could be of poorer quality, although growth data from Swedish schools and child health centers are of high quality (25). In addition, the most important height measurements of the investigation, in connection with the serum testosterone and estradiol assessments, were performed by experienced staff at Queen Silvia Children's hospital or at pediatric endocrine units at local hospitals and university hospitals. Each growth chart has also been checked manually for obvious measuring or registration errors, and the height measurements on which the further calculations are based are rigorously conducted.

5.1.2 Accurate calculation of height velocity

When height measurements are performed with short intervals, there will be considerable variations in height velocity if it is calculated simply by dividing difference in height by elapsed time between measurements. By fitting a polynomial to each child's growth data we avoid some of this problem. Nonetheless, some caution is appropriate in the fitting process when using a high degree polynomial; the 6^{th} degree polynomial used here can take convoluted routes between measurement points. Furthermore, in the beginning and end of the height measurements we have to be alert to

overfitting; to eliminate this risk, no calculations of height velocity were performed before the second or after the penultimate height measurement was recorded, and each fitted curve was visually examined.

5.1.3 Accurate testosterone and estradiol assays

Optimal diagnosis and treatment of children with growth and puberty disorders requires sensitive and specific laboratory methods to measure serum testosterone and 17β-estradiol. This is especially important in the case of 17β-estradiol.
Traditionally, estradiol and testosterone are measured using immunoassays but most assays available on the market are designed to be used in adult sera, for example in fertility investigations in women. These assays are not sensitive enough to measure the low levels of sex steroids, especially estradiol, found in children's sera in prepuberty or early puberty, and they have large intra- and interassay variations in the low concentration range (159, 170).

Mass spectrometry is considered to be the reference method of measuring 17β-estradiol but immunoassays are still widely used. At GP-GRC an ultrasensitive RIA has been developed for pediatric use. The ultrasensitive RIA used in the studies described in this thesis includes a necessary extraction step to reach adequate sensitivity and specificity, as described in the methods section. This assay has been meticulously validated for pediatric use and has been used routinely in clinical practice for more than a decade with 24-hour reference values available for estradiol for all pubertal stages in boys (146) and girls (141, 147). It has been shown that this method can distinguish prepubertal values from early pubertal values (141, 146, 159); it can also be used to determine dose response regarding breast development during hormone replacement therapy in girls (171), as well as during estrogen suppression in girls with precocious puberty (159). The estradiol results for various pubertal stages are comparable to those obtained by gas chromatography tandem mass spectrometry (126).

Regarding testosterone, the method used in these studies has been shown to perform well in routine clinical practice at the laboratory at GP-GRC, and it detects testosterone levels in prepubertal and pubertal children with the same accuracy as liquid chromatography tandem mass spectrometry (172).

Taking these facts together, these methods can be considered accurate for determining estradiol and testosterone levels in early puberty, making it

possible to assess the association between estradiol, testosterone and growth during puberty.

In Paper I and II, samples from 24-hour profiles of estradiol and testosterone were used. The median of the 24-hour profile was calculated in order to confirm that the chosen morning level of estradiol or testosterone was above the median of the 24-hour profile. Despite this rigorous procedure, it is possible to miss the very highest levels of estradiol or testosterone. The highest level in the morning was chosen because an increase in morning levels is associated with pubertal onset (139, 141). This would also be a more convenient sample to be used in the clinics instead of the 24-hour profiles. When studying the decelerating part of pubertal growth, the mean or median of the 24-hour profile is suggested to be used because there is a higher level of sex steroids overall during the 24-hour period and the overall tone of sex steroids is likely to be of importance.

In Paper III and IV, single samples of testosterone were obtained in addition to the 24-hour profiles. They were included in the analyses with the help of extrapolation in order to use as much of the collected data as possible from the study population. In Paper III, the testosterone levels obtained at different time points during the day were extrapolated (as described in the methods section) to allow analysis of the single samples together with the morning levels from the profiles. The larger the reference material, the more accurate these kinds of calculations are, and because the reference population consisted of 87 boys in total, there was some uncertainty in the calculated morning values of testosterone. The accuracy of the input values is of major importance for the accuracy of the outcome values in such a model. If the time point when the single sample is obtained is not accurately registered or is rounded off, the outcome of the model could be affected. As the model is dependent on the testicular volume at the time the sample is obtained, this is also a source of error. Measuring testicular volumes with an orchidometer raises questions of intra- and interindividual observer variability. In this case, if a testicular volume is registered as smaller than it actually is, then the morning value of testosterone will be miscalculated as being lower than expected; equally, if testes are misjudged as larger than they really are, this will lead to a higher morning testosterone value than what is accurate. To avoid miscalculations arising from registration errors and obvious measuring errors, the registered testicular volumes throughout puberty were checked. Thus, although it was not possible to resolve the intra- and inter-observer variability for the boys included in Paper III and IV they were all examined by experienced pediatric endocrinologists.

In Paper IV, the testosterone levels derived from boys with testicular volumes of 15–25 ml and the reference material for these testicular volumes were used to calculate SDS for the obtained testosterone values at time *t*. The SDSs were then compared between groups. The small reference material, n=9, of course brings uncertainty into these calculations. However, figure 2 in Paper IV presents the measured testosterone values graphically for the different groups and no obvious differences in testosterone values between groups are apparent.

5.1.4 Accurate measurement of pubertal growth

According to Karlberg's ICP model (10), the pubertal growth component is similar across children, independent of stature. The combination of differences in the prepubertal childhood component and the constant pubertal component results in different height velocity during puberty. Because growth rate declines with age during the childhood component, a child with early puberty in general has a greater height velocity of total growth during puberty compared to a child with late puberty, due to the differences in the remaining part of the childhood component. Calculating height velocity and assessing its direct relation to testosterone and estradiol levels will result in a large variation between individuals. This prompted us to construct the pubertal growth ratio, which is thought to reflect the total pubertal growth from start of increased height velocity up to PHV, a ratio that is comparable between individuals.

When creating the pubertal growth ratio, the time chosen for calculating prepubertal height velocity was 2.5 years before age at PHV to be sure to cover prepubertal growth and not to miss the start of pubertal growth. We could identify a marginally greater prepubertal height velocity this far back from PHV, as this point could be before the prepubertal dip in height velocity seen in most children; see figure 25, showing height velocity curves for girls and boys, from Tanner et al. (173). However, the same procedure was performed in all children, possibly resulting in an overall higher estimate of prepubertal height velocity compared to the true lowest prepubertal height velocity before pubertal take-off.

Another way of defining prepubertal height velocity is to find the lowest point of the height velocity curve prior to pubertal start. It is possible to find this point through the second derivative of the polynomial fitted to the individual's growth data, similar to the procedure used to find the time of PHV. It is the point where the second derivative passes through zero from negative to positive values prior to the pubertal spurt. Recalculating the pubertal growth

ratios for the children in Paper I and II by finding the prepubertal dip in height velocity resulted in similar EC_{50} values in the dose–response model, namely, girls: estradiol 18 pmol/L (11–31), boys: testosterone 2.8 nmol/L (2.2–3.6), compared to the published EC_{50} values for girls: estradiol 20 pmol/L (13–31), and for boys: testosterone 3.1 nmol/L (2.4–4.2). In two girls and five boys it was not possible to find the prepubertal height velocity dip using the second derivative, and these children were excluded in these analyses.

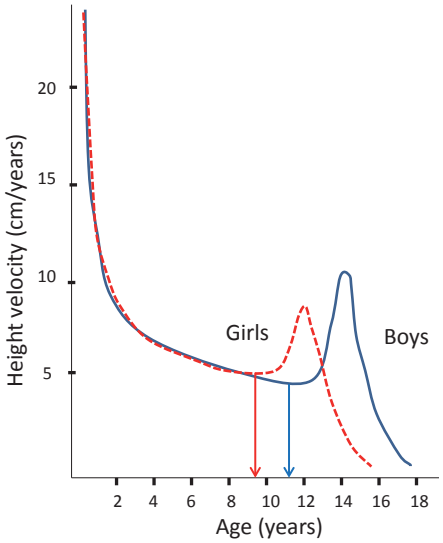


Figure 25. Height velocity in boys (blue line) and girls (dashed red line). Age 2.5 years before PHV is marked with blue line in boys and red line in girls. Modified by permission of Arch Dis Child; Tanner, J.M., R.H. Whitehouse, and M. Takaishi, Arch Dis Child, 1966 (173).

If GH treatment starts close to puberty onset, the catch-up growth from the exogenous GH will contribute to the growth spurt seen during puberty. With our model of calculating height velocity, it is not possible to separate the true endogenous pubertal growth from the GH catch-up growth, which results in measurement of the *total* growth spurt – not the *pubertal* part of the growth spurt. Thus, the pubertal growth ratio will not be calculated correctly when GH start is close to puberty onset, and the ratio cannot be used when studying the association between serum testosterone and increased height velocity in early puberty in these children.

In summary, the construction of the pubertal growth ratio gives us a useful tool for the assessment of growth during puberty and reflects the pubertal growth spurt up to PHV in children without GH treatment and when the timing of GH treatment is well separated from the onset of puberty.

5.2 Estradiol and pubertal growth in girls (Paper I)

The hypothesis in Paper I that estradiol levels in early puberty in girls were associated with increased height velocity up to PHV was confirmed. When analyzing the estradiol levels in relation to pubertal growth, a dose–response relationship was found, with an EC_{50} of 20 (13–31) pmol/L falling well within the range of what is seen in early puberty (141). It was also confirmed that increased height velocity, including its pubertal peak, is an early event in puberty in girls (30, 33, 162).

The children in both Paper I and II were healthy, but with a range of prepubertal height up to ±3 SDS. Nonetheless, the mean value of the height velocity/PHV ratio in both the tall and short children fell within the 95% confidence interval of the mean value for normally growing children from prepuberty up to PHV. This allowed us to include tall, short, and normally growing children in the same study population despite the difference in height velocity. The study population had a delay of approximately one year in both age at PHV and age at menarche compared to reference populations (163, 164). The high proportion of short girls in the study population (55%) could explain the delay in both age at PHV and age at menarche.

Estradiol levels were first correlated with growth during puberty up to PHV and then with the expected height velocity calculated three months after the estradiol sample was obtained; a higher correlation after three months would indicate a delay in the effect of estradiol on growth. The correlation between estradiol and height velocity was in fact stronger without the three-month delay. Hence, the height velocity at the time when estradiol levels were measured was used in the further calculations, and the strongest correlation was found between estradiol and the concomitant height velocity/PHV ratio, rho=0.80, $p<0.01$. By using this ratio we take the individual's maximal growth capacity into account in the correlation with estradiol, which should be more accurate than just using height velocity.

Calculating the EC_{50} was performed both in Paper I and II with three different methods: linear regression, nonlinear regression and through a simple effect max model; all gave similar results, confirming that the calculations were accurate. There was a precipitous rise in the pubertal growth ratio from prepuberty up to PHV, with a concomitant moderate rise in estradiol levels in the lower range of early puberty, which means that estradiol seen in early puberty could be associated with growth close to onset of puberty as well as close to PHV. This reflects the individual differences in estradiol sensitivity and the concomitant effect on longitudinal growth. This could be due to individual variation in the extent of interaction at different levels between

estrogen and GH/IGF-1, or to the direct effect of estrogen on bone growth, or to differences in other growth-stimulating factors.

There was a large range of estradiol levels seen close to PHV, probably due to individual variation in estradiol sensitivity but this could also reflect individual differences in the remaining proliferation capacity of the growth plate. The estradiol level measured at time *t* does not tell us what levels of estradiol the growth plate has been exposed to prior to this time point. Growth plate senescence could be more advanced than expected at a certain serum estradiol level due to prior higher levels of estradiol or possibly a longer period of estradiol exposure, or vice versa: less advanced senescence than expected could be due to lower levels of estradiol or a shorter period of estradiol exposure. There is no information available in the literature about the levels or duration of estradiol exposure in the growth plate required to reach PHV or declining growth and epiphyseal closure. However, it is evident that a less senescent growth plate needs a longer period of estrogen exposure to fuse, compared to a more senescent growth plate (51).

The results from Paper I are concordant with evidence in the literature of a growth-stimulating effect of low levels of estradiol. Increased ulnar growth was seen in girls with Turner syndrome (174) and in boys with delayed puberty (175) when treated with low-dose estrogens corresponding to serum levels of approximately 15 pmol/L. (Extrapolated levels were used because the measurements of estradiol were close to the assay detection limit (176)). This is close to the EC_{50} of estradiol found in Paper I in girls, 20 pmol/L. Furthermore, low-dose estrogen replacement therapy combined with GH treatment in girls with Turner syndrome resulted in improved growth compared to GH treatment alone (177). In addition, low-dose estrogen treatment in a girl with aromatase deficiency was required for normal longitudinal growth and bone maturation, whereas withdrawal of treatment showed increasing bone age delay and a decrease in height velocity (178). In contrast, high-dose estrogen treatment, used for girls with tall stature and patients with aromatase deficiency, leads to maturation of the growth plates and finally fusion (97, 179).

The results from Paper I are concordant with statements that low levels of estradiol seen in early puberty stimulate bone growth, while higher levels are needed for the development of female secondary sex characteristics.

5.3 Testosterone, estradiol and pubertal growth in boys (Paper II)

The fact that height velocity increases later in puberty in boys compared to girls suggests that testosterone levels related to growth, should be found in the early transition to midpuberty in boys, which was the hypothesis in Paper II. This was confirmed in this study, where a serum level of testosterone of 3.1 (2.4–4.2) nmol/L was associated with a 50% increase in growth from prepubertal growth up to PHV. Serum estradiol was somewhat scattered in relation to growth during puberty up to PHV, although an EC_{50} value of 6.5 (3.2–13) pmol/L, was calculated, corresponding to estradiol levels seen in the transition from early puberty to midpuberty.

As described in the introduction, estradiol is of major importance in both genders regarding increased growth during the pubertal growth spurt but also regarding epiphyseal closure and cessation of growth. However, testosterone is able to affect growth directly in the growth plate through the androgen receptor or indirectly through local aromatization into estradiol, which then has a direct effect on the growth plate or through stimulation of GH secretion from the pituitary. The direct effect of androgens on growth could contribute to the steadier dose–response relationship seen between testosterone levels and height velocity than between estradiol levels and height velocity in boys during puberty up to PHV.

The boys with testosterone levels around the calculated EC_{50} value had a mean distance to PHV of 1.5 years. The levels of testosterone a year ahead of PHV presented by Klein et al. (180) correspond to the levels we found closer to PHV. The disparities in our results could reflect differences in the methods of calculating height velocity but also in the methods of determining testosterone levels. The boys in Paper II with testosterone levels above 10 nmol/L were all close to PHV and had testicular volumes above 8 ml which is consistent with boys reaching their PHV at testicular volumes of 10–12 ml (29, 181, 182).

There was a strong correlation between testosterone and height velocity when the testosterone was obtained and a slightly less strong correlation between testosterone and the calculated height velocity expected three months later. Hence, the height velocity at time of obtaining testosterone was used in further calculations. As described in the results chapter, the correlations were recalculated with Spearman's rank correlation coefficient in order to be comparable to the girls' data from Paper I (both presented in table 6). In Paper II, the correlations and regression analyses were made on log-transformed data and, when recalculating with Spearman's rank correlation coefficient,

some correlations were no longer significant, although the pattern was the same. The strongest correlation was found between testosterone levels and the height velocity/PHV ratio, rho=0.90 $p<0.001$. When the boys were divided into groups according to their testicular volume at the time when testosterone was obtained, the strongest correlation was seen in boys with testes between 3–6 ml. The weaker correlation in boys with larger testes could be a statistical artefact that occurs when the rise in testosterone is rather high but the remaining increase in height velocity up to PHV is small. Other growth-stimulating factors also become more important closer to PHV, as GH levels rise significantly and we find larger testicular volumes, > 8 ml (29, 162, 181, 182). As puberty progresses in boys, serum estradiol levels increase and there is a closer correlation between testosterone and estradiol levels measured in serum closer to PHV. We also found a significant correlation between estradiol and the height velocity/PHV ratio in boys with testes >6 ml that was not seen in boys earlier in puberty; this suggests that serum estradiol is a more important growth stimulator closer to PHV.

In men, the local production of estrogen through autocrine and paracrine pathways is of major importance and the circulating levels of lesser importance (142, 183). This means that the levels of estradiol we measure in serum do not correspond very well to the actual levels of estradiol present in peripheral target tissues, which explains the lower calculated EC_{50} value of estradiol in boys compared to what was found in the girls in Paper I. The correlation presented above between increased height velocity and serum testosterone but not estradiol at testicular volumes of 3–6 ml is probably also due to us being unable to mirror the estradiol levels at the target tissues in the measured serum levels.

Nevertheless, the boys with levels of estradiol close to the calculated EC_{50} was comparable to boys studied by Klein et al. (180) regarding estradiol levels about a year ahead of PHV. In addition, all boys close to PHV in our study had estradiol levels >9 pmol/L, which is seen in midpuberty when PHV usually occurs (29, 162, 181). Thus, our results confirm previous findings about estradiol levels and pubertal progression in boys.

5.4 Testosterone and height velocity during puberty in GH-treated short boys (Paper III)

The hypothesis in Paper III was that there would be similar association between testosterone levels and height velocity up to PHV in short boys without deficient GH secretion, with or without GH treatment, compared to the boys studied in Paper II. This hypothesis was based on data for the largest height gain with GH treatment seen in prepuberty, together with the results from Paper IV revealing no indication of negative impact of GH treatment on pubertal onset or progression. The boys in this study group received their GH treatment within a mean of 1.3 (±0.80) years from puberty start, which is worryingly close in terms of pubertal growth assessment. GH treatment is effective in increasing height velocity in most short children without deficient GH secretion during the first year of treatment. The increase in height velocity following start of GH treatment relatively close to puberty onset turned out to be a confounding factor when it comes to assessing pubertal height velocity. How much of the increase is the true endogenous pubertal increase in height velocity and how much is the growth spurt caused by the exogenous GH?

With the model we used to calculate height velocity and PHV, including age at PHV, we could not separate the endogenous pubertal height velocity from the exogenous GH-induced height velocity. This is also obvious in the results from Paper III. The testosterone levels halfway from prepubertal growth up to PHV and close to PHV were found to be lower in boys treated with GH than in untreated boys. However, the GH-treated children were found to have smaller testicular volume in the year before PHV even though their mean testicular volume was larger than in the untreated boys when viewing this in relation to chronological age. These conflicting results suggest that the pubertal height velocity was not calculated accurately. However, the boys with longer elapsed time between GH start and onset of puberty also had a longer time between onset of puberty and PHV, which resembled the untreated boys. In the boys for whom it was possible to calculate individual EC_{50} values, EC_{50} increased with increasing elapsed time between start of GH, puberty and PHV, approaching the levels seen in untreated boys. The conclusion from this is that it does not seem to be the GH treatment per se that is responsible for the results regarding lower testosterone levels associated with height velocity during puberty; rather, it would appear that the timing of GH treatment so close to puberty confounded our calculation of pubertal height velocity. The boys with start of GH treatment well before puberty onset did not differ from the untreated boys in Paper III or the boys in Paper II regarding the testosterone levels associated with growth up to PHV.

The measured growth spurt should be divided into two parts: the GH effect and the "pure" puberty effect. In Karlberg's ICP model (10), the pubertal growth component is constant and the total growth at a given time is a combination of the remaining childhood component and the pubertal component, as mentioned above. With this in mind it is easier to visualize what happens when start of GH treatment is relatively close to puberty, as for some of the boys in our study group. The calculated height velocity will not be the true pubertal height velocity but rather the total height velocity comprised of both the pubertal growth and exogenous GH-induced growth. Different tools are needed to separate these two parts of the calculated height velocity, and these boys' data are to be reanalyzed using the recently developed QEPS model, as will be described in the chapter on future perspectives.

Few studies have focused on the effect of GH treatment on pubertal growth, although there are a few reports of unaffected pubertal growth (184-186) where the pubertal growth has been studied in terms of PHV, age at PHV, duration of puberty, and total pubertal growth. In these studies there were no differences between treated and untreated short children. In the present study the total pubertal growth, measured as difference between adult height and height at onset of puberty, did not differ between treatment groups (untreated: 27.6±4.1 cm, GH_{33}: 30.2±4.3 cm, GH_{67}: 29.6±5.1 cm). The natural growth pattern of ISS children show a somewhat delayed onset of puberty (187) which could result in a lesser gain of pubertal growth according to the ICP model. On the other hand, a spontaneous catch-up growth can be seen in ISS children with non-familial short stature during puberty, with an increase in height SDS from 0.5–1.5 SDS; in contrast, in children with familial short stature, prepubertal height SDS is similar to adult height SDS (187, 188). Thus, there is a variability of spontaneous growth within the ISS group during puberty which could also reflect the variability of the effect of GH treatment on pubertal growth in these children. In the original study from which the children in Paper III and IV derived (158), GH start was relatively close to puberty onset. Despite the timing of GH treatment, there was an increase in adult height of approximately 1 SD compared to the controls, with the greatest effect of GH treatment seen in the high-dose group and in the children with non-familial short stature.
In children with deficient GH secretion, it has been shown that there is a GH dose dependent effect on total pubertal growth although the doses used during puberty did not correspond to the physiological normal rise in GH secretion. However, with the GH doses used the GH dose-dependent effect on growth during puberty is of minor importance compared to the prepubertal years. This has been shown in both randomized controlled dose response trials in children with idiopathic GH deficiency (189-191) and in surveaillence data base

studies (192). Prediction models that predict total pubertal growth in children with idiopathic GH deficiency, ISS and SGA (193, 194) also reveal that the GH dose during puberty contributes to a lesser extent to the total pubertal growth. Taken together, the results from this study correspond well to what is reported in other studies, which suggests that GH treatment does not have a major impact on total pubertal growth.

5.5 Development of secondary sex characteristics and GH treatment in short children (Paper IV)

The aim of Paper IV was to study the possible influence of GH treatment on pubertal development regarding secondary sex characteristics in short children without deficient GH secretion. The concern about the effects of GH treatment on puberty is that it may accelerate sexual maturation, resulting in shortening of the growth period, premature closure of the growth plates and loss of adult height gain. In this study there was no difference regarding timing of the onset of puberty between treated and untreated children. Neither was there any difference in the age at maximum mean testicular volume or menarche, or any acceleration of pubertal maturation in GH-treated children compared to the untreated control group.

In contrast to the concerns about a faster pace of pubertal development, here a significantly slower pace was found among the GH-treated girls in the high-dose group compared to the control group. Although there was a relatively small number of girls in each group and one should thus be cautious when interpreting these results, one speculation as to the causes of the delayed maturation could be altered body composition due to GH treatment, with a relative increase of muscle mass in relation to fat mass (195).

There have also been concerns that GH treatment has negative effects on testicular growth. A study of adult men with small testes who had been treated with GH in childhood suggested poor testicular growth following GH treatment (166). It has also been reported that dogs treated with GH had impaired testicular development (196). This hypothesis of negative effects of GH treatment on testicular growth has since been rejected by other studies of boys on GH treatment (197, 198). In Paper IV, the GH-treated boys had even larger maximum mean testicular volume compared to untreated boys, although their testicular volume in general was smaller than in a Swedish reference population of the same age (15–18 years). The reference population used in Paper IV was a population born in 1956 (162), compensated by 0.5 years for the secular trend in pubertal timing in the population born in 1974 (163). However, when a different reference population is used, the boys do

not appear to have smaller testicular volumes compared to the reference values. Figure 26 shows the mean testicular volume by chronological age in the boys in Paper IV plotted against Zachmann et al.'s testicular growth chart (181). In the study by Zachmann et al., the ellipsoid of the orchidometer that was as large as the testicle examined was registered and in Taranger et al. (162) the ellipsoid nearest the examined testicle was registered. In other words, in Zachmann et al., the registered volume was never larger and often less than the examined testicle and in Taranger et al. the registered volume was sometimes less and sometimes larger than the true volume (162). This can explain the smaller testicular volumes in the testicular chart by Zachmann et al. compared to Taranger et al. This also demonstrates the difficulties in comparing orchidometer measurements of testicular volume made by different observers. However, the children in paper III and IV were examined by trained pediatric endocrinologists in Sweden who mainly measure testicular size using the same procedure as described by Taranger.

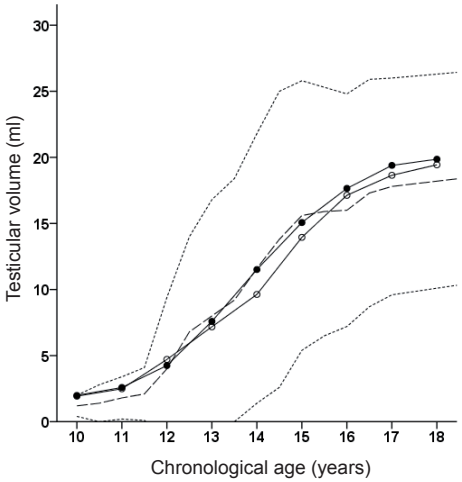


Figure 26. Study population in Paper IV, showing testicular volume by chronological age for GH-treated boys (filled circles) and untreated controls (open circles), plotted against Zachmann et al.'s testicular growth chart. (Dashed line represents mean testicular volume, dotted lines represent ±2 SD.) Modified by permission of Schwabe Verlag. Zachmann et al., Helv Paediatr Acta, 1974 (181).

As a measure of well-functioning testes, testosterone production upon reaching maximum mean testicular volume was measured and did not differ between the groups in Paper IV, although it was significantly higher compared to the reference data. However, a word of caution is appropriate here, as the small size of this reference population, composed of nine boys with testicular volumes of 15–25 ml who had reached adult height (139), makes it hard to draw any reliable conclusions.

There are close interactions between sex steroids and the GH/IGF-1 axis, as described in the introduction, and concerns about disturbing the interactivity with exogenous GH are warranted. Markedly reduced GH secretion is associated with delayed puberty and infertility, which can be restored to

normal with GH treatment (37, 199). Delayed puberty is also found in children with GH insensitivity syndrome (100). These observations indicate important actions of GH and IGF-1 in normal pubertal development and fertility (199). There are also experimental data from animals showing that testosterone secretion and testicular size are reduced in GH-deficient mice. This can be normalized with IGF-1 or GH treatment, which further supports the important role of GH and IGF-1 in gonadal maturation and function (200).

There are conflicting reports in the literature regarding the effect of GH on pubertal development in short children without deficient GH secretion. In Paper IV, some of the reports in this area are summarized in table 3 for boys and table 4 for girls. There are reports of both early (201, 202) and normal (186, 203, 204) initiation of puberty, as well as accelerated (201, 205) and normal pubertal progression (185, 186, 206) with GH treatment in short children mainly defined as ISS. The different results regarding effects of GH treatment on pubertal development in ISS children in the literature could be due to different study designs, small study groups and lack of control groups, a variety of GH doses used with differing timing of start and duration of treatment, and of course the heterogeneity of the ISS group.

As mentioned in the methods section, at the initiation of the original trial from which children in Paper III and IV were derived, the children born SGA were not classified separately from the short children born AGA. In Paper IV, the calculations were performed after dividing the children into SGA and AGA subgroups, as SGA children should be excluded from the children defined as ISS according to the current definition (39, 40). No significant differences were found, although the groups were rather small. In Paper IV, the girls were found to have a delayed onset of puberty compared to a Danish population of girls (165) and a delayed menarche compared to a Swedish population of girls (164), which is in line with what is found in spontaneous pubertal development in children with ISS (187). In contrast, children born SGA are known to have a slightly early to normal onset of puberty and menarche within the normal range (207).

In this study, GH treatment relatively close to puberty did not have a negative impact on the onset or progression of the development of secondary sex characteristics in short children without deficient GH secretion. However, this does not exclude a possible earlier onset of puberty as an effect of GH treatment, as there is a slower tempo of growth with a puberty delay in untreated ISS children. GH treatment this close to puberty could disguise an earlier normal puberty onset.

5.6 GENERAL DISCUSSION

The overall aim of this thesis was to improve current understanding of the association between serum testosterone, estradiol, and pubertal height velocity. The results from the studies in this thesis confirm what is clinically observed in boys and girls at puberty although this has not previously been studied in detail. It is demonstrated here that early pubertal levels of testosterone and estradiol are associated with increased height velocity up to PHV.

The pubertal longitudinal growth spurt is a unique feature in humans and is not found in other mammals or primates. While most mammals undergo sexual maturation when postnatal growth is tailing off, in humans this process is combined with a growth spurt. From an evolutionary and life-history perspective, this delay in somatic growth in humans is believed to be advantageous for essential brain development and it may be a result of natural selection. According to the Darwinian theory, this could be the origin of the human pubertal growth spurt. Taller and stronger men might have been selected together with taller women who were able to give birth to larger babies with larger brains (208-210). However, the physiological and endocrine stimulators of pubertal growth in humans do also exist in other primates, albeit with a different response in longitudinal growth, which suggests different sensitivity between species to growth factors, probably controlled at the genetic level. In addition, there are gender differences within the same species regarding growth (35, 209).

Many biological and physiological processes are similar in men and women and also in boys and girls but others, such as sex differentiation, differ between genders. In children going through puberty there are several clinical observations that are important when discussing levels of sex steroids and longitudinal bone growth:

- the gender difference in onset of the pubertal growth spurt and also the more intense spurt seen in boys,
- the timing of the growth spurt that parallels the onset of estrogen synthesis in girls and in boys, and
- the higher prepubertal levels of estradiol in girls compared to boys.

An important question is when puberty is considered to start. There are different ways of defining onset of puberty. The rise in sex steroids is the first sign of puberty which is then reflected in responses of the "bioassays of sex steroids": increased longitudinal growth and development of secondary sex characteristics. In girls, pubertal onset is defined by pediatric endocrinologists

as attainment of breast stage 2, although an increase in estradiol is seen prior to this and leads to increased height velocity even before breast budding. Boys take longer to reach the growth-stimulating levels of estradiol, because they start at a lower prepubertal base level and are dependent on their poor aromatase capacity in early puberty. However, when boys finally reach the growth spurt it is steeper than in girls, and boys reach a higher PHV. This could partly be due to their less mature growth plates that have not been exposed to estradiol to the same extent as in girls, or perhaps to a difference between the genders in interactions between sex steroids and the GH/IGF-1 axis. It is known that GH signals through the janus kinas (JAK)/signal transducers and activators of transcription (STAT) pathway. In rodents there is emerging evidence of GH-induced gender differences in growth, where STAT5b seems to be of specific importance in male body growth, whereas STAT5a is of importance in both genders (211). Furthermore, estrogen is known to interact with the JAK/STAT pathway (85) and there are likely to be gender differences in response to GH. In addition, the direct effect of androgens also contributes to the increase in height velocity in boys. Girls do not grow at these high velocities and they reach their declining phase of growth at a lower maximal growth rate, which could be due to the strain on the growth plate from the greater exposure of estrogen. Girls also have lower levels of androgens compared to boys during puberty and the growth-stimulating effect of androgens could be negligible.

The routes that estradiol and testosterone follow to exert their effects on growth are complex and occur at different levels. One hypothesis would be that low levels of estradiol stimulate GH secretion and growth is increased due to the rise in both estradiol and GH levels. Depending on the state of growth plate senescence, due to the exposed amount or levels of estradiol, growth will finally decline and stop. This is also supported by results from an animal model, where total ERα inactivation in mice (both brain and bone) affected bone growth and associated alterations in the GH/IGF-1 axis (101). However, in mice with bone-specific ERα inactivation, bone growth was normal but continued after maturation compared to control mice. High-dose estrogen treatment of the control mice reduced growth plate height, in contrast to no effect on the growth plate in mice with bone-specific ERα inactivation. This dual effect of estrogen could explain both the increased height velocity in early puberty and the ending of growth in late puberty. We should be careful when translating animal studies into the situation in humans, because the growth plates in rodents do not fuse directly after sexual maturation as in humans, although high-dose treatment with estrogen results in reduction of growth plate height in mice and rats (102, 103).

However, the evidence of interactions between estrogen and the GH/IGF-1 axis at different levels tells us to be humble and realize that the mechanisms governing growth regulation by sex steroids during puberty are highly complex and not completely understood. In addition, there are other factors underlying normal growth, including other hormones, nutrition, and psychosocial factors.

It is tempting to conclude that the same levels of estradiol are important for regulating growth in both girls and boys and I do think that this is the case. The estrogen receptors are found in the growth plate without gender differences throughout puberty (99), which supports this theory. It is also supported by the gender difference in start of estradiol production that parallels the increase in height velocity. In girls, the measured serum levels of estradiol probably correlate better with the estrogen in action at target tissues such as the brain and the growth plate than in boys, for whom local conversion of androgens into estrogen is of crucial importance. However, there is also evidence in adult women regarding the role of local estrogen in the central control of GH secretion. It is suggested that in women, as in men, aromatase is important in mediating the paracrine control of GH secretion (212). Thus, aromatase is present in the brain in both sexes, where it enhances GH secretion through conversion of testosterone into estradiol, resulting in increased height velocity in early puberty. Aromatase is also present at the growth plate and has been found to be upregulated during puberty, which suggests an important role in the process of epiphyseal fusion (108).

Exposure to endocrine disruptors with estrogenic or antiandrogenic properties also needs to be mentioned, as it adds to the importance of understanding the role of estradiol in prepubertal and early pubertal children. There are reports of prepubertal gynecomastia and precocious puberty in children exposed to potent estrogens through food or cosmetic products. In the last 10–20 years there has been a trend towards earlier pubertal onset in girls, defined as onset of breast development (165, 213). Interestingly, there has not been the same downward shift in age at menarche (213) indicating a prolonged pubertal duration. The earlier onset of breast development (165) could be a result of the environmental factors to which we are exposed. Primed by the endogenous estradiol levels in serum, prepubertal girls may be sensitive to even minor amounts of exogenous estrogenic factors. If there is an effect on breast tissue of these estrogenic factors there may also be an effect on height velocity. In fact, most recent data from Swedish populations indicate that this is happening (personal communication, Albertsson-Wikland, K.). It is interesting to speculate whether exogenous estrogen could increase GH secretion in the same way as endogenous estrogen does. This needs to be further investigated.

6 CONCLUSIONS

From the studies included in this thesis the levels of sex steroids associated with increased height velocity in puberty have been identified, thus contributing to increased knowledge in this field.

- Serum levels of estradiol seen in early puberty are associated with increased height velocity up to PHV in girls

- Serum levels of testosterone seen in early transition to midpuberty are associated with increased height velocity up to PHV in boys.

- Testosterone is the preferred sex steroid for the assessment of increased height velocity during puberty up to PHV in boys.

- There is no indication of negative impact of GH treatment on the onset or progression of the development of secondary sex characteristics in short children without deficient GH secretion

- Similar testosterone levels associated with increased height velocity up to PHV are found in short boys without deficient GH secretion, with or without GH treatment, if start of GH treatment is well separated from puberty onset.

7 FUTURE PERSPECTIVES AND CLINICAL IMPLICATIONS

7.1 Further analyses of children included in this thesis

To find further support for the association between low levels of gonadal steroids and pubertal height velocity found in Paper II, the study population in paper III was also analyzed. In this population of short children, there was no indication that the GH treatment would accelerate pubertal onset or tempo of puberty and there were no signs of testosterone production being affected; this suggested that there would be a similar association between sex steroid levels and growth as that found in Paper II. This seemed to be the case, at least when GH treatment was started well before puberty onset, but it was not applicable to boys with GH start closer to puberty with the growth model we used.
In order to be able to separate the endogenous pubertal growth from GH catch-up growth, we need different tools and we intend to reanalyze the boys' data using a recently developed computerized method of describing pubertal growth. This new model, QEPS, consists of four functions: quadratic, exponential, puberty, and stopping, and gives computerized information on pubertal growth, including start, midpoint, and end of "pure" pubertal growth. It also adds new dimensions of prepubertal growth during the pubertal years by its Q (quadratic) function. This will refine our ability to evaluate any influence of, for example, endogenous or exogenous hormones on both the timing and extent of pubertal growth (8, 214).

The boys' data will also be reanalyzed to calculate free testosterone in addition to total testosterone. It is known that GH treatment can affect the levels of SHBG in children with deficient GH secretion (151) although there is not much evidence in the literature regarding SHBG levels in GH-treated children without deficient GH secretion. It is possible that GH treatment could affect the SHBG levels in these children, resulting in different fractions of free testosterone compared to untreated children. GH treatment could result in a higher fraction of free testosterone. Thus, this could affect our results regarding the association between testosterone and pubertal height velocity, namely that the levels of total testosterone might be similar between untreated and GH-treated boys but the free fraction of testosterone might differ. If there is an effect of GH treatment on SHBG levels in these boys, we will avoid this source of error when using the free fraction of testosterone in the analyses.

7.2 Clinical implications

What is the benefit of increased understanding of low levels of estradiol and testosterone related to increased height velocity during puberty?
The findings in this thesis on the levels of testosterone and estradiol associated with increased height velocity in children in puberty provide a piece of the puzzle that helps the clinician to evaluate growth potential in children with growth and puberty disorders. The information is important when evaluating precocious puberty in girls and delayed puberty in boys. In girls, the low levels of estradiol demonstrated here are associated with increased height velocity, although higher levels are needed for the development of secondary sex characteristics. In boys, the start of testicular enlargement and genital development will be followed by increased height velocity when the levels of estradiol gradually rise and reach growth-stimulating levels.

One important clinical implication of the findings that low levels of estradiol increase height velocity in early puberty is in estrogen replacement therapy (ERT) in hypogonadal girls. We now know that even low levels of estradiol are associated with increased height velocity. In girls without spontaneous puberty, for whom breast development is not the first priority, treatment with low levels of estrogen could be used to stimulate longitudinal bone growth, as is seen close to gonadarche. To mimic the physiological hormonal milieu in early puberty, ERT should start with doses corresponding to serum levels seen in early puberty. Recently published recommendations advise reducing the starting doses of estrogen from 0.08–0.12 µg/kg to 0.05–0.07 µg/kg as ERT in hypogonadal girls, in order to mimic the physiological levels seen during gonadarche (215).

The finding that low levels of testosterone are associated with increased height velocity in boys has already been put to use in the setup of an upcoming clinical trial on pubertal hormone replacement therapy in boys, (EudraCT No 2012-002337-11), helping us to reach similar recommendations for dosages of testosterone in boys as is already available for estrogen in girls.

The increased understanding of estradiol levels related to increased height velocity is also to be used in the future when it will probably be possible to treat children with specific estrogen receptor modulators (SERMs) and address the desired effects to specific target tissues. There is a potential to develop specific ER-targeted therapies, leading to desired effects on growth without negative effects on other tissues. It may become possible to increase height in short children by blocking estrogen effects locally in bone tissue, thus preventing epiphyseal fusion without adverse effects in other estrogen-

dependent systems. Tissue-specific aromatase inhibitors would also contribute to the ability to regulate growth in ways we are unable to do today.

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REFERENCES

1. **Hochberg Z** 2009 Evo-devo of child growth II: human life history and transition between its phases. European journal of endocrinology 160:135-141
2. **Rogol AD, Roemmich JN, Clark PA** 2002 Growth at puberty. J Adolesc Health 31:192-200
3. **Murray PG, Clayton PE** 2013 Endocrine control of growth. American journal of medical genetics. Part C, Seminars in medical genetics 163C:76-85
4. **Tanner JM** 1987 Growth as a mirror of the condition of society: secular trends and class distinctions. Acta paediatrica Japonica; Overseas edition 29:96-103
5. **Ledford AW, Cole TJ** 1998 Mathematical models of growth in stature throughout childhood. Annals of human biology 25:101-115
6. **Cole TJ, Donaldson MD, Ben-Shlomo Y** 2010 SITAR--a useful instrument for growth curve analysis. International journal of epidemiology 39:1558-1566
7. **Cole TJ, Pan H, Butler GE** 2014 A mixed effects model to estimate timing and intensity of pubertal growth from height and secondary sexual characteristics. Annals of human biology 41:76-83
8. **Nierop AFM, Niklasson A, Holmgren A, Gelander L, Aronsson S, Albertsson-Wikland K** 2013 QEPS - a mathematical model describing individual human growth. Horm Res Paediatr 80(suppl 1):152-153
9. **Holmgren A, Nierop AFM, Niklasson A, Gelander L, Aronsson S, Albertsson-Wikland K** 2013 New puberty growth model for estimation of individual pubertal growth parameters and their precision. Horm Res Paediatr 80(suppl 1):172
10. **Karlberg J** 1989 A biologically-oriented mathematical model (ICP) for human growth. Acta paediatrica Scandinavica. Supplement 350:70-94
11. **Tse WY, Hindmarsh PC, Brook CG** 1989 The infancy-childhood-puberty model of growth: clinical aspects. Acta paediatrica Scandinavica. Supplement 356:38-43; discussion 44-35
12. **Hochberg Z** 2008 Juvenility in the context of life history theory. Arch Dis Child 93:534-539
13. **Karlberg J** 1989 On the construction of the infancy-childhood-puberty growth standard. Acta paediatrica Scandinavica. Supplement 356:26-37
14. **Tanner J** 1978 Foetus into man : physical growth from conception to maturity In: Cambridge MA; Harvard University Press
15. **Healy MJ, Lockhart RD, Mackenzie JD, Tanner JM, Whitehouse RH** 1956 Aberdeen growth study. I. The prediction of adult body measurements from measurements taken each year from birth to 5 years. Arch Dis Child 31:372-381
16. **Baker J, Liu JP, Robertson EJ, Efstratiadis A** 1993 Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73-82

17. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A** 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72
18. **Bonapace G, Concolino D, Formicola S, Strisciuglio P** 2003 A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. Journal of medical genetics 40:913-917
19. **Woods KA, Camacho-Hubner C, Savage MO, Clark AJ** 1996 Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. The New England journal of medicine 335:1363-1367
20. **Netchine I, Azzi S, Houang M, Seurin D, Perin L, Ricort JM, Daubas C, Legay C, Mester J, Herich R, Godeau F, Le Bouc Y** 2009 Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. The Journal of clinical endocrinology and metabolism 94:3913-3921
21. **Persson M, Pasupathy D, Hanson U, Norman M** 2011 Birth size distribution in 3,705 infants born to mothers with type 1 diabetes: a population-based study. Diabetes care 34:1145-1149
22. **Verhaeghe J, Van Bree R, Van Herck E, Laureys J, Bouillon R, Van Assche FA** 1993 C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: correlations with birth weight. American journal of obstetrics and gynecology 169:89-97
23. **de Zegher F, Francois I, Boehmer AL, Saggese G, Muller J, Hiort O, Sultan C, Clayton P, Brauner R, Cacciari E, Ibanez L, Van Vliet G, Tiulpakov A, Saka N, Ritzen M, Sippell WG** 1998 Androgens and fetal growth. Hormone research 50:243-244
24. **de Zegher F, Devlieger H, Eeckels R** 1999 Fetal growth: boys before girls. Hormone research 51:258-259
25. **Wikland KA, Luo ZC, Niklasson A, Karlberg J** 2002 Swedish population-based longitudinal reference values from birth to 18 years of age for height, weight and head circumference. Acta Paediatr 91:739-754
26. **Balsamo A, Wasniewska M, Di Pasquale G, Salzano G, Baronio F, Bombaci S, De Luca F** 2006 Birth length and weight in congenital adrenal hyperplasia according to the different phenotypes. European journal of pediatrics 165:380-383
27. **Herber SM, Milner RD** 1984 Growth hormone deficiency presenting under age 2 years. Arch Dis Child 59:557-560
28. **Karlberg J, Albertsson-Wikland K** 1988 Infancy growth pattern related to growth hormone deficiency. Acta paediatrica Scandinavica 77:385-391
29. **Tanner JM, Whitehouse RH** 1976 Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. Arch Dis Child 51:170-179
30. **Marshall WA, Tanner JM** 1969 Variations in pattern of pubertal changes in girls. Arch Dis Child 44:291-303
31. **Marshall WA, Tanner JM** 1970 Variations in the pattern of pubertal changes in boys. Arch Dis Child 45:13-23

32. **Ankarberg-Lindgren C** 2005 Testosterone and 17beta-oestradiol secretion in children and adolescents – assay development, levels for comparison and clinical applications. In: PhD thesis at: Department of Pediatrics. Göteborg: Göteborg University
33. **Coste J, Ecosse E, Lesage C, Chaussain JL, Carel JC** 2002 Evaluation of adolescent statural growth in health and disease: reliability of assessment from height measurement series and development of an automated algorithm. Hormone research 58:105-114
34. **Tanner JM, Whitehouse RH, Marshall WA, Carter BS** 1975 Prediction of adult height from height, bone age, and occurrence of menarche, at ages 4 to 16 with allowance for midparent height. Arch Dis Child 50:14-26
35. **Hochberg Z** 2012 Evo-devo of child growth: treatise on child growth and human evolution. 1 ed. Hoboken: Wiley-Blackwell
36. **Emons J, Chagin AS, Savendahl L, Karperien M, Wit JM** Mechanisms of growth plate maturation and epiphyseal fusion. Hormone research in paediatrics 75:383-391
37. **Tanner JM, Whitehouse RH, Hughes PC, Carter BS** 1976 Relative importance of growth hormone and sex steroids for the growth at puberty of trunk length, limb length, and muscle width in growth hormone-deficient children. The Journal of pediatrics 89:1000-1008
38. **Lindsay R, Feldkamp M, Harris D, Robertson J, Rallison M** 1994 Utah Growth Study: growth standards and the prevalence of growth hormone deficiency. The Journal of pediatrics 125:29-35
39. **Bryant J, Baxter L, Cave CB, Milne R** 2007 Recombinant growth hormone for idiopathic short stature in children and adolescents. Cochrane Database Syst Rev:CD004440
40. **Ranke MB** 1996 Towards a consensus on the definition of idiopathic short stature. Hormone research 45 Suppl 2:64-66
41. **Pedicelli S, Peschiaroli E, Violi E, Cianfarani S** 2009 Controversies in the definition and treatment of idiopathic short stature (ISS). J Clin Res Pediatr Endocrinol 1:105-115
42. **Dahlgren J** 2011 Growth outcomes in individuals with idiopathic short stature treated with growth hormone therapy. Horm Res Paediatr 76 Suppl 3:42-45
43. **Deodati A, Cianfarani S** 2011 Impact of growth hormone therapy on adult height of children with idiopathic short stature: systematic review. Bmj 342:c7157
44. **Wit JM, Reiter EO, Ross JL, Saenger PH, Savage MO, Rogol AD, Cohen P** 2008 Idiopathic short stature: management and growth hormone treatment. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 18:111-135
45. **Albertsson-Wikland K** 2011 Growth hormone in children with idiopathic short stature. Bmj 342:d1248
46. **Kristrom B, Aronson AS, Dahlgren J, Gustafsson J, Halldin M, Ivarsson SA, Nilsson NO, Svensson J, Tuvemo T, Albertsson-Wikland K** 2009 Growth hormone (GH) dosing during catch-up growth guided by individual

responsiveness decreases growth response variability in prepubertal children with GH deficiency or idiopathic short stature. The Journal of clinical endocrinology and metabolism 94:483-490
47. **Nilsson O, Marino R, De Luca F, Phillip M, Baron J** 2005 Endocrine regulation of the growth plate. Hormone research 64:157-165
48. **van der Eerden BC, Karperien M, Wit JM** 2003 Systemic and local regulation of the growth plate. Endocrine reviews 24:782-801
49. **Emons J, Chagin AS, Savendahl L, Karperien M, Wit JM** 2011 Mechanisms of growth plate maturation and epiphyseal fusion. Horm Res Paediatr 75:383-391
50. **Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD, Cutler GB, Jr.** 1994 Catch-up growth after glucocorticoid excess: a mechanism intrinsic to the growth plate. Endocrinology 135:1367-1371
51. **Weise M, De-Levi S, Barnes KM, Gafni RI, Abad V, Baron J** 2001 Effects of estrogen on growth plate senescence and epiphyseal fusion. Proceedings of the National Academy of Sciences of the United States of America 98:6871-6876
52. **Nilsson O, Baron J** 2005 Impact of growth plate senescence on catch-up growth and epiphyseal fusion. Pediatric nephrology 20:319-322
53. **Wit JM, Kamp GA, Rikken B** 1996 Spontaneous growth and response to growth hormone treatment in children with growth hormone deficiency and idiopathic short stature. Pediatric research 39:295-302
54. **Mehta A, Hindmarsh PC, Stanhope RG, Turton JP, Cole TJ, Preece MA, Dattani MT** 2005 The role of growth hormone in determining birth size and early postnatal growth, using congenital growth hormone deficiency (GHD) as a model. Clinical endocrinology 63:223-231
55. **Kerrigan JR, Rogol AD** 1992 The impact of gonadal steroid hormone action on growth hormone secretion during childhood and adolescence. Endocrine reviews 13:281-298
56. **Meinhardt UJ, Ho KK** 2006 Modulation of growth hormone action by sex steroids. Clinical endocrinology 65:413-422
57. **Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, Fujimiya M** 2004 Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 18:439-456
58. **Baumann G** 2001 Growth hormone binding protein 2001. J Pediatr Endocrinol Metab 14:355-375
59. **Ohlsson C, Mohan S, Sjogren K, Tivesten A, Isgaard J, Isaksson O, Jansson JO, Svensson J** 2009 The role of liver-derived insulin-like growth factor-I. Endocrine reviews 30:494-535
60. **Leung KC, Johannsson G, Leong GM, Ho KK** 2004 Estrogen regulation of growth hormone action. Endocrine reviews 25:693-721
61. **Peters CJ, Dattani MT** 2012 How to use insulin-like growth factor 1 (IGF1). Archives of disease in childhood. Education and practice edition 97:114-118

62. **Veldhuis JD, Roemmich JN, Rogol AD** 2000 Gender and sexual maturation-dependent contrasts in the neuroregulation of growth hormone secretion in prepubertal and late adolescent males and females--a general clinical research center-based study. The Journal of clinical endocrinology and metabolism 85:2385-2394
63. **Martha PM, Jr., Rogol AD, Veldhuis JD, Kerrigan JR, Goodman DW, Blizzard RM** 1989 Alterations in the pulsatile properties of circulating growth hormone concentrations during puberty in boys. The Journal of clinical endocrinology and metabolism 69:563-570
64. **Albertsson-Wikland K, Rosberg S, Karlberg J, Groth T** 1994 Analysis of 24-hour growth hormone profiles in healthy boys and girls of normal stature: relation to puberty. The Journal of clinical endocrinology and metabolism 78:1195-1201
65. **Rose SR, Municchi G, Barnes KM, Kamp GA, Uriarte MM, Ross JL, Cassorla F, Cutler GB, Jr.** 1991 Spontaneous growth hormone secretion increases during puberty in normal girls and boys. The Journal of clinical endocrinology and metabolism 73:428-435
66. **Albertsson-Wikland K, Rosberg S, Libre E, Lundberg LO, Groth T** 1989 Growth hormone secretory rates in children as estimated by deconvolution analysis of 24-h plasma concentration profiles. The American journal of physiology 257:E809-814
67. **Albertsson-Wikland K, Rosberg S** 1988 Analyses of 24-hour growth hormone profiles in children: relation to growth. The Journal of clinical endocrinology and metabolism 67:493-500
68. **Juul A, Bang P, Hertel NT, Main K, Dalgaard P, Jorgensen K, Muller J, Hall K, Skakkebaek NE** 1994 Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. The Journal of clinical endocrinology and metabolism 78:744-752
69. **Juul A** 2003 Serum levels of insulin-like growth factor I and its binding proteins in health and disease. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 13:113-170
70. **Blum WF, Albertsson-Wikland K, Rosberg S, Ranke MB** 1993 Serum levels of insulin-like growth factor I (IGF-I) and IGF binding protein 3 reflect spontaneous growth hormone secretion. The Journal of clinical endocrinology and metabolism 76:1610-1616
71. **Ho KY, Evans WS, Blizzard RM, Veldhuis JD, Merriam GR, Samojlik E, Furlanetto R, Rogol AD, Kaiser DL, Thorner MO** 1987 Effects of sex and age on the 24-hour profile of growth hormone secretion in man: importance of endogenous estradiol concentrations. The Journal of clinical endocrinology and metabolism 64:51-58
72. **Faria AC, Bekenstein LW, Booth RA, Jr., Vaccaro VA, Asplin CM, Veldhuis JD, Thorner MO, Evans WS** 1992 Pulsatile growth hormone release in normal women during the menstrual cycle. Clinical endocrinology 36:591-596

73. **Mauras N, Rogol AD, Veldhuis JD** 1990 Increased hGH production rate after low-dose estrogen therapy in prepubertal girls with Turner's syndrome. Pediatric research 28:626-630
74. **Metzger DL, Kerrigan JR** 1994 Estrogen receptor blockade with tamoxifen diminishes growth hormone secretion in boys: evidence for a stimulatory role of endogenous estrogens during male adolescence. The Journal of clinical endocrinology and metabolism 79:513-518
75. **Keenan BS, Richards GE, Ponder SW, Dallas JS, Nagamani M, Smith ER** 1993 Androgen-stimulated pubertal growth: the effects of testosterone and dihydrotestosterone on growth hormone and insulin-like growth factor-I in the treatment of short stature and delayed puberty. The Journal of clinical endocrinology and metabolism 76:996-1001
76. **Veldhuis JD, Metzger DL, Martha PM, Jr., Mauras N, Kerrigan JR, Keenan B, Rogol AD, Pincus SM** 1997 Estrogen and testosterone, but not a nonaromatizable androgen, direct network integration of the hypothalamo-somatotrope (growth hormone)-insulin-like growth factor I axis in the human: evidence from pubertal pathophysiology and sex-steroid hormone replacement. The Journal of clinical endocrinology and metabolism 82:3414-3420
77. **Zachmann M, Prader A, Sobel EH, Crigler JF, Jr., Ritzén EM, Atares M, Ferrandez A** 1986 Pubertal growth in patients with androgen insensitivity: indirect evidence for the importance of estrogens in pubertal growth of girls. The Journal of pediatrics 108:694-697
78. **Carel JC, Lahlou N, Roger M, Chaussain JL** 2004 Precocious puberty and statural growth. Human reproduction update 10:135-147
79. **Tuvemo T, Jonsson B, Gustafsson J, Albertsson-Wikland K, Aronson AS, Hager A, Ivarson S, Kristrom B, Marcus C, Nilsson KO, Westgren U, Westphal O, Aman J, Proos LA** 2004 Final height after combined growth hormone and GnRH analogue treatment in adopted girls with early puberty. Acta Paediatr 93:1456-1462
80. **Juul A** 2001 The effects of oestrogens on linear bone growth. Human reproduction update 7:303-313
81. **Contreras B, Talamantes F** 1999 Growth hormone (GH) and 17beta-estradiol regulation of the expression of mouse GH receptor and GH-binding protein in cultured mouse hepatocytes. Endocrinology 140:4725-4731
82. **Slootweg MC, Swolin D, Netelenbos JC, Isaksson OG, Ohlsson C** 1997 Estrogen enhances growth hormone receptor expression and growth hormone action in rat osteosarcoma cells and human osteoblast-like cells. The Journal of endocrinology 155:159-164
83. **Shao R, Egecioglu E, Weijdegard B, Kopchick JJ, Fernandez-Rodriguez J, Andersson N, Billig H** 2007 Dynamic regulation of estrogen receptor-alpha isoform expression in the mouse fallopian tube: mechanistic insight into estrogen-dependent production and secretion of insulin-like growth factors. American journal of physiology. Endocrinology and metabolism 293:E1430-1442

84. **Sharara FI, Bhartiya D, Nieman LK** 1994 Growth hormone receptor gene expression in the mouse uterus: modulation by gonadal steroids. Journal of the Society for Gynecologic Investigation 1:285-289
85. **Leung KC, Doyle N, Ballesteros M, Sjogren K, Watts CK, Low TH, Leong GM, Ross RJ, Ho KK** 2003 Estrogen inhibits GH signaling by suppressing GH-induced JAK2 phosphorylation, an effect mediated by SOCS-2. Proceedings of the National Academy of Sciences of the United States of America 100:1016-1021
86. **Venken K, Schuit F, Van Lommel L, Tsukamoto K, Kopchick JJ, Coschigano K, Ohlsson C, Moverare S, Boonen S, Bouillon R, Vanderschueren D** 2005 Growth without growth hormone receptor: estradiol is a major growth hormone-independent regulator of hepatic IGF-I synthesis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 20:2138-2149
87. **van den Berg G, Veldhuis JD, Frolich M, Roelfsema F** 1996 An amplitude-specific divergence in the pulsatile mode of growth hormone (GH) secretion underlies the gender difference in mean GH concentrations in men and premenopausal women. The Journal of clinical endocrinology and metabolism 81:2460-2467
88. **Span JP, Pieters GF, Sweep CG, Hermus AR, Smals AG** 2000 Gender difference in insulin-like growth factor I response to growth hormone (GH) treatment in GH-deficient adults: role of sex hormone replacement. The Journal of clinical endocrinology and metabolism 85:1121-1125
89. **Chowen JA, Frago LM, Argente J** 2004 The regulation of GH secretion by sex steroids. European journal of endocrinology 151 Suppl 3:U95-100
90. **Simm PJ, Bajpai A, Russo VC, Werther GA** 2008 Estrogens and growth. Pediatric endocrinology reviews : PER 6:32-41
91. **Liu JL, Yakar S, LeRoith D** 2000 Conditional knockout of mouse insulin-like growth factor-1 gene using the Cre/loxP system. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine 223:344-351
92. **Yakar S, Rosen CJ, Bouxsein ML, Sun H, Mejia W, Kawashima Y, Wu Y, Emerton K, Williams V, Jepsen K, Schaffler MB, Majeska RJ, Gavrilova O, Gutierrez M, Hwang D, Pennisi P, Frystyk J, Boisclair Y, Pintar J, Jasper H, Domene H, Cohen P, Clemmons D, LeRoith D** 2009 Serum complexes of insulin-like growth factor-1 modulate skeletal integrity and carbohydrate metabolism. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 23:709-719
93. **Ohlsson C, Nilsson A, Isaksson O, Lindahl A** 1992 Growth hormone induces multiplication of the slowly cycling germinal cells of the rat tibial growth plate. Proceedings of the National Academy of Sciences of the United States of America 89:9826-9830
94. **Schlechter NL, Russell SM, Spencer EM, Nicoll CS** 1986 Evidence suggesting that the direct growth-promoting effect of growth hormone on cartilage in vivo is mediated by local production of somatomedin. Proceedings of the National Academy of Sciences of the United States of America 83:7932-7934

95. **Wang J, Zhou J, Cheng CM, Kopchick JJ, Bondy CA** 2004 Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. The Journal of endocrinology 180:247-255
96. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. The New England journal of medicine 331:1056-1061
97. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. The Journal of clinical endocrinology and metabolism 80:3689-3698
98. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. The New England journal of medicine 337:91-95
99. **Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J, Ritzén EM, Savendahl L** 2003 Localization of estrogen receptors-alpha and -beta and androgen receptor in the human growth plate at different pubertal stages. The Journal of endocrinology 177:319-326
100. **Laron Z, Sarel R, Pertzelan A** 1980 Puberty in Laron type dwarfism. European journal of pediatrics 134:79-83
101. **Borjesson AE, Lagerquist MK, Liu C, Shao R, Windahl SH, Karlsson C, Sjogren K, Moverare-Skrtic S, Antal MC, Krust A, Mohan S, Chambon P, Savendahl L, Ohlsson C** 2010 The role of estrogen receptor alpha in growth plate cartilage for longitudinal bone growth. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 25:2690-2700
102. **Chagin AS, Lindberg MK, Andersson N, Moverare S, Gustafsson JA, Savendahl L, Ohlsson C** 2004 Estrogen receptor-beta inhibits skeletal growth and has the capacity to mediate growth plate fusion in female mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 19:72-77
103. **Stenstrom A, Hansson LI, Thorngren KG** 1982 Effect of ovariectomy on longitudinal bone growth in the rat. Anatomy and embryology 164:9-18
104. **Chagin AS, Savendahl L** 2007 GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. The Journal of clinical endocrinology and metabolism 92:4873-4877
105. **Windahl SH, Andersson N, Chagin AS, Martensson UE, Carlsten H, Olde B, Swanson C, Moverare-Skrtic S, Savendahl L, Lagerquist MK, Leeb-Lundberg LM, Ohlsson C** 2009 The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. American journal of physiology. Endocrinology and metabolism 296:E490-496
106. **Nilsson O, Weise M, Baron J** 2013 Evidence that estrogen hastens epiphyseal fusion and cessation of longitudinal bone growth by irreversibly depleting the number of resting zone progenitor cells. Horm Res Paediatr 80(suppl1):31-32

107. **Oz OK, Millsaps R, Welch R, Birch J, Zerwekh JE** 2001 Expression of aromatase in the human growth plate. J Mol Endocrinol 27:249-253
108. **Van Der Eerden BC, Van De Ven J, Lowik CW, Wit JM, Karperien M** 2002 Sex steroid metabolism in the tibial growth plate of the rat. Endocrinology 143:4048-4055
109. **Phillip M, Maor G, Assa S, Silbergeld A, Segev Y** 2001 Testosterone stimulates growth of tibial epiphyseal growth plate and insulin-like growth factor-1 receptor abundance in hypophysectomized and castrated rats. Endocrine 16:1-6
110. **Crowne EC, Wallace WH, Moore C, Mitchell R, Robertson WH, Holly JM, Shalet SM** 1997 Effect of low dose oxandrolone and testosterone treatment on the pituitary-testicular and GH axes in boys with constitutional delay of growth and puberty. Clinical endocrinology 46:209-216
111. **Eakman GD, Dallas JS, Ponder SW, Keenan BS** 1996 The effects of testosterone and dihydrotestosterone on hypothalamic regulation of growth hormone secretion. The Journal of clinical endocrinology and metabolism 81:1217-1223
112. **Malhotra A, Poon E, Tse WY, Pringle PJ, Hindmarsh PC, Brook CG** 1993 The effects of oxandrolone on the growth hormone and gonadal axes in boys with constitutional delay of growth and puberty. Clinical endocrinology 38:393-398
113. **Menke LA, Sas TC, de Muinck Keizer-Schrama SM, Zandwijken GR, de Ridder MA, Odink RJ, Jansen M, Delemarre-van de Waal HA, Stokvis-Brantsma WH, Waelkens JJ, Westerlaken C, Reeser HM, van Trotsenburg AS, Gevers EF, van Buuren S, Dejonckere PH, Hokken-Koelega AC, Otten BJ, Wit JM** 2010 Efficacy and safety of oxandrolone in growth hormone-treated girls with turner syndrome. The Journal of clinical endocrinology and metabolism 95:1151-1160
114. **Nilsson KO, Albertsson-Wikland K, Alm J, Aronson S, Gustafsson J, Hagenas L, Hager A, Ivarsson SA, Karlberg J, Kristrom B, Marcus C, Moell C, Ritzen M, Tuvemo T, Wattsgard C, Westgren U, Westphal O, Aman J** 1996 Improved final height in girls with Turner's syndrome treated with growth hormone and oxandrolone. The Journal of clinical endocrinology and metabolism 81:635-640
115. **Haeusler G, Frisch H, Schmitt K, Blumel P, Plochl E, Zachmann M, Waldhor T** 1995 Treatment of patients with Ullrich-Turner syndrome with conventional doses of growth hormone and the combination with testosterone or oxandrolone: effect on growth, IGF-I and IGFBP-3 concentrations. European journal of pediatrics 154:437-444
116. **Alonso LC, Rosenfield RL** 2002 Oestrogens and puberty. Best practice & research. Clinical endocrinology & metabolism 16:13-30
117. **Grumbach MM** 2002 The neuroendocrinology of human puberty revisited. Hormone research 57 Suppl 2:2-14
118. **Fritz MA, Speroff L** 2010 Gonadotropin Secretion Through Fetal Life, Childhood, and Puberty. In: Clinical Gynecologic Endocrinology and Infertility. 8th edition ed: Lippincott Williams & Wilkins

119. **Burger HG, Yamada Y, Bangah ML, McCloud PI, Warne GL** 1991 Serum gonadotropin, sex steroid, and immunoreactive inhibin levels in the first two years of life. The Journal of clinical endocrinology and metabolism 72:682-686
120. **Lee MM** 2003 Reproductive hormones in infant girls--a harbinger of adult reproductive function? The Journal of clinical endocrinology and metabolism 88:3513-3514
121. **Mitamura R, Yano K, Suzuki N, Ito Y, Makita Y, Okuno A** 1999 Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, and testosterone secretion before the onset of male puberty. The Journal of clinical endocrinology and metabolism 84:29-37
122. **Mitamura R, Yano K, Suzuki N, Ito Y, Makita Y, Okuno A** 2000 Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, testosterone, and estradiol secretion before the onset of female puberty in short children. The Journal of clinical endocrinology and metabolism 85:1074-1080
123. **Goji K, Tanikaze S** 1993 Spontaneous gonadotropin and testosterone concentration profiles in prepubertal and pubertal boys: temporal relationship between luteinizing hormone and testosterone. Pediatric research 34:229-236
124. **Albertsson-Wikland K, Rosberg S, Lannering B, Dunkel L, Selstam G, Norjavaara E** 1997 Twenty-four-hour profiles of luteinizing hormone, follicle-stimulating hormone, testosterone, and estradiol levels: a semilongitudinal study throughout puberty in healthy boys. The Journal of clinical endocrinology and metabolism 82:541-549
125. **Klein KO, Baron J, Colli MJ, McDonnell DP, Cutler GB, Jr.** 1994 Estrogen levels in childhood determined by an ultrasensitive recombinant cell bioassay. The Journal of clinical investigation 94:2475-2480
126. **Courant F, Aksglaede L, Antignac JP, Monteau F, Sorensen K, Andersson AM, Skakkebaek NE, Juul A, Bizec BL** 2010 Assessment of circulating sex steroid levels in prepubertal and pubertal boys and girls by a novel ultrasensitive gas chromatography-tandem mass spectrometry method. The Journal of clinical endocrinology and metabolism 95:82-92
127. **Janfaza M, Sherman TI, Larmore KA, Brown-Dawson J, Klein KO** 2006 Estradiol levels and secretory dynamics in normal girls and boys as determined by an ultrasensitive bioassay: a 10 year experience. J Pediatr Endocrinol Metab 19:901-909
128. **Auchus RJ, Rainey WE** 2004 Adrenarche - physiology, biochemistry and human disease. Clinical endocrinology 60:288-296
129. **Nakamura Y, Gang HX, Suzuki T, Sasano H, Rainey WE** 2009 Adrenal changes associated with adrenarche. Reviews in endocrine & metabolic disorders 10:19-26
130. **Tanner JM, Cameron N** 1980 Investigation of the mid-growth spurt in height, weight and limb circumferences in single-year velocity data from the London, 1966-67 growth survey. Annals of human biology 7:565-577
131. **Remer T, Manz F** 2001 The midgrowth spurt in healthy children is not caused by adrenarche. The Journal of clinical endocrinology and metabolism 86:4183-4186

132. **Yen SS, Apter D, Butzow T, Laughlin GA** 1993 Gonadotrophin releasing hormone pulse generator activity before and during sexual maturation in girls: new insights. Human reproduction 8 Suppl 2:66-71
133. **Elias CF** 2012 Leptin action in pubertal development: recent advances and unanswered questions. Trends in endocrinology and metabolism: TEM 23:9-15
134. **Carlsson B, Ankarberg C, Rosberg S, Norjavaara E, Albertsson-Wikland K, Carlsson LM** 1997 Serum leptin concentrations in relation to pubertal development. Arch Dis Child 77:396-400
135. **Vanderschueren D, Vandenput L, Boonen S, Lindberg MK, Bouillon R, Ohlsson C** 2004 Androgens and bone. Endocrine reviews 25:389-425
136. **Sisk CL, Zehr JL** 2005 Pubertal hormones organize the adolescent brain and behavior. Frontiers in neuroendocrinology 26:163-174
137. **Burger HG** 2002 Androgen production in women. Fertility and sterility 77 Suppl 4:S3-5
138. **Ankarberg C, Norjavaara E** 1999 Diurnal rhythm of testosterone secretion before and throughout puberty in healthy girls: correlation with 17beta-estradiol and dehydroepiandrosterone sulfate. The Journal of clinical endocrinology and metabolism 84:975-984
139. **Ankarberg-Lindgren C, Norjavaara E** 2004 Changes of diurnal rhythm and levels of total and free testosterone secretion from pre to late puberty in boys: testis size of 3 ml is a transition stage to puberty. European journal of endocrinology 151:747-757
140. **Kronenberg HM, Melmed S, Polosnky KS, Larsen PR** 2008 Summary of Updated Two-Cell Theory for Ovarian Steroidogenesis. In: Williams textbook of endocrinology. 11th edition ed: Saunders
141. **Norjavaara E, Ankarberg C, Albertsson-Wikland K** 1996 Diurnal rhythm of 17 beta-estradiol secretion throughout pubertal development in healthy girls: evaluation by a sensitive radioimmunoassay. J Clin Endocrinol Metab 81:4095-4102
142. **Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C, Jones M** 2002 Aromatase--a brief overview. Annu Rev Physiol 64:93-127
143. **Grumbach MM, Auchus RJ** 1999 Estrogen: consequences and implications of human mutations in synthesis and action. The Journal of clinical endocrinology and metabolism 84:4677-4694
144. **Brodie A, Inkster S, Yue W** 2001 Aromatase expression in the human male. Mol Cell Endocrinol 178:23-28
145. **Siiteri PK** 1978 Endogenous estrogen production in the young. Pediatrics 62:1134-1137
146. **Ankarberg-Lindgren C, Norjavaara E** 2008 Twenty-four hours secretion pattern of serum estradiol in healthy prepubertal and pubertal boys as determined by a validated ultra-sensitive extraction RIA. BMC Endocr Disord 8:10
147. **Ankarberg-Lindgren C, Norjavaara E** 2009 Estradiol in pediatric endocrinology. Am J Clin Pathol 132:978-980

148. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. The Journal of clinical endocrinology and metabolism 53:58-68
149. **P N-E** 2003 Laurells Klinisk kemi i praktisk medicin. In: Laurells Klinisk kemi i praktisk medicin. 8th ed: Studentlitteratur s 447-450
150. **Norman L** 2009 Disorders of the reproductive system. In: Howes SE ed. Manual of Endocrinology and Metabolism. 4th ed: Charles W Mitchell; 303-304
151. **Belgorosky A, Martinez A, Domene H, Heinrich JJ, Bergada C, Rivarola MA** 1987 High serum sex hormone-binding globulin (SHBG) and low serum non-SHBG-bound testosterone in boys with idiopathic hypopituitarism: effect of recombinant human growth hormone treatment. The Journal of clinical endocrinology and metabolism 65:1107-1111
152. **Leger J, Czernichow P** 1989 Congenital hypothyroidism: decreased growth velocity in the first weeks of life. Biology of the neonate 55:218-223
153. **Buckler JM, Willgerodt H, Keller E** 1986 Growth in thyrotoxicosis. Arch Dis Child 61:464-471
154. **Elias LL, Huebner A, Metherell LA, Canas A, Warne GL, Bitti ML, Cianfarani S, Clayton PE, Savage MO, Clark AJ** 2000 Tall stature in familial glucocorticoid deficiency. Clinical endocrinology 53:423-430
155. **Prader A** 1966 Testicular size: assessment and clinical importance. Triangle 7:240-243
156. **Tanner J, Whitehouse R, Cameron N, Marshall W, Healy M, Goldstein H** 1983 Assessment of skeletal maturity and prediction of adult height (TW2 method): New York: Academic press
157. **Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P** 1991 An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta paediatrica Scandinavica 80:756-762
158. **Albertsson-Wikland K, Aronson AS, Gustafsson J, Hagenas L, Ivarsson SA, Jonsson B, Kristrom B, Marcus C, Nilsson KO, Ritzen EM, Tuvemo T, Westphal O, Aman J** 2008 Dose-dependent effect of growth hormone on final height in children with short stature without growth hormone deficiency. The Journal of clinical endocrinology and metabolism 93:4342-4350
159. **Ankarberg-Lindgren C, Norjavaara E** 2008 A purification step prior to commercial sensitive immunoassay is necessary to achieve clinical usefulness when quantifying serum 17beta-estradiol in prepubertal children. European journal of endocrinology 158:117-124
160. **Bland JM, Altman DG** 1986 Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1:307-310
161. **Ankarberg-Lindgren C, Mahler T, B., Norjavaara E** 2013 Biological reference intervals for serum estradiol and testosterone in children. Hormone research in paediatrics (80 suppl1):179

162. **Taranger J, Engstrom I, Lichtenstein H, Svennberg- Redegren I** 1976 VI. Somatic pubertal development. Acta paediatrica Scandinavica. Supplement:121-135
163. **Liu YX, Wikland KA, Karlberg J** 2000 New reference for the age at childhood onset of growth and secular trend in the timing of puberty in Swedish. Acta Paediatr 89:637-643
164. **Lindgren GW, Degerfors IL, Fredriksson A, Loukili A, Mannerfeldt R, Nordin M, Palm K, Petterson M, Sundstrand G, Sylvan E** 1991 Menarche 1990 in Stockholm schoolgirls. Acta Paediatr Scand 80:953-955
165. **Aksglaede L, Sorensen K, Petersen JH, Skakkebaek NE, Juul A** 2009 Recent decline in age at breast development: the Copenhagen Puberty Study. Pediatrics 123:e932-939
166. **Bertelloni S, Baroncelli GI, Viacava P, Massimetti M, Simi P, Saggese G** 1999 Can growth hormone treatment in boys without growth hormone deficiency impair testicular function? J Pediatr 135:367-370
167. **Voss LD, Bailey BJ, Cumming K, Wilkin TJ, Betts PR** 1990 The reliability of height measurement (the Wessex Growth Study). Arch Dis Child 65:1340-1344
168. **Werther G** 1998 Measuring height: to stretch or not to stretch? Lancet 351:309-310
169. **Gelander L** 1998 Growth in prepubertal children: short term changes and endocrine regulation : the one-year growth study. In: PhD thesis at: Department of Pediatrics. Göteborg: Göteborg University
170. **Bay K, Andersson AM, Skakkebaek NE** 2004 Estradiol levels in prepubertal boys and girls--analytical challenges. Int J Androl 27:266-273
171. **Ankarberg-Lindgren C, Elfving M, Wikland KA, Norjavaara E** 2001 Nocturnal application of transdermal estradiol patches produces levels of estradiol that mimic those seen at the onset of spontaneous puberty in girls. The Journal of clinical endocrinology and metabolism 86:3039-3044
172. **Norjavaara E, Ankarberg-Lindgren C** 2011 Modified Spectria Testosterone RIA detects same testosterone levels in prepubertal and pubertal children as liquid chromatography tandem mass spectrometry. Horm Res Paediatr 76(suppl2):235
173. **Tanner JM, Whitehouse RH, Takaishi M** 1966 Standards from birth to maturity for height, weight, height velocity, and weight velocity: British children, 1965. I. Arch Dis Child 41:454-471
174. **Ross JL, Long LM, Skerda M, Cassorla F, Kurtz D, Loriaux DL, Cutler GB, Jr.** 1986 Effect of low doses of estradiol on 6-month growth rates and predicted height in patients with Turner syndrome. The Journal of pediatrics 109:950-953
175. **Caruso-Nicoletti M, Cassorla F, Skerda M, Ross JL, Loriaux DL, Cutler GB, Jr.** 1985 Short term, low dose estradiol accelerates ulnar growth in boys. The Journal of clinical endocrinology and metabolism 61:896-898
176. **Cutler GB, Jr.** 1997 The role of estrogen in bone growth and maturation during childhood and adolescence. J Steroid Biochem Mol Biol 61:141-144

177. **Ross JL, Quigley CA, Cao D, Feuillan P, Kowal K, Chipman JJ, Cutler GB, Jr.** 2011 Growth hormone plus childhood low-dose estrogen in Turner's syndrome. The New England journal of medicine 364:1230-1242
178. **Janner M, Fluck CE, Mullis PE** 2012 Impact of estrogen replacement throughout childhood on growth, pituitary-gonadal axis and bone in a 46,XX patient with CYP19A1 deficiency. Horm Res Paediatr 78:261-268
179. **Drop SL, De Waal WJ, De Muinck Keizer-Schrama SM** 1998 Sex steroid treatment of constitutionally tall stature. Endocrine reviews 19:540-558
180. **Klein KO, Martha PM, Jr., Blizzard RM, Herbst T, Rogol AD** 1996 A longitudinal assessment of hormonal and physical alterations during normal puberty in boys. II. Estrogen levels as determined by an ultrasensitive bioassay. J Clin Endocrinol Metab 81:3203-3207
181. **Zachmann M, Prader A, Kind HP, Hafliger H, Budliger H** 1974 Testicular volume during adolescence. Cross-sectional and longitudinal studies. Helv Paediatr Acta 29:61-72
182. **Tinggaard J, Mieritz MG, Sorensen K, Mouritsen A, Hagen CP, Aksglaede L, Wohlfahrt-Veje C, Juul A** 2012 The physiology and timing of male puberty. Current opinion in endocrinology, diabetes, and obesity 19:197-203
183. **Grumbach MM** 2000 Estrogen, bone, growth and sex: a sea change in conventional wisdom. J Pediatr Endocrinol Metab 13 Suppl 6:1439-1455
184. **McCaughey ES, Mulligan J, Voss LD, Betts PR** 1998 Randomised trial of growth hormone in short normal girls. Lancet 351:940-944
185. **Hindmarsh PC, Brook CG** 1996 Final height of short normal children treated with growth hormone. Lancet 348:13-16
186. **Rekers-Mombarg LT, Kamp GA, Massa GG, Wit JM** 1999 Influence of growth hormone treatment on pubertal timing and pubertal growth in children with idiopathic short stature. Dutch Growth Hormone Working Group. J Pediatr Endocrinol Metab 12:611-622
187. **Rekers-Mombarg LT, Wit JM, Massa GG, Ranke MB, Buckler JM, Butenandt O, Chaussain JL, Frisch H, Leiberman E** 1996 Spontaneous growth in idiopathic short stature. European Study Group. Arch Dis Child 75:175-180
188. **Wit JM, Clayton PE, Rogol AD, Savage MO, Saenger PH, Cohen P** 2008 Idiopathic short stature: definition, epidemiology, and diagnostic evaluation. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 18:89-110
189. **Mauras N, Attie KM, Reiter EO, Saenger P, Baptista J** 2000 High dose recombinant human growth hormone (GH) treatment of GH-deficient patients in puberty increases near-final height: a randomized, multicenter trial. Genentech, Inc., Cooperative Study Group. The Journal of clinical endocrinology and metabolism 85:3653-3660
190. **Albertsson Wikland K, Alm F, Aronsson S, Gustafsson J, Hagenas L, Hager A, Ivarsson S, Kristrom B, Marcus C, Moell C, Nilsson KO, Ritzen M, Tuvemo T, Westgren U, Westphal O, Aman J** 1999 Effect of growth hormone (GH) during puberty in GH-deficient children: preliminary

results from an ongoing randomized trial with different dose regimens. Acta paediatrica 88:80-84

191. **Sas TC, de Ridder MA, Wit JM, Rotteveel J, Oostdijk W, Reeser HM, Otten BJ, de Muinck Keizer-Schrama SM** 2010 Adult height in children with growth hormone deficiency: a randomized, controlled, growth hormone dose-response trial. Horm Res Paediatr 74:172-181
192. **Ranke MB, Lindberg A, Martin DD, Bakker B, Wilton P, Albertsson-Wikland K, Cowell CT, Price DA, Reiter EO** 2003 The mathematical model for total pubertal growth in idiopathic growth hormone (GH) deficiency suggests a moderate role of GH dose. The Journal of clinical endocrinology and metabolism 88:4748-4753
193. **Wikland KA, Kristrom B, Rosberg S, Svensson B, Nierop AF** 2000 Validated multivariate models predicting the growth response to GH treatment in individual short children with a broad range in GH secretion capacities. Pediatric research 48:475-484
194. **Dahlgren J, Kristrom B, Niklasson A, Nierop AF, Rosberg S, Albertsson-Wikland K** 2007 Models predicting the growth response to growth hormone treatment in short children independent of GH status, birth size and gestational age. BMC medical informatics and decision making 7:40
195. **He Q, Karlberg J** 2001 Bmi in childhood and its association with height gain, timing of puberty, and final height. Pediatric research 49:244-251
196. **Sjogren I, Jonsson M, Madej A, Johansson HE, Ploen L** 1998 Effects of very high doses of human growth hormone (hGH) on the male reproductive system in the dog. Andrologia 30:37-42
197. **Ankarberg-Lindgren C, Norjavaara E, Albertsson-Wikland K** 2002 Short boys treated with growth hormone show normal progression of testicular size and achieve normal serum testosterone concentrations. European journal of endocrinology 146:681-685
198. **Lindgren AC, Chatelain P, Lindberg A, Price DA, Ranke MB, Reiter EO, Wilton P** 2002 Normal progression of testicular size in boys with idiopathic short stature and isolated growth hormone deficiency treated with growth hormone: experience from the KIGS. Hormone research 58:83-87
199. **Hull KL, Harvey S** 2000 Growth hormone: a reproductive endocrine-paracrine regulator? Rev Reprod 5:175-182
200. **Chatelain PG, Sanchez P, Saez JM** 1991 Growth hormone and insulin-like growth factor I treatment increase testicular luteinizing hormone receptors and steroidogenic responsiveness of growth hormone deficient dwarf mice. Endocrinology 128:1857-1862
201. **Kawai M, Momoi T, Yorifuji T, Yamanaka C, Sasaki H, Furusho K** 1997 Unfavorable effects of growth hormone therapy on the final height of boys with short stature not caused by growth hormone deficiency. The Journal of pediatrics 130:205-209
202. **Kamp GA, Waelkens JJ, de Muinck Keizer-Schrama SM, Delemarre-Van de Waal HA, Verhoeven-Wind L, Zwinderman AH, Wit JM** 2002 High dose growth hormone treatment induces acceleration of skeletal maturation and an earlier onset of puberty in children with idiopathic short stature. Arch Dis Child 87:215-220

203. **Crowe BJ, Rekers-Mombarg LT, Robling K, Wolka AM, Cutler GB, Jr., Wit JM** 2006 Effect of growth hormone dose on bone maturation and puberty in children with idiopathic short stature. The Journal of clinical endocrinology and metabolism 91:169-175
204. **Leschek EW, Troendle JF, Yanovski JA, Rose SR, Bernstein DB, Cutler GB, Jr., Baron J** 2001 Effect of growth hormone treatment on testicular function, puberty, and adrenarche in boys with non-growth hormone-deficient short stature: a randomized, double-blind, placebo-controlled trial. The Journal of pediatrics 138:406-410
205. **Loche S, Cambiaso P, Setzu S, Carta D, Marini R, Borrelli P, Cappa M** 1994 Final height after growth hormone therapy in non-growth-hormone-deficient children with short stature. The Journal of pediatrics 125:196-200
206. **Hopwood NJ, Hintz RL, Gertner JM, Attie KM, Johanson AJ, Baptista J, Kuntze J, Blizzard RM, Cara JF, Chernausek SD, et al.** 1993 Growth response of children with non-growth-hormone deficiency and marked short stature during three years of growth hormone therapy. The Journal of pediatrics 123:215-222
207. **Verkauskiene R, Petraitiene I, Albertsson Wikland K** 2013 Puberty in children born small for gestational age. Horm Res Paediatr 80:69-77
208. **Gluckman PD, Hanson MA** 2006 Evolution, development and timing of puberty. Trends in endocrinology and metabolism: TEM 17:7-12
209. **Bogin B** 1999 Evolutionary perspective on human growth. Annual review of anthropology 28:109-153
210. **Hochberg Z** 2011 Evolutionary perspective in child growth. Rambam Maimonides medical journal 2:e0057
211. **Klover P, Hennighausen L** 2007 Postnatal body growth is dependent on the transcription factors signal transducers and activators of transcription 5a/b in muscle: a role for autocrine/paracrine insulin-like growth factor I. Endocrinology 148:1489-1497
212. **Birzniece V, Sata A, Sutanto S, Ho KK** 2010 Paracrine regulation of growth hormone secretion by estrogen in women. The Journal of clinical endocrinology and metabolism 95:3771-3776
213. **Sorensen K, Mouritsen A, Aksglaede L, Hagen CP, Mogensen SS, Juul A** 2012 Recent secular trends in pubertal timing: implications for evaluation and diagnosis of precocious puberty. Horm Res Paediatr 77:137-145
214. **Holmgren A, Nierop AFM, Niklasson A, Gelander L, Aronsson S, Albertsson-Wikland K** 2013 New puberty growth model for estimation of age for peak height velocity compared with a manual method. Horm Res Paediatr 80(suppl 1):177
215. **Ankarberg-Lindgren C, Kristrom B, Norjavaara E** 2014 Physiological Estrogen Replacement Therapy for Puberty Induction in Girls: A Clinical Observational Study. Horm Res Paediatr:Epub ahead of print