

# **Sex steroid secretion during childhood in males**

**- with focus on prematurity, birth size, and  
growth patterns**

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Cover illustration: Linear growth in two boys with Silver-Russell syndrome, plotted in growth charts by Wikland et al. 2002.

Sex steroid secretion during childhood in males – with focus on prematurity, birth size, and growth patterns

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«En barndom varer ikke bare hele livet. Den varer i generasjoner.»

Kari Killén

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## ABSTRACT

**Aim:** The overall aim of the thesis was to evaluate sex steroid secretion patterns during childhood in boys and to study how sex steroid concentrations relate to gestational age, birth weight, growth patterns, and adult height outcome.

**Patients and Methods:** In paper I, 11 growth hormone-treated boys with Silver–Russell syndrome (SRS) who had reached adult height were included. In paper II, two further patients were added. Data on birth characteristics and growth at 6, 8, 10, 12, 14, and 16 years of age were collected retrospectively. Blood samples taken at the same ages, were analyzed for sex steroid concentrations. Two groups were defined and compared, those who reached an unexpected short adult height (non-responders) and those with an expected adult height (responders). Correlations between sex steroid concentrations and adult height were assessed. In papers III and IV, 58 boys born at gestational age 32+0 to 36+6 were followed prospectively. Growth data and sex steroid concentrations were analyzed at 5, 6, 7, 8, and 10 years of age, and correlations between sex steroid concentrations and both birth characteristics and growth patterns were assessed. Mass-spectrometry-based methods were used for sex steroid analysis in all four papers.

**Results:** Paper I showed that non-responders had higher estradiol ( $E_2$ ) at 10–14 years of age and less pubertal height gain compared to responders.  $E_2$  at 10 years of age correlated strongly and negatively with adult height. In paper II, non-responders had higher adrenal androgens from 10–12 years and higher gonadal androgens from 10–14 years of age, compared to responders. There were strong negative correlations between dehydroepiandrosterone-sulfate (DHEAS) (8–12 years), testosterone (10–14 years), and dihydrotestosterone (DHT) (10 and 12 years of age), and adult height. In paper III, DHEAS and androstenedione ( $A_4$ ), correlated with weight at 7–10 years and DHEAS with waist-to-height ratio (WHtR) at 7 and 10 years of age; longitudinal analysis also showed significant associations between weight and WHtR and DHEAS and  $A_4$ . The trajectories of adrenal androgens were established at 5 years of age. Paper IV showed negative correlations between both testosterone at 8 and 10 years and  $E_2$  at 10 years of age and birth weight, both in grams and standard deviation score.

**Conclusions:** Birth weight was inversely associated with concentrations of testosterone from 8 to 10 years and  $E_2$  at 10 years of age in preterm boys. Childhood adrenal androgen concentrations were associated with body weight and WHtR but not with birth weight and adrenal androgen trajectories were established at 5 years of age. In boys with SRS, non-responders had higher concentrations of sex steroids from 10 to 14 years of age. Prepubertal and pubertal sex steroid concentrations correlated inversely with adult height and higher levels of adrenal androgens and earlier increase in gonadal androgens and  $E_2$  were associated with shorter adult height. The results of this thesis suggest that there is a relationship between birth weight, childhood sex steroid secretion patterns and growth patterns both in boys with SRS and in preterm boys without SRS.

**Keywords:** Androgens, birth weight, body height, body weight, child, estrogens, male, mass spectrometry, premature birth, Silver–Russell syndrome.

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# SAMMANFATTNING PÅ SVENSKA

Denna avhandling handlar om hur utsöndring av könshormoner under barndomen hos pojkar kan relateras till födelsevikt, graviditetslängd och tillväxtmönster. Idén till projektet kom av den kliniska observationen att vissa pojkar med den ovanliga diagnosen Silver–Russell syndrom (SRS), som karakteriseras av uttalat låg födelsevikt och kortvuxenhet, växer oväntat dåligt under puberteten och får en kort slutlängd, trots behandling med tillväxtstimulerande hormon (tillväxthormon).

I avhandlingens första studie undersöktes 11 pojkar med SRS. Födelsedata, tillväxtdata och pubertetsbedömningar samlades in från deras medicinska journaler. Vi analyserade också sparade blodprover från åldrarna 6, 8, 10, 12, 14 och 16 år. Pojkarna delades i två grupper; de som hade en oväntat kort slutlängd (6 pojkar) och de som hade en förväntad slutlängd (5 pojkar). När vi jämförde de två grupperna, observerade vi att de med kort slutlängd hade sämre längdtillväxt under puberteten. Tillväxten och utmognaden av skelettet påverkas av det kvinnliga könshormonet östradiol ( $E_2$ ) som också bidrar till att sluta tillväxtzonerna och avsluta längdtillväxten. När vi jämförde nivåerna av  $E_2$  i de två grupperna, hade de med kort slutlängd högre nivåer av  $E_2$  mellan 10 och 14 år. Det fanns också ett starkt samband mellan  $E_2$  vid 10 år och slutlängd när vi analyserade data för hela studiegruppen. Vår bedömning av resultaten var att högre nivåer av  $E_2$  innan pubertetsstart, påverkade längdtillväxten under puberteten negativt och därmed kunde vara en orsak till kortare slutlängd hos pojkar med SRS.

Tillväxten hos pojkar påverkas också av manliga könshormoner som produceras i binjuren och testikeln. Dehydroepiandrosteron sulfat (DHEAS), är det vanligaste könshormonet från binjuren och testosteron är det vanligaste könshormonet från testikeln. Manliga könshormoner från både binjuren och testikeln kan omvandlas till  $E_2$  i kroppen.

I den andra studien undersökte vi om det var binjuren- eller testikelhormonerna som orsakade nivån av  $E_2$  som observerats i den första studien. Till denna studie, kunde vi rekrytera ytterligare två färdigvuxna pojkar med SRS. Båda hade fått oväntat kort slutlängd. Vi undersökte nivåerna av manliga könshormoner vid samma åldrar som i den första studien. Resultaten visade att pojkar med kort slutlängd hade högre nivå av DHEAS vid 10 års ålder och högre nivåer av testosteron mellan 10 och 14 år. När vi analyserade hormonkoncentrationer från hela gruppen, fanns det ett starkt samband mellan DHEAS mellan 8 och 12 år samt testosteron mellan 10 och 14 år och slutlängd. Vår bedömning var att manliga könshormoner både från binjuren och testikeln verkade bidra till nivån av  $E_2$ .

Barn med SRS har oftast uttalad låg födelsevikt och växer dåligt under barndomen. Barn utan SRS, men som fötts för tidigt har också låg födelsevikt men inte alltid dålig tillväxt. Vi ville undersöka om det fanns samband mellan födelsevikt, könshormoner och tillväxt hos pojkar utan SRS, men med låg födelsevikt som hos pojkar med SRS.

I den tredje och fjärde studien ingick 58 pojkar som hade rekryterats vid födelsen. Pojkarna var födda mellan graviditetsvecka 32 och 36 och hade därför låg födelsevikt.

I den tredje studien, analyserade vi nivåerna av manliga könshormoner från binjuren vid 5, 6, 7, 8 och 10 års ålder. Eftersom binjurens utsöndring av könshormoner i andra studier har kopplats till kroppssammansättning, undersökte vi också tillväxtmönster och utveckling av vikt och "waist-to-height ratio" (WHtR) som ett mått på mängden bukfett. Vi observerade att pojkar som redan vid 5 år hade ett högt WHtR, fortsatte att öka sitt WHtR vilket skiljer sig från ett normalförlopp där WHtR i stället sjunker med åldern. Hos alla pojkar ökade nivån av DHEAS med stigande ålder, men hos de som hade högst DHEAS vid 5 år, var ökningen mest tydlig. Vi hittade inga samband mellan nivåerna av könshormoner från binjuren och graviditetslängd eller födelsevikt. Däremot fanns ett samband mellan ökning i både vikt och WHtR och ökning av könshormoner från binjuren.

I den fjärde studien undersökte vi sambanden mellan nivåerna av testosteron och  $E_2$  i samma studiegrupp och vid samma åldrar som i den tredje studien. Vi hittade inga samband mellan dessa hormoner och kroppssammansättning. Däremot fann vi samband både mellan testosteronnivåerna vid 8 och 10 år samt  $E_2$  -nivån vid 10 år och födelsevikt.

Sammanfattningsvis visar resultaten från avhandlingen att det hos de för tidigt födda pojkarna fanns ett samband mellan lägre födelsevikt och högre nivåer av testosteron mellan 8 och 10 år och  $E_2$  vid 10 år. Hos dessa pojkar var högre nivåer av DHEAS från binjuren under barndomen relaterat till högre vikt och WHtR, men inte till födelsevikt eller graviditetslängd. Hos pojkarna med SRS och uttalad låg födelsevikt, var högre nivåer av könshormoner från 10 till 14 år relaterat till sämre längdtillväxt under puberteten och kortare slutlängd. Resultaten tyder således på att det finns ett samband mellan födelsevikt och nivån av könshormoner under barndomen hos för tidigt födda pojkar, och att könshormonerna spelar en viktig roll för såväl längdutveckling under puberteten som slutlängd hos pojkar med SRS.



# LIST OF PAPERS

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

- I. **Kvernebo-Sunnergren K\***, Ankarberg-Lindgren C\*, Åkesson K, Andersson MX, Samuelsson L, Lovmar L, Dahlgren J.  
Hyperestrogenism affects adult height outcome in growth hormone treated boys with Silver-Russell syndrome.  
*Frontiers in Endocrinology 2018; 9:780*
- II. **Kvernebo Sunnergren K**, Ankarberg-Lindgren C, Dahlgren J.  
Adrenal and gonadal activity, androgen concentrations, and adult height outcomes in boys with Silver-Russell syndrome.  
*Frontiers in Endocrinology 2019; 10:829*
- III. **Kvernebo Sunnergren K**, Karlsson AK, Allvin K, Nilsson S, Ankarberg-Lindgren C, Dahlgren J.  
Adrenal androgen trajectories are established during childhood in preterm boys.  
*Acta Paediatrica 2021; 00:1–8. Online ahead of print.*
- IV. **Kvernebo Sunnergren K**, Dahlgren J, Karlsson AK, Nilsson S, Allvin K, Ankarberg-Lindgren C.  
Estrogen and testosterone concentrations during childhood are inversely associated with birth weight in preterm boys.  
*Submitted manuscript*

\* Shared first authorship

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# ABBREVIATIONS

3 $\beta$ -HSD	3 $\beta$ -Hydroxysteroid dehydrogenase
17 $\beta$ -HSD	17 $\beta$ -Hydroxysteroid dehydrogenase
17-OHP	17-hydroxyprogesterone
A <sub>4</sub>	Androstenedione
ACTH	Adrenocorticotrophic hormone
AGA	Appropriate for gestational age
AITT	Arginine-insulin-tolerance test
AMH	Anti-Müllerian hormone
AR	Androgen receptor
BMI	Body mass index
°C	Degrees of Celsius
CRH	Corticotropin-releasing hormone
CV	Coefficient of variation
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone-sulfate
DHT	Dihydrotestosterone
E <sub>1</sub>	Estrone
E <sub>2</sub>	Estradiol
ER $\alpha$	Estrogen receptor alpha
ER $\beta$	Estrogen receptor beta

EUGR	Extrauterine growth restriction
FSH	Follicle-stimulating hormone
G 1-4	Genital stage 1-4 (Tanner stages)
GC-MS/MS	Gas chromatography–tandem mass spectrometry
GH	Growth hormone
GHD	Growth hormone deficiency
hCG	Human chorionic gonadotropin
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
ICP	Infancy–childhood–puberty
IG-DMR	Intergenic differentially methylated region
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
INSL3	Insulin-like peptide 3
IQR	Interquartile range
ITT	Intratesticular testosterone
IUGR	Intrauterine growth restriction
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL	Low density lipoprotein
LH	Luteinizing hormone
LOD	Limit of detection
LOM	Loss of methylation

MPH	Midparental height
MRI	Magnetic resonance imaging
NH-CSS	Netchine–Harbison clinical scoring system
OGTT	Oral glucose tolerance test
P450arom	Cytochrome P450 aromatase
SDS	Standard deviation score
SGA	Small for gestational age
SHBG	Sex hormone-binding globulin
SRS	Silver–Russell syndrome
TW	Tanner and Whitehouse
upd(7)mat	Maternal uniparental disomy of chromosome 7
WHtR	Waist-to-height ratio

## DEFINITIONS IN SHORT

Low birth weight	Birth weight < 2500 grams
Late preterm	Infant born at 34+0 to 36+6 weeks of gestation
Moderately preterm	Infant born at 32+0 to 33+6 weeks of gestation
Non-responder	Patient with adult height > 1 standard deviation score below midparental height
Responder	Patient with adult height $\leq$ 1 standard deviation score below midparental height
Very low birth weight	Birth weight < 1500 grams

# 1 INTRODUCTION

The scope of this thesis was to evaluate patterns of sex steroid secretion during childhood and puberty and to investigate how sex steroid secretion patterns relate to birth characteristics such as gestational age and size at birth, as well as to growth patterns during childhood in boys.

## 1.1 SEX STEROID HORMONES

Human steroid hormones are lipids derived from cholesterol. They are subdivided into *corticoids*, giving rise to glucocorticoids and mineralocorticoids, and *sex steroids*, consisting of androgens, estrogens, and progestins (1, 2). In the context of this thesis, only the action of androgens and estrogens will be discussed.

Sex steroids are crucial for fetal development, pubertal development, skeletal growth, bone health, brain function, and fertility. Figure 1 (next page) gives an overview of sex steroids derived from the adrenal gland during adrenarche and from the testis during gonadarche.

### 1.1.1 THE ADRENAL GLAND

The human adrenal cortex consists of three major zones: *zona glomerulosa*, producing aldosterone under control of the renin-angiotensin system, *zona fasciculata*, secreting glucocorticoids, and *zona reticularis*, synthesizing androgens under the influence of adrenocorticotrophic hormone (ACTH), (3, 4).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis results in pituitary ACTH secretion, which stimulates the adrenal gland to produce glucocorticoids and androgens. Regulatory mechanisms of ACTH secretion include circadian rhythm, stress, and feedback inhibition in response to cortisol (1). However, numerous other endocrine signals, such as prolactin as well as cytokines and the insulin-like growth factor (IGF) system, have been suggested to act as coregulators of adrenal androgen secretion (1).

Adrenal androgens including dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEAS), and androstenedione ( $A_4$ ), have little androgenic activity but are available as circulating precursors for peripheral conversion to more potent androgens, such as testosterone, as well as dihydrotestosterone (DHT) and estrogens such as estradiol ( $E_2$ ) (1, 5). DHEAS is a stable marker of adrenal androgen activity, and the concentration of circulating DHEAS is about 300 times greater than the DHEA concentration (1, 6, 7). A DHEAS receptor has

been anticipated but is not yet identified (8). In children, conversion of DHEA to DHEAS through sulfation prevents androgen effects through conversion of DHEA to  $A_4$  and eventually testosterone, and the amount of testosterone originating from the adrenal androgen secretion is minimal (1, 9), Figure 1.

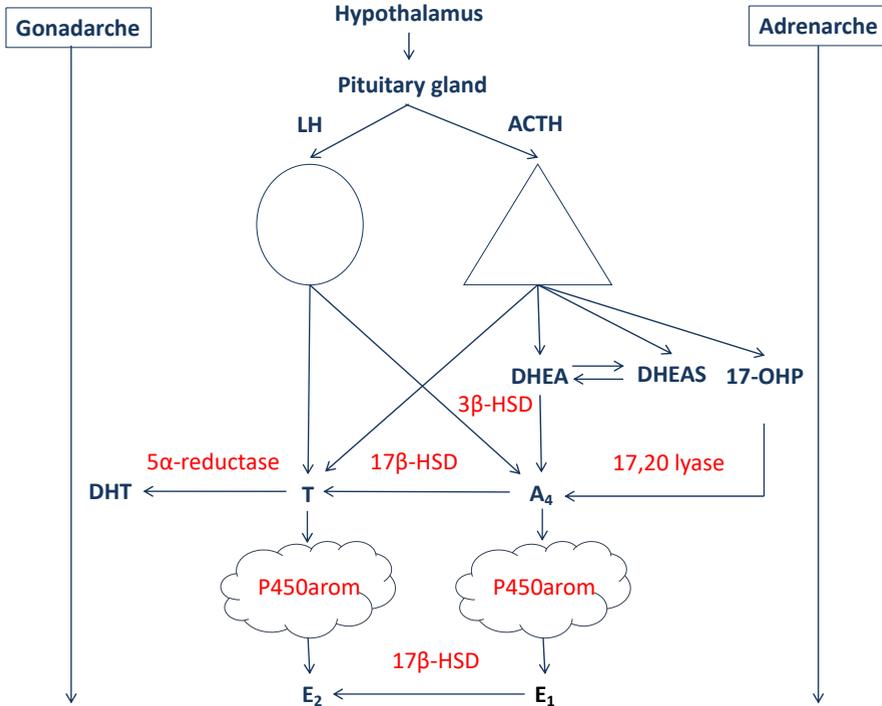


Figure 1: Synthesis of sex steroid hormones during adrenarche and gonadarche. Enzymes are marked in red capitals. Dehydroepiandrosterone (DHEA) is converted to dehydroepiandrosterone sulfate (DHEAS) by sulfotransferase, and DHEAS is converted to DHEA by sulfatase (not shown in the figure). Androgens may be derived either from adrenarche through activation of the hypothalamic-pituitary-adrenal axis, giving rise to adrenal-derived androgens, or from gonadarche through activation of the hypothalamic-pituitary-gonadal axis, giving rise to gonadal-derived androgens. Adrenal and gonadal androgens may further be converted to the biologically potent dihydrotestosterone (DHT) or to estrogens, consisting of estrone ( $E_1$ ) and estradiol ( $E_2$ ). ( $A_4$  = androstenedione; ACTH = adrenocorticotropic hormone; P450arom = Cytochrome P450 aromatase; HSD = hydroxysteroid dehydrogenase; LH = luteinizing hormone; 17-OHP = 17-hydroxy-progesterone; T = testosterone.)

### ***The fetal adrenal gland***

The fetal adrenal gland consists of the *definitive zone* (also called the neocortex) producing aldosterone, which resembles the adult zona glomerulosa, the *transitional zone* producing cortisol, which resembles the adult zona fasciculata responsible for glucocorticoid production, and the *fetal zone* producing androgens, which corresponds to the adult zona reticularis (1, 10). The fetal adrenal gland produces large amounts of DHEAS. At term, the amount of DHEAS secretion exceeds the normal amount of adult secretion, which is reflected by the fact that the relative size of the fetal adrenal gland is 10 to 20 times that of the adult adrenal gland (11). The early stages of adrenal development seem to be independent of ACTH secretion (10). However, ACTH is important for the rapid growth of the adrenal gland during the second and third trimester (10). The fetal zone constitutes 85% of the fetal gland after the first trimester and produces DHEAS, which serves as a precursor for placental estrogen conversion (4, 10). The faster growth of the adrenal glands is seen during the last 6 weeks of gestation (10). Placental corticotropin-releasing hormone (CRH) contributes to fetal adrenal steroidogenesis of both cortisol and DHEAS during the last weeks of gestation (10). The placentas of mothers who suffered from preeclampsia have been shown to have four times higher levels of CRH compared to placentas of healthy mothers (10). It has been suggested that maternal stress leads to a greater fetal exposure to stress hormones, such as cortisol, and thereby increases the risk of intrauterine growth restriction (IUGR) and premature birth as well as an altered set-point of the fetal HPA axis (12).

Due to the loss of placenta-derived factors after birth, the fetal zone undergoes a rapid involution directly after delivery, Figure 2 (next page) (10). Within 6 months of birth, the total size of the fetal gland has decreased by almost 50% due to involution and disappearance of the fetal zone (4). Remaining cell foci from the fetal zone are thought to give rise to the zona reticularis, responsible for androgen secretion from 4 to 5 years of age, resulting in adrenarche (3).

Prematurity is found to be associated with global adrenal deficiency at birth, and lower cord blood concentrations of DHEAS and A<sub>4</sub> are reported in preterm compared to term neonates (13, 14). The expected drop in adrenal androgens seen in term neonates after 3 days after birth is lacking in neonates of gestational age below 33 weeks, who instead present with increased adrenal androgen concentrations (13).

The consequences of disturbed patterns of fetal adrenal androgen secretion and exposure due to preterm birth are not fully understood, but fetal programming has been suggested as a mechanism that potentially affects the set-point of the

HPA axis permanently in response to events early in life (15). Long-term effects of the HPA axis is also suspected to explain the higher adrenal androgens found in young adults born preterm (16). DHEAS levels at 1 year of age predict DHEAS levels at 6 years of age, suggesting that the set-point of the HPA axis is established during infancy (17).

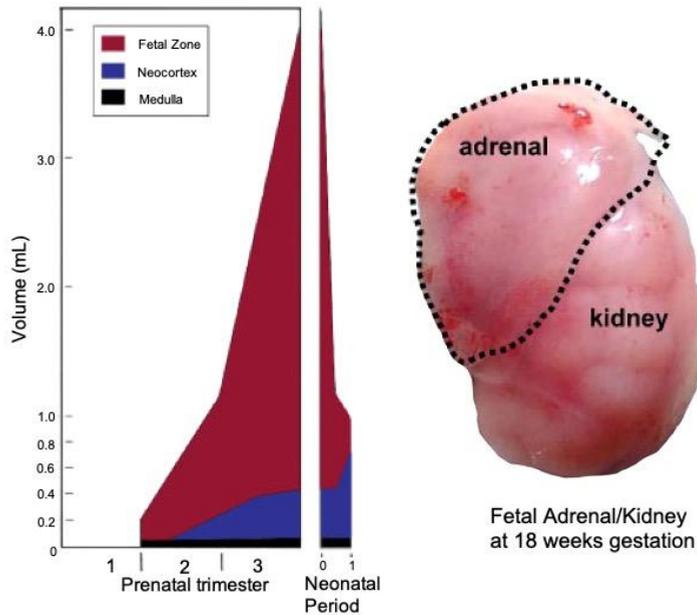


Figure 2: The relative size of the different zones of the fetal gland (left) and the total size of the fetal adrenal gland compared to the fetal kidney at 18 weeks of gestation (right). From Rainey et al. (10), reprinted with permission.

### **Adrenarche and pubarche**

Adrenal androgens are secreted in small amounts during infancy, and DHEAS concentrations increase gradually from 5 years of age and become more evident from 7 to 8 years of age, culminating at late puberty (1, 7). Adrenarche refers to morphological and functional changes of the adrenal cortex leading to increasing production of adrenal androgens, typically around the age of 5 to 8 years (18). DHEAS normally increases 10- to 14-fold over a period of two to three years, starting 12 to 18 months before pubertal onset (4). Even though the definition of adrenarche is based on clinical features of androgen action, DHEAS concentrations above 1.36  $\mu\text{mol/L}$  have been suggested to be compatible with adrenarche (19). However, the clinical signs of adrenarche cannot always be separated from signs of pubertal onset because pubertal onset often occurs simultaneously or soon after adrenarche (18).

Pubarche refers to the appearance of pubic and/or axillary hair due to androgen stimulation of the hair follicles (18, 20, 21). Pubarche may be manifest during adrenarche or gonadarche and is not only dependent on androgen hormone levels but also reflects local enzymatic activity and sensitivity of the androgen receptor (AR) (18, 20, 22). Pubarche is found to take place at an earlier age in overweight and obese boys (23).

Premature adrenarche is defined as the appearance of clinical signs of androgen action such as pubarche before 9 years of age in boys (24). It is a risk factor for the development of metabolic disturbances such as type 2 diabetes and cardiovascular disease in adult life (18, 24). Exaggerated, exacerbated, pronounced, and amplified adrenarche are different terms used by authors to describe the intensity of adrenal DHEAS secretion (18). However, some of these terms are used synonymously with premature adrenarche to refer to the timing rather than the magnitude of adrenarche, and the clinical usefulness of these terms has been questioned. There is a lack of consensus on how to refer to the magnitude of adrenal DHEAS secretion, and the suggested normal upper cut-off levels for prepubertal boys have ranged from 3.0  $\mu\text{mol/L}$  to 6.0  $\mu\text{mol/L}$  (18).

In children with normal birth size, a continuous relationship between DHEAS concentrations and birth weight has been reported (25), and premature adrenarche is associated with small birth size (18). Early postnatal growth is also an important predictor for adrenal function in adolescence, and children born small for gestational age (SGA) with catch-up growth have higher DHEAS levels in adolescence compared to children with normal birth weight (26).

In children, IGF-I concentrations increase naturally with age (27). Children with premature adrenarche have elevated IGF-I concentrations but it is not known if this represents effects of androgen action, growth hormone (GH) effects, or association with the often present overweight and hyperinsulinism in these children (18). At the same time, GH treatment resulting in increased IGF-I concentrations has been proposed to affect the timing and intensity of adrenarche (19, 28).

The role of adrenarche is not fully understood. Increasing DHEAS concentrations have been postulated to trigger pubertal onset (25), illustrated by the early activation of the hypothalamic-pituitary-gonadal (HPG) axis seen in children with inadequately controlled adrenal hyperplasia, compared to children with well-controlled treatment (4).

### ***Adrenal androgens in adult males***

In adult men, DHEAS and DHEA are the most abundant steroid hormones, accounting for 50% of the total androgens (29). Conversion of adrenal A<sub>4</sub> to testosterone accounts for less than 5% of the total testosterone production (3).

In adult men, DHEA affects the immune system, muscle strength, bone density, body fat, and age-related skin atrophy, as well as well-being (30). DHEAS has also been linked to cognitive performance and mental health (31, 32). Furthermore, DHEAS is thought to contribute to regulation of fertility and reproduction through effects on spermatogenesis and modulation of the blood-testis barrier (8).

### **1.1.2 THE GONADAL GLAND**

The two main functions of the adult testis are hormone production and spermatogenesis. Both processes depend on successful activation of the HPG axis as well as functional testicular tissue (22, 33-35). The male gonad consists of three types of cells. *Leydig cells* produce testosterone and, to some extent, A<sub>4</sub> as well as insulin-like peptide 3 (INSL3) responsible for the development of masculine characteristics (35). *Sertoli cells* secrete anti-Müllerian hormone (AMH) and inhibin B (33). *Germ cells* give rise to mature sperm cells through the process of spermatogenesis. In this process, the immature spermatogonium develops to spermatocytes, spermatids, and eventually sperm cells (36-38). Testosterone is peripherally converted to the more potent androgen DHT by 5 $\alpha$ -reductase (39), but in the adult male, 30% is secreted directly by the adrenal or gonadal glands (22). DHT may also be synthesized through a backdoor pathway involving 17-hydroxyprogesterone, androsterone and androstanediol (22). Spermatogenic cell development is totally dependent on somatic cells, and the number of Sertoli cells defines the capacity of sperm production (38).

### ***Gonadarche***

Gonadarche reflects activation of the HPG axis, leading to enlarged testicular volume (40). The HPG axis shows three periods of activation: first, during fetal life, second at mini-puberty during infancy, and last, at pubertal onset, when spermatogenesis is established (41), Figure 3.

### ***HPG activation during fetal life***

The luteinizing hormone (LH) receptor is not active until around 10 weeks of gestation, but Leydig cells synthesize testosterone from around 8 weeks of gestation (42). Initially, testosterone is secreted in response to placental human

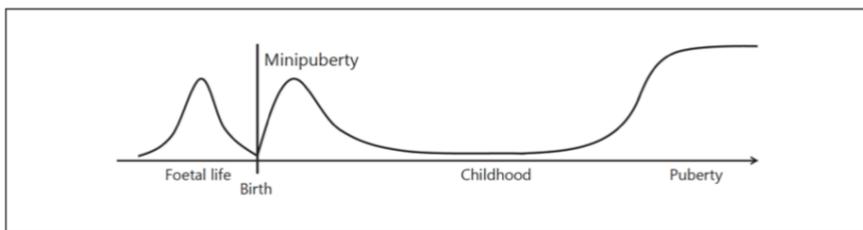


Figure 3: The three periods of hypothalamic-pituitary-gonadal (HPG) axis activation during fetal life, mini-puberty, and puberty. From Kuiri-Hanninen et al. (41), reprinted with permission.

chorionic gonadotropin (hCG) during the masculinization programming window between the 10<sup>th</sup> and 20<sup>th</sup> week of gestation (40, 43). At about 16 weeks of gestation, testosterone secretion reaches adult levels in response to the fetal pituitary secretion of LH, followed by decreasing testosterone levels during the third trimester, most likely due to negative feedback by estrogens from the placenta (22, 40), Figure 4, (next page).

DHT is synthesized locally from fetal testosterone by 5 $\alpha$ -reductase, resulting in differentiation of the urogenital sinus and the male phenotypic external genitalia (22). DHT may also be synthesized from placental androsterone (44). The congenital malformation in which there is an abnormal opening of the urethra on the ventral side of the penis is referred to as hypospadias (44). The incidence of hypospadias and cryptorchidism is higher in boys born SGA, and placental insufficiency in early gestation has been proposed as one possible mechanism affecting both fetal growth and androgen exposure of the fetus (45-49). There is also evidence that prematurity is a risk factor associated with hypospadias and cryptorchidism (46-48). As the fetal Leydig cell matures, it will start the secretion of INSL3, which is important for testicular descent (50). During early fetal development, testosterone and E<sub>2</sub> influence not only various aspects of sexual differentiation but also the maturation of other organs including the lung and kidney, and the prenatal effects of androgens exert a lifelong impact on the expression of genes in the liver thereby affecting the risk of developing the metabolic syndrome and cardiovascular disease in later life (43).

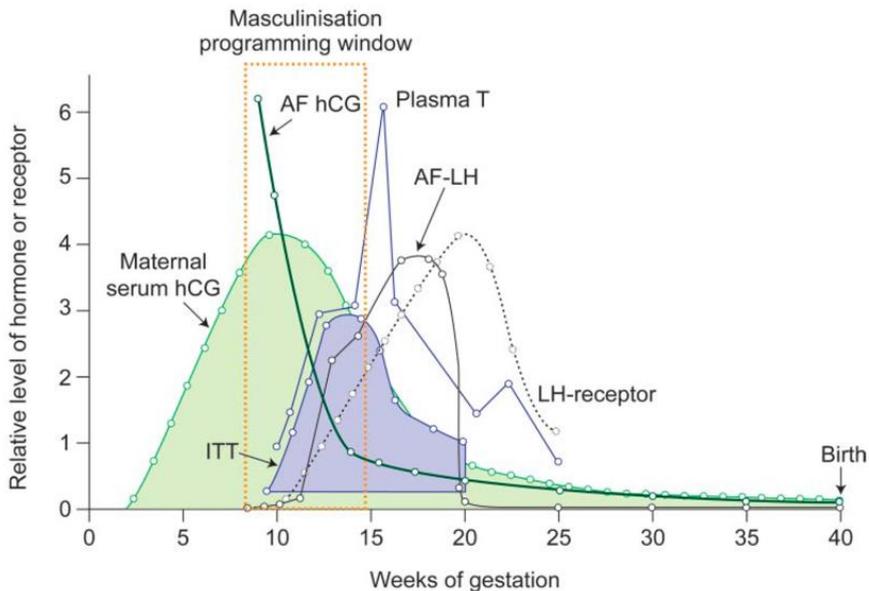


Figure 4: Onset of testosterone production by the fetal testis in relation to human chorionic gonadotropin (hCG) secretion, luteinizing hormone (LH) secretion, LH receptor appearance in the testis, and the time window in which masculinization of the reproductive tract by androgen stimulation occurs. Both intratesticular testosterone (ITT) and plasma testosterone levels are illustrated. Levels of hCG are shown in both maternal serum and amniotic fluid (AF). From Scott et al. (42), reprinted with permission.

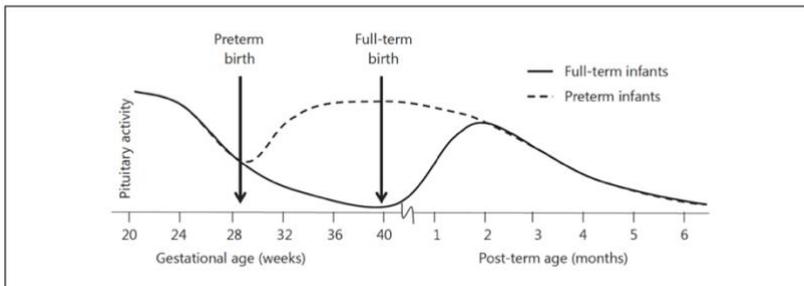
### **HPG activation during mini-puberty**

Gonadotropin levels are low at birth but the negative feedback on the HPG axis will diminish, leading to increasing gonadotropin levels when placental hormones are no longer available after birth (40, 41). During infancy, LH triggers Leydig cells to secrete testosterone, and follicle-stimulating hormone (FSH) stimulates Sertoli cells (33). This phase plays an uncertain role and lasts from the first 30–100 days of life, referred to as the postnatal pituitary-testicular activation period, also known as mini-puberty (36, 50). The mechanism of the following downregulation of circulating LH and FSH during childhood is not fully understood, but other factors than negative feedback by testicular hormones seem to be involved, because downregulation also occurs in anorchid boys (40).

Androgens have the effect of both inducing spermatogenesis and repressing AMH. Even if ARs are present in Leydig cells of the fetal and neonatal testis, Sertoli cells do not express ARs and cannot contribute to germ cell maturation

and AMH repression, despite significant testicular testosterone biosynthesis (51). The total number of Sertoli cells generated during the postnatal period in response to FSH stimulation will, however, directly affect sperm production in adult life, since each Sertoli cell will be able to support a fixed number of germ cells (52). Hormonal stimulation during the first months of life results in phallic growth as well as increased testicular size, reflecting the increasing number of germ cells, Leydig cells, and Sertoli cells (36, 41). From the 6<sup>th</sup> month of age, the HPG axis enters a quiescent period until puberty starts (52).

Premature birth does not seem to affect the timing of the postnatal increase in gonadotropin secretion. However, both the magnitude and the duration of the secretion is affected, Figure 5.



*Figure 5: The effect of preterm birth on the activation of the hypothalamic-pituitary-gonadal (HPG) axis. From Kuiri-Hanninen et al. (41), reprinted with permission.*

The disrupted gonadotropin secretion pattern is reflected by both higher postnatal testosterone levels as well as faster penile and testicular growth after birth in preterm boys compared to full-term boys (53). In boys born SGA, increased postnatal HPG activity with higher FSH as well as testosterone has been reported compared to infant boys with normal birth weight (41).

### **HPG activation during puberty**

Puberty refers to the transition from the immature child to the mature adult capable of reproduction, triggered by activation of the HPG axis leading to gonadarche (40). Puberty normally starts between 9 and 14 years of age, with a mean age of 11.5 years in boys (40). Pubertal development may be assessed by analysis of hormonal changes, testicular volume, and/or the development of secondary sex characteristics such as pubic hair, and the development of the external genitalia according to the Tanner scale (54).

Activation of the HPG axis during puberty is initiated by kisspeptin neurons, localized in the hypothalamus, reducing the inhibition of the gonadotropin-releasing hormone /gonadotropin release, although the mechanisms of this process are not fully understood (55). Obesity is associated with early onset of menarche in girls, but is less of a determinant for age at pubertal onset in boys (43). As already discussed, DHEAS may play a role in activation of the HPG axis. On the other hand, since children with adrenal insufficiency can undergo gonadarche in the absence of adrenarche, adrenal androgens seem not to be necessary for activation of the HPG axis in humans (4).

Clinically, pubertal onset is traditionally defined as testicular volume of more than 3 mL (56). However, biochemically, FSH levels increase up to early puberty without a distinct diurnal rhythmicity, whereas LH levels are low before puberty and develop a marked diurnal rhythm with high nighttime levels even with a testicular volume of only 3 mL (57, 58). These findings are reflected by data showing a significant increase of testosterone when testicular volume increases from 2 to 3 mL but not from 3 to 4 mL (59). Moreover, a study of 100 patients showed that, after reaching testicular volume of 3 mL, further testicular growth and/or pubarche occurs within 6 months in 82% of patients (60). Testicular growth is rapid during puberty, with approximately half of the growth occurring between 12.7 and 14.1 years of age (61).

As already discussed, FSH stimulates Sertoli cells and LH stimulates testosterone secretion from Leydig cells. Furthermore, gonadotropins stimulate the gonads to produce gametes (50). During puberty, testicular testosterone synthesis is required to trigger spermatogenesis and repress AMH production by stimulating AR expression in the Sertoli cells at the onset of puberty (52, 62). Although intratesticular testosterone is needed for successful spermatogenesis, germ cells do not express receptors for either FSH or androgens, suggesting that the effect on spermatogenesis is mediated through mature Sertoli cells via production of other hormones acting in a paracrine, autocrine, or endocrine fashion (33).

### ***Testicular secretion of AMH and inhibin B***

In early fetal life, AMH secretion by Sertoli cells induces the regression of the Müllerian ducts, which inhibits the development of the uterus, fallopian tubes, and upper part of the vagina (40). The onset of AMH secretion is not dependent on FSH stimulation but at later stages of gestation, FSH stimulate Sertoli cell proliferation and upregulation of AMH secretion (33, 40, 63). Testosterone exerts an inhibitory effect on AMH secretion by Sertoli cells but requires functional ARs (33, 63). After birth, AMH peaks at 3 months of life, reflecting the number of functional Sertoli cells present (63, 64). Prepubertal boys with cryptorchidism have actually lower AMH levels compared to normal reference

intervals, possibly reflecting fewer Sertoli cells in these boys (65). After mini-puberty, AMH concentrations rise, reflecting declining FSH levels (64). When the expression of ARs in Sertoli cells increases, AMH secretion is downregulated and AMH reflects the balance of the stimulating effect of FSH and the inhibiting effect of testosterone (33). Sertoli cell maturation is induced by increasing intratesticular testosterone, leading to decreasing serum AMH and increasing inhibin B before serum testosterone increases, Figure 6 (40, 64). Indirectly, AMH concentrations are dependent on the stimulating effect of LH on Leydig cells resulting in testosterone secretion inhibiting AMH secretion (66). From puberty, the function of Sertoli cells is mainly to support spermatogenesis (64).

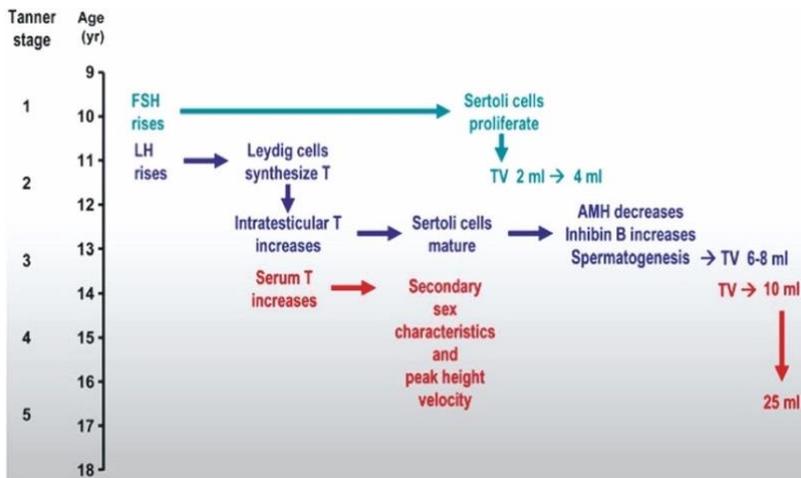


Figure 6: Sex hormone secretion and hormonal effects during Tanner stage 1 to 5 in boys (AMH = anti-Müllerian hormone; FSH = follicle-stimulating hormone; LH = luteinizing hormone; T = testosterone; TV = testicular volume; yr = years). From *Puberty -Physiology and Abnormalities*, Springer Nature (67), reprinted with permission.

During fetal life, Sertoli cells produce inhibin B in response to FSH stimulation but, at the same time, inhibin B suppresses FSH by negative feedback (40). During the first months of life, inhibin B increases and is a useful marker of FSH-dependent Sertoli cell proliferation, reflecting mini-puberty (68). Inhibin B decreases until 2 years of age but is continuously produced by the immature testis and reflects the number of functional Sertoli cells but not germ cells (68, 69). During childhood, inhibin B increases until onset of puberty, with a further increase from Tanner genital stages G1 to G3, reflecting the testosterone-driven maturation of Sertoli cells (40, 68, 70). At pubertal stage G2 there is a

positive relationship between inhibin B and testosterone, but little relationship with FSH. However, from pubertal stage G3 there is a progressively stronger inverse relationship between inhibin B and FSH, reflecting the establishment of a negative feedback loop, and a slight decrease in inhibin B is seen (68, 70). In adult men, inhibin B mirrors the effect of androgens and FSH on spermatogenesis (71).

### ***Testicular volume***

Testicular volume is measured by a Prader orchidometer in the clinical setting and correlates closely with measurements with ultrasonography, although the orchidometer overestimates the testicular volume, especially in small testes (72).

As already mentioned, testicular tissue consists of Leydig cells, Sertoli cells, and germ cells, and when measured with ultrasonography, testicular volume increases slightly from infancy to pubertal onset, due to increase in the Sertoli cell population, Figure 7 (63). During puberty, the number of germ cells increases and, at adult age, germ cells represent most of the gonadal size.

Interestingly, mice over-expressing AR in Sertoli cells are found to have 50% smaller testes with a 70% smaller number of Sertoli cells, which suggests that excessive and premature androgen action may be harmful for testicular development (61). Furthermore, boys born SGA have been found to have smaller adult testicular size, together with lower concentrations of testosterone and higher concentrations of LH (73).

## **1.1.3 SEX STEROID HORMONE-BINDING PROTEINS**

Binding proteins are important for the transport, distribution, metabolism, and biological activity of sex steroid hormones (74). However, the mechanisms of how sex steroid hormone-binding proteins affect sex steroid hormone metabolism are not fully understood (75). The major sex steroid hormone-binding proteins are sex hormone-binding globulin (SHBG) and albumin, which possess varying affinities for different sex steroid hormones, affecting the free circulating proportion of each hormone (74, 75). SHBG is used for calculation of the free androgen index for testosterone and DHT, although the clinical implications in men as opposed to women is questioned, and the free androgen index is mostly used in clinical studies (75, 76).

SHBG is synthesized in the liver, and SHBG levels are influenced both by androgens exerting decreasing effects and by estrogens exerting increasing effects, as well as thyroid hormones and other metabolic factors (76). The affinity for SHBG (in decreasing order) is DHT, testosterone, E<sub>2</sub>, and estrone

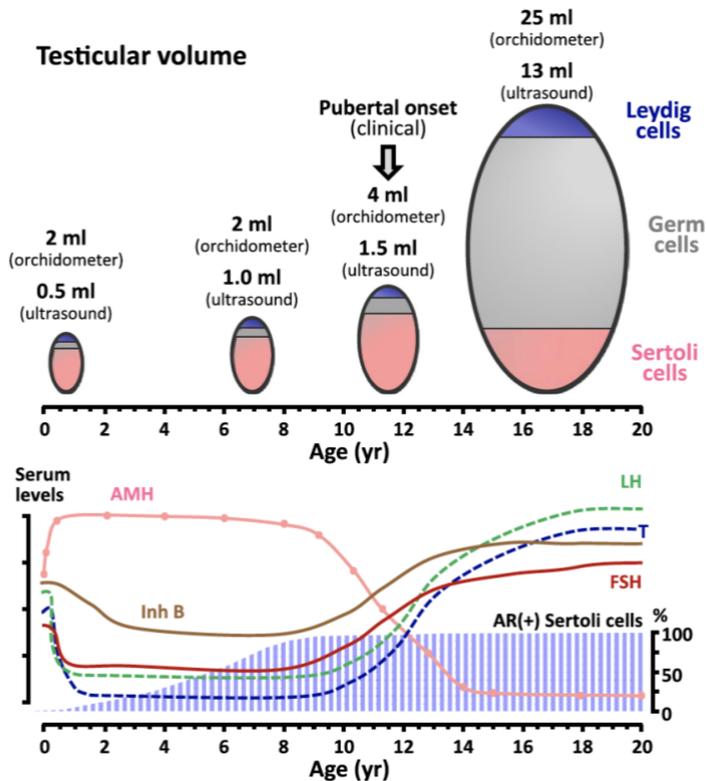


Figure 7: Testicular development from birth to adulthood (yr = years), showing the morphological change of the testicular tissue, along with serum concentrations of anti-Müllerian hormone (AMH), inhibin B (inh B), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (T), as well as the rate of androgen receptor (AR) expression in Sertoli cells. From Edelsztein et al. (63), reprinted with kind permission from Professor Rodolfo Rey.

(E<sub>1</sub>) (76). SHBG actively binds both androgens and estrogens and is synthesized during fetal life, reaching relatively high levels until the onset of puberty, when SHBG levels drop (43). Body composition affects SHBG levels, with a larger amount of adipose tissue in relation to lean muscle leading to lower SHBG (77). Low levels of SHBG are also associated with insulin resistance and the development of type 2 diabetes (78).

### 1.1.4 MECHANISMS AFFECTING SEX STEROID HORMONE CONCENTRATIONS

Multiple organs and enzymes are involved in the synthesis of androgens, Figure 1 (page 2). The amount and profile of sex steroid hormones synthesized by the gonadal and adrenal glands depend on the activity in the HPA and HPG axis, as already discussed. During adrenarche, the adrenal gland is the major provider of androgens (3), and during puberty, the gonads are the dominant source of testosterone. In the adult male, less than 5% of testosterone originates from A<sub>4</sub> (3) and approximately 95% of plasma testosterone is synthesized in the testis, with the remainder originating from the adrenal gland (22). The serum DHT and A<sub>4</sub> concentrations are approximately 10% and 20% of the testosterone concentration, respectively, and 70% of circulating DHT is derived from conversion of testosterone by nongonadal tissues (22).

Another mechanism affecting sex steroid hormone concentrations is the individual half-life for each hormone, which is affected by both the rate of degradation and the rate of peripheral conversion, exemplified by DHEAS having a much longer half-life compared to A<sub>4</sub> and testosterone (1). Adipose tissue possesses endocrine properties through the actions of enzymes such as aromatase, 3 $\beta$ -hydroxysteroid dehydrogenase (HSD), 17 $\beta$ -HSD, and 5 $\alpha$ -reductase (39), and hence, the amount of body fat may influence the profile of sex steroid hormone concentrations. However, the aromatization capacity affecting estrogen synthesis varies with pubertal development and is low at the beginning of puberty (79).

Barriers can also regulate to what extent different tissues are exposed to sex steroids. The blood testis-barrier is an example of a barrier affecting sex steroid concentrations in local tissues (52, 61).

Local regulation of enzymes in different tissues affects the local synthesis of sex steroid hormones, as illustrated by 5- $\alpha$  reductase activity in the skin locally affecting the amount of DHT (22) as well as estrogens produced locally in the growth plate (80).

In the presence of two steroid hormones, the hormone with highest affinity for the enzyme will be metabolized first, which will affect the rate of conversion. An example of this regulatory mechanism is the hormones A<sub>4</sub> and testosterone, which are both metabolized by aromatase and 5 $\alpha$ -reductase, but A<sub>4</sub> possesses a higher affinity and will therefore be metabolized before testosterone, affecting the concentrations of both A<sub>4</sub> and testosterone (81). In conclusion, the regulation of different enzymes is complex and plays a crucial role in sex hormone concentration patterns.

### 1.1.5 ASPECTS OF THE ANDROGEN RECEPTOR

Androgens act through stimulation of the AR and affect several tissues such as skin, hair follicles, secondary sexual organs, bone, muscle, brain, heart, and liver (21, 80, 82, 83). Androgens stimulate the nuclear AR with varying potencies (83). The relative potencies of DHT, testosterone A<sub>4</sub>, and DHEA on the AR have been reported to be 30, 10, 1, and 0.5, respectively (22). In the absence of a functional AR, the male fetus will not be able to develop secondary male characteristics (84). Regulation of AR expression in different cells controls the androgen effects during mini-puberty, as already mentioned.

ARs are found both in the growth plate and osteoblasts, demonstrating an independent pathway for androgen impact on growth and bone health (80). Increased AR activity due to reduced AR gene methylation has been reported to result in premature pubarche, as already mentioned (85). Regulation of AR expression and degree of AR sensitivity seem to play a crucial role for the manifestations of androgen exposure (85, 86).

### 1.1.6 ESTROGENS IN MALES AND ASPECTS OF THE ESTROGEN RECEPTOR

The term estrogen is derived from the two Greek words: *oistros*, meaning “heat, frenzy” (used in the term “estrus” to mean “a periodic state of sexual activity in female mammals”), and *genēs*, meaning “generated”. Historically, estrogens have been regarded as female hormones; however, estrogens also play an important role in male physiology.

In males, estrogens are converted from A<sub>4</sub> and testosterone through the action of the aromatase encoded by the *CYP19A1* gene (39, 87), Figure 1 (page 2). Aromatase is widely expressed in human tissues supplying locally produced estrogens in the central nervous system, adipose and bone tissues, as well as in the placenta, gonads, and hypothalamus. This starts during fetal life, when the adrenal glands are the main source of androgen precursors (50, 88). Before pubertal onset, E<sub>1</sub>, derived from precursors originating from the adrenal gland, is the dominant estrogen (79). However, in adult males, only about 5% of E<sub>1</sub> is produced by the gonads and 95% is derived from circulating androgens (89). Estrogens act through the nuclear receptors estrogen receptor (ER) $\alpha$  and ER $\beta$ , which are also expressed in various tissues, including the bone, brain, liver, adipose tissue, penis, and testis, as well as the vascular, and immune systems (88, 90, 91). In some tissues, cytoplasmic and membrane ERs called G-protein-coupled receptors have also been identified (92, 93). ERs seem to be important for testicular function. ER $\beta$  is the predominant ER found during the neonatal period, with weaker expression throughout infancy and childhood

(50). ER $\alpha$  may regulate Leydig cell function, with estrogens acting as inhibitors of fetal androgens or INSL3, whereas ER $\beta$  seems to affect the determination of the final gonocyte number at birth (84). In bone, the ER $\alpha$  stimulates longitudinal bone growth, growth plate maturation, epiphyseal fusion, and bone mineralization, whereas the role of ER $\beta$  remains unclear (94). In males, E<sub>2</sub> acts in a complex balance with testosterone, aromatase, and ERs, and affects penile development, mood, sexual arousal, libido, erectile function (through effects on vascular permeability), spermatogenesis, skeletal growth, bone metabolism, and metabolic health (88, 91, 92, 95). Interestingly, vasomotor symptoms such as hot flushes in hypogonadal men results from estrogen deficiency and not androgen deficiency (93). On the other hand, E<sub>2</sub> excess in adult males induces hypogonadotropic hypogonadism due to enhanced negative feedback on the pituitary gland (93).

## 1.2 LINEAR GROWTH

Linear growth is a complex process influenced by genetic potential, nutrition, general health status, and various hormones, including sex steroids. Growth charts are important tools for pediatricians when evaluating a child's health, well-being, and development.

### 1.2.1 THE ICP MODEL

The infancy-childhood-puberty (ICP) growth model improved the understanding of the different phases of linear growth and was introduced by Karlberg in the 1980s (96). The model describes three phases reflecting different hormone systems promoting growth, Figure 8.

However, growth is not only dependent on functioning hormonal axes. The genetic component of stature is significant and complex (97). Several other factors affecting the growth plate have also been identified, including paracrine factors, extracellular matrix molecules, and multiple intracellular processes affecting chondrocyte function (97).

#### ***Fetal growth***

Intrauterine growth is rapid, with fetal length velocity at mid-gestation approximately 10 times that of pubertal peak height velocity, and at term, almost 30% of adult height has been reached (98). The pattern of intrauterine weight gain resembles the pattern of length growth, except that the peak weight gain is at approximately 34 weeks of gestation (99).

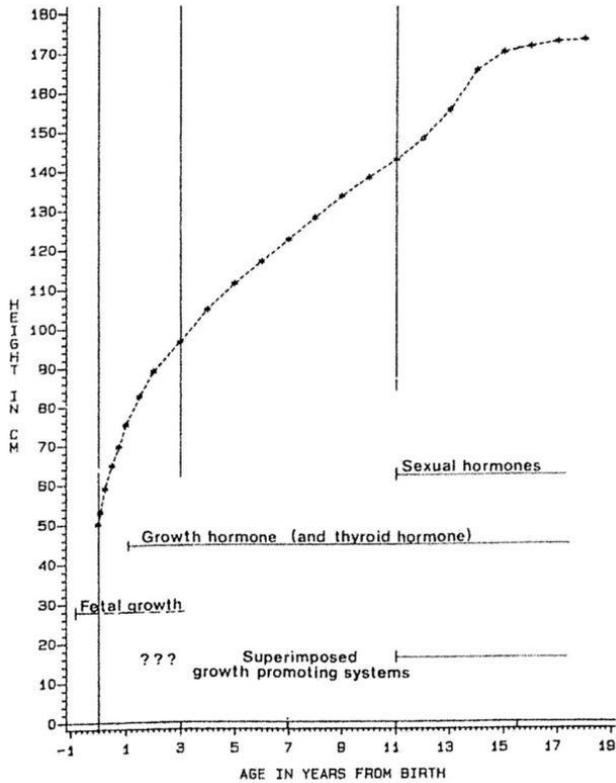


Figure 8: The ICP model, represented by fetal, childhood, and pubertal growth phases promoted by different hormones. From Karlberg (96), reprinted with permission.

The capacity of the mother and placenta to deliver oxygen and nutrients to the fetus is crucial for optimal fetal growth (100). Although no key hormone controlling fetal growth has been identified, the placenta is an important endocrine organ producing GH, estrogens, and lactogen, which all promote fetal growth (100). Placental GH stimulates maternal IGF-I and estrogens, facilitating uterine growth and placental blood flow, inducing maternal insulin resistance, and securing nutrient delivery to the fetus (101). In addition, placental GH increases the uptake of low density lipoprotein (LDL) cholesterol which serves as a precursor for placental steroid production (101). Lactogen is thought to promote early fetal growth and influence fetal secretion of IGF-I and insulin (100, 102). Insulin, IGF-I, and late-gestational secretion of cortisol stimulate fetal growth and fat deposition as well as liver glycogen storage (100, 101).

### ***Infancy growth***

The infancy growth phase starts at birth, when the supply of placental and maternal growth factors through the umbilical cord is discontinued. Growth during the first year of life is in many aspects an extension of the intrauterine growth period from mid-gestation (96, 103). Nutrition is an important regulator of growth during this period, and hormonal regulation is controlled by thyroxine and IGFs (103). From birth, height velocity is fast, followed by rapid deceleration up to about 3 years of age (96). During this period an adaption to the child's genetic height potential is achieved (104).

### ***Childhood growth***

The childhood growth period starts during the second half of the first postnatal year (96). During this period of growth, height velocity declines slowly and, in the presence of normal thyroid function, the main regulator of height growth is GH (96). The pituitary gland secretes GH, which stimulates the growth plate both directly and indirectly through IGF-I secretion by the liver (97). At approximately 7 to 8 years of age, adrenal androgen secretion increases, resulting in a transient increase in height velocity (96).

### ***Pubertal growth***

Pubertal growth is responsible for approximately 20% of adult stature (105). Boys reach peak height velocity at Tanner stage G4, which is later in puberty compared to girls (105). Both the total pubertal growth spurt and the peak height velocity vary negatively with the child's age at pubertal onset (105). During the pubertal growth spurt, sex steroid hormones stimulate linear growth. Both ERs and ARs are found in the growth plate, also enabling the non-aromatizable androgen DHT to facilitate skeletal growth through direct effects (105). Aromatizable androgens have both direct effects on the growth plate and indirect effects through conversion to E<sub>2</sub> affecting the ER, inducing increased activity in the GH-IGF-I axis as well as increased GH sensitivity (105, 106). During puberty, GH secretion increases up to threefold, and IGF-I levels increase even more (105). Despite increasing sex steroid hormone secretion into adulthood, GH secretion declines at the end of puberty (28).

## **1.2.2 BONE AGE**

The process of growth plate fusion is complex and probably involves different growth factors, regulators, and mechanisms, such as hypoxia causing cell death (94). Although the exact mechanisms of epiphyseal fusion are not fully understood, estrogens are known to be important for growth plate maturation and epiphyseal fusion (95).

Bone age, in contrast to chronological age, reflects biological maturation. Chronological age is defined as the time that has passed since the date of birth. However, when assessing a child's height, assessment of bone age may supply information on whether the child is short due to a delay in bone maturation, indicating younger biological age. Several factors such as GH, thyroid hormones, glucocorticoids, body mass index (BMI), chronic illness, and social factors influence bone age (107), and sex steroid hormones constitute an important factor affecting bone age (94).

There are several methods available to assess bone age, such as radiographs of the non-dominant hand, ultrasonography, and magnetic resonance imaging (MRI) (107). In Sweden, the most common methods for clinical use are radiographs of the non-dominant hand, evaluated according to Tanner and Whitehouse (TW) and Greulich and Pyle (108). The assessment of bone age was previously evaluated subjectively, resulting in both intra- and inter observer variability. In recent years BoneXpert has been introduced as an automated method to assess bone age (107).

## **1.2.3 ADULT HEIGHT**

Adult height reflects the potency and added effect of the fetal, childhood, and pubertal growth phases. Nutrition, diseases, genetics, and socioeconomic conditions are all factors found to affect adult height in population-based studies (109). Improvement in living standards is thought to be an important factor contributing to secular changes in adult height (109). Genetics also plays an important role and explains a large component of the normal variation in adult height (97). Age at pubertal onset shows little correlation with adult height, but height at onset of peak pubertal growth is of great importance (110).

## 1.3 BODY COMPOSITION

Anthropometric parameters reflect other aspects of growth than linear growth. Adipose tissue is important for sex steroid hormone transformation and actions, as well as for regulation of the balance between androgens and estrogens through aromatase activity (39). In adult males, obesity is associated to hypogonadism, however, the cause and effect between obesity and hypogonadism are not established and androgen replacement therapy has not been found to reduce the risk of cardiovascular disease (111). Obese children and adolescents are at risk of developing metabolic complications as adults, and visceral fat accumulation is a strong predictive factor for this (112). BMI, waist circumference, and waist-to-height ratio (WHtR) are different methods for the assessment of body composition.

### 1.3.1 BMI

BMI is calculated as weight divided by squared height ( $\text{kg}/\text{m}^2$ ) (113). Even though BMI predicts fat mass with high sensitivity in all age groups, the specificity decreases from childhood to adulthood (114), and BMI does not differentiate between subcutaneous and visceral fat mass (115). BMI correlates strongly with body fat but must be interpreted with caution in athletes because BMI does not distinguish between lean body mass and fat mass (113, 116). In Sweden, BMI curves which provide gender-specific BMI standard deviation score (SDS) are used (117).

### 1.3.2 WAIST CIRCUMFERENCE

There is a lack of consensus as to the landmark for waist circumference measurement (116). In children aged 3 to 19 years, waist circumference is a useful measure of central fat distribution (116) and has been found to be associated with the amount of visceral fat mass (118). In the adult population, health-related cut-offs are used, but in children and adolescents, cut-off limits are based on statistical measures, and different curves for waist circumference are available (114, 119).

### 1.3.3 WAIST-TO-HEIGHT RATIO

WHtR is calculated as waist circumference in cm divided by height in cm (116). WHtR is suggested to be a more precise measure of central fat mass than waist circumference alone, because the magnitude of waist circumference is influenced by height (115). A cut-off of 0.5 to predict increased health risks has been suggested for both children and adults (115). However, WHtR normally does not fall below 0.5 until 5 years of age (120), and WHtR decreases naturally from 5 to 17 years of age (115).

## 1.4 EPIGENETICS

The term *epigenetics* was introduced in the 1950s and describes how genes interact with the environment through modifications of gene expression to produce the individual phenotype (121). Epigenetic mechanisms explain how identical genes are expressed differentially in space and time in different cell types in the same individual (122). DNA is packaged with histones in complexes to form nucleosomes, which build complexes with histones known as chromatin; chromatin is further condensed to form chromosomes (123). Every cell in the body contains the same DNA but, depending on the particular cell-type, different genes are expressed (123).

### 1.4.1 EPIGENETIC MECHANISMS

The most studied epigenetic mechanism in relation to early-life stress is DNA methylation, in which a methyl group binds to the DNA and thereby silences the gene, in contrast to hypomethylation, which activates the gene, transcription (121, 122). Other epigenetic mechanisms are histone modifications or non-coding RNA-associated gene silencing (123).

### 1.4.2 IMPRINTING

Imprinting is an epigenetic phenomenon resulting in monoallelic, parental-specific expression pattern in diploid cells (124). Many imprinted loci show allele-specific DNA methylation as a mechanism of imprinting (125). Normally, both the maternal and paternal copy of a gene have the same potential for activation in any cell but an imprinted gene will express only one parental copy and silence the other parental copy (124). Some genes show tissue-specific imprinting being expressed from both parental genomes in some tissues and exclusively from one parent in other tissues (126). Although imprinted genes account for only 0.5% of the genome, the effects on early fetoplacental development are significant, affecting fetal growth and morphology (15, 125). Disorders of imprinting may be caused by (126):

- a) Deletion or point mutation in imprinted genes.
- b) Two copies of one gene coming from one parent and none from the other (uniparental disomy) as in maternal uniparental disomy of chromosome 7 (upd(7)mat) found in some patients with Silver–Russell syndrome (SRS) (127).
- c) Loss of function in the methylated imprinting control region such as hypomethylation of the H19/IGF-II intergenic differentially methylated region (IG-DMR) in 11p15 seen in some patients with SRS (127).
- d) A random error preventing normal setting of the imprint.

## 1.5 BIRTH SIZE

Birth size may refer to birth weight or length. Genetic and environmental factors, as well as the size of the mother, influence birth size (128-130).

### 1.5.1 SMALL FOR GESTATIONAL AGE

Worldwide, low birth weight (below 2500 grams) is reported in 8%–26% of infants (15). Newborns who are smaller in size than normal for their gestational age are defined as SGA. The definition of SGA has not been consistent. In the 1960s, multiple studies indicated increased mortality in infants with birth weight at or below the 10<sup>th</sup> percentile (131). Defining SGA as 2 SDS or more below the mean, corresponding to 2.3 % of newborns with lowest birth weight, was introduced in the 1960s (131). However, there is an ongoing discussion whether reference charts based on data from a population including all births or standard charts referring to growth data in a healthy population should be preferred (132). In a consensus statement published in 2007, SGA was defined as birth weight and/or birth length at least 2 SDS below the mean for the child's gestational age, and the definition was chosen to identify children in whom ongoing growth assessment was recommended (133). In Sweden, this definition is accepted but worldwide, and in many publications, different definitions of SGA are still used, complicating the interpretation of reported data on the consequences of being small at birth (131). A Swedish population-based study of 3650 healthy children reported that 3.5% were light for gestational age and 3.1% were short for gestational age whereas 1.5% were both light and short at birth (134). When data on gestational age is lacking, the term low birth weight is suggested for newborns with weight below 2500 grams.

### 1.5.2 GROWTH RETARDATION

The term SGA only refers to the infant's size at birth and does not necessarily reflect the fetal growth pattern. IUGR, on the other hand, refers to the presence of a pathophysiologic process inhibiting the fetal growth in utero that enables the fetus to achieve its genetically determined potential size (130, 135). The diagnosis of IUGR is based on slow fetal growth identified by two consecutive ultrasound measurements (133); IUGR affects approximately 8% of pregnancies (102). Impaired fetal growth may be of maternal, placental, or fetal origin, depending on causes that range from genetic to environmental factors (130). The size of the mother is a crucial factor affecting fetal growth and thereby facilitating a normal delivery, which is illustrated by the observation that pregnancies in small mothers after ovum transplantation from large women result in small babies (136). IUGR may induce epigenetic changes with lifelong consequences on growth and health issues (137). An

infant born SGA may or may not have suffered from IUGR, and infants born after a short period of IUGR are not always born SGA. Non-syndromic infants with growth restriction before term age may be classified into three groups; term children born SGA as a result of IUGR, preterm children born appropriate to age (AGA) but who suffer from extrauterine growth restriction (EUGR), and preterm infants exposed to IUGR and EUGR (98).

### **1.5.3 FETAL PROGRAMMING**

Increased death rates from cardiovascular disease in adult life in children with small size at birth and at 1 year of age, reflecting both intrauterine and extrauterine growth, were demonstrated in two large cohorts by Barker et al. (138, 139). Based on these findings, the Barker hypothesis proposed that malnutrition in utero permanently changes the body's structure, function, and metabolism in ways that lead to coronary heart disease in adult life (140). With time, malnutrition in early life has been found to also predispose to obesity, hypertension, hyperlipidemia, and type 2 diabetes in adulthood (141, 142). Boys seem to be more susceptible to placental dysfunction because they grow faster and the placenta thus has less reserve capacity, exposing male fetuses to a greater risk of becoming undernourished (143). Results reported from a cohort born after the Dutch Hunger Winger of 1944-1945 indicate that the timing of intrauterine starvation may affect the risk profile of disease in adult life (144). In the Leningrad Siege study, the cohort had been exposed to starvation in utero or during infancy in the siege of 1941-1944, and results from that cohort, together with results from the Dutch cohort, indicate that postnatal catch-up growth rather than birth size plays a crucial role in development of disease in adult life (145). In support of this hypothesis, patients from a large cohort in Helsinki suffering coronary events as adults, were found to have been small at birth and thin at 2 years of age, thereafter showing rapidly increased BMI (146). Rapid weight gain in early infancy and in childhood confers additional risk of adult morbidity, including decreased insulin sensitivity, even in individuals with normal BMI (15). Altogether, the timing of exposure to poor nutrition in early life, not necessarily reflected by small birth size, and the extent of catch-up growth, seem to be crucial for the development of disease in adulthood (144). However, most of the evidence comes from observational studies and it has been suggested that, taking confounding factors into account, the associations may be small (133).

It has been proposed that nutrition during fetal life, infancy, and early childhood changes gene expression by epigenetic mechanisms involving widespread changes in DNA methylation and thereby establishes functional capacity, metabolic competence, and responses to the later environment (137, 142). The fetus integrates information from the past and postulated future

environmental conditions and adapts through plasticity leading to structural and functional changes (137, 147). This protective mechanism will improve the survival of the fetus given exposure to malnutrition even after birth (147). Three mechanisms have been suggested to explain why the pace and pathway of early growth restriction is reflected in the risk of developing coronary heart disease, type 2 diabetes, stroke, and hypertension (142). First, key organs develop less functional capacity, exemplified by the kidneys in which a reduced number of glomeruli predispose to hypertension (137, 142). Second, the setting of hormones and metabolism, such as the GH-IGF axis, as well as the regulation of sex steroid hormones originating from the adrenal and gonadal glands are affected, especially in children with abnormal weight gain (15, 25, 137, 142). Last, responses to environmental influences in later life may be affected, illustrated by patients who were small at birth and who are found to have persisting alterations in response to stress with higher cortisol levels (142, 148). Tissues and systems are vulnerable to programming during phases of rapid cell replication and therefore different tissues are affected, dependent on which tissue undergoes these critical phases of development at the time of growth restriction (149).

#### **1.5.4 CONSEQUENCES OF BEING BORN SMALL FOR GESTATIONAL AGE**

As already discussed, being born SGA is a risk factor associated with hypospadias and cryptorchidism (45-48).

The incidence of hypospadias and cryptorchidism is higher in boys born SGA, and placental insufficiency in early gestation has been proposed as one possible mechanism affecting both fetal growth and androgen exposure of the fetus (45-48).

Catch-up growth may affect weight, height, or both, and is usually seen in the first months of life in children born SGA (15). The mechanisms of catch-up growth remain unclear, but birth weight, birth length, gestational age, and midparental height (MPH) are important influencing factors (150). The advantages of postnatal catch-up growth are improved neurodevelopment, enhanced immune function and improved adult height; however, the disadvantages seem to be an increased risk of central obesity, impaired glucose tolerance, dyslipidemia, and hypertension (150).

Of children born SGA, 85% to 90% have full catch-up growth for height by the age of 2 years (134, 151). Prematurely born SGA children have a slight decrease in catch-up rate for height at 2 years of age, but a few are still catching up after that age (151). Approximately 10% of infants born SGA remain short

during childhood and adolescence, resulting in short adult height ( $\leq -2$  SDS) (130). Being born SGA for weight carries a five times increased risk of reaching a short adult height, and the risk is seven times higher for those with short birth length (152). Moreover, about 20% of children with short stature are born SGA, and adult height SDS is not likely to increase compared to prepubertal height SDS in this group (153). Children born SGA with catch-up growth during childhood reach a mean adult height of  $-0.7$  SDS compared to normal reference intervals, but SGA children without catch-up growth reach a mean adult height of  $-1.7$  SDS on average (134).

High basal GH levels, low peak amplitude, and high peak frequency between 2 and 6 years of age are reported in children born SGA, indicating an element of GH resistance (15, 153). The European Medicines Agency has approved GH treatment for children born SGA, aged 4 years or older, with height corresponding to  $\leq -2.5$  SDS, growth velocity before treatment  $< 0$  SDS for age, and height SDS  $> 1$  SDS below MPH SDS (133). Short children born SGA are a heterogeneous group and the response to GH treatment is highly variable (137). Factors affecting GH treatment outcome include age and height SDS at start of treatment, MPH, and GH dose (133). Without a height velocity of SDS  $> 0.5$  in the first year of treatment, a decision to discontinue treatment should be considered (133). In Sweden, according to praxis, a height gain of  $> 0.6$  SDS during the first year of treatment is required to justify further GH treatment (108).

DHEAS in adolescent boys is reported to be associated with greater current weight and lower gestational age, birth weight, and birth length (25, 26, 154). In children born SGA, rapid weight gain during infancy is associated with higher DHEAS in adolescence (26), and increased fat mass rather than lean mass affects metabolic health in later life (150). During childhood, both higher levels of IGF-I, insulin, and DHEAS and relatively low levels of SHBG, as well as an increased amount of visceral fat, are reported in children born SGA (155-157). Even in the absence of overweight, children born SGA with spontaneous catch-up growth have more visceral fat by the age of 6 years, compared to AGA controls (156, 158). However, the greatest risk of long-term health consequences is seen in infants who are small at 2 years of age with a catch-up growth in BMI thereafter (159). Despite being significantly shorter and lighter compared to adolescents born AGA, adolescents born SGA are reported to have thicker subscapular skinfolds, and those born SGA without catch-up growth are also reported to have a higher WHtR compared to AGA controls at ages 11 to 14 years (26). Contradicting data have been published regarding whether SGA at birth is a predisposing factor for premature pubarche (160-163).

It has been suggested that pubertal onset in children born SGA might be triggered by rapid weight gain and visceral adiposity inducing insulin resistance (163). However, during mini-puberty, boys born SGA are reported to have higher FSH and testosterone levels compared to boys born AGA, indicating an increased HPG activity during this period (41), and although children born SGA usually reach pubertal onset within the normal range, they are more likely to have earlier pubertal onset with faster progression through puberty, less pubertal growth spurt and, on average, mean adult height approximately 1 SDS lower than children born AGA (134, 163-165). They are at risk of an impaired pubertal growth spurt, even when treated with GH (165), and bone age is not a reliable predictor of adult height potential in these children (130). Despite a normal course of pubertal development, peak pubertal height velocity is reported at an earlier pubertal stage, with shorter duration and earlier fusion of the growth plates resulting in shorter adult height outcome in short children born SGA (166).

Adult men born SGA are less likely to become fathers and are more likely to be diagnosed with infertility compared to men born AGA (167, 168). Studies confirm that men born SGA also are at risk of impaired gonadal function, with reduced testicular volume and decreased semen quality (73, 169, 170). Lower LH and testosterone concentrations have been reported in men born SGA (73).

In conclusion, being born SGA entails a risk of abnormal sex steroid secretion during fetal life, mini-puberty, puberty, and adult life. However, there is a lack of longitudinal studies describing the pattern of sex steroid concentrations during childhood in males with low birth weight (171). It is established that estrogens affect skeletal maturation (95), but whether impaired pubertal height gain is caused by increased estrogen concentrations in this group of patients is unknown.

## 1.6 PREMATURITY

Preterm birth is defined as gestational age below 37 weeks and is reported in 5%–9% of births in developed countries; of these, about 20% occur at 32–33 weeks of gestation, whereas 60%–70% occur at 34–36 weeks of gestation (172). Multiple risk factors including genetic, environmental, and socioeconomic factors, as well as infections and maternal hypertension, are associated with preterm birth (173). Studies reporting data on growth patterns in preterm infants are scarce, often focusing on children with gestational age below 32 weeks or children with very low birth weight (173, 174). Studies focusing on long-term follow-up of these children are lacking because of low survival rates in the past (175).

### 1.6.1 CONSEQUENCES OF BEING BORN PRETERM

As already mentioned, being born preterm is a risk factor associated with hypospadias and cryptorchidism (46, 48), although preterm birth seems to be a weaker risk factor related to hypospadias compared to being born SGA (47).

Preterm neonates, especially very preterm neonates, are exposed to extrauterine life during a critical period of programmed rapid growth in which they are forced to switch from high expenditure growth-promoting actions to survival strategies to cope with the demands of postnatal life (98). Preterm infants, and especially boys, born at gestational age below 34 weeks, with EUGR, defined as weight and/or length  $< -2$  SDS at term, are at risk of growth impairment during childhood (176). EUGR is common in preterm infants, usually followed by catch-up growth although often incomplete (173). The prevalence of short stature at 5 years of age in preterm children with gestational age below 32 weeks, is approximately 10%, which is comparable to the prevalence of short stature in children born SGA (98). Preterm infants born SGA are at higher risk of adult height impairment compared to preterm infants with normal birth weight SDS (16, 173). Interestingly, childhood growth and adult height outcome are similar in preterm individuals born SGA or AGA with evidence of IUGR and in individuals born with very low birth weight (15, 173).

Barker et al. reported no effect of prematurity on the risk of cardiovascular mortality in adult life (139), and IUGR rather than gestational age is thought to increase the long-term risk of developing metabolic disease (15). However, preterm birth, at least before 32 weeks of gestation, is associated with features such as increased abdominal fat, insulin resistance, and hypertension, which might be explained by an overactive HPA axis (16, 175). Catch-up growth in children born preterm results in an increased amount of visceral fat and reduced insulin sensitivity during childhood (174, 177). Also, children born preterm

with rapid gain in weight for length during infancy are at risk of developing cardiovascular disease and type 2 diabetes as young adults (177). However, the effects of catch-up growth in preterm infants compared to SGA infants may be different because normal postnatal growth means a rapid increase in body fat, estimated to 41% compared to 11% of weight gain during intrauterine growth, and catch-up growth in preterm infants may simply reflect the adaptation to extrauterine growth pattern (159).

Prematurity has been found to be a risk factor for precocious pubarche (18, 161). Although premature birth leads to an altered activation of the HPG axis during mini-puberty (41), pubertal onset is reported to be within normal limits in prematurely born boys (173, 178, 179).

Boys born preterm also seem to be at greater risk of infertility as adults (180).

In conclusion, there are indications that preterm males are at risk of pathological exposure to sex steroid secretion during fetal life, mini-puberty and in adult life. However, there is a lack of longitudinal studies assessing sex steroid concentrations during childhood in this population.

## 1.7 SILVER–RUSSELL SYNDROME

SRS is a rare clinical and epigenetic heterogeneous growth disorder characterized by severe IUGR, poor postnatal growth, craniofacial features such as a triangular-shaped face and a broad forehead, body asymmetry, and a variety of minor malformations (SRS, OMIM #180860). The prevalence of SRS is estimated as between 1:30 000 and 1:100 000 (127).

The syndrome was first described in 1953 by Silver et al. (two cases) (181) and in 1954 by Russell (five cases) (182). In 1964, Silver published a detailed series of 29 cases, describing their clinical features (183). Silver summarized the syndrome in both these publications as a combination of body asymmetry, short stature, and signs of sex hormone secretion disturbances, with elevated prepubertal urinary gonadotropins and signs of prepubertal estrogen exposure found in desquamated cells in cytologic analysis of urine in some boys and girls. The term “Silver-Russell syndrome” was introduced by Tanner et al. in 1975 (184).

Six different diagnostic scoring systems have been proposed for the clinical diagnosis of SRS (185). In the absence of stringent diagnostic criteria, the SRS diagnosis has been based on physical characteristics in many studies (186). However, in 2017 a consensus statement was published, offering guidelines

for work-up and diagnosis based on the Netchine–Harbison clinical scoring system (NH-CSS), in which at least four of six criteria are needed for a clinical diagnosis of SRS (127). Epigenetic changes are found in 20%–60% of patients with SRS, underlining the heterogeneity of this syndrome (187-189). Epigenetic changes have also been reported in a patient fulfilling only three of six NH-CSS criteria and thereby not qualifying for a clinical diagnosis of SRS according to the latest consensus statement (127, 190). However, in children born SGA with postnatal growth retardation without additional features of SRS, epigenetic findings have not been found (189, 191). The most common epigenetic change reported in patients with SRS is hypomethylation of the H19/IGF-II IG-DMR in 11p15, referred to as 11p15 loss of methylation (LOM), found in approximately 40% of patients, resulting in growth restriction caused by an imbalance in the expression of the growth promoting paternal IGF-II gene and the growth repressing maternal H 19 gene (127, 187). Another epigenetic change is upd(7)mat, reported in nearly 10% of patients and is believed to cause altered expression of an imprinted growth-regulatory gene or genes (187, 192). Patients carrying upd(7)mat are reported to have a milder phenotype with fewer typical clinical features of SRS (165, 189, 191, 193-195).

### **1.7.1 CONSEQUENCES OF HAVING SILVER–RUSSELL SYNDROME**

Genital abnormalities are reported in 40% of males with SRS (168). Due to severe IUGR, children with SRS are usually born SGA, and one of the diagnostic criteria is postnatal growth failure (127). In a paper published by Tanner et al. in 1975, bone age during childhood was reported to be delayed but it increased faster than chronologic age and, by puberty, bone age had caught up and was on average only 6 months delayed (184). Other studies have also reported bone age to be typically delayed followed by rapid advancement around 8 to 9 years of age (127, 186). In 1995, Wollman et al. published data on spontaneous growth of 386 children with SRS (223 boys), reporting mean adult height in males to be  $152 \pm 7.8$  cm (186). In another study, adult height was reported to be -3.6 SDS in both sexes (196).

GH treatment has a beneficial effect on SRS patients who are reported to reach adult height within the normal range with GH treatment, although adult height does not usually reach MPH (197). When compared to patients born SGA without postnatal catch-up growth, patients with SRS seem to have the same total height gain SDS during GH treatment (165). Although the pubertal growth spurt has been reported to be normal in shape and timing, it has been reported to be small (184), and from pubertal onset until adult height, height SDS declines more in patients with SRS compared to the SGA group (165).

Premature adrenarche is particularly frequent in patients with 11p15 LOM, and earlier adrenarche is followed by early gonadarche in boys with SRS (19, 127). Pubertal onset is reported to be within the normal range, although usually at the younger end of the spectrum and with a faster tempo (127, 184). In those with 11p15LOM, Sertoli cell dysfunction has been reported (198).

Males with SRS seem to be exposed to deviating sex steroid secretions during fetal life and are at risk of premature adrenarche (19, 127), but little is known about longitudinal patterns of sex steroid secretion during childhood and puberty in this group of patients. As early as 1964, Silver described signs of deviating estrogen exposure during childhood in some patients with SRS (183), but due to difficulties in analyzing serum estrogens at low concentrations, this has not been investigated further. It remains unknown whether increased estrogen concentrations explain the finding of peak pubertal height velocity at an earlier pubertal stage and less pubertal height gain in boys with SRS. Studies on reproductive health in adult males with SRS are lacking.

## 2 AIMS

The overall aim of this thesis was to evaluate sex steroid secretion patterns during childhood in one cohort of boys with SRS and one cohort of preterm boys, and to study how the timing and the magnitude of increasing sex steroid secretion was correlated with gestational age and birth weight, as well as how sex steroid secretion patterns related to anthropometric data, growth patterns and adult height.

Paper I: In this study, we aimed to analyze whether adult height was related to estrogen secretion patterns during childhood and puberty, at ages 6, 8, 10, 12, 14 and 16 years in GH-treated boys with SRS.

Paper II: Based on the results of paper I, the aim of this study was to identify the source of  $E_2$  concentrations by analyzing androgen concentrations as well as gonadotropins, AMH and inhibin B concentrations during childhood and puberty at ages 6, 8, 10, 12, 14 and 16 years and to investigate whether androgens were related to adult height in boys with SRS.

Paper III: The aim of this study was to investigate longitudinal adrenal androgen concentrations during childhood at ages 5, 6, 7, 8 and 10 years in a cohort of moderate to late preterm boys and to assess the relationships between adrenal androgen concentrations and gestational age, birth weight, and anthropometric parameters.

Paper IV: In this study, we aimed to analyze longitudinal concentrations of testosterone, DHT,  $E_1$ , and  $E_2$  during childhood in the same cohort and at the same ages as in paper III and to evaluate the relationship between sex steroid hormones and gestational age, birth weight, and anthropometric parameters.

## **3 PATIENTS AND METHODS**

### **3.1 STUDY POPULATION AND STUDY DESIGN**

#### **3.1.1 STUDY DESIGN**

In papers I and II, boys with SRS were studied from 6 to 16 years of age, in a longitudinal retrospective single-center study. Papers III and IV were part of an ongoing prospective, longitudinal, population-based study of 247 children (137 boys) born moderate to late preterm; 58 of these boys were included and studied from 5 to 10 years of age.

#### **3.1.2 STUDY POPULATION (PAPERS I AND II)**

In papers I and II, 19 consecutively referred boys who were born between 1988 and 2004 and diagnosed with SRS at Queen Silvia Children's Hospital, Gothenburg, Sweden, were identified. All patients had been treated with GH. Six patients were lost to follow up. Thirteen patients who had been followed longitudinally from 6 years of age to adult height, agreed to participate in the study. In paper I, 11 patients who had reached adult height were included. In paper II, another two patients who had reached adult height were included, giving a total of 13 boys. The participants were classified as non-responders or responders depending on adult height outcome. Patients with adult height  $>1$  SDS below MPH were defined as non-responders and patients with adult height  $\leq 1$  SDS below MPH were defined as responders. See Table 1 for patient characteristics.

The patients in papers I and II were originally diagnosed according to the clinical characteristics described in the publications by Silver et al., Russell, and Tanner et al. (181-184). Epigenetic work-up was performed in all patients, Table 1. Due to the lack of consensus regarding the diagnosis of SRS, previous studies addressing aspects of SRS have not been consistent in using the NH-CSS criteria suggested in the consensus statement published 2017. In the consensus statement, a diagnosis of SRS requires a minimum of four criteria to be fulfilled, including protruding forehead and relative macrocephaly at birth (127, 186). When reevaluating the diagnosis in our cohort according to the NH-CSS, all patients had at least four out of six criteria consistent with SRS, Table 1 (127). Three non-responders and one responder did not have a protruding forehead, which is assessed subjectively. One of the non-responders without protruding forehead had, however, hemihypotrophy but not relative macrocephaly. Another non-responder without protruding forehead had an epigenetic finding of 11p15 LOM.

Blood samples were missing in one non-responder and one responder at 16 years of age. These two boys also lacked data on bone age at 12 years of age. One of the two non-responders added in paper II also lacked blood samples at 16 years of age, and both of these boys lacked data on bone age at all ages.

*Table 1: Clinical scores according to the NH-CSS (127), presented for each patient*

Patient no.	NR/R	SGA for weight	Postnatal growth failure	Relative macrocephaly at birth	Protruding forehead	Body asymmetry	Feeding difficulty / low BMI at 24 mths	Total NH-CSS score
1	R	1	1	1	1	0	1	5**
2	R	1	1	1	1	1	1	6**
3	NR	1	1	0*	0	1	1	4
4	R	1	1	1	1	0	1	5**
5	NR	1	1	1	0	1	1	5**
6	NR	1	1	1	1	0	1	5
7	R	0	1	1	1	0	1	4
8	NR	1	1	1	1	1	1	6**
9	NR	1	1	1	1	1	1	6**
10	R	1	1	1	0	0	1	4
11	NR	1	1	1	1	0	1	5
12***	NR	1	1	1	1	1	1	6**
13***	NR	1	1	1	0	0	1	4

\*Patient no. 3 had a relative macrocephaly of 1.3 SDS

\*\*Epigenetic findings of 11p15 LOM

\*\*\*Patients no. number 12 and 13 were not included in paper I

(SGA = small for gestational age, BMI = body mass index, mths = months, NH-CSS = Netchine–Harbison clinical scoring system, NR = non-responder, R = responder.)

### **3.1.3 STUDY PROTOCOL (PAPERS I AND II)**

Data on gestational age, birth weight, birth length, and head circumference were obtained from birth charts and converted to SDS according to the gender-specific reference for newborns (199). Body weight, height, and testicular volume were routinely recorded until adult height, which was defined as growth velocity of less than 1 cm/year. Weight SDS and height SDS were calculated, BMI was obtained by dividing weight by square height ( $\text{kg}/\text{m}^2$ ) (113, 200). Pubertal status was assessed according to the largest testicular volume using an orchidometer (201), and signs of pubarche were assessed yearly according to the Tanner scale (54). Pubertal onset was defined as a testis volume of  $\geq 3$  mL. This definition was used because of the substantial evidence indicating HPG-axis activation from this point (57-59, 202). Bone age was assessed from start of GH treatment until 12 years of age, using standardized routine X-rays assessed by a single radiologist using the TW 2 method (203). When there were two X-rays equally close to the target age, bone age was extrapolated from two sets of samples.

GH status was evaluated by both an arginine-insulin-tolerance test (AITT) and a 12- to 24-hour spontaneous GH secretion test. GH deficiency (GHD) was defined as GHmax of  $< 10$   $\mu\text{g}/\text{L}$  in both tests. One non-responder and one responder had only one test performed, in both cases with normal results, thus excluding GHD. All patients were treated with GH due to SGA and/or GHD indication, from an early age (2 to 6 years) until adult height.

Blood samples for hormone analyses, were drawn in the morning (08:00–11:00 a.m.) at the start of GH treatment and yearly during treatment. After separation, sera were stored at  $-80^\circ\text{C}$ . Sex steroids, gonadotropins, AMH and inhibin B were analyzed at ages 6, 8, 10, 12, 14, and 16 years. In paper II, blood samples were also analyzed at a testicular volume of 3-6 mL if not present at these ages and results for SHBG were obtained at the same ages from patient records or analyzed retrospectively. Blood samples for epigenetic analyses and assessment of IGF-I were also analyzed.

### **3.1.4 STUDY POPULATION (PAPERS III AND IV)**

In papers III and IV, 58 boys were recruited from a larger cohort consisting of 247 neonates (137 boys) born at gestational age (weeks + days) 32+0 to 36+6 at the two delivery wards at Sahlgrenska University hospital in Gothenburg, Sweden, between September 2002 and June 2004 (14). Neonates with syndromes, chromosomal abnormalities, severe malformations, or chronic disease were excluded. A total of 340 neonates were not able to participate in the study, and a dropout analysis showed no significant differences in gestational age or birth size in the dropouts compared to the study population

(204). At 5 years of age, the caregivers of 127 children (66 boys) who were not lost to follow-up were asked to continue to participate in the study. Caregivers of 58 boys agreed to further participation, and written informed consent was obtained (118). The gestational age in the cohort of paper III and IV ranged from 32+2 to 36+6 (weeks + days). Eight of 58 boys (14%) were born SGA and 26 boys (45%) had birth weight below 2500 grams. Twelve boys (21%) were exposed to preeclampsia, and one was exposed to hypertonia, of whom three were born SGA for weight. We originally followed the 58 boys to 7 years but extended the follow-up period, first to 8 years and then to 10 years of age.

In paper IV, we had identified two boys who lacked blood samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis but who had blood samples for gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis at 10 years of age; we also identified one boy who had a blood sample for LCMS/MS but not for GCMS/MS analysis at 8 years of age.

A dropout analysis of the 79 boys who were lost to follow-up did not reveal any significant differences in gestational age or birth size.

By the age of 10 years, ten boys were lost to follow-up. Blood samples were analyzed on at least two occasions for each boy, except for one boy who only provided a sample at 5 years of age.

### **3.1.5 STUDY PROTOCOL (PAPERS III AND IV)**

Gestational age was recorded, and birth weight and birth length were measured with the infant in a supine position, using a digital infant scale and measuring tape (14). Gender-specific SDS was calculated for birth weight and birth length according to the Swedish growth reference for newborns (205). Weight, height, and waist circumference were measured every sixth months until the age of 7 years (118) and at ages 8 and 10 years. The boys wore only underwear for body weight measurement, and the measure closest to 0.1 kg using an electronic step scale (SECA 701, Germany) was recorded. Standing height was measured with a precision of  $\pm 1$  mm, using a wall-mounted Ulmer stadiometer. Gender-specific SDS was calculated using a Swedish population-based reference (200). Waist circumference was measured in a standing position at the umbilical level using an inelastic measuring tape. Anthropometric measures were performed three times, and the mean value was calculated. WHtR was calculated by dividing waist circumference (cm) by height (cm). At 8 and 10 years of age, pubertal development was assessed according to the largest testis, using an orchidometer (201), and pubic hair growth was recorded according to Tanner stages at 8 and 10 years of age (54). Four boys were diagnosed with unilateral cryptorchidism.

An oral glucose tolerance test (OGTT) was performed after overnight fasting, at 5 years of age (206).

Venous blood samples for analyses of DHEAS, A<sub>4</sub>, testosterone, DHT, E<sub>1</sub>, E<sub>2</sub>, AMH, and inhibin B were drawn in the morning (08:00–11:00 a.m.) every year from 5 to 8 years and at 10 years of age. At 10 years, blood samples for analyses of FSH and LH were also collected. When blood sampling failed, another attempt was made within six months, and anthropometric measures were collected for further calculations. After separation, sera were stored at -80°C.

## 3.2 METHODS

### 3.2.1 LABORATORY METHODS

#### ***Epigenetic analysis***

DNA was extracted from white blood cells following standard protocol, and a methylation-sensitive multiplex ligation-dependent probe amplification (MS-MLPA) using commercial kits ME30 and ME032 (MRC-Holland; SeqPilot software from JSI Medical Systems GmbH) was run to examine the differently methylated chromosomal regions 11p15, 6q24, 7p12.1, 7q32.2, and 14q32.2. The methylation levels were compared to those of normal control DNA samples. Values below 0.5 on chromosome region 11p15 were considered indicative of LOM. The analyses were conducted at the Department of Clinical Genetics, Sahlgrenska University Hospital, Gothenburg, Sweden.

#### ***Sex steroids analyzed by mass spectrometry***

Serum sex steroid concentrations were determined by mass spectrometry which is an analytical method that determines the mass-to-charge ratio of ions. Figure 9 shows the basic elements of a mass spectrometer. The sample may be turned into a liquid or gaseous form and is ionized through an electron gun. When ionized, the compound is accelerated into vacuum by a negative or positive potential, and in a magnetic field the compounds are divided according to the mass-to-charge ratio.

We used the tandem mass spectrometry-based method in which two mass spectrometers are combined and the compounds are separated through two separate steps. The LC-MS/MS was carried out on an Agilent 1260 LC coupled to an Agilent 6460 triple quadrupole mass spectrometer (Montréal, Canada) and the GC-MS/MS analysis was performed on an Agilent 7890B GC coupled to an Agilent 7000 triple quadrupole mass spectrometer (Montréal, Canada) (79, 207).

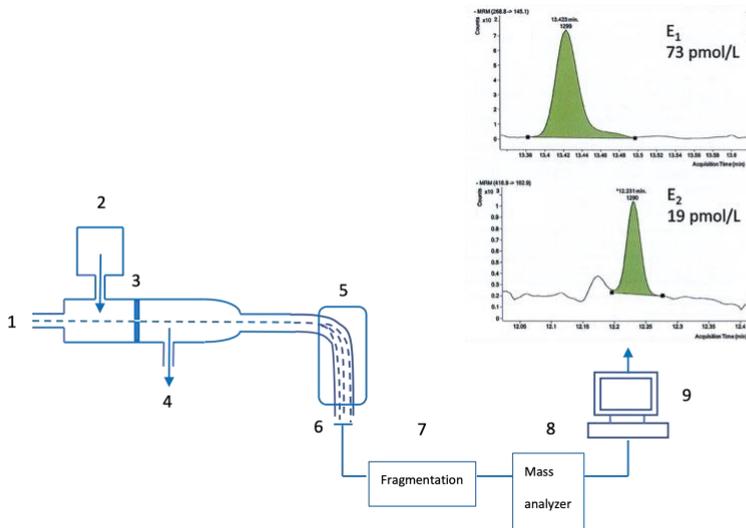


Figure 9: Example of mass spectrometry analysis of estrone ( $E_1$ ) and estradiol ( $E_2$ ) concentrations with results presented as mass spectrums.

- 1: Sample in gas or liquid form
- 2: Ionization (electron gun)
- 3: Accelerating plates
- 4: Vacuum pump
- 5: Magnet
- 6: Detector
- 7: Fragmentation (the most abundant steroids are selected for further fragmentation and analysis in a second mass spectrometer)
- 8: Mass analyzer
- 9: Registration

The compound is selectively ionized, and a precursor ion is generated in the first mass spectrometer. The precursor ions of a specific mass-to-charge ratio are selected and fragmented in the second mass spectrometer to generate a product ion for detection (208). The analyses were performed at our laboratory at the Department of Pediatrics, Sahlgrenska University Hospital, Gothenburg, Sweden.

With commercial direct immunoassays, determination of sex steroid hormones is demanding at low concentrations found in children. Immunoassays tend to overestimate sex steroid concentrations at low concentrations due to assay interference by other endogenous steroids (79, 207). Other advantages of the

mass spectrometry-based method are the capability to analyze several hormones at the same time, with superior sensitivity and specificity, only requiring a sample volume of 50  $\mu\text{L}$  for LC-MS/MS analysis and 200  $\mu\text{L}$  for GC-MS/MS analysis (79, 207).

Generally, the sensitivity of both LC-MS/MS and GC-MS/MS is excellent. Nevertheless, at our laboratory at the Department of Pediatrics, Sahlgrenska University Hospital, Gothenburg, Sweden, only the GC-MS/MS based method was sensitive enough for the determination of  $E_1$ ,  $E_2$ , and DHT concentrations, which are very low in prepubertal children. On the other hand, conjugated and sulfated compounds such as DHEAS could much more easily be analyzed by LC-MS/MS, and GC-MS/MS analysis requires more extensive sample work-up (79, 207). The mass spectrometry-based methods are expensive but are becoming increasingly available in clinical practice.

We used LC-MS/MS for simultaneous analysis of serum  $A_4$  and DHEAS. Limit of detection (LOD) was 0.1  $\mu\text{mol/L}$  for DHEAS and 0.1 nmol/L for  $A_4$ . The total coefficient of variation (CV) for DHEAS was 8% at 0.4  $\mu\text{mol/L}$ , 4% at 1.1  $\mu\text{mol/L}$  and 7% at 3.6  $\mu\text{mol/L}$  and above. For  $A_4$ , the total CV was 21% at 0.2 nmol/L and 12% at 1.2 nmol/L and above.

GC-MS/MS was used for simultaneous analysis of serum testosterone, DHT,  $E_1$ , and  $E_2$ . LOD was 0.1 nmol/L for testosterone, 27 pmol/L for DHT, 9 pmol/L for  $E_1$ , and 2 pmol/L for  $E_2$ . For testosterone, the total CV was 16% at 0.3 nmol/L, 9% at 1.6 nmol/L and 8% at 20 nmol/L. For DHT, the total CV was 15% at 60 pmol/L, 10% at 200 pmol/L and 8% at 800 pmol/L. For  $E_1$ , the total CV was 33% at 11 pmol/L, 14% at 38 pmol/L and 12% at 100 pmol/L. For  $E_2$ , the total CV was 19% at 8 pmol/L and 6% at 36 pmol/L and above.

The free androgen index was calculated as (testosterone (nmol/L)/SHBG (nmol/L))  $\times 10^4$  and (DHT (nmol/L)/SHBG (nmol/L))  $\times 10^4$ .

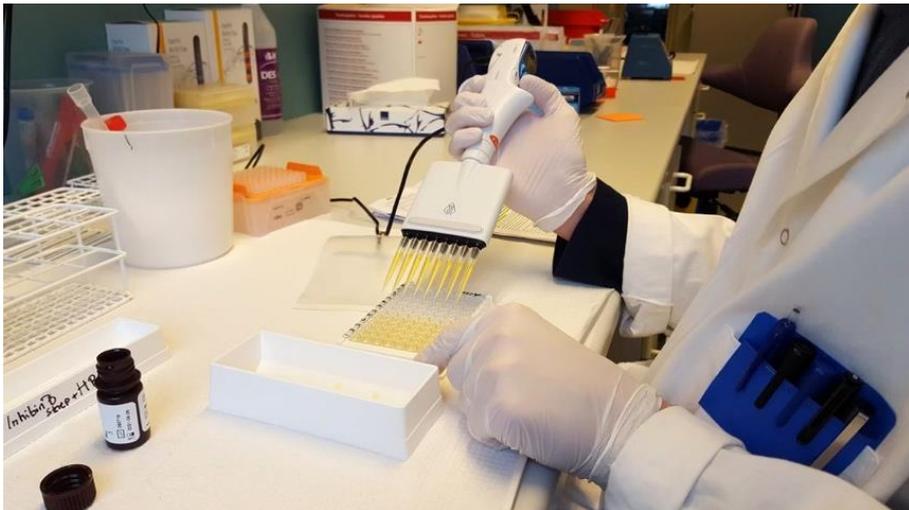
### ***Other hormone analyses***

Serum IGF-I concentrations were determined by radioimmunoassay (Mediagnost GmbH, Tübingen, Germany). The samples were analyzed at our laboratory at the Department of Pediatrics, Sahlgrenska University Hospital, Gothenburg, Sweden. LOD for IGF-I was 0.064  $\mu\text{g/L}$ , and the total CV was 20% at 33  $\mu\text{g/L}$  and 14% at 179  $\mu\text{g/L}$ .

Serum LH and FSH concentrations were determined using microparticle chemiluminescent immunoassay (Architect i2000SR, Abbott Scandinavia) at the Department of Clinical Chemistry, Sahlgrenska University Hospital,

Gothenburg, Sweden. LOD was 0.1 IU/L for LH and 0.05 IU/L for FSH. The total CV was 7% at 7 IU/L and 50 IU/L for LH and 6% at 15 IU/L and 45 IU/L for FSH.

Serum AMH and inhibin B were analyzed using the enzyme-linked immunosorbent assay ELISA (AnshLabs, USA) at our laboratory at the Department Pediatrics, Sahlgrenska University Hospital, Gothenburg, Sweden, Figure 10. The LOD was 0.023 ng/mL for AMH and 1.6 pg/mL for inhibin B. The total CV in the entire range was < 6% for AMH and < 8% for inhibin B. Results from the AMH analysis were compared to normal reference intervals reported by the manufacturer and, because there was a lack of normal reference intervals for inhibin B, normal reference intervals reported by another group were used (70).



*Figure 10: ELISA-based method for the determination of inhibin B concentrations at our laboratory at the Department of Pediatrics, Sahlgrenska University Hospital, Gothenburg, Sweden. (ELISA = Enzyme-linked immunosorbent assay.)*

Serum SHBG was determined using the Cobas 8000 chemiluminescent immunoassay (Roche Diagnostics, Scandinavia AB) at the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden. The LOD was 0.1 nmol/L, and the total CV was 7% at 40 nmol/L and 9% at 100 nmol/L.

### **3.2.2 ORAL GLUCOSE TOLERANCE TEST**

An OGTT was performed at 5 years of age in the preterm cohort in papers III and IV. The boys were asked to drink a solution of 75 g glucose dissolved in 250 mL of water, corresponding to 0.3 g glucose/mL, after overnight fasting. According to bodyweight, 1.75 g of glucose per kg, equivalent to 5.8 mL of the solution per kg, was given. No subject reached the maximum load of 75 g glucose. Blood glucose was determined at start and after 30, 60, and 120 minutes, analyzed by HemoCue 201 RT with an inter-assay variation of 2.1% at 6 mmol/L. Impaired glucose tolerance and type 2 diabetes were defined as a 2-hour post-load blood glucose > 7.8 mmol/L and > 11.1 mmol/L, respectively (206).

### **3.2.3 RADIOLOGY**

To assess bone age, we used the TW2 method, which is considered more reliable and objective compared to the Greulich and Pyle method (107). However, the reliability of the method is influenced by several factors. The TW2 method is based on radiographs of the non-dominant hand in healthy children (203). To what extent factors such as hemihypotrophy, which is seen in some patients with SRS, affects the assessment of bone age is not known. Because the assessment of bone age is based on results from healthy children, there are obvious risks when applied to children suffering from SRS, preterm children, or children with low birth weight. Furthermore, in preterm children, there is a discrepancy between the corrected and chronological age, which might influence the interpretation of the results in these children.

### **3.2.4 STATISTICAL METHODS**

In all paper, we analyzed relationships between hormones and anthropometric data. IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA) was used for statistical calculations in all papers. Origin (OriginLab Corp., Northampton, MA, USA) was used for drawing of figures in papers I, II and IV. The R statistical package (R Foundation for Statistical Computing, Vienna, Austria) was used for regression analyses and drawing of figures in papers III and IV.

Hormone concentrations below LOD were set to LOD/2.

A *p*-value < 0.05 was considered significant.

In papers I and II, the patients were divided into one of two groups according to adult height outcome. The Mann–Whitney U test was used for comparison of the two groups. In paper IV we used the Wilcoxon signed rank test to compare differences in sex steroid concentrations at different ages.

In papers I, III and IV, we calculated Pearson’s correlation coefficient and in paper II we calculated Spearman’s correlation coefficient. In the context of this thesis, a correlation coefficient of 0 to  $< 0.3$  or 0 to  $> -0.3$  was interpreted as a weak correlation, 0.3 to  $< 0.5$  or  $-0.3$  to  $> -0.5$  was interpreted as a moderate correlation, and  $\geq 0.5$  as well as  $\leq -0.5$  was interpreted as a strong correlation.

In paper I, we performed a simple regression analysis and in paper III we performed regression analysis using linear mixed-effect model to evaluate longitudinal data.

The Holm–Bonferroni method was used to correct for multiple testing, when relevant, in paper III.

### **3.2.5 ETHICAL APPROVAL AND INFORMED CONSENT**

All four studies were conducted in accordance with the Declaration of Helsinki.

Before blood sampling, all children were offered local anesthetics. Blood sampling was performed by an endocrine-trained pediatric nurse. All measures and blood sampling were performed in a child-friendly environment.

The protocols of studies I and II were approved by the Central Ethical Review Board in Gothenburg, Sweden (approval no. 449-16). Written informed consent was obtained from the caregivers and retrospectively from the patients after they had reached 16 years of age.

The protocols of studies III and IV were approved by the Central Ethical Review Board in Gothenburg, Sweden (approval no. 297-07) for the age range 4 to 7 years. Amendments for further investigations at 8 years of age (approval no. T675-10) and 10 years of age (approval no. T510-12) were also approved. Written informed consent was obtained from the caregivers.

## 4 RESULTS

### 4.1 BIRTH CHARACTERISTICS AND GROWTH PATTERNS

#### 4.1.1 GESTATIONAL AGE AND GROWTH DATA (PAPERS I AND II)

Anthropometric data for the cohort in paper I are described in paper I. In paper II, a further two patients were added to the cohort. After adding these two patients, the difference in birth weight SDS between non-responders and responders was no longer significant, Table 2. The difference in the birth length SDS remained non-significant. Non-responders had greater weight and height gain from birth to pubertal onset, but responders had greater height gain during puberty and non-responders showed a decline in height SDS during puberty.

*Table 2: Differences in anthropometric measures in 13 growth hormone-treated boys with Silver–Russell syndrome, divided into non-responders and responders.*

Anthropometric measure	Non-responder N = 8	Responder N = 5	<i>p</i> -value
Birth weight SDS	-3.9 (-4.7 to -2.1)	-2.7 (-3.3 to -1.7)	0.078
Birth length SDS	-4.3 (-6.0 to -1.8)	-2.4 (-3.5 to -0.9)	0.056
Δ weight SDS from birth to pubertal onset	2.6 (1.5 to 4.1)	1.4 (0.0 to 2.0)	0.005
Δ height SDS from birth to pubertal onset	3.6 (1.2 to 5.3)	1.9 (0.0 to 2.9)	0.028
Δ height SDS from pubertal onset to adult height	-1.6 (-2.9 to 0.4)	0.1 (-0.1 to 1.1)	0.019

Data are expressed as median and range. The differences between the groups were tested with Mann–Whitney U tests, and a *p*-value of < 0.05 was considered significant. (SDS = standard deviation score.)

## **4.1.2 GESTATIONAL AGE AND GROWTH DATA (PAPERS III AND IV)**

The birth size of the preterm cohort tended to be lower with both lower birth weight SDS and lower birth length SDS, compared to the normal reference intervals.

From 5 to 10 years of age, the height SDS trajectories were stable. Weight SDS showed a discrete increase, with median and range rising from 5 years to 10 years of age. From 7 years of age, no one had a weight SDS < -2 SDS. WHtR was suspected to decline, but boys exceeding a WHtR of 0.5 at 5 years of age tended to show an increased WHtR at 10 years of age.

## **4.2 GENETICS AND BIOMARKERS**

### **4.2.1 EPIGENETICS**

11p15LOM was found in 7 (54 %) of the 13 patients in the SRS cohort. No patient had upd(7)mat.

### **4.2.2 IGF-I**

In the SRS cohort, no significant differences between non-responders and responders were seen in IGF-I status at start or during the first year of GH treatment.

### **4.2.3 SHBG AND FREE ANDROGEN INDEX**

SHBG was used for free androgen index calculations in paper II. The free androgen index for testosterone was higher in non-responders at 10 to 14 years and free androgen index for DHT was higher in non-responder at 10 to 12 years of age. Furthermore, there was a strong negative correlation between free androgen index for testosterone and adult height adjusted for MPH at 10 and 12 years of age, and at 14 years of age the strong negative correlation was of borderline significance ( $p = 0.051$ ). There were no differences in SHBG between non-responders and responders, and no significant correlations between SHBG or the free androgen index for DHT and adult height adjusted for MPH at any age.

#### **4.2.4 GONADOTROPINS**

In the SRS cohort in papers I and II, the LH concentration was higher in non-responders than in responders at 12 years of age. No differences were observed in LH at any other age or in FSH at any age at all. In the cohort in papers III and IV 19 (44%) boys had LH concentrations above LOD. There were inverse moderate correlations between LH at 10 years of age and birth weight in grams and birth weight in SDS. There were no significant correlations between gonadotropins and gestational age or between FSH and birth weight. No significant correlations were observed between gonadotropins and weight SDS, height SDS, or WHtR.

#### **4.2.5 ORAL GLUCOSE TOLERANCE TEST**

The cohort in papers III and IV underwent an oral glucose tolerance test at 5 years of age. Two boys had a pathological post-load blood glucose of 8.2 and 8.3 mmol/L, at two hours. No boy met the criteria for type 2 diabetes.

### **4.3 BONE AGE**

There were no significant differences in bone age between non-responders and responders at any age or at pubertal onset.

### **4.4 ADRENARCHE AND PUBERTAL DEVELOPMENT**

In the SRS cohort, premature adrenarche was only seen in one non-responder, who had reached pubarche just before 8 years of age. Two non-responders and one responder developed pubarche before the onset of puberty and non-responders had a younger age at pubarche.

There was no significant difference in age at pubertal onset defined by a testicular volume of  $\geq 3$  mL, between non-responders and responders in the SRS cohort. However, non-responders had a significantly larger testicular volume at 12 years of age. Only one non-responder who had a testicular volume of 3 mL at 9.6 years, met the definition of pubertal onset with a testicular volume of  $\geq 3$  mL before 10 years of age.

Several patients developed unexpectedly small adult testicular volumes. One responder had a testicular volume of only 8 mL at adult height. Another two non-responders with prepubertal AMH concentrations below normal reference intervals (70), reached an adult testicular volume of 8 mL and one further non-responder reached 12 mL. The remaining boys reached a testicular volume of  $\geq 15$  mL. Two non-responders showed reduced testicular volume at adult height, from 12 mL to 8 mL and from 18 mL to 15 mL, respectively.

In the study population of preterm boys in papers, III and IV, premature adrenarche with the appearance of pubarche before 9 years of age was not seen in any participants.

At 8 years of age, one boy had reached a testicular volume of 3 mL. At 10 years of age, twelve boys had started puberty with a testicular volume of 3–4 mL. Pubarche was observed in only three of these boys.

## 4.5 ADRENAL ANDROGENS

In this section, adrenal androgens refers to DHEAS and A<sub>4</sub>.

### 4.5.1 ADRENAL ANDROGENS AND REFERENCE INTERVALS (PAPERS II AND III)

In paper II, we found increased adrenal androgen secretion in several boys with SRS, compared to normal reference intervals (207), Table 3 (next page). In the presentation of these data, more samples at testicular volume of 8–12 mL, are included when available (two boys), to account for concentrations of DHEAS and A<sub>4</sub> not only at certain ages but also according to normal reference intervals given in relation to testicular volume.

In paper II, we reported higher DHEAS and A<sub>4</sub> concentrations in non-responders at 10 years of age. A<sub>4</sub> concentrations were also significantly higher in non-responders at 12 years of age. No significant differences in adrenal androgen concentrations were found between non-responders and responders at any other age.

Table 3: Adrenal androgen secretion in 13 boys with Silver–Russell syndrome, compared to normal reference intervals.

Testicular volume	1–2 mL		3–6 mL		8–12 mL		15–25 mL	
	DHEAS $\mu\text{mol/L}$	A <sub>4</sub> nmol/L						
Normal reference intervals	0.1-2.8	<0.2-0.8	0.5-4.9	0.2-2.5	0.5-8.1	0.6-3.7	0.2-13.8	0.8-5.0
Patient 1 (R)	0	0	0	0	0	0	0	0
Patient 2 (R)	1	0	0	0	0	0	0	0
Patient 3 (NR)	0	1	0	0	0	0	0	0
Patient 4 (R)	0	1	0	0	0	0	/	/
Patient 5 (NR)	0	1	0	0	0	0	/	/
Patient 6 (NR)	1	1	0	0	0	0	0	0
Patient 7 (R)	0	1	0	0	0	0	-	-
Patient 8 (NR)	0	1	0	0	0	0	/	/
Patient 9 (NR)	1	1	0	1	0	1	/	/
Patient 10 (R)	1	1	1	0	0	0	-	-
Patient 11 (NR)	1	1	1	0	0	0	0	0
Patient 12 (NR)	1	1	1	0	-	-	-	-
Patient 13 (NR)	1	1	1	0	0	0	0	0
Proportion of patients with increased adrenal androgens	7/13 (54%)	11/13 (85%)	4/13 (31%)	1/13 (15%)	0/11 (0%)	1/11 (9%)	0/6 (0%)	0/6 (0%)
Proportion of NRs among those with increased adrenal androgens	5/7 (71%)	8/11 (72%)	3/4 (75%)	1/1 (100%)	-	1/1 (100%)	-	-

Androgen concentrations above normal reference intervals according to testicular volume are denoted as (1); normal concentrations are denoted as (0) (207). Missing values are denoted as (-), and (/) indicates that a testicular volume of  $\geq 15$  mL was not reached. (DHEAS = dehydroepiandrosterone sulfate, A<sub>4</sub> = androstenedione, NR = non-responder, R = responder.)

For the most part, we found adrenal androgen secretion to be within normal reference intervals in the preterm boys (207). At 8 years of age, one boy with a testicular volume of 2 mL had an unexpectedly high A<sub>4</sub> concentration. At 10 years of age, two subjects with a testicular volume of 1 mL and 2 mL had an A<sub>4</sub> concentration above normal reference intervals. DHEAS concentrations above normal reference intervals were only found at 10 years of age, in two boys with a testicular volume of 1 mL and 2 mL, respectively. The boy with a high DHEAS concentration at a testicular volume of 1 mL was one of the two boys with high A<sub>4</sub> concentration at 10 years of age.

#### **4.5.2 ADRENAL ANDROGENS, BIRTH CHARACTERISTICS, AND ANTHROPOMETRY (PAPERS II AND III)**

In this section, anthropometric data refers to weight SDS and WHtR.

In the cohort in paper II, no significant correlations were observed between DHEAS or A<sub>4</sub> at any age and birth characteristics.

In paper III, we reported consistent trajectories of adrenal androgen concentrations, especially for DHEAS, from 5 to 10 years of age. There was a strong correlation between DHEAS and A<sub>4</sub> concentrations throughout childhood. No significant correlations were found between adrenal androgens at any age and gestational age or birth size. One exception was A<sub>4</sub> at 5 years of age, which correlated negatively and weakly with birth length SDS.

DHEAS and A<sub>4</sub> correlated moderately with weight SDS at 7 and 8 years of age. At 10 years of age, DHEAS correlated strongly and A<sub>4</sub> correlated moderately with weight SDS. The correlation between DHEAS and WHtR was weak at 7 years, non-significant at 8 years and moderate at 10 years of age. A<sub>4</sub> did not correlate significantly with WHtR at any age. At 10 years of age, DHEAS correlated strongly ( $r = 0.60, p < 0.001$ ) and A<sub>4</sub> correlated moderately ( $r = 0.42, p = 0.007$ ) with weight gain from birth.

In a linear mixed-effects model, DHEAS and A<sub>4</sub> levels were significantly associated with weight SDS and WHtR SDS, independently of age, Table 4 (next page).

Table 4: The association of adrenal androgens adjusted for age with the predictors weight SDS and WHtR SDS

Predictive variable	DHEAS ( $\mu\text{mol/L}$ ) adjusted for age	A <sub>4</sub> (nmol/L) adjusted for age
Weight SDS	$\beta = 0.22$ $p < 0.0001$	$\beta = 0.04$ $p = 0.002$
WHtR SDS	$\beta = 0.19$ $p < 0.0001$	$\beta = 0.04$ $p = 0.003$

The  $\beta$  value indicates the degree of change in each hormone for each one-unit change in the predictors weight SDS and WHtR SDS. (SDS = standard deviation score, WHtR = waist-to-height ratio, DHEAS = dehydroepiandrosterone sulfate, A<sub>4</sub> = androstenedione.)

### 4.5.3 ADRENAL ANDROGENS, LINEAR GROWTH, AND ADULT HEIGHT (PAPERS II AND III)

In this section, adult height outcome refers to adult height SDS adjusted for MPH SDS.

In paper III, DHEAS correlated weakly with height SDS at 7 years ( $r = 0.29$ ,  $p = 0.041$ ) and moderately at 10 years of age ( $r = 0.37$ ,  $p = 0.020$ ). There were no significant correlations between DHEAS and height SDS at any other age or between A<sub>4</sub> and height SDS at any age.

In paper II, we found strong negative correlations between DHEAS at 8, 10, and 12 years of age and adult height outcome, Table 5. No other significant correlations were observed between DHEAS and adult height outcome at any other age or between A<sub>4</sub> and adult height outcome at any age. In Figure 11, DHEAS concentrations at 10 years of age are plotted against adult height outcome.

Table 5: Significant correlations between DHEAS and adult height SDS adjusted for MPH SDS and  $R^2$  values in 13 boys with Silver–Russell syndrome at 8 to 12 years of age.

Androgen (age in years)	Spearman’s rho ( $r$ )	$R^2$	$p$ -value
DHEAS (8)	-0.72	0.52	0.006
DHEAS (10)	-0.79	0.62	0.001
DHEAS (12)	-0.72	0.52	0.006

A  $p$ -value of  $< 0.05$  was considered significant. (DHEAS = dehydroepiandrosterone sulfate, SDS = standard deviation score. MPH = midparental height.)

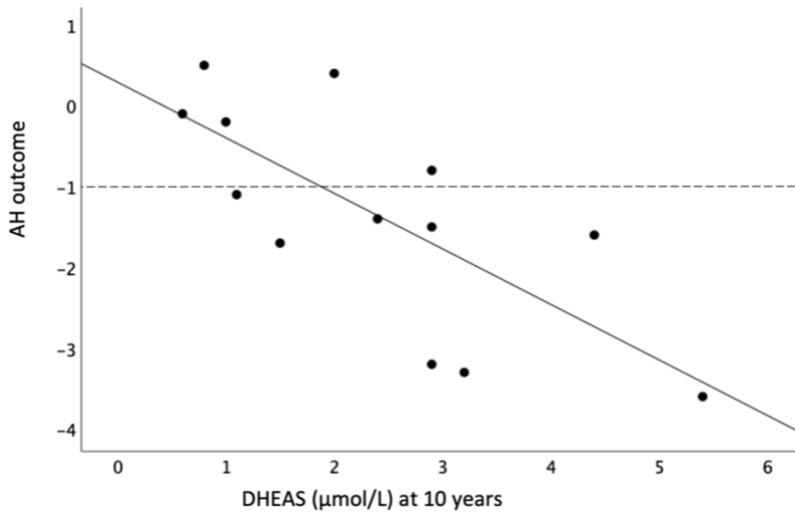


Figure 11: Scatterplot showing dehydroepiandrosterone sulfate (DHEAS) at 10 years of age, plotted against adult height standard deviation score (SDS) adjusted for midparental height SDS (AH outcome), in 13 boys with Silver–Russell syndrome. A dotted line divides non-responders and responders. The solid line is the regression line.

## 4.6 GONADAL ANDROGENS, AMH, AND INHIBIN B

In this section, gonadal androgens refers to testosterone and DHT.

### 4.6.1 GONADAL ANDROGENS, AMH, AND INHIBIN B AND REFERENCE INTERVALS (PAPERS II AND IV)

In paper II, we found increased gonadal androgen concentrations in several non-responders and responders, compared to normal reference intervals (79), Table 6. In the presentation of these data, more samples at testicular volume of 8–12 mL, are included when available (one boy), to compare concentrations of testosterone and DHT, not only at certain ages, but also according to normal reference intervals given in relation to testicular volume.

From 10 to 14 years of age, non-responders had significantly higher concentrations of testosterone. At 10 and 12 years of age, the DHT concentration was also higher in non-responders. No significant differences were seen in concentrations of testosterone or DHT at any other age.

In paper IV, most subjects had normal testosterone concentrations at all ages. However, at 10 years of age, two boys had testosterone concentrations of 1.3 and 0.7 nmol/L, respectively, exceeding normal reference intervals according to testicular volume (79). They both had a testicular volume of 2 mL, with LH of 2.1 and 0.8 IU/L, respectively, and FSH of 2.4 and 5.1 IU/L, respectively. At 10 years of age, the two boys with the highest testosterone concentrations were considered as outliers and their testosterone concentrations at this age were excluded from statistical analyses. No values exceeding normal reference intervals were found for testosterone at any other age or for DHT at any age at all. Testosterone correlated strongly with LH and moderately with FSH at 10 years of age. However, there were no significant correlations between testosterone or DHT and testicular volume at the same age.

Table 6: Gonadal androgen secretion in 13 boys with Silver–Russell syndrome, compared to normal reference intervals

Testicular volume	1–2 mL		3–6 mL		8–12 mL		15–25 mL	
	T nmol/L	DHT pmol/L	T nmol/L	DHT pmol/L	T nmol/L	DHT pmol/L	T nmol/L	DHT pmol/L
Normal reference intervals	0.1–0.5	<27–232	0.1–2.8	77–322	2.1–24.8	151–1315	11.0–26.7	622–1424
Patient 1 (R)	0	0	0	0	0	0	0	0
Patient 2 (R)	0	0	0	0	0	0	0	0
Patient 3 (NR)	0	0	0	0	0	0	0	0
Patient 4 (R)	0	0	0	0	0	1	/	/
Patient 5 (NR)	0	0	0	0	0	0	/	/
Patient 6 (NR)	0	0	1	1	-	-	0	1
Patient 7 (R)	0	0	1	1	0	0	-	-
Patient 8 (NR)	0	0	1	1	0	0	/	/
Patient 9 (NR)	0	0	1	1	0	0	/	/
Patient 10 (R)	0	0	0	0	0	0	-	-
Patient 11 (NR)	0	0	0	0	0	0	0	0
Patient 12 (NR)	1	1	1	1	-	-	-	-
Patient 13 (NR)	1	0	1	1	0	0	0	0
Proportion of patients with increased gonadal androgens	2/13 (15%)	1/13 (8%)	6/13 (46%)	6/13 (46%)	0/11 (0%)	1/11	0/6 (0%)	1/6 (17%)
Proportion of NRs among those with increased gonadal androgens	2/2 (100%)	1/1 (100%)	5/6 (83%)	5/6 (83%)	-	0/1	-	1/1 (100%)

Androgen concentrations above normal reference intervals according to testicular volume are denoted as (1); normal concentrations are denoted as (0) (79). Missing values are denoted as (-), and (/) indicates that a testicular volume of  $\geq 15$  mL was not reached. (T = testosterone, DHT = dihydrotestosterone, NR = non-responder, R = responder.)

In paper II, AMH was within normal reference intervals in all responders (38-332 ng/mL, at < 11.5 years, 1-144 ng/mL at > 11.5 years of age). However, several low values were identified between 6 and 10 years of age, in three out of eight non-responders. From the age of 12 years, AMH was within normal reference intervals in all patients. At 12 years of age, non-responders had significantly lower AMH concentrations compared to responders. Inhibin B concentrations were within normal range (70) in all patients at 6 and 16 years of age. One responder had an inhibin B concentration slightly above normal reference intervals at 8 years of age, and between 10 and 14 years of age, occasional low inhibin B concentrations were observed in two responders and one non-responder. There were no significant differences in inhibin B concentrations between non-responders and responders at any age.

In paper IV, one boy with unilateral cryptorchidism had AMH below normal reference intervals at 5 to 10 years of age. Another two boys had AMH above normal reference intervals at 5 and 10 years of age, respectively. Inhibin B concentrations were below normal range in some (1–3) boys from 5 to 8 years (70) and above normal range in some (3–6) boys from 6 to 10 years of age.

#### **4.6.2 GONADAL ANDROGENS, AMH, AND INHIBIN B, BIRTH CHARACTERISTICS, AND ANTHROPOMETRY (PAPERS II AND IV)**

In this section, anthropometric data refers to weight SDS and WHtR.

In the SRS cohort in paper II, testosterone at 8 years of age, correlated strongly with birth weight in grams ( $r = 0.63$ ,  $p = 0.021$ ), and with gestational age ( $r = 0.56$ ,  $p = 0.046$ ). AMH at 10 years of age, correlated strongly with birth weight SDS ( $r = 0.64$ ,  $p = 0.018$ ). There were no other significant correlations between gonadal androgens or AMH at any other age, or inhibin B at any age, and birth characteristics.

In paper IV, there were no significant correlations between gonadal androgens and birth characteristics before 8 years of age in the preterm cohort. However, there were moderate inverse correlations between testosterone at 8 years of age and birth weight in grams and birth weight SDS. At 10 years of age, testosterone correlated negatively and moderately with gestational age, birth weight in grams and birth weight in SDS. No significant correlations were observed between DHT at any age and birth characteristics.

At 5 years of age, there was a moderate inverse correlation between AMH and gestational age and a weak negative correlation with birth weight in grams but not with birth weight SDS in the preterm cohort. At 7 years of age, AMH also correlated inversely and moderately with gestational age and birth weight in grams but not with birth weight SDS. No significant correlations were observed between AMH and birth characteristics at any other age or between inhibin B and gestational age or birth weight at any age.

There were no significant correlations between testosterone, DHT, AMH, or inhibin B and any anthropometric data at any age in the preterm boys.

### **4.6.3 GONADAL ANDROGENS, AMH, AND INHIBIN B, LINEAR GROWTH, AND ADULT HEIGHT (PAPERS II AND IV)**

In this section, adult height outcome refers to adult height SDS adjusted for MPH SDS.

In paper IV, there was a moderate inverse correlation between AMH and height SDS at 10 years of age. There were no significant correlations between AMH and height SDS at any other age or between gonadal androgens or inhibin B and height SDS at any age.

In paper II, we observed strong inverse correlations between testosterone from 10 to 14 years and adult height outcome and between DHT at 10 and 12 years of age and adult height outcome, Table 7 (next page). There was also a strong positive correlation between AMH at 12 years of age and adult height outcome. No other significant correlations were observed between gonadal androgens or AMH and adult height outcome at any other age or between inhibin B and adult height outcome at any age. Figure 12 (page 55) shows testosterone and DHT at 10 years of age, plotted against adult height outcome.

*Table 7: Significant correlations between testosterone, DHT, and AMH and adult height SDS adjusted for MPH SDS, and R<sup>2</sup> values in 13 boys with Silver–Russell syndrome at 10 to 14 years of age*

Androgen (age in years)	Spearman's rho ( <i>r</i> )	R <sup>2</sup>	<i>p</i> -value
Testosterone (10)	-0.94	0.88	<0.001
Testosterone (12)	-0.70	0.49	0.008
Testosterone (14)	-0.64	0.41	0.018
DHT (10)	-0.62	0.38	0.025
DHT (12)	-0.57	0.32	0.041
AMH (12)	0.62	0.38	0.025

A *p*-value of < 0.05 was considered significant. (DHT = dihydrotestosterone, AMH = Anti-Müllerian hormone, SDS = standard deviation score, MPH = midparental height.)

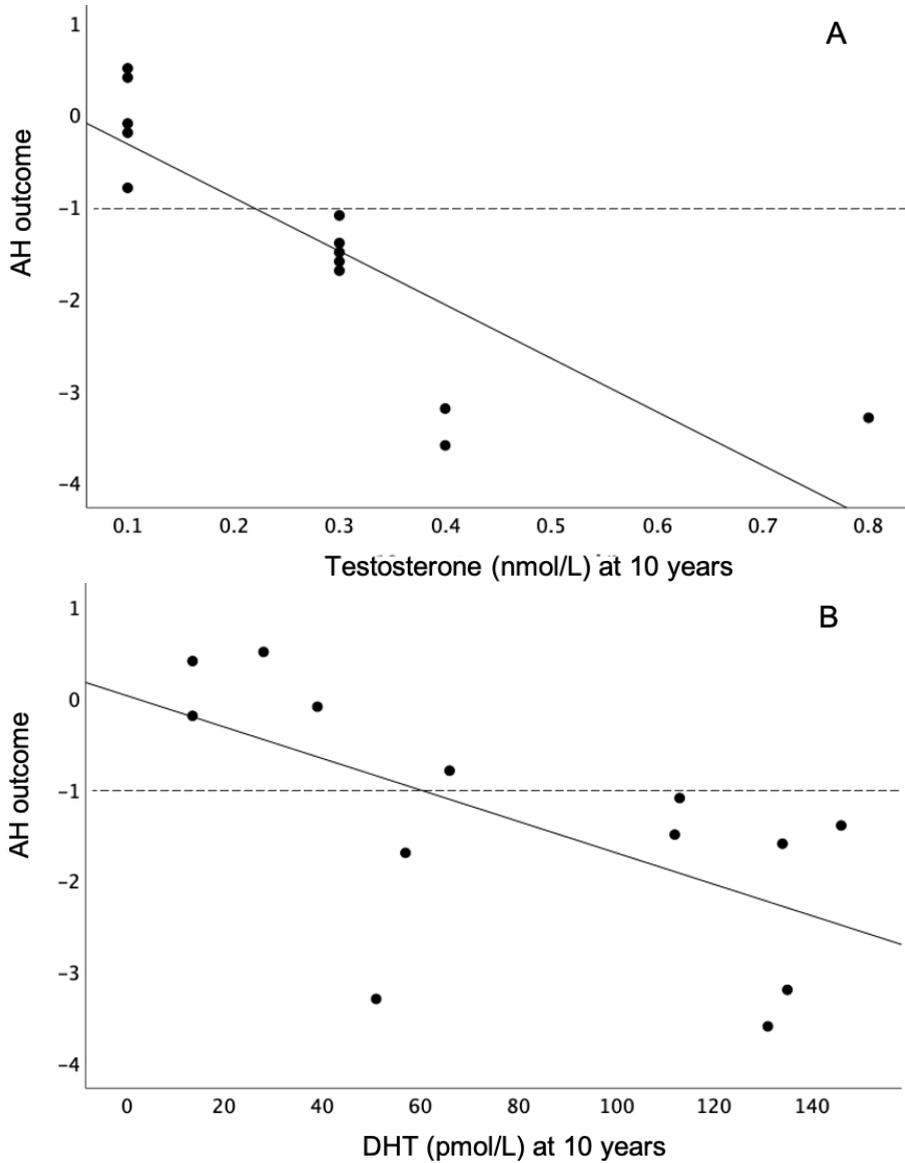


Figure 12: Scatterplots showing concentrations of testosterone (A) and dihydrotestosterone (DHT) (B) at 10 years of age, plotted against adult height standard deviation score (SDS) adjusted for MPH SDS (AH outcome), in 13 boys with Silver–Russell syndrome. A dotted line divides responders and non-responders. The solid line is the regression line.

## 4.7 ESTROGENS

### 4.7.1 ESTROGENS AND REFERENCE INTERVALS (PAPERS I AND IV)

In paper I, we observed increased  $E_2$  concentrations in several boys with SRS, compared to normal reference intervals (79), Table 8. In the presentation of these data, the two patients added to the study population in paper II are included. More samples at testicular volume of 3–6 mL (four boys), and 8–12 mL (one boy) are included when available, to account for  $E_2$  concentrations, not only at certain ages, but also according to normal reference intervals given in relation to testicular volume.

At 10 to 14 years of age, non-responders had significantly higher concentrations of  $E_2$ . Concentrations of  $E_1$  were not significantly higher in non-responders, however,  $E_2/E_1$  ratios were significantly higher in non-responders at 12 and 14 years of age. No other significant differences in estrogen concentrations were found between non-responders and responders at any other age.

In some cases,  $E_1$  exceeded normal reference intervals (79) at 8 and 10 years of age in the cohort of preterm boys. At 8 years of age, there was one boy and at 10 years of age four boys with a testicular volume of 2 mL who had elevated  $E_1$  concentrations. The  $E_2$  concentrations were within prepubertal reference intervals for all boys at both 8 and 10 years of age.

Table 8: Estradiol ( $E_2$ ) secretion in 13 boys with Silver–Russell syndrome, compared to normal reference intervals.

Testicular volume	1–2 mL	3–6 mL	8–12 mL	15–25 mL
Sex steroid	$E_2$ pmol/L	$E_2$ pmol/L	$E_2$ pmol/L	$E_2$ pmol/L
Normal reference intervals	<2–16	<2–23	3–80	23–105
Patient 1 (R)	0	0	0	1
Patient 2 (R)	0	0	0	1
Patient 3 (NR)	0	0	0	0
Patient 4 (R)	0	0	0	/
Patient 5 (NR)	0	0	0	/
Patient 6 (NR)	0	0	-	1
Patient 7 (R)	0	1	0	-
Patient 8 (NR)	0	1	1	/
Patient 9 (NR)	0	1	1	/
Patient 10 (R)	0	0	0	0
Patient 11 (NR)	0	1	0	1
Patient 12 (NR)	0	1	-	-
Patient 13 (NR)	0	1	0	0
Proportion of patients with increased estrogens	0/13 (0%)	6/13 (46%)	2/11 (18%)	4/7 (57%)
Proportion of NRs among those with increased $E_2$	-	5/6 (83%)	2/2 (100%)	2/4 (50%)

Estradiol ( $E_2$ ) concentrations above normal reference intervals according to testicular volume are denoted as (1); normal concentrations are denoted as (0) (79). Missing values are denoted as (-), and (/) indicates that a testicular volume of  $\geq 15$  mL was not reached. (NR = non-responder, R = responder.)

## **4.7.2 ESTROGENS, BIRTH CHARACTERISTICS, AND ANTHROPOMETRY (PAPERS I AND IV)**

In this section, anthropometric data refers to weight SDS and WHtR.

In the study population of 13 boys with SRS, the Pearson correlation coefficient was strongly negative between  $E_2$  at 14 years of age and birth weight SDS ( $r = -0.60$ ,  $p = 0.032$ ). No other significant correlations were observed between estrogens at any other age and birth characteristics.

In paper IV, we found no significant correlations between estrogens and birth characteristics before 8 years of age in the preterm cohort.  $E_1$  at 8 and 10 years of age correlated moderately and inversely with gestational age and birth weight in grams but not with birth weight SDS.  $E_2$  correlated only at 10 years of age with birth weight both in grams and SDS, and those correlations were strong and inverse.

There were no correlations between estrogens at 10 years of age and testicular volume. However,  $E_2$  at 10 years of age correlated with testosterone at the same age ( $r = 0.36$ ,  $p = 0.028$ ) but not with testosterone at 8 years of age.

$E_1$  correlated moderately with weight SDS at 7 and 8 years of age. However, the correlation at 8 years of age was of borderline significance ( $p = 0.050$ ). There were no significant correlations between  $E_1$  and WHtR at any age, and  $E_2$  did not correlate with any anthropometric data at any age.

## **4.7.3 ESTROGENS, LINEAR GROWTH, AND ADULT HEIGHT (PAPERS I AND IV)**

In this section, adult height outcome refers to adult height SDS adjusted for MPH SDS.

In paper IV,  $E_1$  concentrations correlated moderately with height SDS at 7 years of age. There were no other significant correlations between  $E_1$  and height SDS at any other age or between  $E_2$  and height SDS at any age.

At 6 years of age, no patient from the SRS cohort in paper I had a bone age of more than 7 years. However, three non-responders and three responders had a bone age of less than 5 years, ranging from 3.4 to 4.9 years. E<sub>1</sub> at 6 years of age correlated strongly with bone age at the same age and with bone age at 12 years of age, Table 9. There was a borderline significant correlation between E<sub>1</sub> at 10 years of age and bone age at 12 years of age, and a strong correlation between E<sub>1</sub> at 12 years and bone age at 12 years of age; however, the strong correlation between E<sub>2</sub> and bone age at this age was not statistically significant. No other significant correlations were found between estrogens and bone age at any other age or at pubertal onset.

*Table 9: Significant and borderline significant correlations between estrogens and bone age from 6 to 12 years in 11 boys with Silver–Russell syndrome*

Estrogens (years) vs. bone age (years)	Pearson correlation coefficient	R <sup>2</sup>	p-value
E <sub>1</sub> (6) vs. BA (6)	0.63	0.40	0.039
E <sub>1</sub> (6) vs. BA (12)	0.76	0.58	0.018
E <sub>1</sub> (10) vs BA (12)	0.67	0.45	0.050
E <sub>1</sub> (12) vs BA (12)	0.78	0.61	0.012
E <sub>2</sub> (12) vs BA (12)	0.66	0.44	0.053

A p-value of < 0.05 was considered significant. (E<sub>1</sub> = estrone, E<sub>2</sub> = estradiol, BA = bone age.)

When assessing the correlations between estrogen concentrations at different ages and adult height outcome, after adding data from the two boys described in paper II, there were strong inverse correlations between E<sub>2</sub> at 6 to 10 years and at 14 years of age and adult height outcome, Table 10 (next page). There were no significant correlations between E<sub>1</sub> at any age and adult height outcome. In Figure 13, E<sub>2</sub> concentrations at 10 years of age are plotted against adult height outcome.

Table 10: Correlations between  $E_2$  and adult height SDS adjusted for midparental height SDS, and  $R^2$  values in 13 boys with Silver–Russell syndrome at 6 to 16 years of age.

Estrogen (age in years)	Pearson correlation coefficient ( $r$ )	$R^2$	$p$ -values
$E_2$ (6)	-0.58	0.34	0.038
$E_2$ (8)	-0.62	0.38	0.025
$E_2$ (10)	-0.87	0.76	<0.001
$E_2$ (12)	-0.48	0.23	0.096 (ns)
$E_2$ (14)	-0.78	0.61	0.002
$E_2$ (16)	-0.30	0.01	0.41 (ns)

A  $p$ -value of  $< 0.05$  was considered significant. (SDS = standard deviation score,  $E_2$  = estradiol, ns = non-significant.)

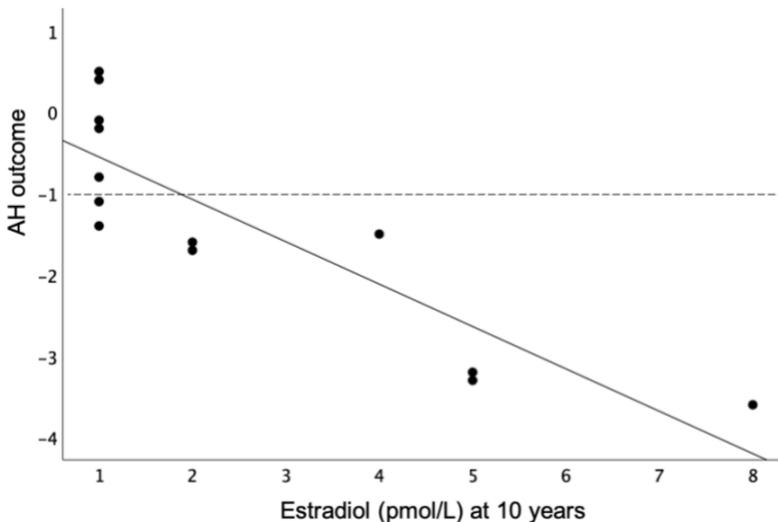


Figure 13: Scatterplot showing concentrations of estradiol at 10 years of age, plotted against adult height SDS adjusted for midparental height standard deviation score (SDS) (AH outcome), in 13 boys with Silver–Russell syndrome. A dotted line divides responders and non-responders. The solid line is the regression line.

## 5 DISCUSSION

### 5.1 MAIN FINDINGS

The main findings of this thesis were that birth weight, both in grams and in relation to gestational age, was inversely associated with concentrations of both testosterone at 8 and 10 years and E<sub>2</sub> at 10 years of age in preterm boys. In contrast, childhood adrenal androgen concentrations were associated with body weight and WHtR, but not with birth weight or gestational age, and the trajectories of adrenal concentrations were established at 5 years of age in preterm boys. Prepubertal and pubertal sex steroid concentrations were inversely correlated with adult height and both higher levels of adrenal androgens and earlier increase in gonadal androgens and E<sub>2</sub> were associated with shorter adult height in boys with SRS.

### 5.2 ADRENAL ANDROGENS

In the cohort of preterm boys, we demonstrated that the trajectories of adrenal secretion were stable, from 5 years of age, especially for DHEAS. This finding is in line with a recent report describing a strong correlation between DHEAS at 1 and 6 years of age (17). The implication of these results is that preterm boys at risk of high DHEAS secretion during childhood, may be identified already from an early age.

A relationship between birth weight, weight gain, body composition and DHEAS secretion in childhood has previously been proposed. Studies of children born SGA and AGA have reported a negative relationship between DHEAS concentrations in childhood and adolescence and gestational age, birth weight and birth length (25, 26, 154). However, in a large study of DHEAS concentrations in children with normal gestational age and birth weight, those with higher DHEAS were not found to have different gestational age or birth weight than those with lower DHEAS concentrations (209). In the SRS cohort, we found that those who did not reach adult height in parity with MPH (non-responders) had higher DHEAS at 10 years and higher A<sub>4</sub> at 10 and 12 years of age, compared to those who reached adult height in parity with MPH (responders). However, there was no significant association between adrenal androgens at any age and birth weight in the SRS cohort or in the preterm cohort, suggesting that birth weight is not important when predicting childhood DHEAS concentrations. In contrast, another study of children born SGA, with a cohort comparable in size to our cohort of preterm boys, found DHEAS concentrations in adolescence to correlate with birth weight SDS (26).

Our cohort of preterm boys was younger when DHEAS concentrations were analyzed, and most of the boys in our cohort were born AGA, which might account for the discrepancy between the findings. Furthermore, in the SGA cohort, weight gain but not birth weight per se, was identified as a predictor for adolescent levels of DHEAS (26), emphasizing the significance of weight gain and body composition as factors affecting DHEAS secretion. The relationship between body composition and adrenal androgen concentrations has also been confirmed by large cross-sectional studies (25, 209). In the SRS cohort, weight gain from birth to pubertal onset was greater in non-responders who also had higher DHEAS at 10 years of age, compared to responders. In the preterm cohort, weight SDS increased from birth to 5 years and from 5 to 10 years of age, and at 10 years of age, DHEAS correlated strongly with weight gain from birth. Notable was also the unexpected increase in WHtR from 5 to 10 years of age in preterm boys with a ratio above 0.5 at 5 years of age, indicating deposition of visceral fat mass in these boys (115), as a decrease in WHtR was expected (120). Children with low birth weight are susceptible to increased amounts of visceral fat, which has been shown in children born SGA with catch-up growth who have been found to be at greater risk of visceral fat deposition, even when overweight is not present (156, 158). We found that both DHEAS and A<sub>4</sub> were positively associated with weight and WHtR when analyzed cross-sectionally and longitudinally. Altogether, the results of this thesis and other studies suggest that DHEAS secretion during childhood and adolescence is related to weight gain and body composition rather than birth weight per se.

There is a discrepancy in data published regarding the risk of developing premature pubarche after being born SGA (160-163). However, both prematurity, and being overweight have been reported to be independent risk factors for precocious pubarche (18, 161). Children with SRS have also been found to be at risk of premature adrenarche (19). In the SRS cohort, clinical signs of adrenarche before the age of 9 years was only identified in one boy. However, at a testicular volume of 1–2 mL, a majority of boys with SRS had DHEAS concentrations above normal reference intervals (207). Of those, 71% did not reach their expected adult height. In early puberty, at a testicular volume of 3–6 mL, 31% of the cohort had increased DHEAS, and 75% of these did not reach their expected adult height. Even though there were some boys in the preterm cohort with higher adrenal androgen concentrations than expected at 8 and 10 years of age, none of them developed pubarche before the age of 10 years. An explanation for the lack of pubarche despite increased DHEAS concentrations may be the function of the androgen receptor in the hair follicles. Not only the androgen levels but also sensitivity of the androgen receptor, based on the degree of androgen receptor methylation, have been shown to be important for the development of pubarche (85). The findings

of this thesis suggest that pubarche is an uncertain sign of both the timing of DHEAS increase and the magnitude of DHEAS concentrations, at least in these two cohorts of Scandinavian boys.

Adrenal androgens naturally increase from 5 years of age, and the increase becomes more apparent from 7 to 8 years of age, culminating at late puberty (1, 7). At 7 and 10 years of age, we reported a positive correlation between DHEAS and height at the same age, in the preterm cohort. This observation is confirmed by results from large cross-sectional studies showing that higher DHEAS concentrations at 7 years of age are associated with greater height at the same age, in children born at term and with normal birth weight (209). In children with premature adrenarche, increased growth velocity and advanced bone age have been reported (24), also suggesting that there is a relationship between DHEAS concentrations, bone age, and height development. The timing of adrenarche has, however, not been found to affect adult height, either in healthy children with premature adrenarche, or in children with SRS (19, 24). At the same time, we found strong negative correlations between DHEAS at 8 to 12 years of age and adult height in boys with SRS, suggesting that those with a more pronounced DHEAS secretion at the age of 8 to 12 years, when a natural increase is expected, are at risk of early bone maturation. To summarize, the magnitude of DHEAS concentrations, rather than the timing of exposure, seems to be of significance for adult height outcome.

### 5.3 GONADAL ANDROGENS

In the boys with SRS, testosterone at 8 years of age correlated positively with both gestational age and birth weight. In the preterm cohort, we found negative correlations between both testosterone at 8 and 10 years of age and birth weight and testosterone at 10 years of age and gestational age. These conflicting findings underline the diversity of the two cohorts and imply that findings from the SRS cohort do not necessarily apply to children without SRS. One explanation for the discrepancy may be that the boys in the SRS cohort had a different sex steroid profile, for instance due to a difference in aromatase activity, compared to the boys in the preterm cohort, but this assumption is speculative.

Although no boys in either of the cohorts met the clinical criteria for precocious puberty defined by a testicular volume of  $> 3$  mL before 9 years of age (40), there were some findings indicating earlier or more intense HPG activation in both cohorts. In the SRS cohort, those who did not reach their expected adult height had higher LH, testosterone, and DHT, lower AMH, and larger testicular volume at 12 years of age, indicating an earlier start or a faster

progression through puberty. In the preterm cohort, LH at 10 years of age correlated negatively with birth weight and there was a strong correlation between testosterone and LH, but not with testicular volume at the same age. An association between birth weight and pubertal development has been implied by other studies. During mini-puberty, increased postnatal HPG activity has been reported in boys born SGA, compared to boys with normal birth weight (41). Other studies have reported pubertal onset within the normal range, but at an earlier age and with a faster tempo, both in short children born SGA and in children with SRS (127, 134, 163, 166). Our findings suggest that boys with SRS and shorter adult height had earlier activation of the HPG axis despite the absence of clinical signs of puberty, compared to those who reached their expected adult height. Findings from the preterm cohort imply that the association between birth weight and regulation of the HPG axis may also be present in children with low birth weight due to prematurity, but this needs to be confirmed in studies on larger cohorts.

The negative correlations observed in the preterm cohort between AMH and gestational age and between AMH and birth weight, were significant at 5 and 7 years of age. One study has reported higher AMH in normal stature prepubertal boys born SGA compared to short prepubertal boys born SGA, but there were no differences when comparing the two SGA groups with a group of boys born AGA (210). Sertoli cell dysfunction has previously been suggested in some boys with SRS (198). Prepubertal AMH concentrations below the normal reference intervals (70) were observed in two boys in the SRS cohort, who later developed an adult testicular volume of < 15 mL. This finding might reflect a smaller number of Sertoli cells, in line with reported lower concentrations of AMH in boys with cryptorchidism (65) and which was observed in one of the four preterm boys with unilateral cryptorchidism. Since germ cells constitute the larger part of the adult testicular volume (63), and the number of germ cells is dependent on the number of mature Sertoli cells (52), a smaller number of Sertoli cells could explain the combination of prepubertal low AMH concentrations and small adult testicular volumes in some of the boys with SRS. Sertoli cell dysfunction might also explain the reported findings of reduced testicular volumes and risk of infertility in adult men born SGA (73, 167, 169, 170). However, whether Sertoli cell dysfunction is associated with low birth weight needs to be explored in larger studies (171).

In the cohort of preterm boys, we did not observe a relationship between gonadal sex steroids from 5 to 10 years of age and linear growth. However, in the SRS cohort, there were strong negative correlations between testosterone at 10 to 14 years and DHT at 10 and 12 years of age and adult height, regardless of prepubertal status, in all except one boy at 10 years of age. This finding suggests that the timing of gonadal androgen exposure is of significance for

adult height outcome. Furthermore, the findings emphasize the need to determine gonadal sex steroids using a sensitive method in boys with SRS when evaluating linear growth and predicting adult height. Gonadal androgens at 10 years of age might be an early marker of HPG activation even if clinical signs of puberty are absent. Relying on testicular volume when assessing pubertal development in boys with SRS may be misleading, with a risk of underestimation, considering the large number of boys with unexpectedly high testosterone concentrations at testicular volumes of 3–6 mL and the smaller adult testicular volumes observed in several of these boys.

## 5.4 ESTROGENS

Henry Silver described signs of estrogen exposure during childhood in prepubertal boys with SRS as early as 1964 (183). Since then, there have been no studies on estrogen exposure in boys with SRS, probably due to the methodological difficulties associated with determining estrogens at low concentrations in children. After almost 60 years, this thesis continues the search for the effects of estrogen exposure in boys with SRS and preterm boys during childhood.

Almost half of the boys in the SRS cohort had unexpectedly high  $E_2$  concentrations at testicular volumes of 3–6 mL, which might reflect underestimation of the testicular size, as already mentioned. High  $E_2$  concentrations were more common in the group with an unexpectedly poor adult height outcome. However,  $E_2$  correlated negatively with birth weight only at 14 years of age, possibly because all the boys in this cohort had pronounced low birth weight as part of the syndrome. At 10 years of age, there was no significant correlation between either  $E_1$  or  $E_2$  and testicular volume in the preterm cohort. However, there was a significant correlation between  $E_2$  and testosterone at this age. It is worth noting that, until 10 years of age, we observed low  $E_2$  concentrations in both cohorts, and it remains unknown whether the pattern of  $E_2$  synthesis during puberty in the preterm cohort resembles the pattern found in non-responders, in the SRS cohort. A relationship between  $E_2$  concentrations and birth weight is suggested by the strong negative correlation between  $E_2$  concentrations at 10 years of age and birth weight, both in grams and birth weight SDS, found in the preterm boys. Another important observation is the finding of unexpectedly high  $E_2$  concentrations in both subgroups of the SRS cohort at adult testicular volume, which reflects the unexpectedly high  $E_2$  concentrations reported in a study of adult men born SGA (211), indicating that males born with low birth weight are at risk of disturbed synthesis of  $E_2$ .

The negative correlation between  $E_1$  at 8 and 10 years of age and both gestational age and birth weight in the preterm cohort, as well as the negative correlation between  $E_2$  at 10 years of age and birth weight in the same cohort, suggests that the prepubertal aromatase activity converting androgens to estrogens may be higher in children with low birth weight.  $E_1$ , which is the dominant circulating estrogen before pubertal onset (79), correlated with current weight at 7 years of age in the preterm cohort, possibly indicating more adipose tissue and increased aromatase capacity in those who were heavier. Contradicting this assumption is the lack of significant correlations between  $E_1$  and WHtR. However, another finding that indirectly implies that those with higher  $E_1$  had more visceral fat was the correlation between  $E_1$  and the 2-hour post-load blood glucose in the OGTT at 5 years of age, since a higher glucose outcome indicates insulin resistance, which is related to the amount of visceral fat (163). The lack of any significant correlation between  $E_2$  and weight is, however, in line with data showing that adult men with obesity have higher  $E_1$  but unaffected  $E_2$  compared to normal-weight men (212), suggesting that the amount of adipose tissue is more related to the conversion rate of  $A_4$  to  $E_1$  than the conversion rate of testosterone to  $E_2$ . The higher  $E_2/E_1$  ratio found in those with shorter adult height in the SRS cohort, suggests that the rate of  $E_1$  to  $E_2$  conversion might be another factor potentially affecting both the  $E_1$  and the  $E_2$  concentrations. Together, these findings suggest that  $E_1$  might be an early indicator of the degree of aromatase activity and estrogen exposure.

In the preterm cohort,  $E_1$  correlated with height at 7 years of age. This finding may indicate a stimulatory effect on linear growth from estrogen exposure (94). Supporting this hypothesis was the finding of consistent correlations between  $E_1$  and bone age at 6 and 12 years of age in the SRS cohort. Boys with SRS and impaired adult height outcome also had significantly higher  $E_2$  concentrations from 10 to 14 years of age compared to those who reached their expected adult height. There were also strong correlations between  $E_2$  from 6 to 14 years of age and adult height, but not between  $E_1$  at any age and adult height. Furthermore, there were no significant correlations between  $E_2$  and height SDS at any age, but those with higher  $E_2$  concentrations from 10 years of age and poor adult height outcome, had greater height gain from birth to pubertal onset, and less pubertal height gain, compared to the responders. These findings indicate that the timing and perhaps the duration of estrogen exposure are significant for the effects on bone maturation, and it is possible that prepubertal estrogen exposure causes bone maturation, affecting the child's growth potential during puberty and adult height outcome. This assumption is in line with observations of pubertal growth in cohorts of children born SGA: they report increased growth velocity before pubertal onset, peak height velocity at earlier pubertal stage, shorter duration of pubertal growth, less pubertal growth spurt, rapid bone maturation through puberty, and

shorter adult height compared to children born AGA (163-166). In healthy children, however, the magnitude of the pubertal growth spurt and peak height velocity vary negatively with age at pubertal onset (105), indicating that chronological age and bone age are comparable before pubertal onset in these children. The results of this thesis and other studies suggest that children with low birth weight are at risk of a more advanced bone age compared to their chronological age at pubertal onset, possibly reducing their linear growth potential during puberty.

## 5.5 BIRTH CHARACTERISTICS

We have studied patterns of sex steroid secretion and growth in two different cohorts with low birth weight in grams. In the 11 boys studied in paper I, we observed a lower birth weight in those who did not reach their expected adult height; however, when adding birth weight data from the two additional boys in paper II, the difference was no longer significant. The results from the preterm cohort imply that birth weight is related to the timing of the increase in gonadal androgen and E<sub>2</sub> concentrations. It is a paradox that not all individuals with pronounced low birth weight, as in the SRS cohort, present with an early increase in sex steroids. Although hypothetical, this might be due to the diverse etiology causing low birth weight in the two cohorts. In patients with SRS, epigenetic changes are identified in many but not all patients who meet the clinical criteria for the syndrome (127, 187-189). SRS seems to be caused by epigenetic dysregulation per se and not necessarily in response to external stimuli. In contrast, the preterm cohort tended to have low birth weight primarily caused by preterm birth, meaning that the majority were born AGA. The etiology of preterm birth is also heterogeneous, ranging from placental insufficiency, potentially affecting the fetus for a longer time, and causing IUGR, to infections, only causing fetal exposure to stress for a shorter period (172, 173).

According to the fetal programming theory, intrauterine exposure to starvation may cause low birth weight and permanently affect regulation of both adrenal and gonadal sex steroids through epigenetic mechanisms (15, 25, 137). In Sweden, undernutrition is uncommon but placental insufficiency may affect the fetal supply of nutrients, causing fetal growth impairment and pathological exposure to sex steroids during the masculinization programming window, resulting in a risk of developing hypospadias and of preterm delivery (45, 46, 48). Being born preterm also results in exposure to stress and entails a potential risk of being exposed to EUGR, which might result in impaired childhood growth (176) and possibly affect hormonal axis in line with the fetal programming theory. Altogether, in the preterm cohort, most boys had low

birth weight due to prematurity, and although the etiology of the prematurity probably was heterogeneous, the cohort was essentially different from the SRS cohort because the preterm boys were mostly born AGA, whereas the SRS cohort were born SGA, and this difference may be important for the programming of the HPA and HPG axis.

## 5.6 STRENGTHS

One major strength of this thesis was the use of the sensitive mass spectrometry-based methods, enabling the analysis of sex steroids at low concentrations and providing several hormone concentrations with high specificity, in the same sample. Another important strength was the longitudinal single-center design of all four studies. An additional advantage was the limited number of skilled pediatric nurses collecting data. The restriction of the study populations to one gender was also a strength in all four papers.

Additionally, a strength in papers I and II was the limited number of dropouts, and in papers III and IV, the prospective study design was an advantage.

## 5.7 LIMITATIONS

A weakness in all the studies was the limited size of the two cohorts. Another common limitation in all four papers was the lack of aged-matched control groups.

Additionally, a limitation in papers I and II was the retrospective study design and in papers III and IV, the number of boys lost to follow-up was a disadvantage.

## 6 CONCLUSION

In conclusion, we have identified relationships between birth weight, patterns of sex steroid secretion during childhood, and growth patterns both in boys with SRS and in preterm boys without SRS.

The results of paper III and IV established a negative association between birth weight and levels of both testosterone and  $E_1$  at 8 to 10 years, and  $E_2$  at 10 years of age in preterm boys while no significant associations were found between birth weight and adrenal androgens.

The results of papers III and IV showed that weight gain, greater body weight and higher WHtR were associated with higher concentrations of adrenal androgens from 7 years of age, but not with gonadal androgens or estrogens in preterm boys. The trajectories of adrenal androgen secretion were established at 5 years of age.

In paper II, we showed that levels of adrenal androgens and gonadal androgens above normal reference intervals were common in boys with SRS and that those with short adult height had higher androgen concentrations from 10 to 14 years of age. A greater magnitude of childhood adrenal androgen secretion and earlier timing of the increase in gonadal androgen concentrations were related to shorter adult height outcome.

In paper I, we demonstrated that  $E_2$  levels above normal reference intervals were common in boys with SRS and that those with short adult height had higher  $E_2$  concentrations from 10 to 14 years of age. Earlier timing of the increase in  $E_2$  concentrations was related to short adult height outcome. We also showed that boys with SRS are at risk of developing low adult testicular volume.

The implications of these findings are:

Boys with low birth weight may be at risk of an earlier increase in testosterone and estrogen concentrations which might cause a faster bone maturation resulting in an impaired pubertal growth spurt and shorter adult height.

Boys with increasing weight SDS, greater body weight, and increased amount of visceral fat mass may be at risk of increased childhood adrenal androgen concentrations and boys with risk of higher adrenal androgen secretion may be identified before adrenarche.

A sensitive method for the evaluation of the level of sex steroids in boys with SRS is needed, to avoid relying on testicular size when assessing pubertal development.

This thesis shows that estrogens which are traditionally considered to be female hormones, also play an important role in males, both during childhood and puberty.

Prospective studies with appropriate control groups and longer follow-up periods are needed to establish whether boys and girls with lower birth weight but without prematurity or SRS, are at risk of higher pubertal levels of sex steroids and whether the levels of prepubertal sex steroids influence the pubertal growth spurt and adult height.

## 7 FUTURE PERSPECTIVES

This thesis has answered some questions but also raised several new ones. The unanswered questions are interesting topics for further research:

- What is the pattern of sex steroid secretion during puberty, and how does sex steroid secretion during puberty relate to birth characteristics in preterm boys?
- What is the pubertal height gain and adult height outcome in the preterm cohort?
- How does sex steroid secretion relate to birth weight and growth patterns in children born at term?

Other related areas which need to be explored:

- What is the relationship between sex steroid concentrations in girls and birth weight, gestational age, and growth patterns?
- Are there implications of an early rise in sex steroids on metabolic health?
- Considering the complexity of sex steroid regulation, what are the implications of hormonal treatment of adolescents with gender dysphoria?

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