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Tanja Kuiri-Hänninen

Postnatal Hypothalamic-Pituitary-Gonadal Axis Activation (i.e., Minipuberty) in Full-term and Preterm Infants

Longitudinal Assessment of Hormone Levels and Target Tissue Effects

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ABSTRACT:

Shortly after birth, the hypothalamic-pituitary-gonadal (HPG) axis transiently activates, and gonadal hormone levels increase to adult levels. This activity peaks during the first months of life and then decreases towards the age of six months. After this, the HPG axis remains silenced until the onset of puberty. The mechanisms and significance of this minipuberty remain poorly understood. So far, minipuberty has been better described in boys than in girls, and it is considered to have a role in normal male reproductive development. Prematurity has been associated with increased hormonal activity in minipuberty, but the underlying mechanism and biological significance of this is unclear.

The aim of this work was to evaluate and compare the reproductive hormone levels in urine (i.e., luteinizing hormone, follicle-stimulating hormone (FSH), testosterone, estradiol, and prostate-specific antigen (PSA)) and serum (i.e., anti-Müllerian hormone, (AMH)) and their effects in target tissues between full-term (n=58) and preterm infants (n=67) in a prospective, longitudinal setting. Hormone levels and clinical findings were determined monthly from one week to six months of age. The majority of the infants (n=99, 79%) were re-evaluated at the corrected age of 14 months. The results were analyzed both according to calendar and postmenstrual age to account for the immaturity of the preterm infants.

Gonadotropin and testosterone levels were significantly higher, and the target tissue effects (i.e., testicular and penile growth) were significantly faster in preterm than in full-term boys during the minipuberty. The peak LH and testosterone levels were observed at one month of age in both full-term and preterm boys, which is earlier than previously reported. The levels of PSA transiently increased, indicating androgen effects in the prostate. In both sexes, androgen-dependent cutaneous manifestations, sebaceous gland hypertrophy, and acne were observed during minipuberty.

In preterm girls, FSH levels were extremely high after birth, but they decreased to similar levels as in full-term girls around the expected date of delivery. This decrease was associated with the maturation of antral follicles in ovarian ultrasonography and increase of the follicle-derived AMH levels. Estradiol levels in girls were higher than in boys, but the levels varied and did not show a similar peak as testosterone levels in boys. Estrogen target tissues (i.e., mammary glands and uterus) were stimulated in full-term girls at birth by the high intrauterine estrogen levels, and no further growth was observed. In preterm girls, the size of the mammary glands and the uterus were positively correlated with estradiol levels.

In conclusion, the postnatal activity of the HPG axis was associated with target tissue effects in both sexes. Maturational factors affected both the hormone levels and the biological effects in the target tissues. The possible long-term significance of the observed differences in minipuberty between full-term and preterm infants requires further studies.

National Library of Medicine Classification: WS 410, WS 420, WK 501, WK 900
Medical Subject Headings: Anti-Mullerian Hormone; Estradiol; Follicle Stimulating Hormone; Gonads; Hypothalamo-Hypophyseal System; Infant, Premature; Luteinizing Hormone; Prostate-Specific Antigen; Sexual Development; Testosterone; Longitudinal Studies

Kuiri-Hänninen, Tanja

Imeväisiän hypotalamus-aivolisäke-sukurauhanen-akselin aktivaatio eli minipuberteetti täysiaikaisina ja keskosina syntyneillä lapsilla: Hormonitasot ja kohdekudosvaikutukset pitkittäisasetelmassa.

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TIIVISTELMÄ:

Pian syntymän jälkeen hypotalamus-aivolisäke-sukurauhanen (HPG)-akseli aktivoituu ja sukupuolihormonien tasot nousevat hetkellisesti aikuisen tasoja vastaaviksi. Aktiivisuus on voimakkainta ensimmäisinä elinkuukausina ja hiipuu kohti puolen vuoden ikää, minkä jälkeen HPG-akselin toiminta on vaimeaa murrosiän alkuun saakka. Tämän minipuberteetiksi kutsutun ilmiön mekanismit sekä merkitys tunnetaan puutteellisesti. Minipuberteetti on kuvattu paremmin pojilla kuin tytöillä ja sillä ajatellaan olevan merkitystä normaaliin miehen lisääntymisterveyteen johtavassa kehityksessä. Keskoslapsilla on raportoitu korkeampia sukupuolihormonitasoja minipuberteetissa kuin täysiaikaisena syntyneillä lapsilla, mutta tämän syytä tai merkitystä ei tiedetä.

Tämän työn tavoitteena oli kuvata ja verrata minipuberteetin aikaisia sukupuolihormonitasoja virtsassa (luteinisoiva hormoni (LH), follikkelia stimuloiva hormoni (FSH), testosteroni, estradioli, prostata-spesifinen antigeeni (PSA)) ja seerumissa (anti-Müllerin hormoni (AMH)) sekä kliinisiä ilmentymiä kohdekudoksissa täysiaikaisten (n=58) ja keskoslasten (n=67) välillä pitkittäisaineistossa. Hormonitasoja sekä niiden kohdekudosvaikutuksia määritettiin kuukausittain viikon iästä puolen vuoden ikään saakka. Suurin osa lapsista (n=99, 79 %) tutkittiin uudelleen 14 kuukauden korjatussa iässä. Tulokset analysoitiin sekä kalenteri- että kehitysiän mukaan.

Keskospojilla todettiin täysiaikaisena syntyneitä poikia korkeammat gonadotropiini- ja testosteronitasot ja myös kohdekudosvaikutukset eli kivesten ja peniksen kasvu oli heillä nopeampaa. LH- ja testosteronitasojen huippu nähtiin kuukauden iässä sekä täysiaikaisilla että keskospojilla, eli varhaisemmin kuin aiemmissa tutkimuksissa on todettu. Pojilla androgeenivaikutus näkyi myös PSA-tasojen nousuna. Sekä tyttö- että poikavauvoilla havaittiin androgeeniriippuvaisia ihomuutoksia eli talirauhasten kasvua ja aknea.

Keskostyttöjen FSH-tasot nousivat hyvin korkeiksi syntymän jälkeen, mutta laskivat täysi-aikaisten tyttöjen tasolle lasketun ajan vaiheilla. Samanaikaisesti havaittiin ultraäänitutkimuksissa munarakkuloiden kasvua ja myös munarakkulaperäisen AMH:n tasot nousivat. Tyttöjen estradiolitasot olivat korkeampia kuin pojilla, mutta tasoissa esiintyi vaihtelua eikä vastaavanlaista huippua kuin poikien testosteronitasoissa havaittu. Täysiaikaisilla tytöillä estrogeenien kohdekudokset eli kohtu ja rintarauhaset olivat stimuloituneet ennen syntymää eikä kasvua havaittu enää syntymän jälkeen. Keskostytöillä rintarauhasen ja kohdun koko korreloivat positiivisesti estradiolitasojen kanssa.

Yhteenvetona voidaan todeta, että minipuberteetilla on vaikutuksia sukupuolihormonien kohdekudoksissa kummallakin sukupuolella. Kehitysikä vaikutti sekä hormonien tasoihin että kohdekudosvaikutuksiin. Täysiaikaisten ja keskoslasten erilaisen minipuberteetin mahdolliset pitkäaikaisvaikutukset vaativat lisätutkimuksia.

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Kuopio, May 2015

Tanja Kuiri-Hänninen

List of the original publications

This dissertation is based on the following original publications:

I Kuiri-Hänninen T, Seuri R, Tyrväinen E, Turpeinen U, Hämäläinen E, Stenman UH, Dunkel L and Sankilampi U. Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. *J Clin Endocrinol Metab 2011;96:98-105*

II Kuiri-Hänninen T*, Kallio S*, Seuri R, Tyrväinen E, Liakka A, Tapanainen J, Sankilampi U and Dunkel L. Postnatal developmental changes in the pituitary-ovarian axis in preterm and term infant girls. *J Clin Endocrinol Metab 2011;96:3432-9*

III Kuiri-Hänninen T, Haanpää M, Turpeinen U, Hämäläinen E, Dunkel L and Sankilampi U. Transient Postnatal Secretion of Androgen Hormones Is Associated with Acne and Sebaceous Gland Hypertrophy in Early Infancy. *J Clin Endocrinol Metab. 2013;98:199-206*

IV Kuiri-Hänninen T, Haanpää M, Turpeinen U, Hämäläinen E, Seuri R, Tyrväinen E, Sankilampi U and Dunkel L. Postnatal ovarian activation has biological estrogen effects in infant girls. *J Clin Endocrinol Metab. 2013;98:4709-16*

*Shared first authorship

Contents

Abbreviations

AGA	Appropriate for gestational age
AMH	Anti-Müllerian hormone
AUC	Area under the curve
BPD	Bronchopulmonary dysplasia
CHH	Congenital hypogonadotropic hypogonadism
cM14	Corrected age of 14 months
CV	Coefficient of variation
D7	Day seven
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
HPG axis	Hypothalamic-pituitary-gonadal axis
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry method
HSD	Hydroxysteroid dehydrogenase
INSL3	Insulin-like factor-3
IVH	Intraventricular hemorrhage
LGA	Large for gestational age
LH	Luteinizing hormone
LOQ	Limit of quantification
M1(–M6)	Month one (to six)
MGD	Mammary gland diameter
NEC	Necrotizing enterocolitis
PDA	Patent ductus arteriosus
PSA	Prostate-specific antigen
RDS	Respiratory distress syndrome
SD	Standard deviation
SGA	Small for gestational age
SGH	Sebaceous gland hypertrophy
SHBG	Sex-hormone binding globulin
TTN	Transient tachypnea of neonatorum
UGT	Uridine diphosphate glucuronyltransferase

1 Introduction

Puberty is preceded by two periods of activity of the hypothalamic-pituitary-gonadal (HPG) axis in early life. The first period of activity occurs during fetal life and is strongest at the midgestation; it then decreases towards term due to suppressive effects of the placental hormones, especially estrogens (Takagi et al. 1977, Debieve et al. 2000). By the time of birth, the HPG axis is silenced. When the placental restraint is removed at delivery, the second period of activity commences soon after birth. This postnatal activity of the HPG axis, also referred to as minipuberty, peaks during the first months of life and then diminishes towards the age of six months (Forest et al. 1974, Andersson et al. 1998). After this, the HPG axis remains quiescent for the childhood years until it is reawakened at the onset of puberty.

Minipuberty was first described in humans already 40 years ago (Forest et al. 1973a, Faiman & Winter 1971); however, underlying mechanisms and its biological significance remain still incompletely understood. In infant boys, increase in gonadotropin levels results in activation and proliferation of testicular Leydig and Sertoli cells and increase in testicular volume (Main et al. 2006). Proliferation of Sertoli cells at this time might be important for subsequent sperm production in adulthood (Sharpe et al. 2003). Following the luteinizing hormone (LH) surge, testosterone levels transiently increase to pubertal values in 1-3 month old boys (Forest et al. 1974). These high levels might have a role in the development of male genitalia, since a positive correlation has been found between penile growth and testosterone levels at three months of age (Boas et al. 2006) and genital involution has been reported in infant boys with hypogonadotropic hypogonadism who lack the surge (Main et al. 2000). In addition, androgens secreted at this phase may play a role in priming target tissues for subsequent growth and maturation as well as programming brain functions, such as initializing feedback loops and influencing behavioural traits (Hines 2008, Sharpe 2006, Mann & Fraser 1996). On the other hand, the biological significance of high testosterone levels has also been questioned, since sex-hormone binding globulin (SHBG) levels rise concomitantly, leading to a low level of free testosterone that is considered biologically active (Bolton et al. 1989).

Minipuberty and its possible consequences in girls are even more obscure than in boys. The pattern of the postnatal gonadotropin surge differs between girls and boys: in boys LH levels predominate, whereas in girls follicle-stimulating hormone (FSH) levels are higher than LH levels and may remain elevated until 2-3 years of age before declining to prepubertal levels (Andersson et al. 1998, Penny et al. 1974). Unlike the uniformly elevated testosterone and inhibin B levels in infant boys, levels of ovarian hormones estradiol and inhibin B in infant girls have been heterogeneous and ranged from unmeasurable to high (Chellakooty et al. 2003). The role of postnatal HPG axis activation in female reproductive development is currently not understood.

Preterm birth, defined as birth before the completion of 37 weeks of pregnancy, has been associated with increased and prolonged postnatal gonadotropin and sex steroid secretion compared with full-term infants, although the differences between preterm and full-term infants have not been studied in a longitudinal setting (Greaves et al. 2008b, Tapanainen et al. 1981b, Shinkawa et al. 1983, Forest et al. 1980, Chellakooty et al. 2003). The reason for this increased activity is unknown, but immaturity of the hypothalamic feedback mechanisms has been suggested. Today, 5-10% of all newborns are born prematurely and babies born as early as 23-26 weeks of gestation survive. Little is known about the consequences of such extreme prematurity on reproductive development. In addition to possibly altered minipuberty, premature infants lack the high intrauterine estrogen levels of the last trimester in utero. The possible biological effects and consequences of increased HPG axis activation in premature infants compared with full-term infants have not been studied so far.

Consequently, although minipuberty is a physiological phase in human development and has been recognized for 40 years, there are still open questions on hormonal activity during minipuberty, and its biological role and consequences. Especially in females, even the physiological changes in hormonal levels during infancy are still poorly defined, not to mention their biological effects. Previous studies on minipuberty have been mainly cross-sectional, but because of the dynamic changes in hormone levels during the first months of life, longitudinal hormone profiles would provide a more reliable picture of the hormonal activity. The general aim of this thesis was to characterize and compare the postnatal HPG axis activation and its biological effects in full-term and premature boys and girls in a prospective, longitudinal setting. This study also serves as a pilot study for future studies on the possible life-long role of the first postnatal activation of HPG axis, i.e., minipuberty. These studies might include investigations on the effects on reproductive development, childhood growth, bone mineralization, childhood psychosexual development, timing of puberty, sexual behaviour, and reproductive capacity in adulthood.

2 Review of the literature

2.1 OVERVIEW OF THE HPG AXIS

The hypothalamus, pituitary, and gonads form a functional unit, the HPG axis, which controls the reproductive functions in both sexes. Activity of the HPG axis changes across the life-span: robust activity in the mid-gestational fetus is dampened by the time of birth, reactivated for the first postnatal months, silenced for the childhood years, and then gradually increased again marking the onset of puberty (Figure 1). After the reproductive years, activity decreases again during old age. Besides controlling the development and maturation of the gonads, secondary sexual characteristics, sex-typed behaviour, and fertility, HPG axis activity is also associated with linear growth, body composition, bone mineral density, and metabolism.

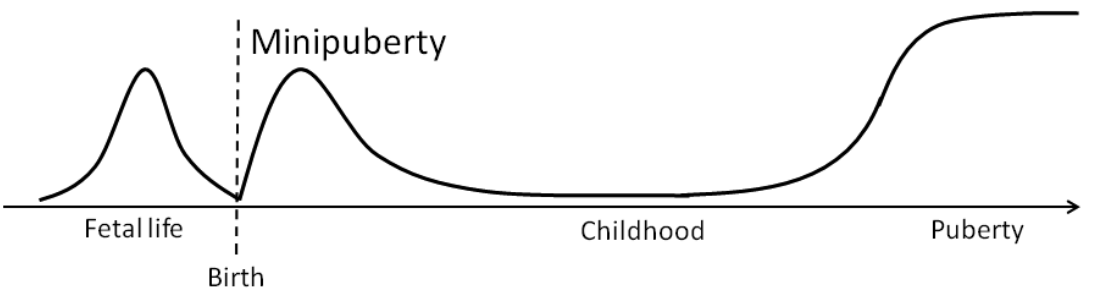

Figure1. Three periods of HPG axis activity: the first during fetal life, the second during the first months of life (minipuberty), and the third at the puberty and onwards.

2.1.1 Hypothalamus and pituitary

The function of the HPG axis is controlled by pulsatile secretion of the gonadotropin-releasing hormone (GnRH) from the specific hypothalamic cells, the GnRH neurons. The activity of the GnRH neurons is modulated by a complex network of stimulatory and inhibitory cues from the brain and periphery (Christian & Moenter 2010, Plant 2008). GnRH is delivered from the hypothalamus via the portal circulation to the anterior pituitary where it induces the synthesis and release of the gonadotropins, LH, and FSH by specific cells expressing the GnRH receptor, the gonadotropes.

LH and FSH are heterodimeric glycoproteins that consist of a common α-subunit and a hormone specific β-subunit. Following the GnRH pulses, they are released in pulses into general circulation. The GnRH pulse frequency determines the ratio of the two gonadotropins: faster GnRH pulses favour LH synthesis, and slower pulses favour FSH synthesis (Kaiser et al. 1997) whereas continuous GnRH secretion leads to suppression of gonadotropin levels (Belchetz et al. 1978). Because GnRH has a short half-life in circulation (2-4 minutes) and is produced and utilized mainly inside the brain, monitoring of its episodic secretion in peripheral blood is not practical. Instead, since each GnRH pulse regenerates a pulse of gonadotropins, measuring of peripheral blood LH levels in approximately 10-minute intervals has been used to monitor GnRH pulse activity. LH is more suitable than FSH for this purpose because of its shorter half-life (20 minutes vs. 3-4 hours) (Hayes & Crowley 1998). LH and FSH are secreted in

urine, and urinary levels have been shown to reflect well the levels in serum (Demir et al. 1994, Kuijper et al. 2006). LH and FSH stimulate the gonadal functions by binding to their cognate receptors in the gonadal cells.

2.1.2 Testis

The main functions of mature testis (i.e., the production of sperm and secretion of androgens) are specific to two testicular compartments. Sperm is produced in the seminiferous tubules consisting of the Sertoli and germ cells, and androgen biosynthesis takes place in the Leydig cells located in the interstitial tissue (Figure 2). Pituitary FSH stimulates the Sertoli cell functions including secretion of AMH and inhibin B, and LH stimulates the Leydig cells which produce testosterone and insulin-like factor 3 (INSL3). AMH and inhibin B are dimeric glycoprotein hormones and INSL3 is a peptide hormone structurally related to insulin. AMH and its receptor (type II AMH receptor) are important in male reproductive development (see 2.2.2). Testosterone suppresses AMH secretion and consequently AMH levels decrease during puberty and are low in adult men (Aksglaede et al. 2010). Inhibin B is important in the regulation of pituitary FSH secretion. INSL3 has a role in testicular descent (see 2.2.2). The INSL3 receptor, RXFP2, is expressed in germ cells but its role there is not yet properly understood (Ivell et al. 2013). In addition to FSH action, testosterone is needed for sufficient sperm production. The seminiferous tubule compartment accounts for the majority (80-90%) of the testicular volume, which therefore is considered as a direct surrogate of the sperm production capacity.

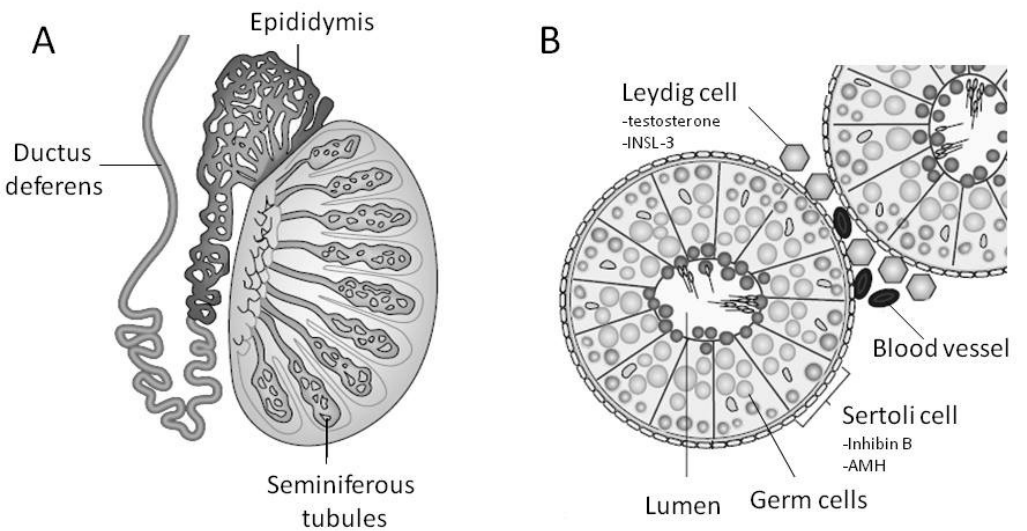

Figure 2. Organization of the testis. A) Cross-section through a testis showing the macroscopic structure and localization of the seminiferous tubules, the epididymis and the ductus deferens. B) Cross-section through a testicular tubule showing the organization of the Sertoli, Leydig and germ cells. The main products of the Leydig and Sertoli cells are listed. Modified from Cooke & Saunders 2002.

2.1.3 Ovary

The key functions of the ovary are to produce viable oocytes for fertilization and to secrete hormones that prepare the female body for reproduction. The functional unit of the ovary is a follicle consisting of an oocyte surrounded by granulosa and theca cells (Figure 3). The ovarian reserve is formed of dormant primordial follicles in which an oocyte is surrounded by one layer of flat granulosa cells. A follicle goes through the primordial, primary and secondary stages (i.e., pre-antral development) before becoming an antral follicle. This initial follicular growth is very slow, and in humans it has been estimated to take about six months for a primary follicle to reach the antral stage and diameter of 2-5 mm (Gougeon 1996). Gonadotropins are not essential in early folliculogenesis, but at later stages of follicular development FSH becomes essential for its growth (Gougeon 1996). During each menstrual cycle, some of the antral follicles are recruited by FSH for further growth. One will become a leading follicle which finally ovulates, while other recruited follicles atrophy (McGee & Hsueh 2000). Hormonal changes during the menstrual cycle mirror the changes in follicular growth and differentiation.

While in the testis, testosterone production in Leydig cells occurs solely under the influence of LH, in ovarian follicles both LH and FSH and two cell types are needed for the production of the main estrogenic hormone, estradiol (reviewed in Edson et al. 2009). LH stimulates the steroidogenesis in the follicular theca cells that lack aromatase, the enzyme required for estrogen synthesis. Consequently, theca-cell-derived androgens are delivered to the adjacent granulosa cells that express aromatase activity under FSH stimulation and are capable for estrogen synthesis.

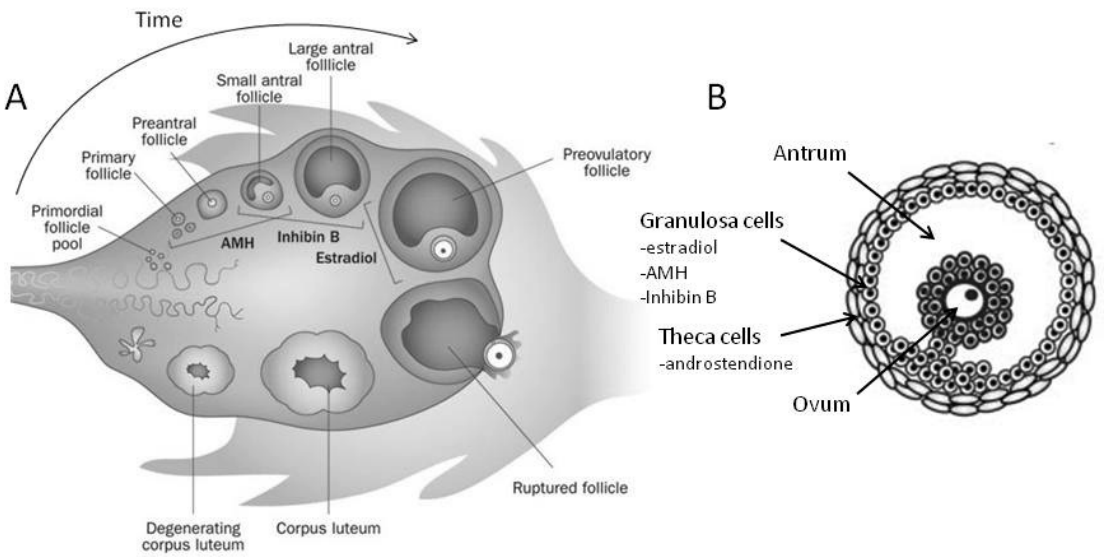

Figure 3. A) Organization of the ovary showing phases of follicular development from primordial follicle to corpus luteum. AMH is secreted mainly by preantral and small antral follicles, inhibin B by small and large antral follicles and estradiol by large, preovulatory follicles. B) Organization of the graafian follicle showing granulosa and theca cell layers, the fluid filled antrum and the oocyte. The main products of the granulosa and theca cells are listed. Modified from Visser et al. 2012.

In mid-cycle, rising estradiol levels result in a LH surge that triggers ovulation. The ovulated follicle becomes corpus luteum and produces progesterone in response to LH stimulation. In addition to estrogens, ovarian granulosa cells produce peptide hormones such as activins, inhibins, and follistatin. Granulosa cells of pre-antral and small antral follicles secrete AMH (Rajpert-De Meyts et al. 1999, Rey et al. 2000, Andersen & Byskov 2006). AMH restrains primordial follicle activation in mice (Durlinger et al. 1999, Durlinger et al. 2002, Carlsson et al. 2006), and in human ovaries in vitro (Carlsson et al. 2006), and might inhibit the stimulatory effect of FSH (Durlinger et al. 2001). In women, serum AMH levels correlate well with the number of antral follicles in ovarian ultrasonography (de Vet et al. 2002, van Rooij et al. 2002, Hansen et al. 2011), and both are used as markers of ovarian reserve.

2.1.4 Hormonal feedback system of the HPG axis

The secretion rate of gonadal steroid hormones and inhibin B is controlled by the feedback effects on the hypothalamus and pituitary. In males, testosterone and its metabolite estradiol suppress GnRH and gonadotropin secretion in the hypothalamus and the pituitary (Hayes et al. 2000, Pitteloud et al. 2008), and inhibin B suppresses pituitary FSH secretion. In females, estradiol suppresses hypothalamic GnRH secretion during the follicular phase, but at mid-cycle, high estradiol levels stimulate GnRH secretion resulting in a LH surge that triggers ovulation (i.e., positive feedback). In women, estrogens have also direct pituitary effects on gonadotropin secretion (Shaw et al. 2010). Progesterone slows down the frequency of pulsatile GnRH secretion during the luteal phase of the menstrual cycle. Follistatin, inhibin A, and inhibin B suppress FSH secretion in women.

2.1.5 Metabolism and peripheral effects of gonadal steroids

Gonadal steroid hormones induce the sex-specific physical changes at puberty and maintain the normal reproductive functions in adulthood. Besides the gonads, smaller amounts of sex steroids are secreted by the adrenal glands, and some target tissues are also able to locally synthesize potent sex hormones from inactive circulating adrenal precursor hormones, such as dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), and androstenedione (reviewed in Labrie 2014). The major steroidogenic pathway to androgens and estrogens is presented in Figure 4. Peripheral effects of gonadal hormones in the target tissues are influenced by the rate of secretion, transport to the target tissue, peripheral metabolism in the target tissue, receptor activity and availability, and rate of excretion.

2.1.5.1 Androgens

Testosterone produced by the testicular Leydig cells is the main circulating androgenic hormone in males and circulates in nanomolar concentrations. In addition to testis, minor amount of testosterone is produced by the adrenal glands, and some peripheral tissues are able to metabolize circulating androgen precursors to potent androgens for local utilization. In females, most of the testosterone is produced from adrenal and ovarian precursors in peripheral tissues.

In circulation, testosterone is mainly bound to plasma proteins and only 0.5-3% is in its free form. Approximately 30-44% of testosterone is bound to SHBG and 54-68% to albumin. Binding of testosterone with SHBG is tight and, according to the free hormone hypothesis, prevents its biological availability for target tissues. In contrast, binding with albumin is loose, and therefore the albumin-bound testosterone in addition to the free form is considered biologically active and classified as bio-available testosterone. However, the free hormone-hypothesis has been challenged, and some target tissues may also be able to utilize carrier-bound steroids (Hammes et al. 2005).

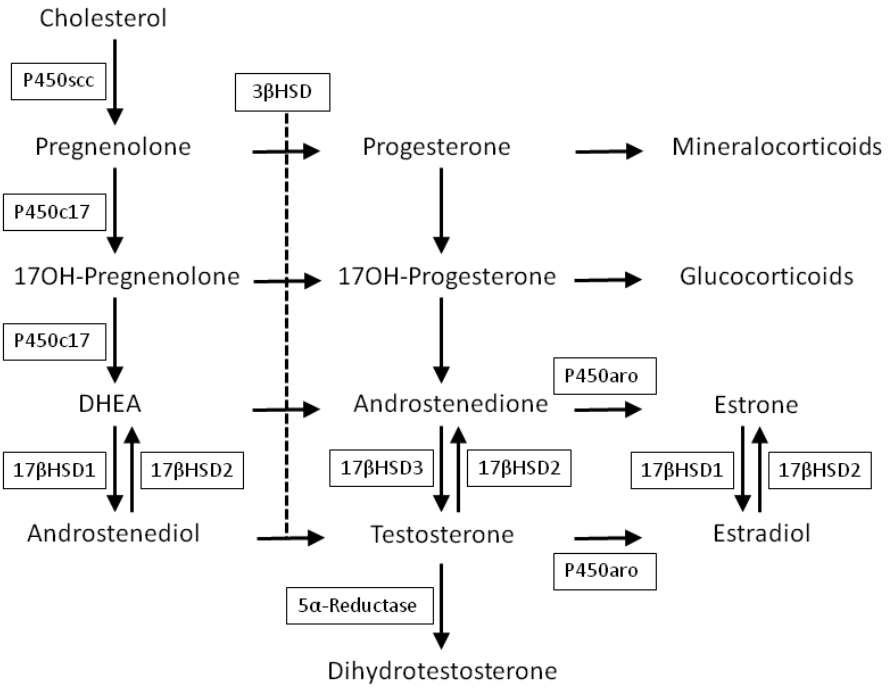

Figure 4. Major steroidogenic pathways of androgens and estrogens. Key enzymes are shown inside boxes, and arrows indicate the direction of synthesis. Modified from Miller & Auchus 2011.

Bio-available circulating testosterone enters the target cells by passive diffusion and binds to the intracellular androgen receptor. Binding results in changes of DNA transcription and protein synthesis. Some of the effects of testosterone are mediated after its active metabolism to dihydrotestosterone (DHT) or to estradiol. DHT is formed in target tissues by the enzyme 5α-reductase. DHT is the most potent endogenous activator of the androgen receptor, and it binds to the androgen receptor with a higher affinity and greater stability than testosterone. Local aromatization of testosterone by the aromatase enzyme results in the formation of estradiol, the most powerful natural ligand of estrogen receptors α (ERα) and β (ERβ). Expression of 5α-reductase and aromatase is tissue-specific and changes during development.

Androgens are essential for phenotypic male development. Androgens induce penile and scrotal growth, enlargement of the prostate and seminal vesicles, and production of accessory sexual gland secretions and seminal fluid. At puberty, testosterone is required to initiate and maintain spermatogenesis. In addition to the male reproductive tract, androgen target tissues include the brain, skin, skeleton, muscle, hematopoietic system, and adipose tissue (Sinha-Hikim et al. 2004, Abu et al. 1997, Matsumoto et al. 2013, Shahani et al. 2009). In the brain, androgens exert both organizational (Lombardo et al. 2012) and activational effects and are associated with sexual and aggressive behaviour (reviewed in Hines 2010).

Androgens are metabolized in the peripheral tissues and in the liver by CYP-enzymes (phase I reactions) and then conjugated mostly with glucuronides and in a smaller proportion with sulphates (phase II reactions) to increase water-solubility before excretion into bile and urine (Alcorn & McNamara 2002, Chouinard et al. 2008). The metabolic pathways for testosterone in men are shown in Figure 5. Uridine diphosphate glucuronyltransferase (UGT) 2B7, UGT2B15, and UGT2B17 are isoenzymes that are important in androgen glucuronide conjugation; UGT2B17 is the most important in testosterone glucuronidation (Turgeon et al. 2001). It is detectable in various tissues including the liver, kidney, uterus, placenta, mammary gland,

adrenal gland, skin, testis, and prostate (Belanger et al. 1998, Nakamura et al. 2008). UGT activity in the liver microsomal suspension of fetuses and newborns is lower than in that of adults (Leakey et al. 1987, Ekstrom et al. 2013). UGT2B15 and B17 are primary androgen-regulated genes, and the androgen receptor is required for both their basal expression and their androgen-regulated expression (Bao et al. 2008).

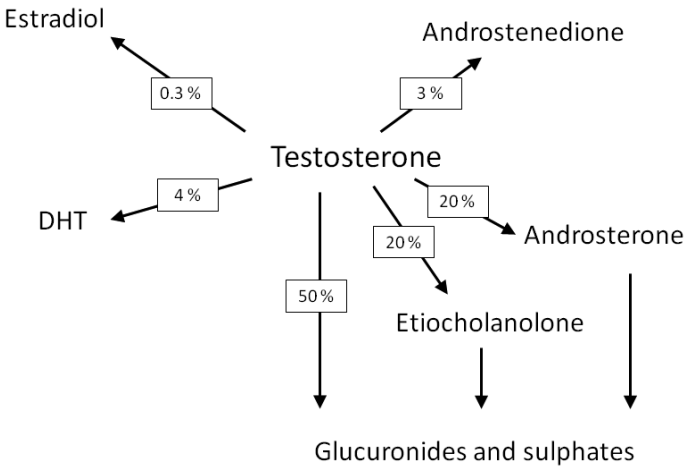

Figure 5. Metabolic pathways of testosterone in human males. Modified from Becker 2001.

2.1.5.2 Estrogens

The three estrogens produced by the ovaries are estrone, estradiol, and estriol. Estradiol is the most potent natural estrogen, whereas estrone and estriol are considered weak estrogens. During the reproductive years, estradiol is the main estrogen, and its serum levels change according to menstrual cycle with the highest levels at the end of the follicular phase. Estriol levels increase during pregnancy, and estrone is the most abundant estrogen in circulation after menopause. Estrone can be converted in peripheral tissues to estradiol by the enzyme 17-β-hydroxysteroid-dehydrogenase (HSD17B). Estradiol circulates in picomolar concentrations and is mostly bound to albumin and SHBG.

Estrogen action is mediated mainly by two intranuclear receptors, ERα and ERβ, which are encoded by separate genes (Burns & Korach 2012, Heldring et al. 2007). Both receptors are widely expressed in the body and present tissue-specific distribution (Bottner et al. 2014) even during the fetal period (Brandenberger et al. 1997, Takeyama et al. 2001). Estradiol is the most powerful endogenous activator of both ERα and ERβ. Classical tissues where ERα mediates growth are the uterus and mammary glands. In addition, estrogens stimulate endometrial thickening and maturation of the epithelium in the vulva and vagina. Besides the female reproductive tract, estrogen signalling has a role in bone growth and bone mineral density, epiphyseal closure, lipid metabolism, energy expenditure, and glucose homeostasis, coagulation, fluid balance, and in cardiovascular and nervous systems (Bulun 2014, Barros & Gustafsson 2011).

Estrogens can be metabolized by hydroxylation to catechol estrogens and further to methoxyestrogens. Estrogens are also conjugated in the liver and several peripheral tissues with sulphates and to a lesser degree with glucuronides before excretion in bile and urine (Raftogianis et al. 2000).

2.2 PRENATAL DEVELOPMENT AND FUNCTION OF THE HPG AXIS

2.2.1 Hypothalamus and pituitary

During early embryogenesis, GnRH neurons migrate from the nasal placode to the anterior hypothalamus (Cariboni et al. 2007), and GnRH is detected in the fetal hypothalamus by 14-16 weeks (Quinton et al. 1997, Guimiot et al. 2012). Kisspeptin and its receptor KISS1R are involved in the regulation of the fetal GnRH neuron activity (Guimiot et al. 2012).

LH and FSH are detected in the fetal anterior pituitary and within circulation by 12-14 weeks of gestation (Asa et al. 1986, Clements et al. 1976, Kaplan & Grumbach 1976); by that age fetal gonadotropes respond to GnRH stimulus in vitro (Asa et al. 1991). The exact time when the pituitary gonadotropin secretion comes under the control of the hypothalamic GnRH neurons is not fully clear. Vascular connections are already present at the end of the first trimester (Thliveris & Currie 1980), but the maturation of the portal vascular system continues to the latter part of the pregnancy (reviewed in Forest 1985). In anencephalic fetuses lacking the hypothalamus but with the pituitary intact, the gonadotrope development is normal up to 17-18 weeks of gestation but they are almost absent after 32 weeks (Pilavdzic et al. 1997), suggesting that hypothalamic input is required after midgestation for maintaining the gonadotropes. The development of different components of the HPG axis is presented in Figure 6.

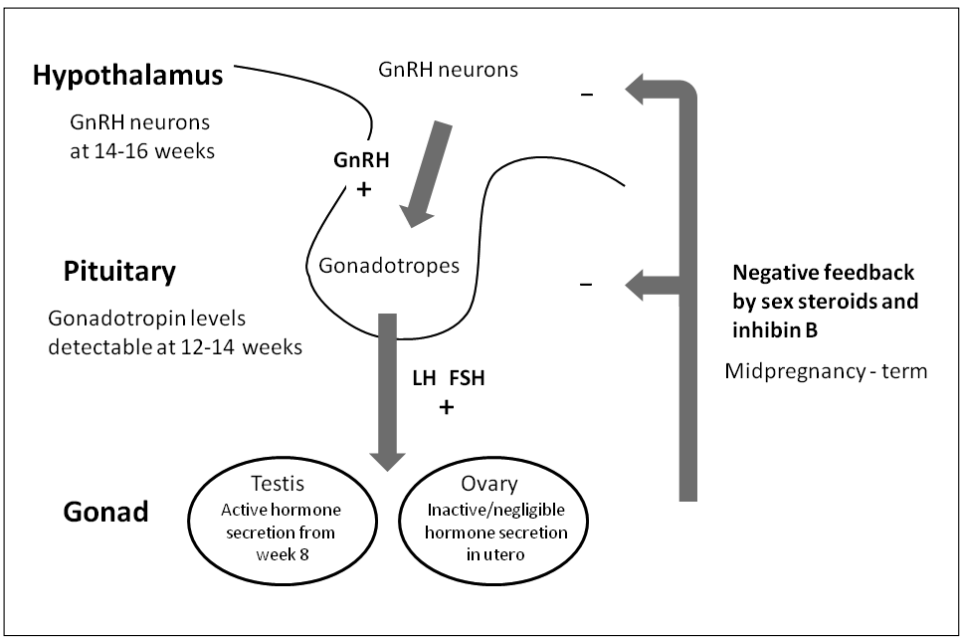

Figure 6. Prenatal development of the HPG axis.

During the first half of pregnancy, female fetuses have more gonadotropes (Asa et al. 1986), greater pituitary content of the gonadotropins (Siler-Khodr & Khodr 1980, Guimiot et al. 2012, Kaplan & Grumbach 1976), and higher serum gonadotropin levels (Clements et al. 1976, Kaplan & Grumbach 1976) than male fetuses. This sex difference has been suggested to be due to the negative feedback effects by the fetal testicular hormones. At midgestation, gonadotropin levels in female fetuses are very high, resembling the levels of castrated adults or postmenopausal women (Reyes et al. 1973, Debieve et al. 2000, Beck-Peccoz et al. 1991, Guimiot et al. 2012). In male fetuses, LH levels are higher than FSH levels (Debieve et al. 2000, Beck-Peccoz et al. 1991, Guimiot et al. 2012, Takagi et al. 1977). In both sexes, gonadotropin levels decrease towards the

end of gestation (Guimiot et al. 2012) and are low at term (Debieve et al. 2000, Beck-Peccoz et al. 1991), probably because of the negative feedback effects mediated by the high placental estrogen levels.

2.2.2 Male reproductive organs

Fetal testis differentiates early and already secretes testosterone and AMH at week 8 of fetal development. Testosterone production by the fetal testis at this stage of development is essential for the masculinisation of the fetus and probably occurs under the influence of high human chorionic gonadotropin (hCG) levels, since pituitary gonadotropins become detectable only later. Testosterone induces the development of the male internal genitalia epididymis, vas deferens, and seminal vesicles from the Wolffian ducts (reviewed in Hannema & Hughes 2007, Rey & Grinspon 2011). Formation of DHT is required for the development of the prostate, penis, and scrotum. A positive correlation has been reported in penile length and gestational age in premature newborns at birth (Tuladhar et al. 1998). AMH causes the regression of the Müllerian ducts and prevents the formation of a uterus and fallopian tubes. Initial testicular development takes place intra-abdominally, and the descent of the testes into the scrotum occurs in two phases. The first transabdominal phase is completed by 15 weeks of gestation (reviewed in Virtanen et al. 2007). This phase is independent of androgens, but INSL3 seems to be important. By binding to its receptor RXFP2, INSL3 anchors the testis to the gubernacular ligaments in the inguinal region (Ivell et al. 2013). The second inguinoscrotal phase is usually completed by the end of the 35th week of gestation, and this phase is androgen dependent (reviewed in Virtanen et al. 2007).

Testosterone levels are high in male fetuses between 11-17 weeks, reaching adult values (Reyes et al. 1974, Takagi et al. 1977, Tapanainen et al. 1981a, Svechnikov & Soder 2008). After this, testosterone levels decrease towards term, but at 17-24 weeks, free testosterone levels are still higher in male than in female fetuses (Beck-Peccoz et al. 1991). From 24 weeks to term pregnancy, available data on fetal testosterone levels are mainly based on umbilical cord samples at birth, and while some studies have reported higher levels in males than in females (Bolton et al. 1989, Herruzo et al. 1993, Keelan et al. 2012, Troisi et al 2003), some have found no sex difference (van de Beek et al. 2004, Takagi et al. 1977). Recent meta-analysis is in favour of higher cord testosterone levels in newborn boys than in girls (Barry et al. 2011). The changes in fetal testosterone levels in males are adjacent to the changes in the number of fetal Leydig cells (Codesal et al. 1990). Inhibin B levels are higher in male than female fetuses (Morpurgo et al. 2004, Debieve et al. 2000) and in cord blood at term (Wallace et al. 1997), indicating intrauterine Sertoli cell activity. Sertoli cell activity probably decreases from midpregnancy towards term as lower inhibin B levels (Debieve et al. 2000) and inhibin immunoreactivity (Massa et al. 1992) have been reported at term than at midpregnancy.

2.2.3 Female reproductive organs

The embryonic differentiation of the ovary occurs a week later than that of the testis. Folliculogenesis is initiated in the fetal ovary around 15 weeks post-conception when the first primordial follicles are formed (Kurilo 1981, Peters et al. 1978). The first follicles start to grow immediately after their formation. Follicular development to the antral stage has been observed during late pregnancy (Kurilo 1981, Forabosco & Sforza 2007, Cole et al. 2006, Vaskivuo et al. 2001). Ovarian volume increases almost linearly from 15 weeks of gestation to term (Sforza et al. 2004). In anencephalic female fetuses, normal follicular development up to 34 weeks of gestation has been reported, but after this age, larger, growing follicles seen in healthy female fetuses have been absent in anencephalic girls (Baker & Scrimgeour 1980). This finding suggests that ovarian development up to the seventh month of pregnancy occurs independently of the stimulation by the fetal hypothalamus.

The pool of primordial follicles serving as the ovarian reserve for the whole life span is formed during the fetal period, and the amount of oocytes is highest at midgestation, reaching

6-7 million. After this, the ovarian follicle pool is decreased due to apoptosis and atrophy. At birth, there are about 1 million oocytes left, and the amount declines to 300 000-400 000 at the time of puberty. Only 300-400 follicles will then ovulate before menopause occurs (Oktem & Oktay 2008). Follicular development from a primordial follicle to the small antral stage is a continuous process beginning already during fetal period and continuing until depletion of the follicle pool. The factors that regulate the recruitment of resting primordial follicles to the pool of growing follicles are not completely understood, but since primordial follicles lack the FSH receptor, FSH does not seem to play a role (Oktay et al. 1997).

The ontogenesis of steroid production in fetal human ovaries is not completely understood. Even though the enzymatic capacity of the fetal ovary to convert androgens to estrogens has been observed at the end of the first trimester (George & Wilson 1978, Fowler et al. 2011), the ovarian estrogen production is considered minimal during fetal life. Serum estradiol levels are high in both female and male fetuses during late gestation because of placental estradiol production (Shutt et al. 1974). AMH expression is very low in fetal ovaries (Voutilainen & Miller 1987, Modi et al. 2006).

Expression of ERα and ERβ in fetal ovaries has been detected around midgestion (Vaskivuo et al. 2005, Fowler et al. 2011), and high intra-uterine estrogen levels may have a role in prenatal ovarian development as has been shown in non-human primates in whom deprivation of the intrauterine estrogen during the latter part of pregnancy led to an approximately 50% reduction in the number of primordial follicles compared with normal pregnancies (Zachos et al. 2002).

The ovary is not required for the differentiation of internal or external female genitalia. In the absence of AMH, Müllerian ducts develop into fallopian tubes, the uterus, and the upper portion of the vagina. The lower portion of the vagina is derived from the urogenital sinus. ER is expressed in the fetal uterus beginning from the early second trimester (13-15 weeks of gestation) (Glatstein & Yeh 1995), and uterine size increases with advancing gestation (Sulak et al. 2007, Soriano et al. 1999).

At midgestation, both androgen and estrogen receptors have been detected in the female external genitalia; this suggests that intrauterine estrogens might have a role in female external genitalia development (Kalloo et al. 1993). The presence of the androgen receptor, on the other hand, explains how female external genitalia may be masculinized in the presence of abnormally high intrauterine androgen levels such as in congenital adrenal hyperplasia.

2.2.4 Feto-placental unit

During pregnancy, the placenta secretes many hormones and growth factors that modulate the function of the HPG axis in both the mother and the fetus. The placenta secretes the third gonadotropin, hCG, which is structurally similar to LH, binds to LH receptor, and has similar biological effects as LH. The half-life of hCG is 24 hours, which is markedly longer than that of LH. The fetal levels of hCG rise early in gestation, peak at around 8–12 weeks of gestation, and then decrease towards term but remain at considerable levels until late gestation (Clements et al. 1976, Varvarigou et al. 2009). The role of hCG in the fetus is not completely understood, but it may have widespread effects as the receptor for hCG and LH is also expressed in non-gonadal fetal tissues (Abdallah et al. 2004).

Placental steroid production increases towards the end of gestation, and estrogen and progesterone levels are high in both maternal and fetal circulation during the late pregnancy (Reyes et al. 1974, Troisi et al. 2003, Nagata et al. 2006). The placenta is an incomplete steroidogenic organ, and it lacks some important steroidogenic enzymes. Therefore, placental estrogen and progesterone production is dependent on precursor hormones synthesized in both the mother and the fetus. In the fetus, the fetal zone of the adrenal cortex produces large amounts of DHEAS that serve as precursors for placental estrogen production. The factors that regulate the function of the fetal adrenal zone are not completely understood. The fetal zone involutes after birth, but this involution is probably developmentally programmed since in premature infants, the secretion of adrenal androgen precursors continues at a comparable level

until term (Bolt et al. 2002, Heckmann et al. 2006, Midgley et al. 1996). Increasing estrogen and progesterone levels during the latter part of pregnancy probably suppresses the activity of the fetal HPG axis and results in low gonadotropin levels by the end of gestation. After birth, estrogens, progesterone, and hCG levels are cleared from the newborn's circulation during the first postnatal days (Bidlingmaier et al. 1973).

2.3 POSTNATAL ACTIVATION OF THE HPG AXIS: "MINIPUBERTY"

2.3.1 Hormonal changes associated with minipuberty

2.3.1.1 Gonadotropins

In the cord blood of term newborns, FSH and LH levels are low in both sexes (Winter et al. 1975, Debieve et al. 2000, Takagi et al. 1977, Varvarigou et al. 2009). The levels remain low for the first postnatal days but begin to increase around one week of age (Takagi et al. 1977, Winter et al. 1975, Schmidt & Schwarz 2000, Bergada et al. 2006). hCG levels are relatively high in cord blood (Varvarigou et al. 2009, Furuhashi et al. 1982), but they are then cleared from the circulation during the first days of life (Takagi et al. 1977, Winter et al. 1975, Bidlingmaier et al. 1973). FSH and LH levels peak between 1 week and 3 months of age (Winter et al. 1975, Andersson et al. 1998). At this time, FSH levels are higher in females, and LH levels predominate in males (Andersson et al. 1998, Schmidt & Schwarz 2000, Bergada et al. 2006, Shinkawa et al. 1983, Ibanez et al. 2002, Sir-Petermann et al. 2007, Burger et al. 1991, Belgorosky et al. 1996a). In boys, LH and FSH levels decrease by 6–9 months of age, but in girls FSH levels remain elevated longer, up to 3–4 years of life (Winter et al. 1975, Andersson et al. 1998, Faiman & Winter 1971, Penny et al. 1974).

Cord blood gonadotropin levels are higher in premature than full-term infants (Massa et al. 1992, Shinkawa et al. 1983, Tapanainen et al. 1984). In premature girls, the postnatal gonadotropin surge is increased and prolonged in comparison to full-term girls (Shinkawa et al. 1983, Tapanainen et al. 1981b, Greaves et al. 2008b). However, in premature boys, postnatal gonadotropin levels have been reported in the same range as in full-term boys (Shinkawa et al. 1983, Tapanainen et al. 1981b). Consequently, LH and FSH levels are higher in female premature infants than in male premature infants (Tapanainen et al. 1981b, Greaves et al. 2008b, Shinkawa et al. 1983). Increased postnatal FSH levels have also been reported in infants who were born small for gestational age (SGA) (Ibanez et al. 2002).

Pituitary activity in infancy is pulsatile (Waldhauser et al. 1981), and peripheral LH pulses have been observed by the first day of life (de Zegher et al. 1992). In a group of infant boys with uni- or bilaterally undescended testes, the pituitary response to GnRH increased after birth, was maximal at 1–3 months of age, and then decreased towards the age of one year (Tapanainen et al. 1982).

2.3.1.2 Gonadal hormones

Testosterone

In cord blood, testosterone levels are higher in boys than in girls (Garagorri et al. 2008, Forest et al. 1973b, Pang et al. 1979, Barry et al. 2011). In girls, testosterone levels decrease during the first weeks after birth and then remain low (Garagorri et al. 2008, Forest et al. 1974, Kulle et al. 2010). However in boys, testosterone levels start to increase after one week of age, peak between 1–3 months of age, and decline to prepubertal levels by six months of age (Forest et al. 1973b, Bergada et al. 2006, Andersson et al. 1998, Burger et al. 1991, Hammond et al. 1979, Bolton et al. 1989, Kulle et al. 2010, Pang et al. 1979, Winter et al. 1976, Gendrel et al. 1980). After this, there is no sex difference in testosterone levels until the onset of puberty (Kulle et al. 2010, Forest et al. 1973b, Courant et al. 2010, Ilondo et al. 1982). In autopsy samples, testicular testosterone concentration is increased in boys aged 1–3 months to pubertal levels and thereafter decreases until 6 months of age (Bidlingmaier et al. 1983).

In premature boys, testosterone levels are higher than in full-term boys during the first months of life (Forest et al. 1980, Tapanainen et al. 1981b). Also in SGA boys, increased testosterone levels compared to full-term boys have been reported (Forest et al. 1980).

The biological activity of testosterone during the first months of life has been questioned, since the levels of SHBG increase concomitantly and lead to low levels of free testosterone (Bolton et al. 1989). In addition, no increase was observed in salivary testosterone levels that are considered to reflect the free-hormone levels in longitudinal samples from infant boys (Huhtaniemi et al. 1986). In infant boys, SHBG levels are higher than in adult men, but total testosterone and non-SHBG-bound testosterone levels are lower than in men (de Ronde et al. 2005). SHBG levels increase in both sexes during the first months of life (Bolton et al. 1989), and at three months of age the levels are similar in both sexes (Schmidt et al. 2002). No difference has been observed in binding of testosterone with SHBG between full-term and preterm infants during the first months of life (Forest et al. 1980).

Estradiol

Placental estradiol levels are high in the newborn cord blood of both sexes and decrease rapidly during the first postnatal days (Winter et al. 1976, Bidlingmaier et al. 1973, Kenny et al. 1973, Nagata et al. 2006, Trotter et al. 1999, Troisi et al. 2003). Higher estradiol levels have been reported in girls during the first months of life than later in childhood, but these levels have been extremely variable and ranged from undetectable to very high (Winter et al. 1976, Kuhnle et al. 1982, Burger et al. 1991). In a post-mortem study, estradiol concentrations in the ovarian tissue were higher during the first six months of life than between 6–24 months of life (Bidlingmaier et al. 1987). At three months of age, higher estradiol levels have been observed in girls than in boys (Schmidt et al. 2002), and in one study, premature girls had higher levels than in full-term girls (Chellakooty et al. 2003). The reason for the large inter-individual variability in the levels is not understood, but cyclic ovarian activity has been suggested. However, there are no previous longitudinal studies to evaluate this. Size at birth might influence postnatal estradiol levels, and higher estradiol levels have been reported in SGA and large for gestational age (LGA) girls than in appropriate for gestational age (AGA) girls after a GnRH agonist test (Sir-Petermann et al. 2007), although the reported non-stimulated levels have not been significantly different (Sir-Petermann et al. 2007, Sir-Petermann et al. 2010, Ibanez et al. 2002). With very sensitive methods, higher estradiol levels in girls than in boys have also been found during the later prepubertal period (Courant et al. 2010).

Gonadal peptide hormones

AMH

In boys, AMH levels increase after birth to peak levels at two to three months of age and then decline to the age of one year (Aksglaede et al. 2010). In infant girls, a similar pattern in AMH levels during the first months of life has been reported, but the levels are significantly lower than in boys (Hagen et al. 2010). Higher AMH levels have been reported in SGA and LGA girls than in AGA girls at two-three months of age, suggesting altered follicular development in them (Sir-Petermann et al. 2007, Sir-Petermann et al. 2010). AMH levels in premature girls have not been studied before.

Inhibins

In girls, inhibin A levels are high after birth and then decrease to the second month of life (Bergada et al. 2002). At three months of age, inhibin A is undetectable in most girls (Chellakooty et al. 2003). In infant boys, inhibin A levels have been undetectable (Bergada et al. 1999). Inhibin B levels increase in boys from cord blood to three months of age to supra-adult levels and then decrease by 15 months of age (Andersson et al. 1998). In girls, inhibin B levels are low at birth but increase during the first months of life and then decrease again towards one year of age, showing inter-individual variation in levels (Andersson et al. 1998).

INSL3
In boys, INSL3 levels are higher in cord blood and at three months of age than later in prepuberty (Bay et al. 2007). INSL3 has not been detectable in infant girls (Bay et al. 2007).

2.3.1.3 Androgens from the fetal adrenal cortex

The fetal zone of the adrenal cortex produces large amounts of precursor hormones for the placental estrogen synthesis during pregnancy. The fetal zone regresses after birth, but during the first weeks of life in full-term infants—and even longer, close to the term age in preterm infants—the levels of steroid hormones from the fetal adrenal cortex are elevated (Bolt et al. 2002, Heckmann et al. 2006, Midgley et al. 1996, Garagorri et al. 2008). The role of these precursor hormones in the physiology of the newborn is not well understood, and it is not known whether these precursor hormones are converted into active sex hormones in the target tissues of the newborn infants.

The contribution of adrenal and testicular tissues to circulating androstenedione and testosterone levels in infant boys have been evaluated in postmortem samples by Bidlingmaier et al. (Bidlingmaier et al. 1986, Bidlingmaier et al. 1983). These studies conclude that the testis is the source of the transiently elevated testosterone levels during the first months of life, and androstenedione levels are mainly derived from the adrenals. Androstenedione concentrations in adrenal glands of infant boys decreased from the first week towards the end of the first year in parallel with the involution of the fetal cortex. Adrenal testosterone concentration was approximately 15% of that of androstenedione and similarly decreased towards the end of first year.

The high levels of steroid hormones from the fetal adrenal cortex in the neonatal blood might confuse the results of direct immunoassays, which have been shown to overestimate the testosterone levels (Fuqua et al. 1995, Tomlinson et al. 2004) and also estradiol levels (Diver 1987); therefore, chromatographic purification prior to analysis is important.

2.3.2 Clinical features associated with minipuberty

2.3.2.1 Male reproductive organs

Testis
Testicular volume has been shown to increase during the first months after birth in studies using ultrasonography (Cassorla et al. 1981, Main et al. 2006, Kuijper et al. 2008) or orchidometer (Cassorla et al. 1981) and also in autopsy material (Muller & Skakkebaek 1984, Siebert 1982, Bidlingmaier et al. 1983, Berensztein et al. 2002). In a cross-sectional sonographical study, testicular volume increased from birth to five months of age (from 0.27 cm^3 to 0.44 cm^3) and then decreased to 0.31 cm^3 at nine months of age (Kuijper et al. 2008).

Increase in the number of germ cells (Muller & Skakkebaek 1984), Leydig cells (Codesal et al. 1990), and Sertoli cells (Cortes et al. 1987) has been observed during the first postnatal months. The androgen receptor is not expressed in Sertoli cells during infancy, explaining why spermatogenesis is not initiated (Berensztein et al. 2006, Chemes et al. 2008, Boukari et al. 2009). This lack of androgen receptor is thought to also explain the absent suppression of AMH levels by testosterone during the postnatal testosterone surge (Boukari et al. 2009, Chemes et al. 2008).

Penis
In a large group of Danish and Finnish boys, a positive correlation was found between the increase in penile length from birth to three months of age and serum total and free testosterone measured at three months of age (Boas et al. 2006). Penile growth rate was higher from birth to three months of age than later in infancy (1 mm/month from birth to three months of age) (Boas et al. 2006).

Prostate
Secretion of PSA is androgen dependent and requires local DHT action. Previous data on PSA secretion in infancy are scarce. Positive PSA staining in prostatic tissue has been reported in infants less than six months old (Goldfarb et al. 1986). Sato et al. (Sato et al. 2007) analyzed serial urinary samples of six infants until the age of 18 weeks and found measurable PSA in some samples.

Scrotal hair
Transient isolated scrotal hair in infancy has been reported, typically appearing between 3 to 6 months of age and disappearing around one year of age (Janus et al. 2013, Bragonier et al. 2005, Papadimitriou et al. 2006).

2.3.2.2 Female reproductive organs
Ovary
In histological studies, antral follicles have been common findings in ovaries of post-mortem newborn girls (Polhemus 1953, deSa 1975, Forabosco & Sforza 2007, Channing et al. 1984, Lintern-Moore et al. 1974). Increase in follicular development during the first postnatal months has been described, and in prepubertal ovaries, a multicystic appearance was most frequently seen at the age of four months (Polhemus 1953). It is generally believed that ovulation does not occur in the fetal or infant ovary. However, a single case report describes the presence of corpus luteum in the ovary of a deceased premature newborn girl (Miles & Penney 1983).

In sonographical studies, small ovarian cysts have been reported in approximately 80% of girls less than two years of age (Cohen et al. 1993, Gilchrist et al. 2010), and larger cysts were seen more often during the first than the second year of life (Cohen et al. 1993). In ovarian follicular fluid of autopsied infants, estradiol and inhibin concentrations were increased during the first 60 days of life and decreased thereafter (Channing et al. 1984).

Increase in ovarian volume from birth to two months of age and decrease thereafter has been shown by sonographical imaging in a small group of infant girls in a semi-longitudinal setting (Nguyen et al. 2011). Another sonographical study reported tendency toward larger ovarian volumes during the first than the second year of life (Cohen et al. 1993).

Uterus
Placental estrogens stimulate uterine growth in the fetus and uterine size is larger in full-term newborn girls than later in childhood (Haber & Mayer 1994, Nguyen et al. 2011). Uterine size decreases rapidly during the first months of life (Haber & Mayer 1994, Nguyen et al. 2011) and more slowly until about four years of age (Griffin et al. 1995). After this, uterine size starts to increase steadily until puberty (Griffin et al. 1995). A midline endometrial echo was visible in six out of ten infant girls aged less than six months of age, and after this it was seen only close to 12 years of age (Griffin et al. 1995). The role of endogenous postnatal estrogens in uterine growth of infant girls has not been previously studied, although one study has evaluated the effects of postnatal estradiol and progesterone replacement on uterine volumes in premature girls (Trotter et al. 1999). In this study, hormone replacement resulted in significant increase in uterine volumes compared with the control group.

Vulvar epithelium
Estrogens induce the maturation of the squamous epithelium of the female genital tract, which can be observed as a presence of superficial cells in a smear test. The maturation from parabasal through intermediate to superficial cell type after hormonal stimulus takes about 4–5 days, so any changes in hormone levels can be detected quite quickly. Vulvar and vaginal epithelia are similar, and cytological changes during the menstrual cycle are parallel in them (Tozzini et al. 1971).

The hormonal cytology of a newborn vagina resembles that found in the mother shortly before delivery (Sonek 1969), indicating similar hormone effects in the epithelium. The percentage of superficial cells in the vaginal epithelium decreased after delivery and were absent by the tenth postnatal day. Intermediate cells were observed in a small percentage of cases after 30–60 days of life, but later in childhood, the vaginal epithelium is in the atrophic stage with chiefly parabasal cells found until the age of 8–9 years (Sonek 1969).

The maturation index expresses the maturation of the epithelium as a percentile relationship of parabasal cells to intermediate cells to superficial cells. At the time of ovulation, the maturation index could be 0:35:65 and in postmenopause 90:10:0. The maturation value is calculated as 0*parabasal cells+0.5*intermediate cells+1*superficial cells. The maturation value is between 50 and 95 in normal women and a maturation value below 50 indicates varying degrees of atrophy. In a small study, the maturation index of vaginal wall cells was clearly higher in newborn girls than in one-month old girls, whereas somewhat higher values were observed in girls aged two to six months (Bernbaum et al. 2008).

2.3.2.3 Mammary glands

Most full-term newborns have some palpable breast tissue present (Jayasinghe et al. 2010, McKiernan & Hull 1981). At birth, there is no sex difference in the mammary gland size, but later in infancy, the mammary gland size is larger in girls than in boys (Jayasinghe et al. 2010, McKiernan & Hull 1981, Schmidt et al. 2002). Contrary to full-term infants, palpable breast tissue is not usually present in preterm infants at birth. This is probably explained by the finding that ERα is detected in fetal mammary glands only from 30 weeks of gestation onwards (Keeling et al. 2000, Friedrichs et al. 2007). Some preterm infants might show mammary gland development later during the first postnatal months (McKiernan 1984). In some infants, transient secretion of milk has been observed during the first postnatal weeks (McKiernan & Hull 1981, Madlon-Kay 1986, Francis et al. 1990). In histological studies, a well-formed lobular pattern with ductal structures containing secretions has been described in infant mammary glands (McKiernan et al. 1988, Anbazhagan et al. 1991). No differences in the histological structure of the infant mammary gland have been reported between girls and boys (Anbazhagan et al. 1991).

2.3.2.4 Cutaneous manifestations

Androgens are essential for sebum production, and increasing levels of adrenal and gonadal androgens are associated with sebaceous gland hypertrophy (SGH) and acne during adrenarche and puberty (Shaw 2002, Deplewski & Rosenfield 2000, Pochi et al. 1977, Stewart et al. 1992, Lucky et al. 1994, Mourelatos et al. 2007). However, the relationship between circulating androgen levels and acne severity in adolescence and adulthood has been difficult to prove, and other factors clearly play a role (reviewed in Shaw 2002, Thiboutot 2004). Skin possesses the enzymatic machinery for the local synthesis of potent androgens from circulating precursor steroids, and these local factors might be more important than the circulating hormone levels in the development of acne (Labrie et al. 2000). Sebum secretion is already active during the first months of life (Agache et al. 1980, Henderson et al. 2000), and SGH and acne-like skin eruptions are observed in infant boys and girls. However, the role of androgens in the etiology of these conditions in infancy has only been speculated (McKiernan & Spencer 1981, Agache et al. 1980, Lucky 1998, Duke 1981, Hello et al. 2008).

The previous literature on acne in infancy consists mainly of case reports and retrospective studies; there are no studies comparing the presence of acne or SGH with androgen levels in infants. In the literature, acne occurring in infancy has been divided into two entities, neonatal and infantile acne, distinguished by the time of onset and clinical features (Lucky 1998, Herane & Ando 2003, Jansen et al. 1997). The definition of neonatal acne includes inflammatory, papulopustular facial eruptions with few comedones beginning during the first weeks of life and spontaneously recovering during the first months of life. Acne appearing after 3-6 months

of age has been classified as infantile acne and may present as comedonal and inflammatory lesions including nodules. Infantile acne has been described to be more persistent than neonatal acne, and treatment with topical or oral antibiotics is sometimes needed (Cunliffe et al. 2001, Hello et al. 2008). The estimated prevalence of neonatal acne has been around 20% (Jansen et al. 1997), but the prevalence of infantile acne is unknown. It has been estimated that both neonatal (Katsambas et al. 1999) and infantile acne (Cunliffe et al. 2001, Hello et al. 2008) are more common in boys, but this has never been studied properly. The role of androgens in the etiology of neonatal acne has been questioned, and instead it has been suggested that it is an inflammatory reaction to Malassezia yeast (Rapelanoro et al. 1996, Bernier et al. 2002).

2.3.3 The mechanisms and significance of minipuberty

The underlying mechanisms and significance of minipuberty remain poorly understood. Postnatal HPG axis activation is also observed in other primates, and the mechanisms and consequences of altered minipuberty have been studied in non-human primates. In addition, hormone levels during minipuberty have been described in several human disorders affecting reproductive development, and these observations provide insight into its potential mechanisms and role in reproductive physiology.

2.3.3.1 Primate studies

The effects of reversible suppression of the postnatal HPG axis activation for the first 3-4 postnatal months have been studied in non-human primates using GnRH agonists (leading to pituitary desensitization via down-regulation of receptor numbers) or antagonists (binding with high affinity to GnRH receptors and reducing available sites for native GnRH). In these studies, early postnatal GnRH antagonist treatment significantly reduced testicular weight but had only a minor effect on germ cell numbers (Sharpe et al. 2003). In GnRH agonist treated animals, testis volume was decreased compared with controls during the first two months of life, but at four months of age the groups were comparable (Liu et al. 1991). GnRH antagonist treated monkeys had lower FSH and inhibin B levels (Mann et al. 1997), more atrophy of the Leydig cells (Prince et al. 1998), and less Sertoli cells than controls in infancy (Sharpe et al. 2000). Phallic length was decreased in GnRH agonist treated animals during the first six months of life compared with controls (Brown et al. 1999, Liu et al. 1991). GnRH agonist treatment for the first four months of age was associated with delayed puberty and attenuated rise in peripubertal testosterone levels (Mann et al. 1989). As adults, the central neural system of these monkeys exhibited subnormal sensitivity to excitatory amino acids (Mann et al. 1993). Neonatally castrated monkeys showed less sexual and aggressive behavior than prepubertally castrated monkeys or monkeys castrated during adulthood (Dixson 1993). However, no effect on reproductive behavior in adulthood was observed in another study after neonatal GnRH antagonist treatment (Lunn et al. 1994).

In addition to these effects of postnatal HPG activation on the male reproductive tract, effects on other organ systems have been suggested (reviewed in Mann & Fraser 1996). Neonatal treatment with GnRH agonist or antagonist has altered early postnatal programming of immune function (Mann et al. 1994). These effects have been probably independent of the effects on gonadal function (Mann et al. 2000, Mann et al. 1994). During the first six months of life, GnRH agonist treatment had no effect on somatic growth (Brown et al. 1999, Liu et al. 1991, Lunn et al. 1994) nor on bone density (Liu et al. 1991), but in adult monkeys with blocked postnatal HPG axis activation, diminished growth and skeletal mineralization has been reported (Mann et al. 1993). This might be due to retarded sexual development. Higher leptin levels throughout development were observed in GnRH antagonist treated monkeys than in controls (Mann et al. 2002), suggesting that the treatment might alter body composition.

2.3.3.2 Experiments of nature in humans

In complete androgen insensitivity syndrome, lower postnatal LH and testosterone levels compared with healthy infant boys have been reported (Bouvattier et al. 2002). However, those infants with partial androgen insensitivity syndrome had normal or high testosterone and LH levels (Bouvattier et al. 2002). These findings suggest a role for androgen signalling in postnatal HPG axis activation. In aromatase deficient infants with low estrogen levels, gonadotropin levels are elevated in infant girls but not in boys (Belgorosky et al. 2003, Deladoey et al. 1999). This suggests that estrogens have a more important role in the HPG axis negative feedback in infant females than in males.

In Turner's syndrome, infant girls with the 45,X karyotype have higher FSH levels than healthy girls, and the levels remain elevated up to six years of age. In contrast, girls with other Turner karyotypes or mosaicism have close to normal FSH levels, suggesting retained ovarian feedback effects on pituitary FSH secretion in these girls (Fechner et al. 2006). In contrast, infant boys with Klinefelter syndrome (more than one X chromosome) have been reported to have normal levels of inhibin B, AMH, and INSL3, suggesting normal Sertoli and Leydig cell function in infancy, although elevated LH (Aksglaede et al. 2007) and FSH levels (Cabrol et al. 2011, Aksglaede et al. 2007) have also been reported. Testosterone levels in these boys have been reported to be normal (Cabrol et al. 2011) or slightly elevated (Aksglaede et al. 2007).

In girls with congenital adrenal hyperplasia (CAH), perinatal androgen levels are high because of increased adrenal androgen production. In CAH girls, higher LH levels have been reported during the first months of life compared with healthy girls of similar age (Belgorosky et al. 1996b). FSH levels on the other hand were similar as in healthy girls, but LH/FSH ratio was closer to that in healthy boys. Consequently, high perinatal androgen levels might modulate gonadotropin secretion in these girls.

In boys with congenital adrenal hypoplasia due to mutations in the NROB1 gene and DAX-1 deficiency, the postnatal HPG axis activation appears to be normal, although in these patients sexual development is impaired at puberty due to hypogonadotropic hypogonadism (Galeotti et al. 2012).

In congenital hypogonadotropic hypogonadism (CHH), both fetal and postnatal pituitary gonadotropin secretion is low. During fetal life, placental hCG stimulates the testis, resulting in masculinisation of the external genitalia. However, later in development LH is needed for further growth of the penis and testicular descent. Consequently, boys with CHH may present a micropenis and often also undescended testes at birth. In boys with hypogonadotropic hypogonadism, the lack of postnatal HPG axis activation has been associated with involution of the external genitalia after birth (Main et al. 2000). Hormone therapy has been used to induce penile growth and descent of the testis in infant boys with hypogonadtropic hypogonadism (Bouvattier et al. 2011).

In cryptorchid boys, higher FSH and LH levels, lower inhibin B levels, and reduced levels of INSL-3 in relation to LH have been observed at three months of age compared to healthy controls (Bay et al. 2007). Reported testosterone levels in cryptorchid boys have been normal (Suomi et al. 2006), lower (Pierik et al. 2009), or similar as in controls (Barthold et al. 2004). Decreased serum androgen bioactivity has been observed in infant boys with at least one undescended testis (Raivio et al. 2003).

2.3.4 Open questions on minipuberty

To summarize, according to previous observational studies in humans and experiments in non-human primates, postnatal HPG axis activation obviously results in testicular activation, and proliferation of Sertoli cells during this period is probably important for future reproductive capacity. Association of testosterone levels at three months of age with early penile growth (Boas et al. 2006) and involution of the penis and scrotum in boys with hypogonadotropic hypogonadism in infancy (Main et al. 2000) suggests a role for postnatal testosterone in "stabilizing" male genitalia. However, other possible effects of the postnatal testosterone surge

in boys have remained unknown. Analogous to true puberty, androgens secreted early in life might also have effects on linear growth, skeletal development, body composition, and psychosexual development. However, evaluation of the possible consequences of minipuberty would require adequate quantification of its magnitude. Previous studies on hormone levels during minipuberty have been almost completely cross-sectional, and hence the inter-individual differences in timing, duration, and magnitude of the minipuberty have remained unknown. Serial blood sampling from healthy infants is problematic because of its invasiveness and therefore non-invasive urine or salivary sampling would be more appropriate; however, these methodologies are not yet in routine use.

Because of the large variability in reported estradiol levels during infancy, profiling of longitudinal hormone levels is essential to gain a better understanding of the nature of ovarian activity in infant girls and thereby to learn about the possible consequences of minipuberty on female reproductive development. As estrogen target tissues, such as mammary glands and the uterus, are already stimulated at birth by the high intrauterine estradiol levels, differentiating the effects of endogenous estrogens during the first months of life is complicated. Longitudinal follow-up of the changes in estrogen target tissues might allow recognition of the biological effects of endogenous estrogens in infant girls.

The reason for the reported higher levels of reproductive hormones in premature than in full-term infants is unclear, and because longitudinal studies have been lacking, the differences in timing, duration, or magnitude of minipuberty between full-term and premature infants have remained unclear. Moreover, no previous data exist on the possible biological consequences of these higher levels in either premature boys or girls.

The importance for gaining better understanding of the perinatal hormonal milieu in normal and altered situations (e.g., prematurity) is highlighted by the current concept of developmental origins of health and disease theory (Gluckman et al. 2008). This theory has its basis in the large epidemiological studies that link perinatal events with long-term health consequences. According to this theory, adverse effects during critical developmental periods can permanently reprogram normal physiological responses resulting in increased vulnerability to metabolic and hormonal disorders in adulthood. According to present comprehension, this critical developmental period includes both the fetal period and early infancy. Cardiovascular disease, metabolic abnormalities such as insulin resistance and obesity, polycystic ovary syndrome, osteoporosis, and certain cancers are conditions, that have been linked to perinatal events (Abbott & Bacha 2013, Gluckman et al. 2008). Notably, sex hormones are associated with all of these conditions and perinatal programming of the HPG axis function and responsiveness of sex hormone target tissues might play a role in the mechanism behind observed pathologies.
The exact mechanism of the reprogramming is not yet understood but seems to involve epigenetic mechanisms (Gluckman et al. 2008). Intrauterine growth retardation followed by accelerated weight gain during the postnatal months (Kerkhof & Hokken-Koelega 2012), abnormal hormonal exposure during a critical developmental window (Zambrano et al. 2014), and prematurity (Hofman et al. 2006) are considered factors contributing to reprogramming. In epidemiological studies, premature birth has been associated with reduced reproduction rate (Swamy et al. 2008, deKeyser et al. 2012), increased risk of pregnancy complications (Boivin et al. 2012, a Rogvi et al. 2012), increased risk of testicular cancer (Crump et al. 2012), precocious pubarche (Neville & Walker 2005), and decreased insulin sensitivity (Hofman et al. 2004, Tinnion et al. 2014). Understanding the differences in the perinatal hormonal milieu and HPG axis activity in premature infants compared with healthy full-term infants will form the basis for the future studies evaluating the possible role of early sex hormones in the pathogenesis of these long-term consequences.

3 Aims of the study

The general aim of this study was to quantify and compare the changes in the HPG axis activity during the first six months of life in full-term and preterm infants in a longitudinal, prospective setting, and to examine the associated biological effects in hormonal target tissues. One aim was also to implement methodology for measurements of low hormone levels in non-invasive pediatric samples.

Specifically, the aims were the following

1) Examine the differences in the timing and magnitude of the postnatal gonadotropin and testosterone secretion in full-term and preterm boys, and evaluate the associated biological effects by measuring the changes in testicular and penile growth and PSA secretion (Study I)
2) Evaluate the effects of prematurity on the postnatal activation of the pituitary-ovarian axis by measuring longitudinal FSH and AMH levels and comparing these levels with sonographically determined changes in ovarian morphology (Study II)
3) Examine the effects of postnatal androgens in the skin by comparing urinary DHEAS and testosterone levels with the presence of sebaceous gland hypertrophy and acne during the first six months of life in full-term and preterm infants (Study III)
4) Characterize the postnatal activation of ovarian steroidogenesis by longitudinal urinary estradiol measurements and to evaluate the biological estrogen effects in the mammary glands, uterus, and vulvar epithelium in full-term and preterm girls (Study IV)

4 Subjects and methods

4.1 STUDY POPULATION

Pregnant women were recruited at the maternity clinics of Kuopio health centers and at the maternity outpatient clinic and maternity wards at Kuopio University Hospital between August 2006 and March 2008. Mothers were recruited into three cohorts, and the final cohort of the baby was determined after birth according to the length of gestation and proportionate birth size (i.e., AGA, defined as birth length and weight between -2 SD and +2SD or SGA, defined as birth length and/or weight ≤ -2 SD according to the Finnish birth size reference (Pihkala et al. 1989)). The recruitment criteria for the mothers and for the allocation of the newborns in final cohorts is shown in Figure 7. All eligible mothers were asked to participate in the study and the aim was to complete the six month follow-up in at least 20 infants in each cohort.Altogether 172 mothers were recruited, and 113 mothers and their 125 babies completed the six-month follow-up. Both parents signed an informed consent of the baby's participation in the study after the baby was born.

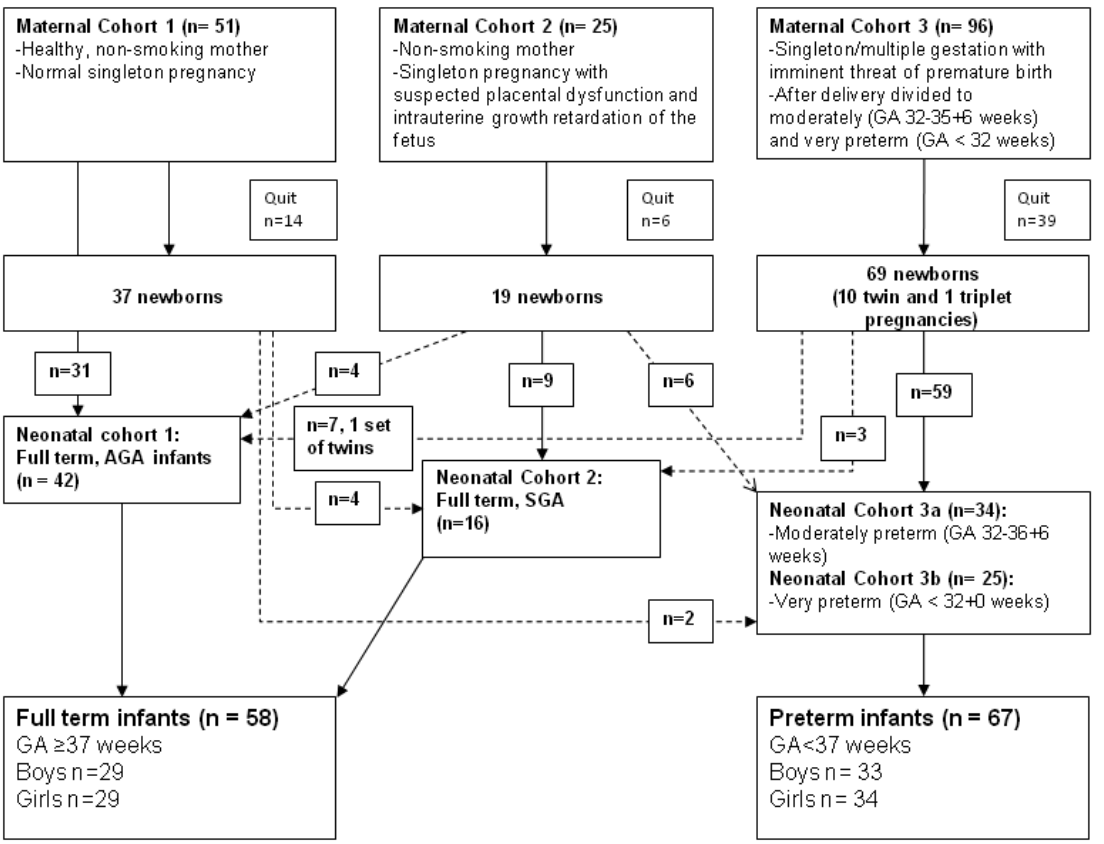

Figure 7. The flowchart of the study population. The number of those who quit in each cohort includes also infant drop-outs.

4.1.1 Full-term infants

Altogether 58 infants (29 boys and 29 girls) born at/after 37 weeks of gestation completed the study. Nine boys were born small for gestational age. In the others, birth size was appropriate for gestational age. One set of twin girls was included in this cohort. Seven girls were born SGA, and one girl was LGA (birth length > +2 SD scores). Three boys and 5 girls had received antenatal betamethasone treatment because of earlier threat of premature birth.

4.1.2 Premature infants

Sixty-seven infants (33 boys and 34 girls) born before 37 weeks of gestation were included in this cohort. Forty-two infants were born at/after 32+0 weeks of gestational age (19 boys and 23 girls) and 25 before 32 weeks of gestational age (14 boys and 11 girls). Twelve of the boys and 15 of the girls were born SGA. There were 9 sets of twins (three sets of boys, 4 sets of girls and two sets with a girl and a boy) and one set of triplets (girls). Seven boys and 11 girls had not received antenatal betamethasone treatment.

Table 1. Characteristics of the full-term and preterm infants. Data are expressed as median and range or as n and % (within the group). TTN, transient tachypnea of the newborn; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; BPD, bronchopulmonary dysplasia (diagnosis at 36 weeks of gestational age); IVH, intra-ventricular hemorrhage; NEC, necrotizing enterocolitis.

Total n=125	**FT girls**	**FT boys**	**PT girls**	**PT boys**
n	29	29	34	33
Gestational age (weeks)	39.5 (37.0–41.7)	39.8 (37.1–42.1)	32.9 (24.7–36.7)	31.8 (24.7–36.6)
Birth weight (g)	3375 (2070–4750)	3275 (1910–4420)	1765 (530–2720)	1695 (550–2850)
Birth length (cm)	49.2 (44.0–54.0)	49.1 (42.0–53.0)	41.8 (28.5–47.0)	41.3 (30.0–48.0)
Birth weight SDS	-0.6 (-2.8–1.9)	-0.9 (-3.7–1.5)	-1.7 (-4.8–0.5)	-1.25 (-3.7–1.5)
Birth length SDS	-0.9 (-3.6–2.3)	-1.0 (-4.7–1.1)	-1.5 (-6.9–2.1)	-0.9 (-4.6–2.1)
TTN (n)	2 (6.9%)	1 (3.4%)	5 (17.2%)	5 (15.2%)
Hyperbilirubinemia (n)	4 (13.8%)	0	13 (38.2%)	15 (45.5%)
RDS (n)	0	0	6 (17.6%)	14 (42.4%)
PDA (n)	0	0	5 (14.7%)	8 (24.2%)
BPD (n)	0	0	2 (5.9%)	3 (9.1%)
IVH gradus III-IV (n)	0	0	0	2 (6.1%)
NEC (n)	0	0	2 (5.9%)	2 (6.1%)

In studies I, III and IV, preterm infants are presented as one group, but in study II, premature girls were divided in two groups: near-term girls whose gestational age at birth was between 34+0 and 36+6 weeks, and very preterm girls with gestational age less than 34 weeks. This partition in study II was performed because in preliminary analyses, the outcome measures were strongly dependent on gestational age also within the preterm group. Thirty-four weeks

was chosen for cut-off gestational age, because it allowed allocation of equal number of girls in both groups.

4.2 METHODS

Outline of the study
Study outline is presented in Figure 8. The first examination was performed at the age of seven days (D7) and then monthly from one month to six months of age (M1–M6). Re-examination at the corrected age of fourteen months (cM14; fourteen months from the expected date of delivery, see Figure 9A) was performed for 99 infants (25 full-term girls, 22 full-term boys, 28 preterm girls, and 24 preterm boys).

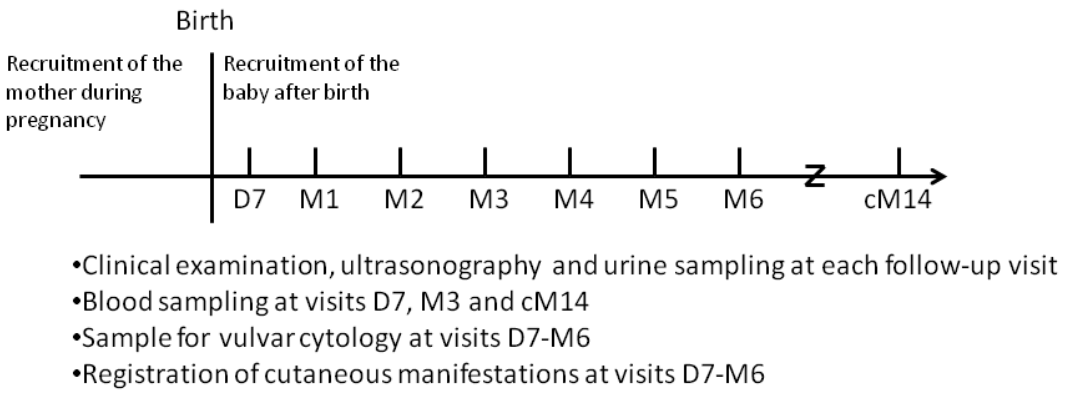

Figure 8. Outline of the study.

4.2.1 Antropometric measurements
At each follow-up visit, the recumbent length, weight, head circumference, mammary gland diameter, and areolar diameter were determined in all infants. In boys, the penile length and testicular length and width were measured. All measurements were repeated three times, and the mean was used in the analyses. In girls, a sample for vulvar cytology was obtained at visits from D7 to M6. In all infants, facial cutaneous changes were registered and photographed at follow-up visits from D7 to M6.

The recumbent length was measured by an infantometer (Holtain limited, Crymmych, Pembs, U.K.) to the nearest 0.1 cm. If the examination was performed in the neonatal intensive care unit, a portable infantometer was used. Recumbent weight was measured with a baby scale (Seca, Mod. 727 Hamburg, Germany) to the nearest 0.005 kg. Head circumference was measured with a metal tape to the nearest 0.1 cm. For very preterm babies in the neonatal intensive care unit, the measurements taken by nurses close to the examination day were used when the baby's condition did not allow taking extra measurements.

The diameters of the mammary glands were measured with a slide gauge to the nearest 0.1 cm. A mammary gland diameter less than 0.3 cm was considered unmeasurable and was marked as 0. The mean of left and right mammary gland measurements was used in the analyses.

The penile length was measured as described by Boas et al. (Boas et al. 2006). The flaccid, non-streched length was measured with a ruler to the nearest 0.1 cm from the symphysis pubis to the tip of the glans excluding the foreskin.

The length and width of both testicles were measured with a ruler to the nearest 0.1 cm. The volume of the testis was calculated by using the formula length (mm) x width (mm) x width (mm) x $\pi/6$. The position of each testicle was also recorded as described by Boisen et al. (Boisen et al. 2004). Non-palpable, inguinal, suprascrotal, or high scrotal position was considered as undescended and retractile and normal scrotal as descended.

4.2.2 Cytological samples of vulvar epithelium

The samples for vulvar cytology were obtained by gently wiping the inner surface of the labia minora with a cotton stick wetted in physiological saline. The sample was placed on a sheet of glass and fixed in 98% ethanol for 15 minutes. After staining, conventional cytomorphometric methods were used for the estimation of hormonal effects on epithelial maturation. One hundred cells were counted and classified as parabasal, intermediate, or superficial cells.

4.2.3 Registration of cutaneous manifestations

Four benign cutaneous changes typical for infancy were distinguished from each other and recorded. These included SGH, milia, erythema toxicum neonatorum, and acne. SGH was defined as white-yellowish, regularly spaced papules without inflammation, most often seen in the central part of the face. Milia were defined as discrete, firm, small (diameter < 2 mm) white papules. Erythema toxicum neonatorum was defined as discrete papules or pustules with surrounding erythema of 1–3 cm in diameter occurring during the first week of life. SGH, milia, and erythema toxicum neonatorum were recorded as present or absent. Acne was defined as more than five inflamed papules, pustules, or comedones on the face. The severity of acne was divided into three categories: 5–10 papules, 11–50 papules, and >50 papules.

4.2.4 Ultrasonographic measurements

Three paediatric radiologists performed the ultrasonographical measurements. All measurements were done with an ATL HDI 5000 (Philips Medical Systems). The measurements were done with the child lying supine and the assistant gently holding the child in place. Warmed ultrasound gel was used. The measurements were repeated three times and the mean was used for the analyses.

The length of the penile corpora cavernosa was determined by using a 7.5 MHz linear transducer probe by the previously described method (Smith et al. 1995). The length was measured longitudinally on the dorsal surface of the flaccid penis. The measurement line was drawn along the echogenic line of the urethra.

The length and width of the testis were measured in a single longitudinal plane. The epididymis was not included in the measurements. The position of the testis (scrotum/ inguinal canal) was also determined. The same formula was used to calculate the volume of the testis as described above for manual measurements.

The length of the uterus and the thickness of the fundus and the corpus were measured in the longitudinal section. Along the transverse section, the maximum width of the corpus was measured. If the endometrial lining was visible it was registered, and its thickness was measured.

If the ovaries were identifiable, their length and width were measured in a single longitudinal plane. Ovarian volume was calculated as length (mm) x width (mm) x width (mm) x $\pi/6$. The visible ovarian follicles were counted, and the diameters of ≥ 6 mm were registered.

4.2.5 Urine samples and assays

Spot urine samples were collected at every follow-up visit. In most cases, a plastic urine collection bag was used. In some cases the urine samples were obtained by a straight catch into a plastic cup during the examination of the baby. Urine samples were divided in aliquots and stored in -70°C until analyses.

All urinary analytes were corrected for creatinine to adjust the results for urine concentration. Urinary creatinine was analyzed by an enzymatic method before the urine was stored in -70°C. Urinary creatinine was measurable in all samples and did not show constant differences between full-term and preterm infants or boys and girls.

4.2.5.1 Urinary gonadotropin assays

Urinary LH and FSH levels were quantified with a sensitive time-resolved immunofluorometric assay (AutoDELFIA, Wallac, Turku, Finland) adapted for measurements of urinary samples. The detection limit of the LH assay was 0.05 IU/l, and the inter-assay coefficient of variation (CV) was <4% in the concentration range of 0.3–42 IU/l. For FSH, the detection limit was 0.05 IU/l, and the inter-assay CV <5% in the concentration range of 2–78 IU/l.

4.2.5.2 Urinary testosterone assay

Urinary testosterone was measured with high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) after enzymatic hydrolysis of glucuronides. The method is described in more detail in Study I. The majority of the testosterone in urine was in conjugated form and only a small fraction (~1%) was unconjugated; thus, testosterone detected by the assay mostly came from testosterone glucuronide. The limit of quantification (LOQ) was 0.2 nmol/l.

4.2.5.3 Urinary DHEAS assay

To cleave steroid sulphates, urine samples were subjected to solvolysis, and urinary DHEA concentrations were measured by HPLC-MS/MS. The contribution of unconjugated DHEA and DHEA glucuronide to the measured DHEAS was very small. The majority of the DHEA in urine was in conjugated form, and unconjugated fraction was undetectable. In the assay the recovery of DHEA glucuronide was only 1.8-3.8%. Thus, DHEA detected by the assay mostly came from DHEAS. The method is described in more detail in Study III. The between-run imprecision (CV %) for urinary DHEAS was 7.6% at 85 nM, 6.2% at 343 nM and 2.7% at 1119 nM. The LOQ was 10 nmol/l.

4.2.5.4 Urinary estradiol assay

Urinary estradiol was measured by HPLC-MS/MS. Prior to the mass spectrometric analysis, all urine samples were subjected to enzymatic hydrolysis to cleave estradiol glucuronide and sulphate conjugates. Thus, urinary total estradiol was measured, including both urinary free estradiol and conjugated forms. The method is described in more detail in Study IV.

The relative recoveries of five spiked urine samples were 98.8–102.8% for 500 pmol/l of estradiol glucuronide and 82.3–93.9% for 500 pmol/l of estradiol sulphates. The between-run imprecisions (CV %) for urinary total estradiol were 8.8% at 159 pmol/l, 5.0% at 990 pmol/l and 3.3% at 4686 pmol/l (n = 10). The LOQ was 40 pmol/l (1:2 diluted urine). Urinary estradiol levels were under the quantification limit of the assay in 86.6% of all measurements in boys and in 38.0% of all measurements in girls.

4.2.5.5 Urinary PSA assay

Urinary total and free PSA were analyzed by the Wallac Prostatus PSA Free/Total Autodelfia assay (PerkinElmer-Wallac, Turku, Finland). The detection limit for total PSA was 0.1 μg/l and 0.01 μg/l for free PSA. In the concentration range 0.5–50 μg/l, the intra-assay CVs were 4.3–18.8% for total PSA and 4.1–7.8% for free PSA (Mitrunen et al. 1995).

4.2.6 Blood samples and AMH assay

Blood samples for AMH measurements were obtained from girls at one week and three months of age and at the corrected age of 14 months. In preterm babies the amount of the blood sample was proportioned with the weight of the infant. After centrifugation, serum was aliquoted and frozen in -70°C.

Serum AMH concentrations were determined by using an enzyme immunometric assay (Diagnostic Systems Laboratories, Webster, TX). Intra- and inter-assay coefficients of variation were 4.6% and 8.0%. The minimum detection limit was 0.006 ng/ml.

4.2.7 Statistical analyses

The results were analyzed according to chronological age (D7–M6) and, to account for the immaturity of the premature infants, according to postmenstrual age as well. These age definitions are clarified in Figure 9A. Consequently, the data were categorized according to postmenstrual age in four-week intervals beginning from 26 weeks of postmenstrual age (illustrated in Figure 9B).

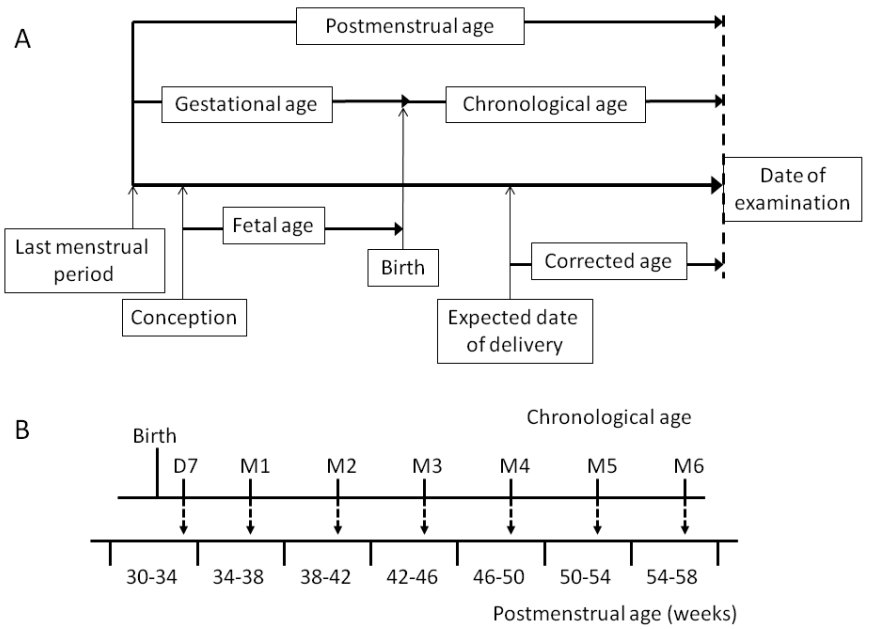

Figure 9. A. Age terminology in the perinatal period (modified from Engle & American Academy of Pediatrics Committee on Fetus and Newborn 2004).
B. Categorization of the results according to postmenstrual age. As an example, categorization of the results of an infant born on 32 weeks of gestation is shown.

Comparisons of hormone levels and other continuous variables between and within groups were carried out using a mixed models analysis. A group, time point, or postmenstrual age category and birth weight and length in SD scores were included in the model as fixed effects. The subject and twinness were included as random effects. Birth size SD scores were included in the model since size at birth has been suggested to influence hormone levels (Forest et al. 1980, Sir-Petermann et al. 2010, Chellakooty et al. 2003, Ibanez et al. 2002) and both cohorts

included SGA infants. The hormonal data were right-skewed, and therefore they were transformed to achieve normality of residuals in the mixed models analyses. All results of urinary analytics that were under the detection limit of the assays were artificially set to zero because setting these results on the detection limit would have falsely elevated or decreased the values after creatinine correction. In order to avoid zero values from dropping out when logarithmic transformation was needed, a constant value was added to all measurements before taking the logarithms. Interobserver variation in the sonographic measurements was taken into account by including the observer as a fixed factor in the analysis.

A summary measure to describe the average hormonal concentration from D7 to M6 was calculated to enable the use of correlation analysis between hormone levels and testicular and penile growth in Study I. Consequently, an average level for each hormone in each infant was defined by calculating the area under a curve (AUC) with the trapezoid rule and dividing it by total time. Those for whom either the first or the last measurement or the expected peak value at M1 was missing were excluded from AUC calculations. Consequently, average hormone levels were calculated for 24 full-term and 21 preterm boys. The associations of average hormone levels with each other and with testicular and penile growth percentages were tested using Spearman's correlation.

The proportions of girls with and without antral follicles (marked as 1 and 0, respectively)—also including those measurements where ovaries were not identified—were compared by using generalized estimating equations with binary logistic assumptions, with similar adjustments as in the mixed models analysis mentioned above (Study II). This same method was used to compare the proportions of infants with or without SGH or acne at each time point (Study III) and the proportions of girls with superficial epithelial cells (Study IV). Fisher's exact test was used to compare the duration of SGH and acne and the severity of acne between groups (Study III).

SPSS software version 17.0 or 19.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. P values <0.05 were considered statistically significant.

4.3 ETHICAL CONSIDERATIONS

Consent for the study plan was obtained from the Ethical Review Board of Kuopio University Hospital (Permission number 26/2006, dated 14.3.2006). Both parents signed an informed written consent for the participation of the infant. The procedural pain associated with venipunctures was reduced by using oral sucrose with or without a pacifier at the time of D7 and M3 blood sampling. At cM14, topical analgesia was used. In sick premature infants, the measurements and the samplings were performed only if the child's clinical condition allowed for it.

5 Results and discussion

5.1 HORMONE LEVELS

5.1.1 Urinary gonadotropins

Boys (Study I, Figure 10)

Urinary LH was measurable in all except one sample, and FSH was measurable in all samples in boys. In the full-term boys, urinary LH levels were highest from D7 to M1 and then decreased significantly towards M6 (P<0.0001). FSH levels decreased steadily from D7 to M6 (P<0.0001). In preterm boys, both LH and FSH levels increased from D7 to peak at M1 (P<0.0001 for the increase) and then decreased to M6 (P<0.0001). Consequently, there was a clear increase in gonadotropin levels from D7 to M1 in preterm boys, but in full-term boys the levels were high already at D7, and no subsequent rise was observed, which is in accordance with a previous study on serum gonadotropin levels in full-term boys (Bergada et al. 2006).

At D7, LH levels were not significantly different between full-term and preterm boys, while FSH levels were significantly lower in preterm boys (P=0.002). However, after D7, LH levels were higher in preterm than in full-term boys from M1 to M4 (P=0.002–<0.0001) and at M6 (P=0.017) and FSH levels from M1 to M3 (P=0.03–0.002). Consequently, gonadotropin levels in minipuberty were significantly higher in premature boys than in full-term boys, which had not been reported earlier.

To evaluate the effect of immaturity of the preterm infants in the observed difference in postnatal gonadotropin levels between the groups, comparisons were also made according to postmenstrual age (Figure 10). In these analyses, LH and FSH levels were higher in preterm than in full-term boys only between 38–42 postmenstrual weeks (P<0.0001 for LH and P=0.037 for FSH), but not after this. This suggests that postmenstrual rather than chronological age determines the duration of the postnatal pituitary activity and that the differences in gonadotropin levels according to chronological age observed between full-term and preterm boys are explained by a "time-shifting" of the axis in preterm boys.

Girls (Study II, Figure 11)

Urinary FSH was measurable in all of the samples in girls. FSH levels in girls were compared between full-term, near-term (gestational age H34+0–H36+6), and very preterm (gestational age <H34+0) girls from D7 to M6 and also at cM14. In full-term girls, FSH levels were highest at D7 and M1 and then decreased by M2 (P=0.04). From M2 to cM14, FSH levels in full-term girls remained fairly constant and no statistically significant changes were observed.

Compared with full-term girls, FSH levels were significantly higher in near-term girls until M1 (P<0.0001 at D7 and P<0.0001 at M1) and in very preterm girls until M3 (P<0.0001–0.001). From D7 to M1, FSH levels between near-term and very preterm girls were not significantly different, but at M2 and M3 very preterm girls had higher FSH levels than near-term girls (P=0.0001). At M1, very preterm girls had up to 18-fold higher FSH levels than full-term girls and 5-fold higher FSH levels than near-term girls. FSH levels were similar in full-term and very preterm girls from M4 to cM14, but in near-term girls FSH levels were significantly lower than in full-term girls from M4 to M6 (P=0.001–0.007).

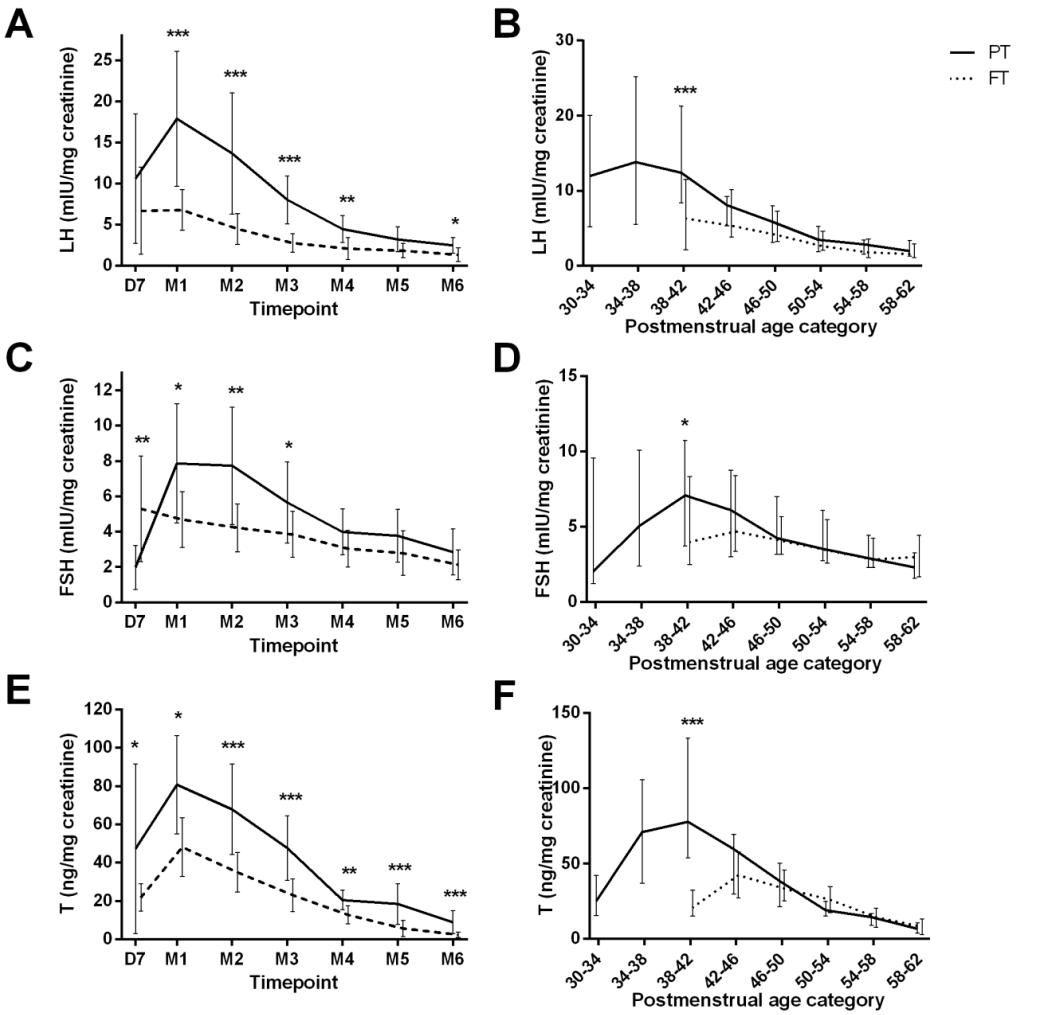

Figure 10. Urinary LH (A), FSH (C), and testosterone (E) levels in full-term (FT) and preterm (PT) boys from one week of age (D7) to six months of age (M1–M6) according to chronological age (time from birth). Lines present medians and error bars present quartiles. The same hormone levels are presented according to postmenstrual age in panels B (LH), D (FSH), and F (testosterone). The asterisks present the statistical significance for the difference between hormone levels in FT and PT boys in the mixed models analyses. *P<0.05, **P<0.01, ***P<0.001.

According to postmenstrual age, highest urinary FSH levels were seen before the postmenstrual age of 40 weeks, and after this age the levels declined steeply. FSH levels were significantly higher in near-term and very preterm girls than in full-term girls between 38–42 weeks (P=0.0001). In near-term girls, FSH levels were lower than in full-term girls from 54 to 62 weeks of postmenstrual age (P=0.0005–0.001), whereas the levels in full-term and very preterm girls were similar after 42 weeks. At cM14, FSH levels did not differ significantly between the three groups.

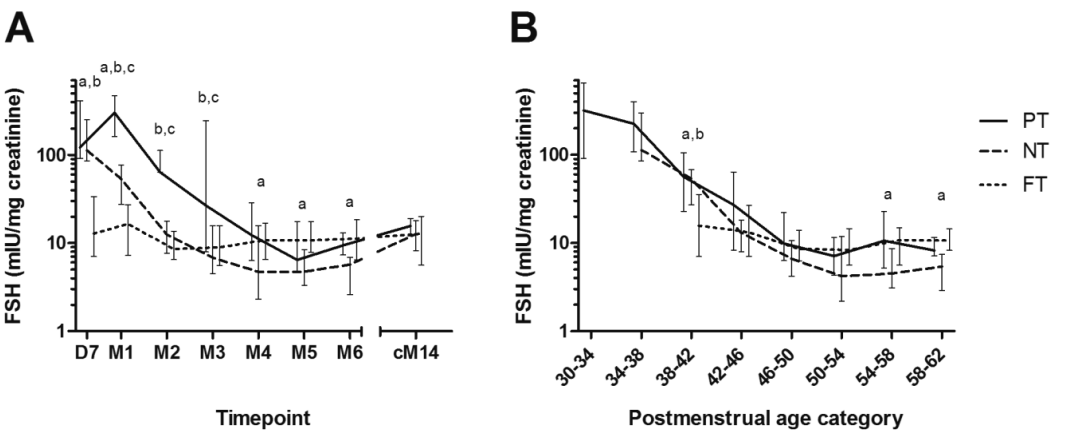

Figure 11. A) Urinary FSH levels (medians and quartiles) in preterm (PT), near-term (NT) and full-term (FT) girls from one week (D7) to six months of age (M1–M6) and at fourteen months of corrected age (cM14). B) Urinary FSH levels presented according to postmenstrual age in the same groups of girls. Letters represent the statistically significant difference between groups ($P<0.05$): a: FT vs. NT; b: FT vs. PT; c: NT vs. PT.

These results confirmed the earlier reports on increased postnatal FSH surge in prematurely born girls compared with full-term girls (Shinkawa et al. 1983, Tapanainen et al. 1981b, Greaves et al. 2008b). The analysis according to postmenstrual age revealed that the very high, postmenopausal FSH levels in prematurely born girls decline close to the levels in full-term girls around term age. This was a novel finding and indicates a clear change in the pituitary activity in girls at that postmenstrual age, possibly reflecting the maturation of the negative feedback mechanisms. A similar sharp decrease in urinary FSH levels at term age in premature girls has also been reported later by de Jong et al (de Jong et al. 2013).

In accordance with previous studies (Andersson et al. 1998, Schmidt & Schwarz 2000, Bergada et al. 2006, Shinkawa et al. 1983, Ibanez et al. 2002, Sir-Petermann et al. 2007, Burger et al. 1991, Belgorosky et al. 1996a), FSH levels in full-term girls were significantly higher than in full-term boys ($P<0.0001$ at every timepoint from D7 to M6). The effect of prematurity on FSH levels was stronger in girls than in boys, since median FSH levels in premature girls were remarkably higher than in premature boys (100-fold higher between 30–34 postmenstrual weeks, 20-fold higher between 34–38 postmenstrual weeks, and 5-fold higher between 38–42 postmenstrual weeks). This sex difference is probably explained by the later maturation of the negative feedback system mediated by the gonads in girls than in boys and is discussed more in section 5.1.2.2.

5.1.2 Gonadal hormones

5.1.2.1 Urinary testosterone

The examination of longitudinal urinary testosterone levels in each group revealed a subset of infants with very low levels or undetectable levels at every time point. On the other hand, all other urinary analytics were in the same range as in the rest of the infants of the same group; this suggests that low urinary testosterone levels were not associated with altered gonadotropin levels or renal function. Review of the results according to postmenstrual age showed two distributions of longitudinal testosterone levels, more clearly in boys (Figure 12).

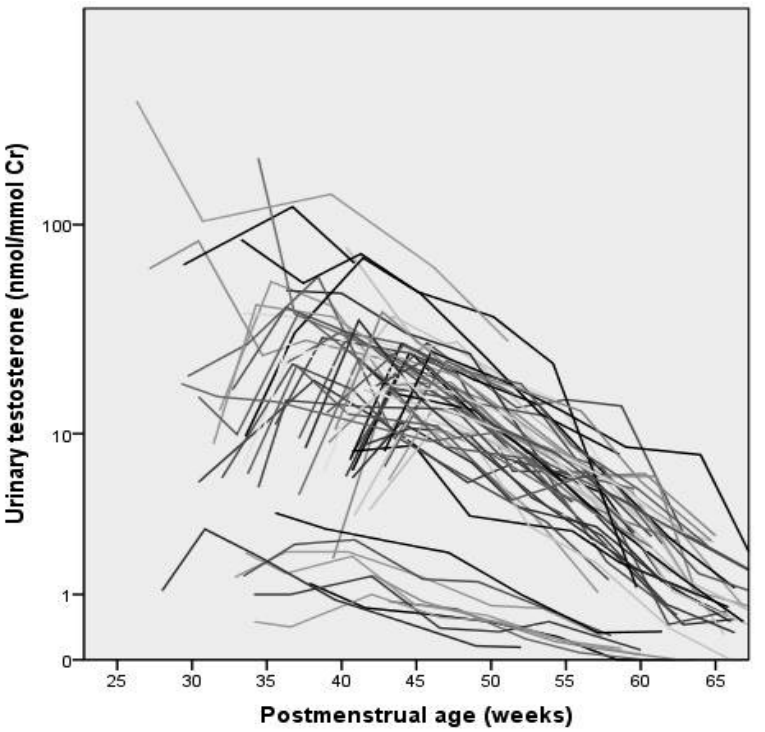

Figure 12. Two distributions of longitudinal urinary testosterone levels in boys. The lower distribution probably presents levels of those infants with the homozygous deletion of UGT2B17 gene that results in significant reduction of urinary testosterone excretion. Note the logarithmic scale.

This observation was in line with the previously reported differences in urinary testosterone levels between individuals with at least one copy of the UGT2B17 gene and individuals with homozygous deletion of this gene (Juul et al. 2009). The UGT2B17 gene codes the most important testosterone-glucuronidating enzyme UGT2B17, and homozygous deletion of this gene results in the lack of this enzyme and significantly lower urinary testosterone glucuronide levels than in those with one or two copies of this gene. This homozygous deletion has been estimated to be present in approximately 10% of Caucasians (Wilson et al. 2004). Despite the reduced capability to excrete testosterone as glucuronide in urine, blood levels of testosterone or serum androgenic activity in individuals with homozygous deletion of UGT2B17 are not higher than in those with normal UGT2B17 function (Ekstrom et al. 2011). It is assumed that different metabolic pathways are used to excrete testosterone in individuals with homozygous deletion of the UGT2B17 gene, but this is not yet fully understood (Schulze et al. 2011). Consequently, because of the different metabolic fate of testosterone, urinary testosterone glucuronide levels in these subjects underestimate the circulating testosterone levels when compared with subjects with normal testosterone glucuronidation capacity.

Because of the clear difference in urinary testosterone levels without any abnormality in any other measured hormone levels or physical findings, these infants with very low urinary testosterone levels were most likely carriers of homozygous deletions of the UGT2B17 gene and these infants were excluded from those analyses involving urinary testosterone levels (Study I and III). Among these infants were three full-term boys, seven preterm boys, and five preterm girls (12% of the total study population). The finding that there were two sets of twins, of whom both siblings presented very low urinary testosterone levels, supported the genetic background

of the observed difference. Unfortunately, because of the lack of adequate DNA samples, we could not verify the suspected homozygous deletion of the UGT2B17 gene.

Boys (Study I & III, Figure 10)
Testosterone levels increased significantly from D7 to M1 (P<0.0001) in both full-term and preterm boys and then decreased quite steadily to M6 (P<0.0001). The peak testosterone levels were observed in both groups at M1, which is earlier than reported in previous cross-sectional studies on serum levels displaying the peak testosterone levels at 1–3 months of age (Tapanainen et al. 1981b, Forest et al. 1974).

Testosterone levels were higher in preterm than in full-term boys at every time point from D7 to M6 (P=0.034–<0.0001), supporting the findings in previous cross-sectional studies (Forest et al. 1980, Tapanainen et al. 1981b). However, after adjusting for postmenstrual age, preterm boys only had higher testosterone levels between 38 to 42 postmenstrual weeks (P<0.0001); after that, there was no significant differences between the groups. The pattern of urinary testosterone levels followed closely the pattern of urinary LH secretion.

Our results indicate that regardless of developmental age at birth, the pituitary-testicular-axis activates soon after birth, and peak testosterone levels are seen approximately at one month of age. The magnitude of the pituitary-testicular-axis activation appears to depend on the developmental age, so that the highest pituitary and testicular activity are seen in those that are most premature. According to postmenstrual age, the suppression of the axis occurs at a similar developmental age in both full-term and preterm boys.

Higher androgen levels in preterm than in full-term boys in early infancy raise a question about the possible organizational and programming effects of hyperandrogenism in preterm infants. Through programming, hyperandrogenism in infancy could influence the characteristics of growth, body composition, fat distribution, blood pressure, and lipid and glucose metabolism thereby contributing to risk factors of many chronic diseases. In addition, androgens have organizational effects in the brain (Hines 2008), and abnormally high testosterone levels might affect neural and behavioral development in preterm boys. These possible effects require further studies in prematurely born boys later in life.

Girls (Study III)
Median urinary testosterone levels were 1.2–8-fold lower in full-term girls than in full-term boys (P<0.0001–0.03). This sex difference was most pronounced at M2 and M3 and decreased towards M6. In preterm infants, the difference between sexes was not significant at D7, but after D7 boys had 1.6–3.4-fold higher urinary testosterone levels than girls (P≤0.001).

In full-term girls, urinary testosterone levels decreased steadily from D7 to M6. In preterm girls, urinary testosterone levels increased significantly from D7 to M1 (P=0.001) and then decreased until M6. From M1 to M6, preterm girls had significantly higher urinary testosterone levels than full-term girls. The urinary testosterone in girls is probably mainly of adrenal origin or metabolized from adrenal precursors in peripheral tissues. In accordance with this, the urinary DHEAS levels, originating from the adrenals, were higher in preterm than in full-term girls as well, (see 5.1.3.). The possible biological effects of high androgen levels in preterm girls are not known. However, transient clitoral hypertrophy reported in some premature girls could be associated with these high androgen levels (Midgley et al. 1990, Dumont et al. 2009, Greaves et al. 2008a).

5.1.2.2 Urinary estradiol (Study IV, Figure 13 and 14)
Girls
Estradiol levels were low at one week of age in both full-term and preterm girls (Figure 14 A) but increased to M1 and were significantly higher than in boys from M2 in full-term (P<0.0001) and from M1 in preterm infants (P<0.0001). This observed sex difference in postnatal estradiol levels is in agreement with previous cross-sectional (Winter et al. 1976, Schmidt et al. 2002,

Bidlingmaier et al. 1973) or semilongitudinal findings (Kuhnle et al. 1982) and indicates the activation of ovarian steroidogenesis in early infancy. After six months of age, median estradiol levels decreased to cM14, but in 61.5% of girls levels were detectable even at cM14. The observed rise in urinary estradiol levels during the first six months of life and the consequent decrease to cM14 is in agreement with Bidlingmaier et al., where increased ovarian estradiol concentrations were demonstrated during the first six months of life compared to the end of the first year in post-mortem samples (Bidlingmaier et al. 1987).

In contrast to the clear peak in testosterone levels in boys during the first months of life, individual estradiol levels in infant girls showed considerable fluctuation, and no uniform temporal pattern could be distinguished (Figure 13). This fluctuation in levels is in line with previous cross-sectional studies that showed that measured estradiol levels in infant girls have ranged from very high to unmeasurably low (Chellakooty et al. 2003, Winter et al. 1976, Kuhnle et al. 1982). Because of this fluctuation, it has not been clear whether the postnatal rise in estradiol levels occurred only in some individuals. In the present data, there were only two girls out of 63 (3.2%) who had undetectable estradiol levels in all samples during the follow-up, indicating that postnatal activation of ovarian steroidogenesis is a general event in girls. Only four girls had detectable estradiol levels in all samples. Because of the changing levels, ovarian activity in infancy cannot be adequately described by singular estradiol measurements.

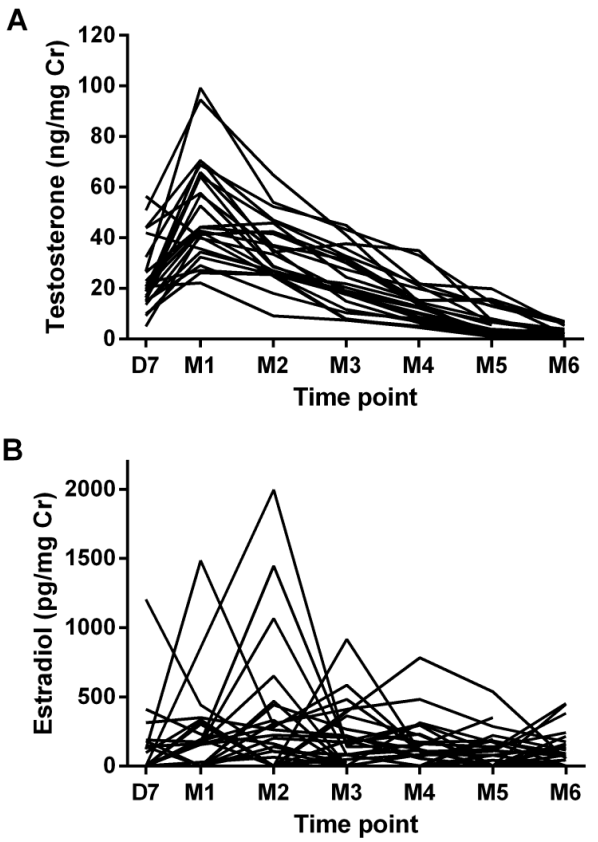

Figure 13. The pattern of individual testosterone levels in full-term boys (A) and estradiol levels in full-term girls (B). In boys, there was a uniform temporal pattern in testosterone levels, but estradiol levels in girls were fluctuating, and no uniform pattern could be recognized.

Premature girls had significantly higher urinary estradiol levels than full-term girls at M1, M4, and M5 (P≤0.03). However, inspection of the estradiol levels according to postmenstrual age showed that the levels were clearly affected by postmenstrual age: estradiol levels in preterm girls remained low until 38 weeks then increased and were significantly higher than in full-term girls from 38–46 weeks postmenstrual age (P<0.0001–0.03) (Figure 14 B). These higher estradiol levels in premature than in full-term girls suggest increased ovarian activity after premature birth, which is in accordance with previous data, that report higher serum estradiol levels in premature than in full-term girls at three months of age (Chellakooty et al. 2003). Because FSH has a central role in ovarian estradiol production, higher FSH levels in premature than in full-term girls probably contribute to the elevated estradiol levels. However, despite the high FSH stimulation, ovarian estradiol production in preterm girls does not appear to begin properly before close to term. This could reflect the developmental time-schedule of ovarian follicles as estradiol synthesis is most powerful in large antral follicles, and these follicles are usually observed in fetal ovaries only at late pregnancy. As estradiol levels increased, high FSH levels decreased, suggesting suppressive effects of estradiol on FSH secretion. Consequently, extremely high FSH levels in preterm girls before term might be in part due to the lack of negative feedback effects of ovarian estradiol. Central inhibitory mechanisms seem to be already operational in premature girls by the beginning of the last trimester as evidenced by the capacity of exogenous estrogen to suppress gonadotropin levels in premature girls around 30 weeks of gestational age (Trotter et al. 1999). In addition to estradiol, inhibin B levels are important in suppressing FSH secretion, but inhibin B measurements were not included in the present work.

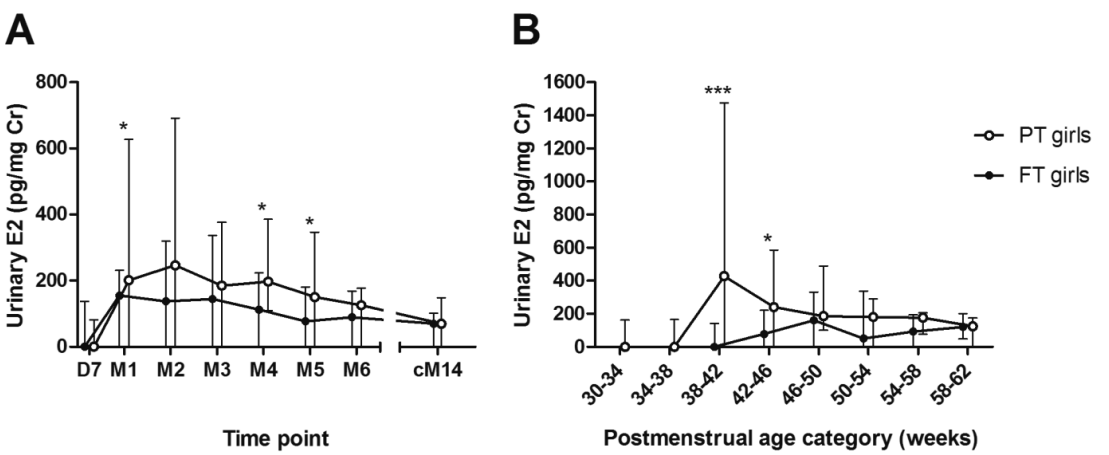

Figure 14. A) Urinary E2 levels (medians and quartiles) in full-term (FT) and preterm (PT) girls from one week (D7) to six months (M1–M6) of age and at fourteen months of corrected age (cM14). B) Estradiol levels presented according to postmenstrual age. The asterisks present the statistical significance for the difference between urinary estradiol levels in FT and PT girls in the mixed models analyses. *P<0.05, **P<0.01, ***P<0.001.

Boys

Only some boys had measurable levels of estradiol, and there were no statistically significant differences between full-term and preterm boys. Most (71%) of the detectable levels of urinary estradiol in boys were seen before M3 and probably originate from the testes in association with the postnatal testosterone surge or are aromatized from testosterone in peripheral tissues. This

agrees with a previous finding of elevated estradiol levels in the testicular tissue of infant boys during the first months of life in comparison to levels after three months of age (Bidlingmaier et al. 1987).

5.1.2.3 Serum AMH levels (Study II, Figure 15)
AMH levels were lowest at D7 and increased by M3 in full-term, near-term, and preterm girls. An 8-fold increase was seen in full-term girls, a 17-fold increase in near-term girls, and a 7-fold increase in very preterm girls (P<0.0001 for all). In full-term and near-term girls AMH levels decreased from M3 to M14 (P=0.016 in full-term girls and P=0.001 in near-term girls), but in very preterm girls, the level remained the same. At D7, the median AMH level in full-term girls was 1.5-fold higher than in near-term girls and 2-fold higher than in very preterm girls. At M3, AMH levels in full-term and near term girls were similar, but in very preterm girls, AMH levels were 2.5- to 3-fold lower. At cM14, AMH levels were similar in the three groups. There was a significant negative correlation between AMH and FSH levels at D7 (Spearman's rho -0.42, P=0.001) and M3 (Spearman's rho -0.62, P<0.0001).

The increase of serum AMH levels after birth demonstrates postnatal proliferation of granulosa cells and activation of ovarian follicular development. A similar postnatal peak in AMH levels has been reported previously in full-term girls (Hagen et al. 2010). Higher AMH levels in full-term than in near term and very preterm girls at D7 probably indicates larger granulosa cell mass and more advanced follicular development prior to birth.

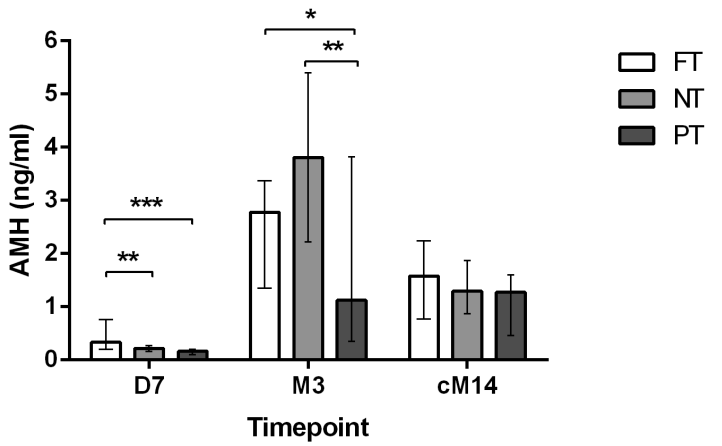

Figure 15. Serum AMH levels in full-term (FT), near-term (NT), and preterm (PT) girls at one week of age (D7), three months of age (M3), and at corrected age of 14 months (i.e., 14 months from the expected date of delivery). Asterisks indicate the significance for the difference between groups in a mixed models analysis. *** P<0.001, ** P<0.01, *P<0.05. Bars present medians, and error bars present upper and lower quartiles.

5.1.3 Urinary DHEAS (Study III)
Urinary DHEAS levels from D7 to M6 did not show significant sex differences in either full-term or preterm groups. However, preterm infants had significantly higher DHEAS levels than full-term infants from D7 to M5 in girls and from D7 to M4 in boys. In full-term infants, median urinary DHEAS levels were highest at D7 then decreased and were below the quantification limit of the assay by M2. In preterm infants, median urinary DHEAS levels remained high from D7 to M2 and then decreased to levels below the quantification limit of the assay by M4 in preterm boys and by M5 in preterm girls. After correction for postmenstrual age, DHEAS levels

were significantly higher in preterm infants than in full-term infants from 38 to 46 postmenstrual weeks (P<0.001–0.045). Median DHEAS levels in full-term boys and girls as well as in preterm boys fell below the quantification limit of the assay by 46 weeks of postmenstrual age. In preterm girls, median DHEAS levels were measurable longer, until 54 weeks of postmenstrual age.

Higher and prolonged postnatal secretion of DHEAS in preterm than in full-term infants suggests prolonged postnatal activity of the fetal adrenal cortex in preterm infants, which is in line with previous studies (Heckmann et al. 2006, Forest et al. 1980, Midgley et al. 1996, Grueters & Korth-Schutz 1982, Wallace et al. 1987). At least in boys, this postnatal secretion of DHEAS seems to decrease at the same postmenstrual age as in full-term infants, suggesting that the involution of the fetal adrenal cortex may be developmentally regulated.

Because urinary testosterone (representing mostly testosterone glucuronide) levels originate in part from the peripheral conversion of androgen precursors such as DHEAS, higher urinary testosterone levels in preterm than in full-term infants might be explained in part by higher levels of DHEAS. Especially in preterm girls, high postnatal urinary testosterone levels are probably metabolized from adrenal precursors in peripheral tissues. Supporting this, urinary testosterone and DHEAS levels correlated significantly (Spearman's rho 0.43–0.72; P=0.024–0.0001) in preterm girls from D7 to M6.

5.2 EFFECTS ON TARGET TISSUES

5.2.1 Male reproductive organs (Study I)

5.2.1.1 Penile length

The penile length and the sonographically measured length of the corpus cavernosum increased in both full-term and preterm boys from D7 to M6 (P<0.0001); the increase was greatest during the first months after birth, which is in accordance to a previous study (Boas et al. 2006). Penile length was larger in full-term than in preterm boys from D7 to M2 (P=0.042–<0.0001), but after this there was no significant difference between the groups. Faster penile growth in preterm than in full-term boys led to catch-up in penile length earlier than catch-up in height or weight in comparison with full-term boys. The mean increase in penile length was 7.0 mm (SD 1.9 mm) in full-term and 10.1 mm (SD 3.7 mm) in preterm boys and in the length of corpus cavernosum 12.3 mm (SD 3.3 mm) in full-term and 15.7 mm (SD 6.5 mm) in preterm boys. From M6 to cM14 the length of the corpus cavernosum did not change significantly in either group. According to postmenstrual age, there were no significant differences in penile length or in the length of the corpus cavernosum between the groups.

Maximum penile growth percentage correlated positively with the average urinary testosterone levels ($AUC_{D7\text{-}M6}$ divided by total time) (Spearman's rho: 0.47, 95% CI: 0.21–0.67, P=0.001) indicating that postnatal testosterone surge is associated with penile growth in infant boys. This is in accordance with an earlier study, where a positive correlation was found between penile length and serum testosterone levels at three months of age (Boas et al. 2006). In addition, supporting the role of postnatal HPG axis activation in penile development, poor phallic growth has been described in hypogonadal male infants (Main et al. 2000).

5.2.1.2 Testicular volume

Testicular volume increased significantly (P<0.0001) from D7 to M5 in both groups. In full-term boys the median testicular volume at D7 was 0.19 ml (quartiles 0.15 and 0.26 ml) and increased to 0.44 ml (0.35–0.48ml) at M5. In preterm boys, median testicular volume was 0.08 (0.06–0.11) ml and increased to 0.37 (0.28–0.50) ml at M5. In both groups, testicular volume decreased from M5 to cM14 (to 0.33 [0.22–0.43] ml in full-term boys, P=0.001, and to 0.30 [0.24–0.38] ml in preterm boys P=0.003). Consequently, the increase in median testicular volume was approximately 2-fold in full-term and 4-fold in preterm boys. Testicular volume was significantly larger in full-term than in preterm boys from D7 to M5 (P=0.047–<0.0001).

However, according to postmenstrual age, there were no significant differences in testicular volumes between the groups.

The testicular volumes and the pattern of testicular growth in full-term boys were similar to those reported for full-term infant boys by Kuijper et al. (Kuijper et al. 2008). Compared with the testicular volumes in a larger cohort of Finnish boys (Main et al. 2006), the volumes in our study were in the same range at birth, but larger at three months of age. The reason for this difference is not clear but could be due to our smaller sample size.

Maximum testicular growth percentage correlated positively with average testosterone and LH and FSH levels (assigned as $AUC_{D7\text{-}M6}$ and divided by total time). The increase in testicular size probably reflects the proliferation of Sertoli cells in the seminiferous tubules after FSH stimulation (Chemes & Chemes 2001). A positive correlation has been found between testicular volume and inhibin B, a Sertoli cell product stimulated by FSH, in infancy (Main et al. 2006). In this regard, higher FSH levels in preterm than in full-term boys from M1 to M3 might contribute to their faster testicular growth during the first postnatal months.

In full-term boys, testicular position was scrotal in all but one boy from D7 to M6. Also in this boy with unilaterally undescended testis at D7, spontaneous descent occurred by M2. In preterm boys, undescended testis was observed in 9/24 (37.5%) at D7, 5/23 (21.7%) at M1, and 1/24 (4.2%) at M2. By M3 testes were scrotal in all preterm boys. At cM14, undescended testis was observed in two full-term and one preterm boy. Testicular descent is normally completed during the last trimester of pregnancy and therefore cryptorchidism is a common finding in premature newborn boys (Damgaard et al. 2008). Androgens are essential for the last phase of testicular descent, and in this regard high postnatal testosterone levels in preterm boys might have a role in completion of the testicular descent.

5.2.1.3 Urinary PSA levels

The results of total and free urinary PSA were similar, and because the detection limit of the assay for free PSA was 10-fold lower than that for total PSA, only data for free urinary PSA are presented. Urinary free PSA levels increased in both full-term and preterm boys during the first months of life ($P<0.001$). In full-term boys, free PSA levels increased to peak at M2 and then decreased and were undetectable by M5. In preterm boys, free PSA levels remained elevated longer, and by M6 levels were still detectable in most preterm boys. By cM14, free PSA levels also decreased in preterm boys. Consequently, the changes in urinary PSA levels followed the changes in urinary testosterone levels in both groups. According to postmenstrual age, there were no statistically significant differences in free PSA levels between full-term and preterm boys. PSA and testosterone correlated positively from M4 to M6 in the whole group (Spearman's rho: 0.45 – 0.6; $P=0.002–0.0001$).

PSA secretion in prostate epithelial cells is androgen dependent, and an increase in PSA levels in infant boys indicates androgen effects in the prostate. Our findings are in line with previous studies, reporting positive PSA staining in prostatic tissue in infants less than six months old (Goldfarb et al. 1986) and measurable PSA in small number of urinary samples in infants (Sato et al. 2007). In our data, PSA was detected at least once during the first six months of life in all but one of the 50 boys studied. The reason for the later onset of urinary PSA secretion seen in preterm boys is not clear, but it could be explained by developmentally regulated androgen responsiveness of prostatic tissue. In addition, the smaller prostate size in preterm boys compared with full-term boys might explain the lower PSA levels in the preterm group.

5.2.2 Female reproductive organs (Study II and IV)

5.2.2.1 Ovarian volume and morphology

The median ovarian volume in full-term girls increased from D7 to M3 and then slowly decreased to cM14 (Figure 16). In near-term and very preterm girls, there were fewer observations, but the temporal pattern was similar to that in full-term girls. The changes

between the time points within the groups or differences between the groups were not statistically significant, probably because of the large variation and the small number of observations. Ovarian volume correlated positively with the number of antral follicles (Spearman's rho: 0.67; P<0.0001). The ovarian volumes in full-term girls in our study are similar as reported earlier for infant girls (Cohen et al. 1993), and the temporal changes are in line with the findings of a previous semilongitudinal study performed on a smaller number of infant girls (Nguyen et al. 2011).

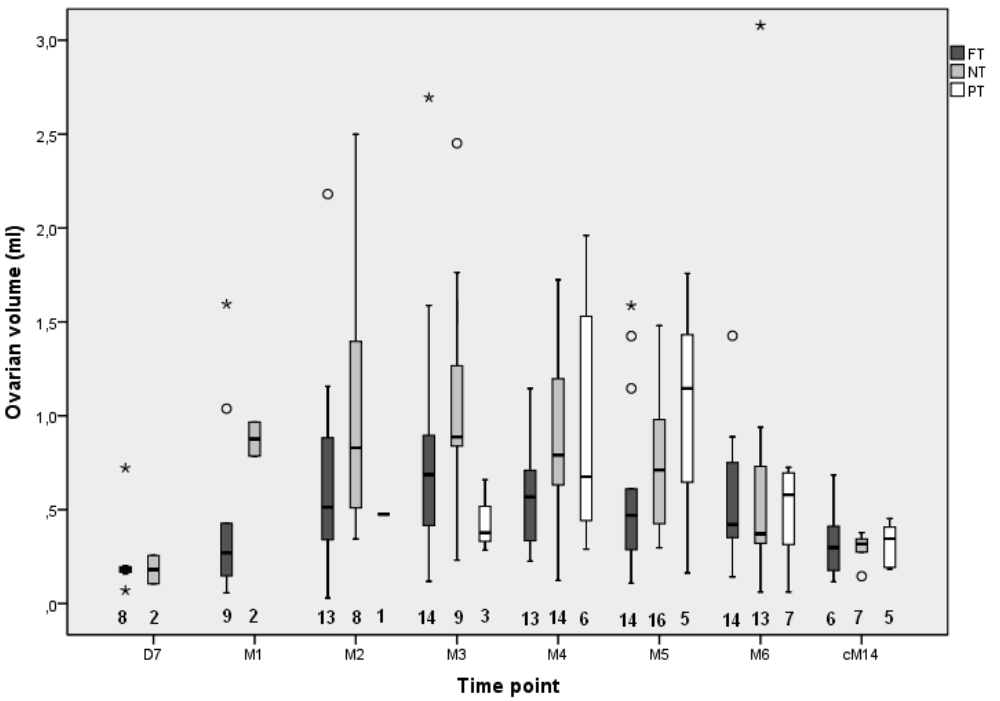

Figure 16. Ovarian volume from D7 to cM14 in full-term (FT), near-term (NT) and preterm (PT) girls. If both ovaries were visible for measurement, the mean of left and right was used. The number of observations is presented below each bar.

Antral follicles were seen in transabdominal sonography in 58 out of 63 girls in the study (92.1%). The proportion of girls with visible antral follicles increased significantly in full-term (from 31.0% at D7 to 60.7% at M4, P=0.006), near-term (from 5.9% at D7 to 93.8% at M5, P<0.0001), and very preterm (from 0% at D7 to 53.3% at M6, P=0.0003) groups during the first six months of life and then decreased to cM14 in full-term (16.0%, P<0.0001 for the decrease from M4 to cM14) and near-term girls (52.9%, P<0.0001 for the decrease from M5 to cM14, P=0.008). In very preterm girls, the proportion of those with antral follicles was similar at M6 and cM14 (45.4%). Large antral follicles (diameter >6mm, indicative of FSH action) were seen in 47 girls (74.6%). Also the proportion of girls with large antral follicles increased during the first months of life in all three groups: from 3.4% at D7 to 27.6% at M1 (P=0.036) in full-term girls, from 0% at D7 to 43.8% at M3 in near-term girls (P=0.005) and from 0% at D7 to 28.6% at M4 (P=0.03) in very preterm girls. In the full-term and near-term groups, the proportion of those with large antral follicles decreased to cM14: 8.0% in full-term girls (P=0.017) for the decrease from M2 and 5.9% in near-term girls (P=0.02) for the decrease from M2. At cM14 there was no difference between the groups. These findings in full-term girls are in line with previous histological (Polhemus 1953, deSa 1975, Forabosco & Sforza 2007, Channing et al. 1984, Lintern-

Moore et al. 1974) and sonographical studies (Cohen et al. 1993, Gilchrist et al. 2010), whereas in prematurely born girls, no previous data exist.

The number of antral follicles observed per examination varied over time in each group. Highest numbers were observed at M2 in full-term (median 4, range 0–10 follicles) and near-term girls (median 5, range 2–9 follicles) and at M5 in very preterm girls (median 3, range 3–6 follicles). In full-term and very preterm girls, significantly fewer follicles were seen at cM14 than at M2 (at cM14 median 1, range 0–4 follicles in full-term [P=0.001 for the decrease from M2]; and median 2, range 1–3 follicles in near-term girls [P=0.02 for the decrease from M2]).

Antral follicles were not seen before the postmenstrual age of 36 weeks. This is probably explained by the natural developmental time schedule of the ovarian follicle. Folliculogenesis is initiated in fetal ovaries at around 15 weeks post-conception (Kurilo 1981) and occurs according to a strict biological time schedule involving primordial, preantral, and antral stages before reaching the preovulatory size. The exact time required for early follicular growth up to the preantral stage is not known in humans, but development from the preantral to the large antral stage takes about 9 weeks (Gougeon 1996). This explains the observed delay in follicular maturation in relation to the increase of FSH in near-term and preterm girls compared with full-term girls, because, at the time of birth, follicular development was delayed by approximately five weeks in near-term and 9 weeks in very preterm girls as compared with full-term girls.

FSH levels were highest before the postmenstrual age of 40 weeks, at the time when AMH levels and the number of antral follicles were lowest. AMH levels increased concomitantly with the increase in the number of antral follicles and were then decreased at cM14 in a manner similar to the number of antral follicles. A positive correlation was observed between AMH levels and the number of antral follicles (Spearman's rho: 0.53; P<0.0001) indicating that an increase in AMH levels reflects increase in follicular growth and granulosa cell mass.

The underlying mechanism leading to the elevated and prolonged FSH surge in preterm girls has not been well defined. In the present longitudinal data, extremely high FSH levels in premature girls were observed at the time when the ovarian folliculogenesis was still immature, i.e., antral follicles were not observed in ultrasonography, and AMH levels were low. FSH levels then declined steeply in premature girls, close to the levels observed in full-term girls, at the time when the first antral follicles appeared in ovarian ultrasonography, and AMH levels began to increase. There was a negative correlation between FSH levels and antral follicle number (Spearman's rho: -0.35; P<0.0001) and AMH (Spearman's rho: -0.65; P<0.0001). The secretion of estradiol is positively related to the size of the follicle and takes place mostly in large antral follicles (Andersen & Byskov 2006). In the present data, estradiol levels in premature girls remained low until close to term and an increase in levels was observed as antral follicles became visualized in the sonography, suggesting that these events are interrelated. Consequently, insufficient negative feedback inhibition mediated by the ovarian estrogens on gonadotropin secretion may be an explanation for the extremely high FSH surge in premature girls. The negative feedback on FSH at the central level seems to be already operational at the beginning of the last trimester, as evidenced by the capacity of exogenous estrogen to suppress gonadotropin secretion in premature girls at around the gestational age of 30 weeks (Trotter et al. 1999). In addition to estradiol, inhibin B has a central role in FSH negative feedback, and its levels are also positively associated with the size of the follicle (Andersen et al. 2010). Inhibin B measurements were not included in the present results.

Although increased follicular growth in infancy has been reported as early as in the 1950's in postmortem samples (Polhemus 1953), the present study is the first one that demonstrated the temporal relationship between the postnatal FSH surge and activation of the ovarian follicular development within the same population of full-term and prematurely born infant girls.

5.2.2.2 Uterine size

Uterine length in full-term girls was largest at D7 and then steadily decreased to M3 (P<0.001). Subsequently, uterine length remained fairly unchanged until cM14. The coronal measure did

not change between D7 to cM14. Fundus was largest at D7 and then decreased to M2 (P<0.0001) but did not change after that. Corpus was largest at D7 and then decreased to M2 (P<0.0001) but did not change after that.

There were no changes in uterine measures in the preterm girls according to calendar age. According to postmenstrual age, uterine length increased significantly from 34–38 weeks to the maximum at 38–42 weeks (P=0.008) in preterm girls, but never reached the largest size of full-term girls. In contrast to the decrease of uterine length observed in full-term girls from 38 to 54 weeks, uterine length of preterm girls remained similar from 38 to 62 weeks postmenstrual age. By cM14, uterine length in preterm girls had decreased from the maximum at 38–42 weeks (P=0.001). The coronal measure or the fundus did not show statistically significant changes. The corpus increased in size from 30–34 weeks to 38–42 weeks (P=0.032). At cM14, the corpus was smaller than at 38–46 and 50–54 weeks (P=0.0001–0.045). According to postmenstrual age, uterine length was significantly larger in full-term than in preterm girls from 38 to 46 weeks. From 50 to 62 weeks, median uterine length was larger in preterm than in full-term girls, but this difference was not statistically significant. After adjustment for each infant's actual length, uterine length was larger in full-term girls at D7 (P=0.021), but at M3, M5, and M6 it was significantly larger in preterm than in full-term girls (P=0.018, 0.005, and 0.019, respectively).

The postnatal regression of uterine size in full-term girls is in line with previous data and reflects the withdrawal of high intrauterine estrogen levels (Haber & Mayer 1994). Apparently endogenous estradiol production in full-term girls was not sufficient enough to stimulate further growth of the uterus during the postnatal HPG axis activation and any small effects would have been masked behind the residual effects of prenatal estrogens. A similar decrease in uterine size was not observed in preterm girls, in whom uterine size slightly increased according to postmenstrual age, although this increase was not statistically significant. In preterm girls, the categorized uterine length was positively associated with urinary estradiol levels, suggesting that postnatal endogenous estradiol production probably stimulates uterine growth in them.

5.2.2.3 Vulvar cytology

The proportion of intermediate cells dominated throughout the follow-up period in both full-term and preterm girls (median 90–97.5% from D7–M6). Superficial cells comprised 0–10% of cells in full-term and 0–40% of cells in preterm girls. The maturation values ranged from 15 to 55 in FT and from 32.5 to 70 in PT girls. Median maturation values were highest at D7 in both groups. In FT girls, maturation value at D7 was higher than at M1 (P=0.043), but did not change significantly after that. In PT girls, the changes in maturation value were not significant. Maturation values were significantly higher in PT than in FT girls at follow-up visits from D7 to M6 (P<0.001–0.46). According to postmenstrual age, the proportion of girls with superficial cells was significantly larger in preterm than in full-term girls after 42 weeks. Because estrogens are needed for full maturation of the vulvar epithelial cells, these findings suggest increased local estrogen effects in preterm girls compared with full-term girls during the first six months of life. There were no previous studies that compare vulvar cytology between full-term and premature girls during minipuberty.

5.2.3 Mammary glands (Study IV)

In full-term infants, the mammary gland diameter (MGD) was largest at one week of age and decreased significantly (P<0.01) by the age of two months in both sexes. This is in accordance to previous studies (Jayasinghe et al. 2010, McKiernan & Hull 1981) and indicates proliferating effects of the high placental estrogens on the mammary glands during late gestation (Nagata et al. 2006, Kenny et al. 1973, Shutt et al. 1974). Even though these high estradiol levels are cleared from the newborn's circulation during the first days of life (Winter et al. 1976, Bidlingmaier et al. 1973), the residual effects in target tissues seem to disappear more slowly. This probably explains why no sex difference in MGD in full-term infants was seen during the first months of

life in the present or previous studies (Schmidt et al. 2002, Jayasinghe et al. 2010, McKiernan & Hull 1981). After two months of age, MGD continued to decrease in full-term boys, but in full-term girls, MGD remained enlarged and was significantly larger than in full-term boys from the age of four to six months (P<0.001), suggesting that endogenous estradiol production in infant girls has biological effects in the mammary glands.

In contrast to full-term infants, MGD in preterm infants was small at one week of age, and in preterm boys no further growth was observed. In preterm girls, MGD increased after the age of one month and was larger than in preterm boys from the age of two to six months (P<0.0001). The lack of mammary gland development in preterm infants at birth is probably explained by the developmental expression of ERα in fetal mammary glands only from about 30 weeks of gestation (Keeling et al. 2000). MGD in preterm girls continued to grow until M6, and from the age of four to six months MGD was significantly (P<0.0001–0.025) larger in preterm than in full-term girls. Even after correction for postmenstrual age, MGD was larger in preterm than in full-term girls from 46 weeks onwards (P<0.001–0.02). The increase of MGD in preterm girls paralleled the increase in estradiol levels, and categorized MGD was significantly positively associated with urinary estradiol levels. These findings indicate that postnatal ovarian activity and increased estradiol levels have biological effects in the mammary glands.

5.2.4 Androgenic cutaneous manifestations (Study III)

Androgen effects in the skin were evaluated by observations of two androgen-associated cutaneous changes in the skin: SGH and acne. SGH was observed in 24/26 of full-term boys (92.3%), 24/28 of full-term girls (85.7%), 20/22 of preterm boys (90.9%), and in all preterm girls. SGH was present already at D7. By M1, the proportion of full-term infants with SGH decreased to 19%, and after this, SGH was observed only in one full-term infant. In preterm infants, SGH persisted longer than in full-term infants and was observed at M1 in 54.5% of preterm boys and 80.8% of preterm girls (P=0.046 for the difference between sexes) and at M2 in 27.3% of preterm boys and in 41.7% of preterm girls (P=0.3). The occurrence of SGH seemed to closely follow the declining levels of DHEAS. SGH was related to the maturation of the infant, because according to postmenstrual age the occurrence of SGH was mainly limited to postmenstrual age less than 46 weeks in both full-term and preterm infants.

Acne was observed at least once in 49/54 of full-term infants (90.7%) and in 36/48 of preterm infants (75.0%) (P=0.06 for the difference between full-term and preterm groups). Sex differences were not observed. Acne appeared by M1 in most of the full-term cases (71.4%) but only in 5.6% of preterm cases. In preterm infants, the number of new cases was highest at M2 (24 new cases, 66.7% of all preterm cases). In full-term infants, acne was most commonly seen at M1 (in 63.5%) and M2 (in 67.9%). Full-term infants had more acne papules than preterm infants (>10 papules in 81.5% full-term vs. 43.8% preterm infants, P<0.0001; >50 papules in 29.6% full-term vs. 8.3% preterm infants, P=0.011). The duration of acne was not significantly different between sexes or between full-term and preterm infants (median number of follow-up visits with acne 2 [range 0–5] in full-term and 1 [range 0–4] in preterm, P=0.26). Acne was not observed before 37 postmenstrual weeks.

Because there were no sex differences in either SGH or acne despite of the clear sex difference in urinary testosterone levels, and since preterm infants had less acne despite of higher DHEAS and testosterone levels than full-term infants, the possible associations of SGH and acne with androgen levels were evaluated in separate groups. In all four groups, urinary DHEAS and testosterone levels were significantly higher when SGH was present than when SGH was not observed (P<0.01). In each of the four groups, the peak urinary DHEAS and testosterone levels preceded the peak occurrence of acne, suggesting a delayed effect. When the androgen levels of the preceding follow-up visit were compared between acne severity categories (grouped as no acne [less than five papules], 5–10 papules, and more than 10 papules), both DHEAS and testosterone levels were the lowest when acne was not present and increased according to acne severity category.

Consequently, these longitudinal findings indicated that postnatal androgen secretion is associated with SGH and acne in infants. Whereas the presence of SGH closely followed the changes in urinary DHEAS levels, the onset and the severity of acne were influenced by developmental factors. Namely, despite the high urinary androgen levels in preterm infants after birth, acne was observed only at the same postmenstrual age as in full-term infants and was less severe. This might be explained by a developmental change in the local androgen sensitivity or production. In fact, it has been described that the expression of 5α-reductase 1 and 2—enzymes that convert testosterone to the most powerful androgen, DHT—is developmentally regulated in the skin, and they both are transiently expressed in the newborn skin (Thigpen et al. 1993). However, the exact developmental window of expression of these isozymes during the perinatal period is not properly known, and many other factors might contribute to the observed pattern of acne in infants.

Based on the present longitudinal data, both SGH and acne were more common than previously reported (Rivers et al. 1990, Ferahbas et al. 2009, Monteagudo et al. 2011), and in contrast to previous knowledge, there was no sex difference in the occurrence or the severity of acne at this age.

5.3 METHODOLOGICAL CONSIDERATIONS

In order to quantify longitudinal hormone profiles during minipuberty, we chose urinary hormone analysis over serum analysis because urine samples could be obtained non-invasively, which allowed for frequent sampling from small infants. The use of creatinine correction did not affect the results; the differences between groups were identical if raw hormone values were used. In previous studies, a good correlation between serum and urine gonadotropin levels has been observed in pediatric population (Demir et al. 1994, Kuijper et al. 2006) and the measured urinary gonadotropin levels in this study most likely reflect the circulating gonadotropin levels.

The situation is more complex regarding the relationship between urinary and serum steroid hormones and their association with biological effects in the target tissues. Steroid hormones are largely metabolized in the target tissues and the liver, and this local metabolism can result in activation or inactivation of the parent hormone before excretion of the metabolites in urine as water-soluble conjugates, mainly as glucuronides or sulphates (Figures 4 and 5). Therefore, for example urinary testosterone glucuronide is not solely derived from circulating testosterone but partly from peripheral conversion of precursors, such as DHEAS, DHEA, and androstenedione in the liver, and also in other peripheral sites that possess the required enzymatic machinery (i.e., steroid sulfatase, 3βHSD, 17βHSD, and UGTs) such as the skin. This is the case especially in women, who have low circulating testosterone levels, and a substantial amount of testosterone (and presumably testosterone glucuronide) is formed peripherally. The proportion of testosterone glucuronide originating from the peripheral tissues in relation to testosterone glucuronide formed from circulating testosterone is therefore significantly larger in women than in men with very high circulating testosterone. Thus, because urinary testosterone glucuronide is formed from both circulating and peripherally formed T, it probably overestimates the levels of circulating testosterone in both sexes, but especially in women, whose testosterone is mostly formed from precursors in target tissues. The exact contribution of each peripheral site to testosterone glucuronide formation is not known. In infants, and especially in premature infants, the peripheral androgen metabolism probably differs from that in adults because developmental changes take place in both phase I and phase II pathways (reviewed in Alcorn & McNamara 2002) and because of very high input of precursor hormones from the involuting fetal adrenal cortex. Furthermore, the glucuronide conjugation capacity of the liver is significantly lower in early infancy than in adults (reviewed in de Wildt et al. 1999). Thus, in regards to the present results, urinary testosterone glucuronide in infants originates from both the plasma testosterone and peripherally synthesized testosterone, but the contribution of each of these sites is impossible to determine and might differ between girls and

boys and full-term and premature infants. Consequently, changes in urinary testosterone glucuronide levels therefore reflect the changes in overall androgen production and exposure rather than only the circulating testosterone levels. On the other hand, significant correlation between urinary LH and testosterone in boys suggests that most of the detected urinary testosterone in them is of testicular origin as LH has no influence on peripheral androgen metabolism.

Previously, urinary testosterone glucuronide levels have been shown to increase according to age and pubertal stage and correlate significantly with serum testosterone levels in pubertal boys (Juul et al. 2009). This study also pointed out that the UGT2B17 del/del genotype greatly determines the levels of urinary testosterone glucuronide. Consequently, interpretation of low urinary testosterone glucuronide levels requires the consideration of the effect of the UGT2B17 del/del genotype. In the present study, infants with repeatedly very low urinary testosterone levels without associated abnormalities in other measured hormones, urinary creatinine or biological effects were considered probable carriers of this deletion and were excluded from the analyses regarding testosterone levels. Unfortunately, due to lack of adequate DNA samples we were unable to verify this.

6 Summary and conclusions

This study provided novel information on the postnatal HPG axis activation in full-term and preterm infants. Simultaneous longitudinal measurements of hormone levels and biological effects in target tissues enabled the evaluation of their associations in a manner not previously performed. Analysis of the results both according to chronological and postmenstrual age clarified the differences and similarities in the postnatal function of the HPG axis between full-term and premature infants. The main findings are listed below in more detail.

1. Postnatal pituitary activation was both increased and prolonged in premature infants compared with full-term infants when the analysis was based on chronological age (i.e., time from birth). However, analysis based on postmenstrual age (i.e., age from the last menstruation of the mother) revealed that the gonadotropin levels in premature infants actually decline to similar levels as in full-term infants at the same developmental stage. These results suggest that the duration of the postnatal HPG axis activation is developmentally regulated. Previous studies have shown that intrauterine gonadotropin levels during the last trimester of full-term pregnancy are low due to inhibition by the placental hormones. Together, these findings point out that at a similar developmental stage, gonadotropin stimulation is very different in premature infants and in full-term infants that grow in utero until the expected date of delivery.

2. The postnatal gonadotropin surge was associated with activation of gonadal steroid production in both boys and girls, but the pattern of gonadal steroid levels was affected by both sex and maturity. In boys, testosterone levels increased and decreased in accordance with LH levels, and higher LH levels in premature boys resulted in higher testosterone levels in them than in full-term boys. However, in girls, estradiol levels fluctuated probably reflecting the growth and atrophy of the ovarian follicles. In premature girls, initially extremely high FSH levels declined to similar levels as in full-term girls as term age approached. Estradiol levels in premature girls were low when FSH levels were high but increased simultaneously as FSH levels decreased. This interrelationship of FSH and estradiol levels suggests that the high FSH levels reflect the lack of negative feedback effects mediated by the ovarian estrogens and that follicular development to the stage that enables estradiol production occurs only close to term age. Although estradiol levels in premature girls initially remained low, there was a peak in estrogen levels around term age, when the levels were significantly higher than in full-term girls of same postmenstrual age. The preceding increase of FSH stimulation in preterm girls was probably associated with the increased estradiol levels.

3. Activation of gonadal steroid hormone production was associated with biological effects in target tissues in both boys and girls. In boys, testosterone levels were associated with penile growth and transient increase in PSA levels, indicating androgen actions in the prostate. In full-term infants, mammary glands at birth were stimulated by intrauterine estrogens, making it difficult to discern the effects of endogenous estrogens. However, the persistence of the mammary glands for a longer period than in boys suggests effects of endogenous estrogen production in full-term girls. In premature girls, mammary glands were small at birth but increased in size during the following months in association with rising estradiol levels. Uterine size decreased in full-term girls during the first postnatal months, but in preterm girls uterine size slightly increased and was associated with estradiol levels.

4. Reflecting the hormonal activity of the fetal zone of the adrenal glands, urinary DHEAS levels remained detectable to the age of one month in full-term infants, but persisted longer in premature infants. A decrease in DHEAS levels was observed at a similar age in full-term boys and girls and preterm boys, but in preterm girls, DHEAS secretion was prolonged. In addition to urinary testosterone levels, also DHEAS levels were associated with the presence of SGH and acne, suggesting a biological role for postnatal adrenal androgen secretion.

5. Urinary analysis of reproductive hormone levels in infants proved to be a practical non-invasive option for invasive blood sampling. Analysis of urinary rather than serum samples may offer certain advantages, such as being less prone to pulsatile changes in hormone levels which is important for example in gonadotropin measurements. In the interpretation of urinary steroid levels, it must be considered that most of these hormones in urine are presented as metabolized conjugates and thus inter-individual differences in the metabolic pathways may play a role. The previously described deletion of UGT2B17 enzyme that results in very low urinary testosterone glucuronide levels despite of normal serum testosterone levels is a good example of this. In the present study we were able to discern two distributions in urinary testosterone profiles and persistently low levels were assumed to be caused by UGT2B17 deletion and these infants were excluded from further testosterone analyses. A limitation of our study is that we were not able to confirm this mutation in a genetic analysis.

6. Minipuberty provides a time-window for evaluation of the HPG axis functionality before puberty. Longitudinal characterization of the gonadotropin and sex steroid levels in the present study has provided new information about the hormonal patterns including the timing of the peak hormone levels and the decrease in hormonal activity according to developmental rather than calendar age in premature infants. Consequently, these results may aid in defining aberrant HPG axis activity in infancy and thus facilitate early diagnosis of HPG axis disorders.

The present results form the basis for future studies evaluating the possible long-term consequences of altered minipuberty in premature infants. These might include effects on reproductive development, childhood growth, bone mineralization, childhood psychosexual development, timing of puberty, sexual behaviour, and reproductive capacity in adulthood.

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Postnatal Hypothalamic-Pituitary-Gonadal Axis Activation (i.e., Minipuberty) in Full-term and Preterm Infants

The hypothalamic-pituitary-gonadal axis transiently activates soon after birth. The biological role of this minipuberty has remained poorly understood, especially in girls. This longitudinal study investigated and compared the reproductive hormone levels and associated target tissue effects during minipuberty in 125 full-term and preterm boys and girls. Longitudinal findings showed that both the hormone levels and the biological effects in minipuberty were influenced by maturational factors.

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