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AND DEMIR Clinical Use of Urinary Gonadotropin Determinations in Children and Adolescents 85/2015

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85/2015

AND DEMIR

Clinical Use of Urinary Gonadotropin Determinations in Children and Adolescents

CHILDREN'S HOSPITAL
DEPARTMENT OF CLINICAL CHEMISTRY
HELSINKI UNIVERSITY HOSPITAL & HUSLAB
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN CLINICAL RESEARCH
UNIVERSITY OF HELSINKI

CLINICAL USE OF URINARY GONADOTROPIN DETERMINATIONS IN CHILDREN AND ADOLESCENTS

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Academic dissertation

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To my family

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ABSTRACT

This study was undertaken to assess the feasibility of non-invasive sampling and assay of urinary gonadotropins for clinical evaluation of pubertal development. In the first study, the concentrations of LH and FSH in concurrent serum and first-morning-voided (FMV) urine samples of 820 children (486 boys and 334 girls, age 0-17 years) were determined with time-resolved immunofluorometric assay (IFMA). The detection limit of IFMA was 0.018 IU/L for FSH, 0.015 IU/L for LH and 0.012 IU/L for LHspec. It was possible to measure the low prepubertal LH and FSH concentrations reliably in these samples due to the high sensitivity and low detection limits of IFMA. The correlation between serum and urinary gonadotropin values was high ($r=0.751$; $p<0.001$ for FSH and $r=0.720$; $p<0.001$ for LH), and the urinary and serum concentrations were close to each other. Correcting urinary gonadotropin concentrations on the basis of urinary density or creatinine did not improve the correlation. Age-related changes in urinary LH and FSH (U-LH and U-FSH) were examined. The concentrations of U-LH and U-FSH decreased from birth until the child was a few months old, after which the upper range of the U-LH levels of girls remained stable at below 0.5 IU/L until age 9 years and of boys below 1.0 IU/L until age 11 years. The upper range of the U-FSH levels of girls remained below 3.0 IU/L until age 10 years and of boys below the same concentration until age 12 years. The median U-LH concentration during the prepubertal period was about 0.06 for girls and 0.07 for boys. For the boys, this figure rose 10-fold by age 11, 40-fold by age 12 and 50-fold by age 13-14. The overall increase in the median U-LH concentrations was 75-fold from 5 to 15 years and 35-fold from Tanner stage G1 to G5. The corresponding figures for girls were 30-fold by age 11, 70-fold by age 12 and 90-fold by age 14; the overall increase in median U-LH concentrations was 90-fold from 5 to 15 years and 40-fold from Tanner stage B1 to B5 times. These finding indicate that the U-LH concentrations of FMV samples obtained from clinically prepubertal children reflect pubertal levels. The age-related changes in U-FSH concentrations were similar for boys and girls; the only difference was that the levels were generally higher for girls, in particular between ages 2–8 years. U-FSH reached a 5-fold level compared to prepubertal levels by the end of the puberty in both sexes. FMV U-LH, U-FSH and their ratios correlated well with the corresponding basal and GnRH-stimulated serum concentrations ($P<0.001$). Receiver operating characteristic (ROC) curve analyses of urinary and serum LH and FSH concentrations showed that FMV U-LH and U-LH/U-FSH performed equally well as the GnRH test for differentiating early puberty (Tanner 2) from prepuberty (Tanner 1) [area under the curve (AUC) 0.768-0.890 vs. 0.712-0.858]. FMV U-LH and U-LH/U-FSH performed equally well as basal S-LH for predicting a pubertal GnRH test result (AUCs 0.90–0.93). Among the tests studied, only FMV U-LH differentiated the transitions from Tanner stage 1 to 2 and Tanner stage 2 to 3 ($p<0.001$ for boys and $p=0.003$ for girls). Again, this corroborates that FMV U-LH is the most reliable tool for evaluation of pubertal development. Therefore, FMV urinary LH determinations, which are non-invasive and, at most, minimally stressful for the child patient, can be used for preliminary diagnostic evaluation of pubertal development. It reduces the need for S-LH determinations and the GnRH stimulation tests, both invasive procedures.

TIIVISTELMÄ

Tämän tutkimuksen tarkoituksena oli arvioida virtsan gonadotropiinimäärityksen (kajoamaton tutkimus) käyttökelpoisuutta puberteettikehityksen kliinisessä arvioinnissa. Ensin tutkimme puberteetin eri vaiheissa olevilta 820 lapselta (486 poikaa ja 334 tyttöä, ikä 0–17 vuotta) otettujen seerumi- ja aamuvirtsanäytteiden LH- ja FSH-pitoisuudet käyttäen aikaerotteista immunofluorometristä määritystä (IFMA). Erittäin herkän määritysmenetelmän toteamisrajat olivat seuraavat: FSH 0,018 IU/L, LH 0,015 IU/L ja LHspec 0,012 IU/L. Menetelmän herkkyyden ja matalien toteamisrajojen ansiosta sillä oli mahdollista mitata luotettavasti esipuberteettivaiheen matalahkoja LH- ja FSH-pitoisuuksia virtsa- ja seeruminäytteistä. Seerumin ja virtsan gonadotropiiniarvot korreloivat voimakkaasti (FSH: $r = 0,751$, $p < 0,001$; LH: $r = 0,720$, $p < 0,001$), ja pitoisuudet virtsassa ja seerumissa olivat lähellä toisiaan. Virtsan gonadotropiinipitoisuuksien korjaus virtsan tiheyden tai kreatiniiniarvon perusteella ei parantanut korrelaatiota. Tutkittaessa virtsan LH- ja FSH-pitoisuuden (U-LH ja U-FSH) muutoksia iän myötä todettiin, että U-LH-arvot laskevat ensin muutaman kuukauden ikään asti ja sen jälkeen ylimmät arvot ovat tytöillä 9 vuoden ikään asti alle 0,5 IU/L ja pojilla 11 vuoden ikään asti alle 1,0 IU/L, kun taas ylimmät U-FSH-arvot ovat tytöillä 10 vuoden ja pojilla 12 vuoden ikään asti alle 3,0 IU/L. U-LH-pitoisuuden mediaani oli esipuberteettivaiheessa tytöillä noin 0,06 ja pojilla noin 0,07. Poikien arvo kasvoi 11 vuoden ikään mennessä 10-kertaiseksi, 12 vuoden ikään mennessä 40-kertaiseksi ja 13–14 vuoden ikään mennessä 50-kertaiseksi. U-LH-pitoisuuden mediaani kasvoi 5 ja 15 ikävuoden välillä kaikkiaan 75-kertaiseksi ja Tannerin asteesta G1 asteeseen G5 siirryttäessä 35-kertaiseksi. Tytöillä arvo kasvoi 11 vuoden ikään mennessä 30-kertaiseksi, 12 vuoden ikään mennessä 70-kertaiseksi ja 14 vuoden ikään mennessä 90-kertaiseksi. U-LH-pitoisuuden mediaani kasvoi 5 ja 15 ikävuoden välillä kaikkiaan 90-kertaiseksi ja Tannerin asteesta B1 asteeseen B5 siirryttäessä 40-kertaiseksi. Nämä havainnot osoittavat selvästi, että kliinisesti esipuberteettivaiheessa olevien lasten aamuvirtsanäytteen LH-pitoisuudet ilmentävät jo puberteettivaiheen tasoa. U-FSH-pitoisuuksien muutokset iän myötä olivat pojilla ja tytöillä hyvin samanlaiset. Ainut ero oli, että tytöillä pitoisuudet olivat yleensä suuremmat varsinkin 2–8 vuoden iässä. Kummankin sukupuolen arvot viisinkertaistuivat puberteetin loppuun mennessä. Aamuvirtsan LH, FSH ja niiden suhteet korreloivat hyvin vastaavien lähtötasolla saatujen ja GnRH-stimulaatiokokeessa saatujen seerumiarvojen kanssa ($P < 0,001$). Virtsan ja seerumin LH- ja FSH-pitoisuuksien ROC-käyrän (receiver operating characteristic) analyysi osoitti, että varhainen puberteetti (Tanner 2) pystyttiin erottamaan esipuberteetista (Tanner 1) yhtä hyvin aamuvirtsan LH-arvojen ja U-LH/U-FSH-suhteen kuin GnRH-testin perusteella [käyrän alla oleva pinta-ala (AUC) 0,768–0,890 / 0,712–0,858]. Aamuvirtsan LH ja U-LH/U-FSH ennustivat pubertaalisen GnRH-testin tulosta yhtä hyvin kuin S-LH (AUC 0,90–0,93). Tutkituista testeistä vain aamuvirtsan LH erotti muutoksen Tannerin asteesta 1 asteeseen 2 yhtä hyvin kuin muutoksen asteesta 2 asteeseen 3 ($p < 0,001$ pojilla ja $p = 0,003$ tytöillä), ja sekin osoittaa, että aamuvirtsan LH on luotettavin keino arvioida puberteettikehitystä. Siksi aamuvirtsan LH-määritystä voidaan käyttää puberteettikehityksen alustavaan diagnostiseen arviointiin, mikä vähentää kajoavan S-LH-mittauksen ja GnRH-stimulaatiokokeiden tarvetta.

LIST OF ORIGINAL PUBLICATIONS

1. **Demir A**, Alfthan H, Stenman U-H, Voutilainen R. 1994 A clinically useful method for detecting gonadotropins in children: assessment of luteinizing hormone and follicle-stimulating hormone from urine as an alternative to serum by ultrasensitive time-resolved immunofluorometric assays. Ped Res 36: 221-226
2. **Demir A**, Dunkel L, Stenman U-H, Voutilainen R. 1995 Age-related course of urinary gonadotropins in children. J Clin Endocr Metab 80: 1457-1460.
3. **Demir A**, Voutilainen R, Juul A, Dunkel L, Alfthan H, Skakkebaek NE, Stenman U-H. 1996 Increase in first-morning voided urinary luteinizing hormone levels precedes the physical onset of puberty. J Clin Endocrinol Metab. 81: 2963-2967.
4. **Demir A**, Voutilainen R, Stenman U-H, Dunkel L, Albertsson-Wikland K., Norjavaara E. 2015 First-morning-voided urinary gonadotropin measurements as an alternative to the GnRH test in the evaluation of pubertal development. In press: Horm Res Paediatr. DOI: 10.1159/000440955.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

ARC	Arcuate nucleus
AUC	Area under the curve
AVPV	Anteroventral periventricular nucleus
B	Tanner breast stage
BSA	Bovine serum albumin
CDGP	Constitutional delay of growth and puberty
CV	Coefficient of variation
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
E	Estradiol
FMV	First-morning-voided
FSH	Follicle-stimulating hormone
G	Tanner genital stage
GFR	Glomerular filtration rate
GH	Growth hormone
GP-GRC	Gothenburg Pediatric Growth Research Center
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HH	Hypogonadotropic hypogonadism
HPG	Hypothalamic-pituitary-gonadal
h	hour
ICMA	Immunochemiluminometric assay
IFMA	Immunofluorometric assay
KP	Kisspeptin
LH	Luteinizing hormone
LHcβf	Core fragment of LH β-subunit
ME	Median eminence
MKRN3	Makorin ring finger protein 3
min	minute
PH	Tanner pubic hair stage
RIA	Radioimmunoassay
ROC	Receiver operating characteristic
SD	Standard deviation
S-FSH	Serum FSH
S-FSHmax	Maximal S-FSH concentration (in the GnRH test)
S-LH	Serum LH
S-LHmax	Maximal S-LH concentration (in the GnRH test)
T	Testosterone
U-FSH	Urinary FSH
U-LH	Urinary LH
U-LHspec	Intact LH, its free ß-subunits and certain fragments of LH in urine
WADA	World Anti-Doping Agency

INTRODUCTION

Determinations of urinary gonadotropin levels have been used to evaluate gonadotropin secretion in children since the 1960s. Initially, bioassays (Fitschen & Clayton, 1965) were used. In the 1970s, Kulin and co-workers used radioimmunoassay (RIA) to measure FSH and LH in timed 3- to 24-h urine collections obtained from healthy infants and children and from patients with disorders of sexual maturation (Kulin, Bell, Santen, & Ferber, 1975; Kulin & Santner, 1977). In order to quantify the low concentrations in prepubertal children, they concentrated the gonadotropins by aceton precipitation of timed urine samples. Their study showed that the amount of U-LH excreted by prepubertal children was about 3% of the amount excreted by adults and of U-FSH about 9% (Kulin & Santner, 1977).

Until the 1990s, the RIA methods were not sensitive enough to measure gonadotropin concentrations in biological samples obtained from prepubertal children unless the samples were extracted and concentrated (Rifkind, Kulin, Rayford, Cargille, & Ross, 1970; Kulin et al., 1975; Kulin & Santner, 1977; Apter, Pakarinen, & Vihko, 1978; Bourguignon, Vanderschueren-Lodeweyckx, Reuter, Vrindts-Gevaert, Gerard, & Franchimont, 1980; Girard & Hadziselimovic, 1987; Maesaka, Suwa, Tachibana, & Kikuchi, 1990b; a). S-LH and S-FSH concentrations measured by RIA showed 2- to 6-fold increases during pubertal development, but their concentrations overlapped significantly by pubertal stage, which rendered the evaluation of pubertal progress based on these assays, in fact, impossible (Faiman & Winter, 1974; Apter et al., 1978; Sizonenko, 1978). Development of ultrasensitive immunoassays based on time-resolved fluorometry made it possible to determine the low prepubertal gonadotropin concentrations in serum and urine (Apter, Cacciatore, Alfthan, & Stenman, 1989; Dunkel, Alfthan, Stenman, & Perheentupa, 1990a; Dunkel, Alfthan, Stenman, Tapanainen, & Perheentupa, 1990b; Wu, Butler, Kelnar, Stirling, & Huhtaniemi, 1991; Dunkel, Alfthan, Stenman, Selstam, Rosberg, & Albertsson, 1992; Goji & Tanikaze, 1992). These assays were then used to show that a rapid increase in daytime serum LH (S-LH) levels from below 0.2 IU/L to more than 0.5 IU/L (biochemical puberty) goes hand in hand with the emergence of the clinical signs of female puberty at around age 10-11 (clinical puberty).

The onset of pubertal maturation is initiated by gonadotrope activation characterized by increased pulse amplitude and frequency with higher bursts of LH than of FSH secretion during night-time (Jakacki, Kelch, Sauder, Lloyd, Hopwood, & Marshall, 1982). This is an amplification of an existing circadian pattern of gonadotropin secretion. Thus, it is possible to evaluate pubertal development by taking samples at 10-20 min intervals for several hours during sleep (Apter et al., 1989; Apter, Butzow, Laughlin, & Yen, 1993; Manasco, Umbach, Muly, Godwin, Negro-Vilar, Culler, & Underwood, 1995). However, for practical reasons, this approach is of limited use.

Basal S-LH concentrations are superior to basal S-FSH concentrations for reflecting the course of pubertal development. For instance, in girls, the increase in S-LH concentrations from the prepubertal level to the adult level is 50-100-fold; the corresponding increase in S-FSH is only 10-fold (Apter et al., 1989).

Because the low basal LH and FSH levels in prepubertal children could not be measured practically by the available RIA methods until late 1980s, the higher gonadotropin concentrations produced by gonadotropin releasing hormone (GnRH) in the stimulation test have been used to evaluate gonadotropin secretion and pubertal development. The GnRH test has been the gold standard for evaluation of hypothalamic-pituitary-gonadal (HPG) function in pubertal disorders for over 40 years (Kastin, Schally, Gual, & Arimura, 1972; Kastin, Schally, Schalch, Korenman, Miller, Gual, & Perez-Pasten, 1972). However, the GnRH stimulation tests are invasive, involve adequately trained personnel for repeated blood sampling in hospital facilities and require several FSH and LH measurements. This results in high costs. Several research groups have aimed at simplifying the test, e.g., by simply determining S-LH in a single blood sample drawn at a selected time point (Cavallo, Richards, Busey, & Michaels, 1995; Kandemir, Demirbilek, Ozon, Gonc, & Alikasifoglu, 2011; Kim, Kee, Seo, Yang, Chae, & Kim, 2011), but this approach has not become clinically established.

Information on integrated gonadotropin secretion can be obtained by measuring gonadotropins in urine samples collected over a certain period of time. Maesaka and colleagues have shown that urinary gonadotropin concentrations measured in monthly urine samples from patients with idiopathic precocious puberty follow a similar pattern as in healthy subjects matched for pubertal stage (Maesaka et al., 1990a).

An alternative to night-time blood collections (which reflect the nocturnal increase in gonadotropin secretion) is to use first-morning-voided (FMV) urine.

In the studies in this thesis, we have investigated whether FMV urine could be used to evaluate physiological and disturbed pubertal development, provided that the gonadotropin concentrations in FMV urine really reflect the concentrations in serum and provide meaningful information about the secretion of LH and FSH.

REVIEW OF THE LITERATURE

1.1 Gonadotropins

1.1.1 Structure and function of gonadotropins

The gonadotropins which regulate pubertal development are luteinizing hormone or lutropin (LH) and follicle stimulating hormone or follitropin (FSH). These hormones are secreted by the gonadotropes, which compose 7% - 15% of the cells in the anterior pituitary (Marshall, 2001). Both gonadotropins are synthesized and secreted in a pulsatile fashion in response to the pulsatile release of GnRH (Clarke & Cummins, 1982), an oligopeptide of hypothalamic origin, into the portal venous system of the pituitary gland. Pituitary gonadotrope activity and GnRH secretion are regulated by negative feedback governed mainly by gonadal steroids. In addition, inhibin B, produced by Sertoli cells within seminiferous tubules in males and ovarian granulosa cells in females, inhibits FSH secretion from the pituitary and thus controls separately the serum concentrations of LH and FSH. Activin, on the other hand, exerts an opposite effect and stimulates FSH biosynthesis and release from the gonadotropes of the pituitary gland (Ying, 1987).

LH and FSH are heterodimeric glycoproteins composed of two noncovalently bound dissimilar subunits, α and β (Shome & Parlow, 1973; Ward, Reichert, Liu, Nahm, Hsia, Lamkin, & Jones, 1973; Shome & Parlow, 1974a; b; Sairam & Manjunath, 1983; Ryan, Keutmann, Charlesworth, McCormick, Milius, Calvo, & Vutyavanich, 1987). The smaller α-subunit consists of 92 amino acid residues and is common to the glycoprotein hormone family: LH, FSH, human chorionic gonadotropin (hCG) and thyroid stimulating hormone (TSH). The β-subunits have different amino acid sequences; this facilitates receptor-specific binding and activity of the intact heterodimer. Due to the differences in the amino acid sequence specific antibodies to each hormone may be generated (Pierce, Bahl, Cornell, & Swaminathan, 1971; Rathnam & Saxena, 1971; Vaitukaitis & Ross, 1974; Pierce & Parsons, 1981). The β-subunits of human FSH and LH contain 117 and 121 amino acids, respectively (Gharib, Wierman, Shupnik, & Chin, 1990). The β-subunits have an identical amino acid sequence within the family of glycoprotein hormones, but there is a 15 to 45 per cent variation in carbohydrate composition and content (Vaitukaitis & Ross, 1974). The subunits with a higher content of carbohydrate (in particular, sialic acid) residues have a lower rate of metabolism and thus a longer half-life in the circulation. The relatively slow clearance rate of FSH (half-life 3-4 h) compared to LH (half-life 20 min) is attributed to the relatively higher content of sialic acid of FSH (de Leeuw, Mulders, Voortman, Rombout, Damm, & Kloosterboer, 1996). The half-life of hCG, which presents with a highly sialylated molecular structure, is the longest (24 h) among the glycoprotein hormones. The presence of terminal sialic residues and/or sulfated sugars provides protection of the hormone against degradation in the circulation. Treatment of glycoprotein hormones with neuraminidase (sialidase) shortens their circulatory half-life. Interestingly, sialylated recombinant LH and sulfated LH isolated from urine have the same half-lives (le Cotonnec, Loumaye, Porchet, Beltrami, & Munafo, 1998).

Bioactivity and the net molecular charge of the gonadotropins are affected by variations in the *N*-linked carbohydrate side chains of the β-subunit (Haavisto, Pettersson, Bergendahl, Virkamaki, & Huhtaniemi, 1995; Huhtaniemi, 2000). The pituitary gonadotropin molecules comprise differently glycosylated 'isoforms', the compositions of which vary according to the physiological state. The biological potencies depend on the variation in the composition of the carbohydrate moieties (Jeffcoate, 1993; Lambert, Talbot, Anobile, & Robertson, 1998) which changes during puberty (Phillips, Albertsson-Wikland, Eriksson, & Wide, 1997).

Synthesis of gonadotropin β-subunits is determined by the pattern of the GnRH pulse frequency. Slower GnRH pulses favor FSH synthesis and release, while faster pulses favor LH synthesis and release (Filicori, Santoro, Merriam, & Crowley, 1986; Shupnik, 1990; Haisenleder, Dalkin, Ortolano, Marshall, & Shupnik, 1991; Burger, Dalkin, Aylor, Haisenleder, & Marshall, 2002). Receptor binding requires that the αβ-dimers are intact (Ryan et al., 1987). Gonadotropin secretion is further regulated by positive and negative feedback mechanisms (Figure 1).

FSH exerts its effects by binding to a specific receptor on the membrane of its target cells, the follicular (granulosa) cells in the ovary and the Sertoli cells in the testis. The metabolic effects and stimulation of steroid secretion are induced by activation of adenylate cyclase and increased cAMP production (Knecht, Amsterdam, & Catt, 1981), which are eventually translated to follicular growth and preparation of the follicle for the ovulation-inducing action of LH in the ovaries. FSH induces biosynthesis and secretion of estrogens (mainly estradiol) by facilitating aromatization of androgens, which are produced in the theca cells under the influence of LH, into estradiol 17β (Armstrong & Papkoff, 1976; Leung & Armstrong, 1980). FSH receptors are present only in the follicular cells of the ovary. On the other hand, LH receptors are found in the theca, interstitial and luteal cells, and to varying degrees also in follicular cells (Richards, 1980). In the male, FSH acts on the Sertoli cells and induces synthesis of an androgen-binding protein that appears to be involved in transporting testosterone to the seminiferous tubules and epididymis. This is essential for spermatogenesis which requires the presence of high local levels of testosterone (Means, Dedman, Tash, Tindall, van Sickle, & Welsh, 1980). FSH stimulates the growth of seminiferous tubules, which facilitates the initiation of spermatogenesis. A lack of FSH causes testis atrophy and cessation of sperm production. FSH also stimulates estradiol production in isolated Sertoli cells, but the importance of this for male physiology is unclear. In males, FSH and testosterone mediate the maturation of the Sertoli cells and the development of the functions that contribute to the initiation and maintenance of spermatogenesis. Spermatogenesis is promoted by testosterone, whereas meiotic division and formation of late spermatids to spermatozoa require FSH stimulation. However, maintenance of spermatogenesis, once initiated, does not necessarily require FSH (Depenbusch, von Eckardstein, Simoni, & Nieschlag, 2002).

LH expresses its biological effect by binding to membrane receptors in the target organs, which activates adenylate cyclase and augments cAMP formation (Marsh, 1975; Dufau, Tsuruhara, Horner, Podesta, & Catt, 1977; Dufau, 1988). cAMP formed by LH receptor induction mediates the action of LH. It converts acetate to squalene (the precursor for

cholesterol synthesis) (Morris & Gorski, 1973) and cholesterol to 2α-hydroxycholesterol (Conti, 2002), and facilitates the use of circulating LDL-cholesterol for steroid synthesis (Hu, Zhang, Shen, & Azhar, 2010). All these steps are essentials for the formation of ovarian and testicular sex steroids.

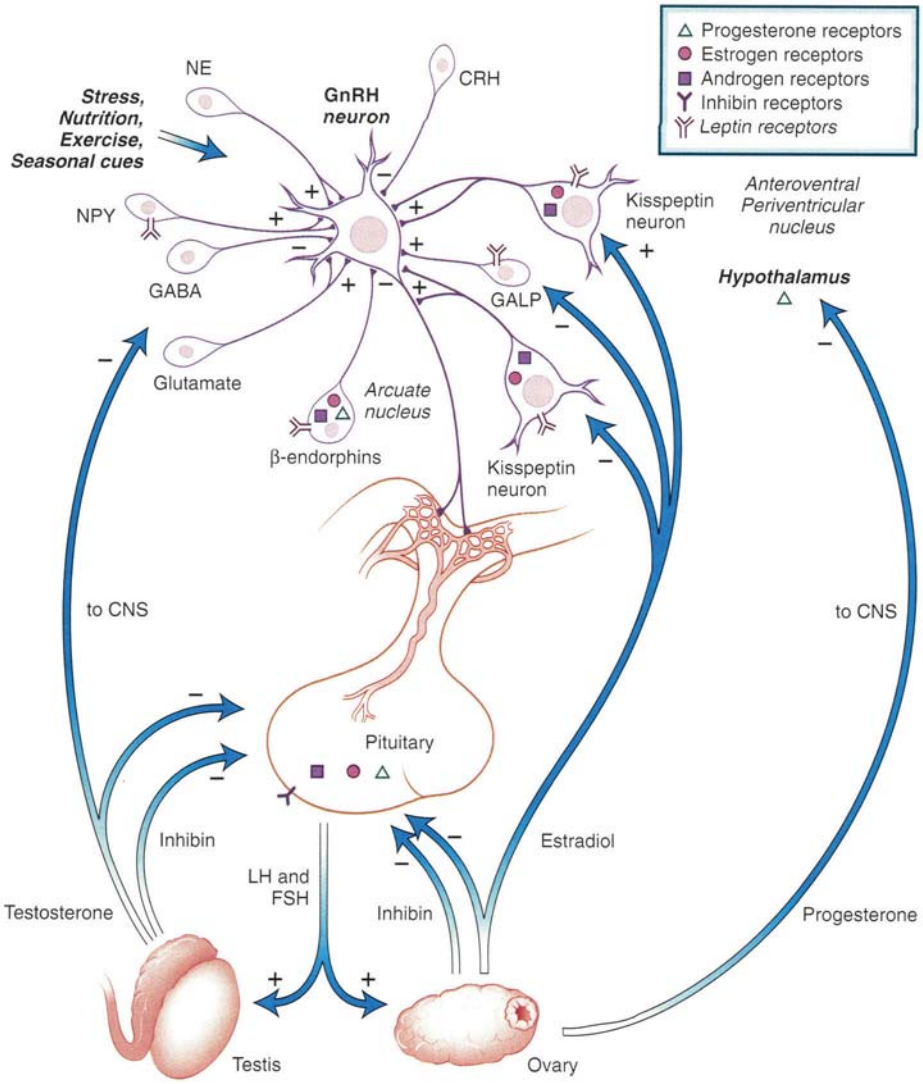

Figure 1. Regulation of the hypothalamic-pituitary-gonadal axis. Neural systems regulate gonadotropin-releasing hormone (GnRH) secretion and gonadal steroid hormones provide feed-back at the level of the hypothalamus and pituitary. CNS, central nervous system; CRH, corticotropin-releasing hormone; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; GALP, galanin-like peptide; LH, luteinizing hormone; NE, norepinephrine; NPY, neuropeptide Y. *(From Williams textbook of endocrinology, 2011—12th ed. ISBN: 978-1-4377-0324-5 / Shlomo Melmed et al.) Reproduced with permission from the author.*

In males, the main function of LH is to stimulate production of testosterone in the Leydig cells of the testes, which promotes spermatogenesis together with FSH. In females, LH increases the production of androgens and androgen precursors, e.g., androstenedione, dehydroepiandrosterone and testosterone by stimulating the theca cells. Occupancy of a rather small portion of the LH receptors of the Leydig cells produces a full steroidogenic response (Huhtaniemi, Clayton, & Catt, 1982). The presence of an excess of (unoccupied) LH receptors on the corpus luteum or Leydig cell surface may therefore enhance the sensitivity of the cellular responses to low gonadotropin concentrations (Catt & Dufau, 1973).

1.1.2 Structure of FSH and LH in the pituitary, plasma and urine

Characterization of immunoreactive FSH by gel filtration and electrophoresis revealed no significant differences between FSH in pituitary extracts, serum and urine (Maffezzoli, Kaplan, & Chrambach, 1972; Talas, Midgley, & Jaffe, 1973). These studies showed also that renal filtration did not damage the structure of the FSH molecule. Also the immunoreactive FSH peaks were the same regardless of the source of the urine sample, be it postmenopausal women, men or children (Maffezzoli et al., 1972; Beitins, Derfel, O'Loughlin, & McArthur, 1977).

Characterization of LH in urinary extracts by gel infiltration revealed several immunoreactive peaks detected by RIA. In addition to the expected molecular weight range, peaks with lower molecular weights were identified especially in urine. This indicates that subunits or fragments are formed as serum LH breaks down during renal excretion (Young, Harsoulis, Kuku, & Fraser, 1975). These breakdown products can be detected only by some LH assays (Kovalevskaya, Birken, O'Connor, Schlatterer, Maydelman, & Canfield, 1995).

1.1.3 Determination of gonadotropins

Development of methodologies for gonadotropin determinations

The first methods for determination of hormones were bioassays. *In vivo* biological assays were employed for the first time in the 1930s (Evans, Meyer, Pencharz, & Simpson, 1932). The result was quantified by the biological response (e.g., ovarian, uterine or prostate growth) in test animals. However, test results were often inconsistent, since there were variations in sample extraction procedures and in the responses of the test animals.

Many studies from the 1950s and 1960s concluded that gonadotropins are not detectable by bioassays in the urine of prepubertal children (Albert, 1956; Johnsen, 1959; Rosemberg, 1960; Wilkins, 1965). The presence of gonadotropins in prepubertal children was even considered unphysiological (Johnsen, 1959), but some studies since the 1940s indicated that gonadotropins are indeed present in the urine also before puberty (Catchpole & Greulich, 1943; Brown, 1958; Fitschen & Clayton, 1965). Nevertheless, until the mid-1960s it was generally believed that prepubertal children did not have detectable gonadotropin concentrations in urine or plasma.

The first RIA was developed by Yalow and Berson in 1959 (Yalow & Berson, 1959), after which bioassays were gradually replaced by immunoassays. This revolutionized research as well as the clinical practice of endocrinology. Decades later some authors still argued that concurrent utilization of *in vitro* bioassays and immunoassays is advantageous (Wide & Hobson, 1983), but immunoassays have become the dominating method for clinical diagnostics.

The first immunoassay for LH developed by Wide at al. was based on agglutination inhibition and antigen-coated red blood cells (Wide & Gemzell, 1962). In this assay, LH cross-reacts with hCG. The first radioimmunoassay of LH that was sensitive enough for determination of LH in serum and urine without concentration of the samples was published in 1966 (Midgley, 1966; Odell, Ross, & Rayford, 1966). These assays were based on the use of radiolabeled hCG as a tracer and anti-hCG antibodies which reacted equally with LH. Odell et al. reported that LH is detectable by RIA in the serum of children older than 1 year (Odell et al., 1966). This observation generated further studies on urinary assays. When large volumes of urine were used, it was possible to detect bioassayable concentrations of total gonadotropins (Kulin, Rifkind, Ross, & Odell, 1967), including LH and FSH, in the urine of prepubertal children (Rifkind, Kulin, & Ross, 1967; Kulin, Rifkind, & Ross, 1968). The presence of gonadotropins in prepubertal urine was confirmed by bioassays and RIAs (Fitschen & Clayton, 1965; Kulin et al., 1967) (Bagshawe, Wilde, & Orr, 1966).

In 1969, Wide et al. described the so-called sandwich assay. This is basically a different assay principle based on the detection of "reagins" with an allergen bound to a solid phase and a labeled "antireagin" for detection (Wide, Bennich, & Johansson, 1967; Wide, 1969).

In 1970, Rifkind et al. quantified urinary FSH and LH by RIA (Rifkind et al., 1970). Methods that involved extraction and concentration were later used to improve sensitivity, and timed urine samples were used for better accuracy (Kulin et al., 1975). The gonadotropin concentrations in prepubertal urine remained, however, below the detection limits of RIA despite extraction and concentration. In addition, non-specific interference may have overestimated the concentrations of RIA-detectable gonadotropin concentrations in serum (Apter et al., 1989; Chappel, 1990; Haavisto, Dunkel, Pettersson, & Huhtaniemi, 1990; Jaakkola, Ding, Kellokumpu-Lehtinen, Valavaara, Martikainen, Tapanainen, Ronnberg, & Huhtaniemi, 1990; Huhtaniemi, Ding, Tähtelä, & Välimäki, 1992).

Total gonadotropin excretion over 24 hours has been determined by assessment of FSH and LH in urine samples collected over 24 hours or shorter times (Kulin et al., 1975; Kulin & Santner, 1977; Santen & Kulin, 1986). In 1980, Bourguignon et al. showed that there is a morning increase in gonadotropin secretion at the time of onset of puberty. They also showed that gonadotropin excretion follows a circadian pattern by analysis of unextracted urine samples collected as timed fractions of 24-h urine (Bourguignon et al., 1980). Girard et al. proposed the use of FMV urine as an alternative to the 24-h output, because the 24-h urine collection procedure is cumbersome and may be unreliable in children (Girard, Baumann, & Ruch, 1980). A study by Girard and Hadziselimovic advocated urinary gonadotropin determinations in FMV urine for diagnosis and treatment follow-up of various pubertal disorders. They also employed extraction methods on urine samples for

increasing the sensitivity of the assay (Girard & Hadziselimovic, 1987). Maesaka et al., who used polyclonal double antibody RIA for analysis of urine samples extracted by ammonium sulfate, reported a high correlation between gonadotropin concentrations measured in FMV and 24-h urine (Maesaka et al., 1990b).

From the 1970s to the 1990s, radioimmunoassay was widely used use for determining LH and other glycoprotein hormones. However, this methodology had some limitations. RIA used antigens and antibodies labeled with radioactive isotopes with short half-lives. Highly specific antibodies were also a prerequisite, which was particular challenging for specific determinations of hCG and LH. In addition, the pulsatile secretion of FSH and LH complicated interpretation of the results of single samples (Santen & Bardin, 1973; Smith, Tcholakian, Chowdhury, & Steinberger, 1974). No less than 18 samples over a 6-hour interval or pooling of three blood samples obtained at 20-min intervals were proposed by various investigators for obtaining more reliable results (Santen & Bardin, 1973; Goldzieher, Dozier, Smith, & Steinberger, 1976).

RIA was the dominating method for hormone determination until the beginning of the 1990s, but alternatives were being developed in the 1970s: enzyme immunoassays. Before that, Catt and Tregear showed that antibodies adsorbed to the wall of plastic tubes could bind antigens (Catt & Tregear, 1967). The use of such solid phase antibodies simplified RIAs. Labeling of antibodies with enzymes was used for immunohistochemistry in 1969 (Avrameas, 1969). A similar approach was taken for serologic assays in 1971 by Engvall et al., who called the method ELISA, enzyme-linked immunosorbent assay. ELISA was based on coating plastic with antigens or antibodies to produce a solid phase (Engvall & Perlmann, 1971). Subsequently, immunoassay has been facilitated by development of monoclonal antibodies (Kohler & Milstein, 1975) and highly sensitive non-radioactive labeling techniques (Ekins, Edwards, Jackson, & Geiseler, 1984).

In 1968, Miles and Hales introduced sandwich assays with one antibody adsorbed to a solid phase and another radiolabeled antibody recognizing a different epitope for detection (Miles & Hales, 1968). The introduction of sandwich assays (also called two-site immunometric assays) improved the detection 10- to 100-fold. A further improvement in assay sensitivity was achieved with the use of time-resolved fluorometry for labeling the tracer antibody (Soini & Hemmila, 1979; Soini & Kojola, 1983; Ekins et al., 1984).

Assays for LH and FSH by dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) were made commercially available by LKB Wallac. This technique provided high sensitivity (Lovgren, Hemmila, Pettersson, Eskola, & Bertoft, 1984; Bador, Dechaud, Claustrat, & Desuzinges, 1987). The assay is based on a sandwich technique in which two monoclonal antibodies are directed against two separate sites on the gonadotropin molecule (Lovgren et al., 1984). In contrast to polyclonal RIAs, this method allows measurement of intact molecules, and even isolated subunits as a supplementary feature. Also, very small sample volumes (25 μL) are sufficient for running immunofluorometric assays (IFMAs) in contrast to the larger volumes (100-250 μL) required for RIAs. The reaction kinetics allows for a broad calibration curve and a wide range of sample concentrations without a need for dilution. In 1990s, the DELFIA LH assay came in two formats which varied with

respect to the duration of the incubation time, the regular LH assay and the so-called LH Spec assay. Many studies (Dunkel et al., 1990a; Dunkel et al., 1992; Kletter, Padmanabhan, Foster, Brown, Kelch, & Beitins, 1993b; Saketos, Sharma, Adel, Raghuwanshi, & Santoro, 1994) used the DELFIA LH assay which required a 2-h initial incubation time. A potential limitation of this assay was the high degree of cross-reactivity with hCG, which was fortunately not an issue in pediatric endocrinology. This problem was eliminated with the introduction of the LH Spec assay (Pettersson, Ding, & Huhtaniemi, 1991; Taylor, Khoury, & Crowley, 1994).

In the 1980s, two-site immunometric assays for gonadotropins with significantly improved sensitivities were described (Stenman, Alfthan, Myllynen, & Seppala, 1983; Lovgren et al., 1984; Odell & Griffin, 1987). The use of monoclonal antibodies improved discrimination between LH and hCG (Pettersson, Siitari, Hemmila, Soini, Lovgren, Hanninen, Tanner, & Stenman, 1983; Soos & Siddle, 1983; Haavisto, Pettersson, Bergendahl, Perheentupa, Roser, & Huhtaniemi, 1993). With an assay using time resolved fluorescence for detection, Apter et al., showed that the prepubertal LH concentrations of girls are very low and often undetectable (Apter et al., 1989). This indicated that previously reported LH measurements, which were based on competitive RIAs, overestimated LH (Wennink, Delemarre-van de Waal, van Kessel, Mulder, Foster, & Schoemaker, 1988; De Hertogh, Wolter, Van Vliet, & Vankrieken, 1989). Stenman et al. showed that this method could also be used on urine samples (Stenman, Alfthan, Koskimies, Seppala, Pettersson, & Lovgren, 1985). Pettersson et al. also reported that radioimmunoassays overestimate the true LH concentrations either due to non-specific artifacts (matrix effects) or to cross-reactivity with free alpha subunits (Pettersson & Soderholm, 1991), which are present in both serum and urine (Prentice & Ryan, 1975; Ishibashi, Yamaji, Takaku, Teramoto, & Fukushima, 1987; Silva de Sa, Matthews, & Rebar, 1988; Landy, Schneyer, Whitcomb, & Crowley, 1990). This led to a critical evaluation of the validity of the results obtained with the first-generation immunoassays (Chappel, 1990; Jaakkola et al., 1990).

Haavisto et al. compared LH assay results obtained by an *in vitro* bioassay, a conventional RIA and an IFMA in their abilities to discriminate between CDPG and HH (Haavisto et al., 1990). The bioassay and IFMA after GnRH stimulation provided significant distinction between CDPG and HH, whereas the RIA failed. The correlation of LH levels detected by bioassay was higher with IFMA than RIA, which apparently was due overestimation of low LH levels because of limited sensitivity and cross-reaction with free LH subunits. Using different antibodies, Lövgren had developed two IFMA methods (Lovgren et al., 1984), one which detected intact LH, the other intact LH as well as the free LH β-subunit. The correlation of LH levels measured by the two IFMAs in serum samples was excellent (Haavisto et al., 1990; Pettersson & Soderholm, 1990). This finding showed that serum does not contain any substantial concentrations of free LH β-subunits. The LH levels obtained by IFMA correlated highly also with those obtained by an *in vitro* bioassay but not with those obtained by conventional RIA (Haavisto et al., 1990). However, the ratio of bioactivity to immunoreactivity measured by RIA increased on GnRH stimulation and during normal pubertal development, whereas the ratio of bioactivity to immunoreactivity measured by IFMA under the same conditions remained the same (Haavisto et al., 1990).

When ultrasensitive time-resolved IFMA was used to determine S-LH and S-FSH, the levels were low but detectable in serum samples obtained from prepubertal girls (Apter et al., 1989). Gonadotropin IFMAs have been successfully used to determine very low S-FSH and S-LH levels (Apter et al., 1989; Dunkel et al., 1990a; Dunkel et al., 1990b; Haavisto et al., 1990; Wu et al., 1991; Dunkel et al., 1992; Goji & Tanikaze, 1992). Children at early pubertal stages have higher serum gonadotropin concentrations than children prepuberty; however, these concentrations are still much lower than those in adults (Apter et al., 1989; Dunkel et al., 1992; Bordini, Littlejohn, & Rosenfield, 2009; Mortensen, Ehrmann, Littlejohn, & Rosenfield, 2009; Zimmer, Ehrmann, & Rosenfield, 2010). RIAs were not sensitive enough for evaluation of prepubertal gonadotropin secretion. Since the beginning of the 1990s, highly sensitive immunofluorometric and chemiluminometric assays have been routinely used for clinical research and practice. IFMA has been used to examine the pulsatile nature of gonadotropin secretion in healthy and hypogonadal prepubertal boys with primary testicular failure (Dunkel et al., 1990a).

Assessment of pubertal hormone concentrations requires consideration of diurnal and cyclic changes. Gonadotropin concentrations determined in clinically pubertal subjects during daytime may not catch the increase during the early stages of puberty because of cyclic and diurnal variations. The GnRH test was therefore the method of choice for evaluation of pubertal development (Cavallo & Zhou, 1994) and the diagnosis of pubertal disorders. An S-LHmax level greater than 4.0 IU/L after GnRH or GnRH-agonist administration has been regarded as the sign of evoked puberty (Carel, Eugster, Rogol, Ghizzoni, Palmert, Antoniazzi, Berenbaum, Bourguignon, Chrousos, Coste, Deal, de Vries, Foster, Heger, Holland, Jahnukainen, Juul, Kaplowitz, Lahlou, Lee, Lee, Merke, Neely, Oostdijk, Phillip, Rosenfield, Shulman, Styne, Tauber, & Wit, 2009; Bordini, Littlejohn, & Rosenfield, 2010).

1.2 Clinical aspects of puberty

1.2.1 Hormonal development during healthy puberty

Maturation of the HPG axis triggers the development of puberty in healthy children (Grumbach, 1975). Gonadal function matures during a period starting with sexual differentiation and development of the HPG system. This starts during the prenatal period and continues during infancy (Grumbach & Kaplan, 1974; Kaplan, Grumbach, & Aubert, 1976; Kaplan & Grumbach, 1978; Grumbach & Gluckman, 1994). It continues through a juvenile pause followed by maturation of the HPG axis during puberty (Reiter & Grumbach, 1982; Grumbach & Kaplan, 1990) (Figures 2 and 3). Increasing kisspeptin secretion induced by hypothalamic GPR54 signaling leads to secretion of GnRH (Shahab, Mastronardi, Seminara, Crowley, Ojeda, & Plant, 2005). GnRH-secreting neurons are activated by kisspeptin either indirectly via interneurons or directly by acting on gonadotropes (Han, Gottsch, Lee, Popa, Smith, Jakawich, Clifton, Steiner, & Herbison, 2005; Messager, Chatzidaki, Ma, Hendrick, Zahn, Dixon, Thresher, Malinge, Lomet, Carlton, Colledge, Caraty, & Aparicio, 2005; Nazian, 2006).

Fetal life

The fetal pituitary gland contains FSH and LH by 10 weeks from conception. Gonadotropin secretion begins at 11-12 weeks. The gonadotropin content of the pituitary gland increases at 25-29 weeks (Grumbach & Kaplan, 1974; Kaplan et al., 1976; Kaplan & Grumbach, 1978; Grumbach & Gluckman, 1994; Grumbach, 2005). Fetal S-LH and S-FSH concentrations reach maximum levels at midgestation and decrease gradually until term. As the negative feedback system and sex steroid receptors in the hypothalamic-pituitary axis develop, serum FSH and LH concentrations of the fetus decrease towards term (Grumbach & Kaplan, 1974; Kaplan et al., 1976; Gluckman, Marti-Henneberg, Kaplan, & Grumbach, 1983). Additionally, inhibin B plays a secondary role in suppressing FSH levels (Grumbach & Gluckman, 1994; Grumbach, 2005). Activin A, detectable in fetal serum samples, has not been shown to be involved with any endocrine change. Female fetuses have a higher pituitary content of gonadotropins, in particular during the first half of gestation (Kaplan et al, 1976; Huhtaniemi, 1989) and a higher serum concentration of LH and FSH (Reyes, Winter, & Faiman, 1973) than their male counterparts. This is due to the negative feedback effects of fetal testicular hormones on LH and FSH. At midgestation, S-LH and S-FSH concentrations in female fetuses increase markedly and reach the same levels as postmenopausal women or agonadal adults. The FSH concentrations exceed those of LH in female fetuses, whereas the opposite is true for male fetuses (Takagi, Yoshida, Tsubata, Ozaki, Fujii, Nomura, & Sawada, 1977; Debieve, Beerlandt, Hubinont, & Thomas, 2000). Towards term, FSH and LH concentrations decrease and become almost undetectable due to inhibition by placental estrogens (Beck-Peccoz, Padmanabhan, Baggiani, Cortelazzi, Buscaglia, Medri, Marconi, Pardi, & Beitins, 1991);(Debieve et al., 2000).

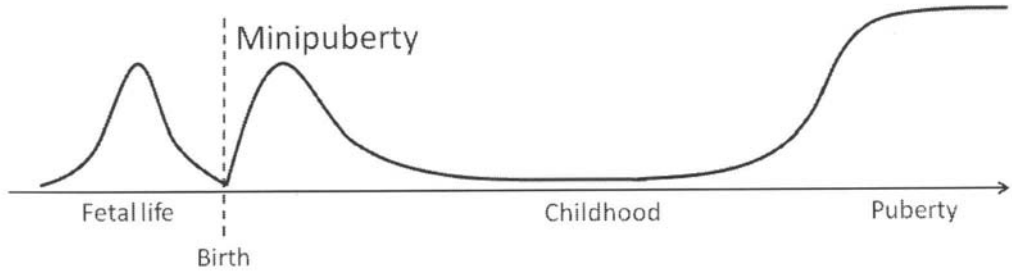

Figure 2. Three periods of hypothalamic-pituitary axis activity. During fetal life, the activity peaks at midgestation and diminishes towards term, probably due to suppression induced by placental hormones and especially by estrogens. At birth, gonadotropin concentrations are low, but the hypothalamic-pituitary axis becomes active again at about 1 week after birth. This neonatal gonadotropin surge, or minipuberty, leads to activation of gonadal hormone secretion in both sexes. The activity peaks when the infant is 1–3 months of age, whereafter the hypothalamic-pituitary activity gradually decreases and remains low until reactivation when it is time for the onset of puberty. *Reprinted by permission from (Kuiri-Hanninen et al, 2014). © Horm Res Paediatr 2014, Copyright Clearance Center, License ID: 3645570771359.*

Early infancy - minipuberty

In both sexes, high levels of placental estrogens cause low S-FSH and S-LH concentrations in cord blood due to the inhibition involved. Interestingly, the S-LH concentration in peripheral blood increases abruptly (about 10-fold) right after birth in the male newborn but not in the female. This LH burst occurs within a few minutes after birth and raises the serum testosterone levels during the first 3 hours of postnatal life. The levels remain high for about half a day (Corbier, Dehennin, Castanier, Mebazaa, Edwards, & Roffi, 1990; Grumbach & Kaplan, 1990; Kuiri-Hanninen, Sankilampi, & Dunkel, 2014).

A hypothalamic GnRH pulse generator is clearly active by postnatal day 12. During the first postnatal days, S-LH and S-FSH concentrations are low in both sexes (Winter, Faiman, Hobson, Prasad, & Reyes, 1975; Debieve et al., 2000) because hormones of placental origin are eliminated from the circulation in relation to their half-lives (Bidlingmaier, Wagner-Barnack, Butenandt, & Knorr, 1973; Winter et al., 1975). S-FSH and S-LH concentrations rise during the first postnatal week and peak in children aged 1–12 weeks (Winter et al., 1975; Andersson, Toppari, Haavisto, Petersen, Simell, Simell, & Skakkebaek, 1998; Bergada, Milani, Bedecarras, Andreone, Ropelato, Gottlieb, Bergada, Campo, & Rey, 2006; Kuiri-Hanninen, Kallio, Seuri, Tyrvainen, Liakka, Tapanainen, Sankilampi, & Dunkel, 2011a; Kuiri-Hanninen, Seuri, Tyrvainen, Turpeinen, Hamalainen, Stenman, Dunkel, & Sankilampi, 2011b). This neonatal-to-midinfancy surge of pulsatile gonadotropin secretion (as well as of sex hormones and inhibin production) is due to an increase in the GnRH pulse amplitude and presents as a period of 'minipuberty', which may cause vague clinical symptoms [reviewed in (Kuiri-Hanninen et al., 2014)]. During these early weeks and months of life, S-LH predominates in boys and S-FSH in girls (Shinkawa, Furuhashi, Fukaya, Suzuki, Kono, & Tachibana, 1983; Andersson et al., 1998; Ibanez, Valls, Cols, Ferrer, Marcos, & De Zegher, 2002; Bergada et al., 2006). The mean S-LH levels are higher in boys than in girls (Kuiri-Hanninen et al., 2014), while the mean S-FSH levels are higher in girls than in boys during the newborn period and the first few years of life (Kuiri-Hanninen et al., 2014). The S-LH concentrations in both sexes and S-FSH concentrations in girls decline after age 6–9 months, but high concentrations of S-FSH prevail in girls for a longer period of time–from an average of age 18 months to an average of 4 age years (Winter et al., 1975; Andersson et al., 1998; Kuiri-Hanninen et al., 2011a; Kuiri-Hanninen et al., 2011b).

Juvenile pause

When boys are approximately 6 months and girls 2–3 years, serum gonadotropin concentrations decrease to low levels that persist until the HPG axis becomes activated and chemical puberty ensues. During the juvenile pause, the hypothalamic GnRH pulse generator is relatively but not completely silent, since it produces low amplitude and low frequency GnRH discharges (Grumbach, 1978; Reiter & Grumbach, 1982; Apter et al., 1989; Grumbach & Kaplan, 1990; Apter et al., 1993; Yen, Apter, Butzow, & Laughlin, 1993; Grumbach & Gluckman, 1994; Terasawa & Fernandez, 2001; Plant, 2008).

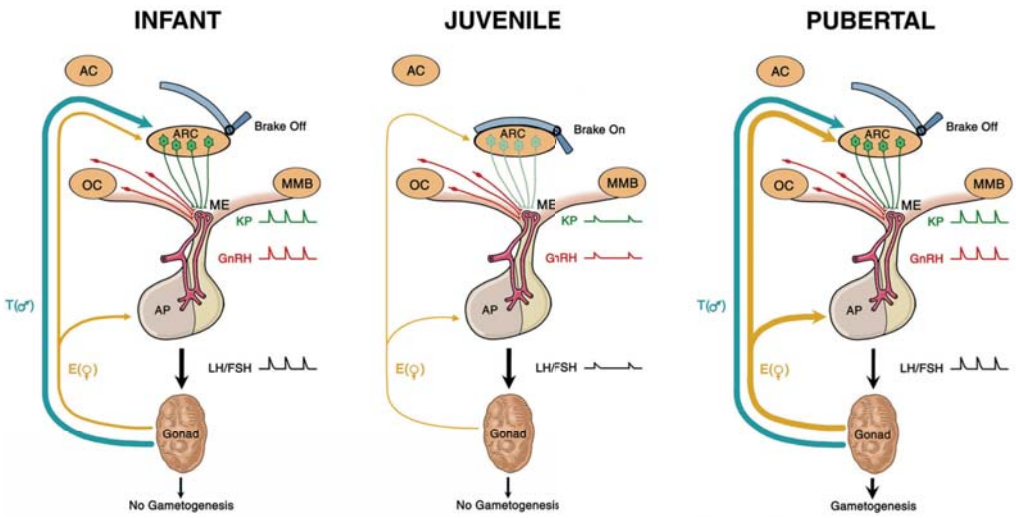

Figure 3. Model of puberty timing control in primates. The role of kisspeptin (KP, green) signaling is thought be critical for the neural machinery and essential for generation of pulsatile GnRH (red) secretion in the hypothalamus. The GnRH pulse generator resides in the arcuate nucleus (ARC) and the signaling is relayed to GnRH terminals in the median eminence (ME) by KP projections arising from the perikarya in the ARC. During infancy (left panel), the GnRH pulse generating activity in the ARC leads to intermittent release of KP in the ME and then to the release of GnRH into the portal circulation. This drives pulsatile secretion of LH and FSH. During transition from infancy to the juvenile phase of development (middle panel), a central inhibition (neurobiological brake) controls the ARC GnRH pulse generating mechanism and suppresses the pulsatile release of KP in the ME. Reduced GnRH release leads to a hypogonadotropic state in the juvenile period. Puberty is triggered when the neurobiological brake is removed and GnRH pulse generation and intermittent release of KP in the ME is reactivated (right panel). In this model, the secret of primate puberty lies in the nature of the neurobiological brake, i.e., the timing of its function during infancy and its release at the end of the juvenile period. The thickness of the blue (T, testosterone) and amber (E, estradiol) arrows indicate negative feedback by the testis and ovary, respectively, and reflect the degree of gonadal steroid inhibition exerted on LH secretion during the three stages of primate development. AC, anterior commissure; AP, anterior pituitary gland, ARC, arcuate nucleus; OC, optic chiasm; ME, median eminence; MMB, mamillary body. *Modified from (Terasawa, Guerriero, & Plant, 2013).*

Highly sensitive and specific assays have been used to detect LH and FSH pulses with low amplitudes throughout the juvenile pause, during which the activity of the GnRH pulse generator is low (Kelch & Marshall, 1990; Apter et al., 1993; Yen et al., 1993; Mitsushima, Hei, & Terasawa, 1994; Mitsushima, Marzban, Luchansky, Burich, Keen, Durning, Golos, & Terasawa, 1996; Wu, Butler, Kelnar, Huhtaniemi, & Veldhuis, 1996; Albertsson-Wikland, Rosberg, Lannering, Dunkel, Selstam, & Norjavaara, 1997; Terasawa & Fernandez, 2001). GnRH stimulation causes only a modest response in prepubertal children except in infancy; this response becomes stronger as puberty approaches, is high during puberty (Grumbach, Roth, & Kaplan, 1974; Grumbach & Kaplan, 1990) and increases during adulthood (Keye

& Jaffe, 1975; Yen, Lasley, Wang, Leblanc, & Siler, 1975). Ultrasensitive LH and FSH assays (Apter et al., 1993; Albertsson-Wikland et al., 1997; Mitamura, Yano, Suzuki, Ito, Makita, & Okuno, 1999; 2000) have been used to confirm the existence of pulsatile gonadotropin secretion in prepuberty and indicate that the basal levels of LH are much lower than previously reported (Mitamura et al., 1999; 2000). Thus, a functional GnRH deficiency appears to regulate the hormonal status in prepubertal children (Grumbach et al., 1974; Reiter & Grumbach, 1982; Watanabe & Terasawa, 1989; Grumbach & Kaplan, 1990). An increased amplitude of LH and FSH secretion bursts appears at night-time in prepubertal boys and girls by 5 years of age. There are LH pulses that occur mainly after falling asleep but not before slow-wave sleep begins (Shaw, Butler, McKinney, Nelson, Ellenbogen, & Hall, 2012). This type of sleep is known as deep sleep, and, taken together, these observations indicate that deep sleep is closely associated with triggering of the GnRH pulse generator. The amplitude and frequency of these secretion peaks increase with the progression of pubertal development (Apter et al., 1989; Apter, 1993; Mitamura et al., 1999; 2000).

The main factor inhibiting pubertal development during the juvenile pause between age 4 and 11 years appears to be a GnRH pulse generator inhibiting process, which is not associated with any steroid hormone (Grumbach et al., 1974; Grumbach & Kaplan, 1988; Grumbach & Kaplan, 1990; Terasawa & Fernandez, 2001; Plant, 2008). Progressive impairment of this mechanism awakens the GnRH pulse generator as puberty approaches and proceeds.

Gonadotropins (FSH rather than LH) are secreted in high amounts in infants with primary hypogonadism (e.g., Turner syndrome) and in prepubertal children, which indicates that a rather sensitive negative feedback mechanism restrains secretion of LH and FSH even when low levels of steroid hormones are secreted from the gonads of healthy prepubertal children (Burr, Sizonenko, Kaplan, & Grumbach, 1970; Conte, Grumbach, Kaplan, & Reiter, 1980; Grumbach & Kaplan, 1990). Administration of gonadal steroids in small doses blocks entirely gonadotropin secretion in childhood, indicating that the impact of negative inhibition by gonadal steroids on the HPG axis is rather strong (approximately 6 to 15 times stronger than in adults) (Kelch, Kaplan, & Ghumbach, 1973; Grumbach et al., 1974; Grumbach & Kaplan, 1990).

Onset of puberty

S-FSH levels are higher than the S-LH levels in prepubertal children of both sexes (Kaplan et al., 1976), and higher amplitudes of LH pulses than of FSH during sleep is a phenomenon that is regarded as the strongest indicator of puberty onset. Activation of the slumbering (partially silent) GnRH pulse generator results in increasing serum concentrations of LH, which heralds the onset of puberty at the biochemical level (Wu et al., 1996). Initially, the inhibition of the hypothalamic GnRH pulse generator is gradually decreased and LH secretion during sleep increases in prepubertal boys and girls (Boyar, Rosenfeld, Kapen, Finkelstein, Roffwarg, Weitzman, & Hellman, 1974).

With the approach of puberty, pulsatile secretion of GnRH escalates in parallel with the increases in LH reserves and sensitivity of the pituitary to GnRH as the inhibition on

the hypothalamic GnRH pulse generator lifts (Germak & Knobil, 1990). Endogenous, increasing GnRH secretion at puberty primes the gonadotropes (Roth, Kelch, Kaplan, & Grumbach, 1972; Grumbach et al., 1974), which increases the sensitivity of the pituitary to endogenous GnRH (as in the case of enhanced release of LH at nighttime during the course of normal puberty) and to exogenous GnRH (as in the response to intravenous GnRH during the prepubertal period) (Boyar, Finkelstein, Roffwarg, Kapen, Weitzman, & Hellman, 1972; Job, Garnier, Chaussain, & Milhaud, 1972; Roth et al., 1972; Boyar, Finkelstein, David, Roffwarg, Kapen, Weitzman, & Hellman, 1973; Roth, Grumbach, & Kaplan, 1973; Boyar et al., 1974; Kapen, Boyar, Freeman, Frantz, Hellman, & Weitzman, 1975; Kelch, Clemens, Markovs, Westhoff, & Hawkins, 1975; Mitamura et al., 2000; Styne & Grumbach, 2002). Alternating rounds of disinhibition and reactivation of GnRH secretion occur before full-blown pubertal development gets established (reactivation episodes mainly during night towards late prepuberty) (Conte, Grumbach, & Kaplan, 1975; Conte et al., 1980; Reiter & Grumbach, 1982; Grumbach & Kaplan, 1990; Terasawa & Fernandez, 2001; Plant, 2008). This increases the GnRH pulse amplitudes and causes pattern changes and a gradual increase in the LH pulses during the early hours of sleep as of the onset of puberty (Boyar et al., 1973; Judd, Parker, Siler, & Yen, 1974; Boyar, Wu, Roffwarg, Kapen, Weitzman, Hellman, & Finkelstein, 1976; Wennink et al., 1988; Mitsushima et al., 1994; Mitsushima et al., 1996). Nocturnal LH secretion influences gonadal sex steroid secretion differently in boys and girls: peaks of estradiol occur during mid-day whereas peaks of testosterone occur almost instantly after sleep (Mitamura et al., 1999; 2000). Secretion of LH predominates during daytime as pubertal development gets established. Girls after menarche no longer present with this diurnal variation (Mitamura et al., 2000). Adult sex steroid concentrations, regulated by a moderate diurnal variation, are highest on awakening (Mitamura et al., 1999; 2000).

Later pubertal development

As puberty proceeds the amplitude and frequency of gonadotropin peaks escalate and daytime gonadotropin secretion increases (Boyar et al, 1972; Boyar et al, 1974; Dunkel et al, 1990a; Dunkel et al, 1990b; Hale et al, 1988; Hassing et al, 1990; Jakacki et al, 1982; Wennink et al, 1988). As increase in GnRH pulse frequency results in increased secretion of gonadotropins, favoring LH over FSH, which is one of the typical endocrine markers of pubertal development. This finding was confirmed by the finding that the FSH/LH ratio increased as GnRH stimulation became less frequent (once per hour instead of one every 3 hours) in adult rhesus monkeys with ablative hypothalamic lesions which eliminated the endogenous GnRH secretion (Wildt et al, 1981).

During late prepubertal and pubertal periods, the increased amplitude, not the difference in pulse frequency, of the GnRH pulses increases gradually the LH pulses in both sexes (Mitamura et al, 1999; Mitamura et al, 2000; Wu et al, 1996). In other words, an increased serum LH response to GnRH is a finding associated with emerging puberty.

The main inhibitive mechanism limiting gonadotropin secretion in pubertal children appears to be the negative feedback exerted by gonadal steroids and inhibin, since primary hypogonadism raises serum gonadotropin concentrations (Plant, 2008). Both testosterone

and estradiol derived from aromatization of testosterone exert a negative feedback on the hypothalamus, and this suppresses pulsatile GnRH secretion (Pitteloud, Dwyer, DeCruz, Lee, Boepple, Crowley, & Hayes, 2008b; a). Aromatization of testosterone to estradiol is a prerequisite for testosterone to elicit negative feedback inhibition on pituitary LH and FSH secretion (Pitteloud et al., 2008b; a). Inhibin B is the main physiologic negative feedback regulator of FSH secretion, since suppression of FSH by estradiol is modest when inhibin B levels are in the normal range and becomes prominent only when inhibin B activity is absent, which is the case for men with anorchia or testes presenting with a non-functional seminiferous epithelium (Boepple, Hayes, Dwyer, Raivio, Lee, Crowley, & Pitteloud, 2008).

Central precocious puberty may occur when a prepubertal child has previously been exposed to excessive levels of androgens from an endogenous or exogenous source (e.g., after initiation of glucocorticoid therapy for congenital virilizing adrenal hyperplasia, or after removal of a sex steroid–secreting adrenal or gonadal neoplasm) (Reiter, Grumbach, Kaplan, & Conte, 1975). Most patients with a premature adrenarche, who secrete excessive amounts of adrenal androgens for their age, enter puberty and experience menarche in the same age as their unaffected counterparts (Grumbach, 1977). Moreover, prepubertal children who present with low or absent adrenal androgen secretion as a result of chronic adrenal deficiency have normal pubertal development, if they get adequate replacement therapy for the missing adrenal steroids (Grumbach, 1977).

Cross-sectional and longitudinal studies have documented that an increase in serum concentrations of the precursors of adrenal androgens (dehydroepiandrosterone [DHEA], dehydroepiandrosterone sulfate (DHEAS) and androstenedione) precedes the rise in gonadotropin and gonadal steroid levels by approximately 2 years in both sexes. High levels of these precursors are maintained throughout puberty (Sizonenko & Paunier, 1975; Grumbach, 1977; Reiter, Fuldauer, & Root, 1977) and they peak at age 20 to 30 years, after which a gradual decrease takes place (Sklar, Kaplan, & Grumbach, 1980). The adrenarchal increase in DHEA and DHEAS secretion bears no association with the pituitary gonadotropes being more sensitive to GnRH stimulation (Reiter, Kaplan, Conte, & Grumbach, 1975) or with sleep-associated LH secretion; actually, this occurs usually at an age when the HPG axis function is the lowest (Grumbach, 1977). The significance of the adrenarche for regulation of puberty is controversial. In addition to being a precursor for biologically active androgens (testosterone, dihydrotestosterone) and estrogens (estradiol) through peripheral conversion (Voutilainen & Jaaskelainen, 2015), DHEA is a neurosteroid, the concentrations of which increase as the cerebral cortex matures from age 6 years to the mid-20s. DHEA(S) plays an important role in the evolution of the adrenarche: it supports cortical development in the brain and facilitates memory functions which regulate social interaction outside the immediate family and acquaintances (Campbell, 2006).

1.2.2 Genetic basis: Neuroendocrine control of onset of puberty

Mutations in KISS1R gene encoding the receptor for kisspeptins, also known as GPR54 cause hypothalamic hypogonadism (Seminara, Messager, Chatzidaki, Thresher, Acierno, Shagoury, Bo-Abbas, Kuohung, Schwinof, Hendrick, Zahn, Dixon, Kaiser, Slaugenhaupt, Gusella, O'Rahilly, Carlton, Crowley, Aparicio, & Colledge, 2003; Shahab et al., 2005). This

observation paved the way for further studies which then identified several other genes, e.g., TAC3, TAC3R and LEPR, that regulate the onset of puberty (Sykiotis, Pitteloud, Seminara, Kaiser, & Crowley, 2010). These genes are organized as functional webs that regulate the various steps which initiate the pubertal process.

Neurons secreting gonadotropin-releasing hormone (GnRH) are believed to be subjected to persistent trans-synaptic inhibition during the juvenile pause, which comes to an end as this inhibition expires. As expiration of inhibitory inputs alone cannot trigger the onset of puberty, an excitatory input to GnRH neurons is required. In fact, kisspeptin neurons are the major component of this excitatory input, as kisspeptin signaling is a prerequisite for the onset of puberty (Seminara et al., 2003). Pulsatile GnRH release in both sexes is triggered by activity of kisspeptin neurons of the arcuate nucleus (ARC) in the hypothalamus. The preovulatory surge of gonadotropins is dependent on kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) of the hypothalamus, which are almost absent from males (Pinilla, Aguilar, Dieguez, Millar, & Tena-Sempere, 2012).

The mechanism triggering the onset of puberty functions in a transcriptional model. An inhibition–activation model would be too simplistic because the network of genes involved in the pubertal onset mechanism facilitates the primary excitatory system (kisspeptin neurons) that controls pulsatile GnRH secretion. However, also an epigenetic regulation is known to be at least as essential and as effective (Lomniczi, Loche, Castellano, Ronnekleiv, Bosch, Kaidar, Knoll, Wright, Pfeifer, & Ojeda, 2013a).

At the point when central puberty is initiated, promoter DNA methylation of Eed and Cbx7 genes takes place. This decreases the expression of these genes and increases the dissociation of their protein products from the Kiss1 promoter. This occurs in parallel with an increase in the expression of Kiss1 and changes in the chromatin status of the Kiss1 promoter. Inhibition of DNA methylation counters the peripubertal decline in Eed and Cbx7 expression and blocks the removal of EED and CBX7 from the Kiss1 promoter, which arrests the onset of puberty (Lomniczi et al., 2013a).

Many cases of familial central precocious puberty are caused by mutations in the MKRN3 gene (Abreu, Dauber, Macedo, Noel, Brito, Gill, Cukier, Thompson, Navarro, Gagliardi, Rodrigues, Kochi, Longui, Beckers, de Zegher, Montenegro, Mendonca, Carroll, Hirschhorn, Latronico, & Kaiser, 2013), a gene encoding transcriptional repressors. The decrease in hypothalamic expression of Mkrn3 in mice undergoing prepubertal development and MKRN3 deficiency in patients with precocious puberty constitute firm evidence that MKRN3 represses the onset of puberty (Abreu et al., 2013). These observations are supported by a longitudinal study by Hagen et al., who reported that serum MKRN3 concentrations decline prior to the onset of puberty and throughout puberty in healthy girls (Hagen, Sorensen, Mieritz, Johannsen, Almstrup, & Juul, 2015). The negative correlation between MKRN3 and gonadotropins in the same study further supports MKRN3 as a major regulator of hypothalamic GnRH secretion during childhood. Another supporting finding was that subjects with early onset of puberty have undetectable or low MKRN3 levels (Hagen et al., 2015).

These findings demonstrate that genes from the zinc finger family regulate the timing of puberty onset through repression of downstream targets, rather than by activating GnRH neurons (Lomniczi, Wright, Castellano, Sonmez, & Ojeda, 2013b). These findings support the notion that the timing of puberty is regulated by transcriptional repression, rather than by some on-off switch in the neuroendocrine circuitry (Lomniczi et al., 2013a).

According to this new view on the central and peripheral mechanisms responsible for initiating puberty (Figure 4), the central control of puberty is provided by upstream mechanisms that involve gene silencing instead of inhibition occurring at the level of trans-synaptic communication.

Physiological (biochemical/neuroendocrine) onset of puberty before or along with the first clinical signs of puberty should be evaluated closely by the clinician studying the start and development of puberty. Molecular genetic (neuroendocrine) regulation behind the initial activation and sustained activity of the HPG axis and GnRH pulse generator eventually establishes the nature of this unique physiological process.

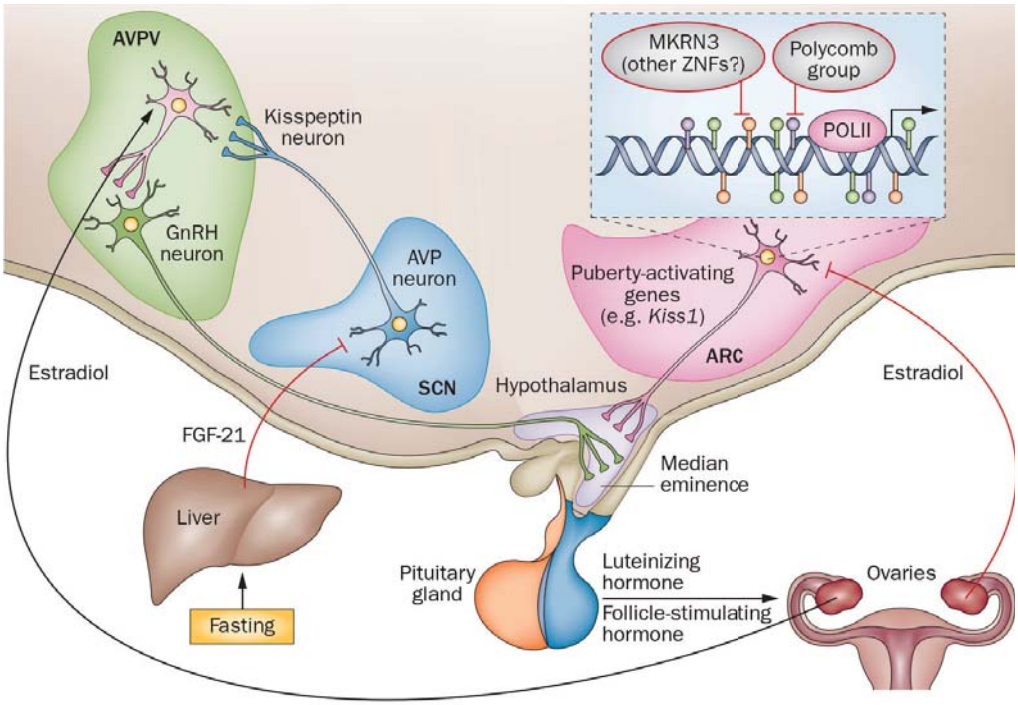

Figure 4. Repressive control of puberty. In the ARC, two gene-silencing systems are involved–the polycomb group complex and MKRN3. At the AVPV level, the completion of puberty is delayed by FGF21. Abbreviations: ARC, arcuate nucleus; AVP, vasopressin; AVPV, anteroventral periventricular nucleus; FGF21, fibroblast growth factor 21; MKRN3, makorin ring finger protein 3; SCN, suprachiasmatic nucleus; ZNFs, member of the zinc finger family of proteins. *Reprinted by permission from (Ojeda & Lomniczi, 2014). © Nat. Rev. Endocrinol. 2014, Copyright Clearance Center, License ID: 3645741168018.*

1.2.3 Physical development during normal puberty

The onset of puberty in the Caucasian population occurs at age 9-14 years in boys and 8-13 years in girls (Carel et al., 2009). The age at onset of puberty has decreased over the last 100 years (Parent, Teilmann, Juul, Skakkebaek, Toppari, & Bourguignon, 2003; Euling, Selevan, Pescovitz, & Skakkebaek, 2008; Biro, Galvez, Greenspan, Succop, Vangeepuram, Pinney, Teitelbaum, Windham, Kushi, & Wolff, 2010), but methodological issues hinder researchers from establishing whether this trend continues or not. Excess adiposity in girls may falsely be interpreted as a clinical sign of puberty onset, but there are studies suggesting that adiposity may facilitate, at least slightly, the onset of puberty in some girls (Rosenfield, Lipton, & Drum, 2009).

Tanner staging, the gold standard for sexual maturity rating is an instrument for following a child's advancement through puberty by physical examination. Breast (female) or genital (male) and pubic hair development are graded using separate stages. Pituitary gonadotropin and subsequent gonadal sex steroid secretion control male genital and female breast development, whereas adrenal and gonadal androgens regulate pubic hair development in both sexes (Reynolds, 1951; Marshall & Tanner, 1969; 1970; Herman-Giddens, Bourdony, Dowshen, & Reiter, 2010). Interestingly, there are reports that the increase in androgen levels occurs earlier in girls than in boys (Winter & Faiman, 1972; 1973), although this has not been confirmed by later studies. Tanner stage 1 corresponds to the prepubertal status. Thelarche (Tanner stage B2) and testicular enlargement (Tanner stage G2) reveal that pubertal development has started (gonadarche) and will progresses to stage 3 through 5.

Girls

Although breast budding is the usual sign heralding puberty, an increase in height velocity may also be an early marker for the onset of female puberty (Bourguignon, 1991). Early breast budding may be initially be unilateral for many months, which causes concern for both the subject and her parents. However, the situation is benign and transient. On the other hand, fat deposition (adipomastia) should not be confused with breast tissue development. The distinction is best made by careful examination of the subareolar region during palpation of the breast bud. Enlargement of the papilla of the nipple occurs continuously during pubertal development. The increase in the nipple diameter during breast stage 1 to 3 is quite small (3 to 4 mm) and becomes prominent only after breast stage 3. This parameter provides an objective criterion to differentiate between stage 4 and stage 5 (the final diameter is approximately 9 mm) (Rohn, 1987). The timing of the menarche is usually closely associated with bone development, i.e., fusion of the second and first distal phalanges and the appearance of the first sesamoid of the thumb (Bjork & Helm, 1967). In most cases, this corresponds to Tanner breast stage 4. The 95th percentile for menarche is 14.5 years, and a diagnosis of primary amenorrhea can be made if menses have not started at 16 years (Practice Committee of American Society for Reproductive Medicine, 2008).

Boys

The first histologic sign of spermatogenesis occurs between ages 11 and 15 years. Spermaturia is indeed one of the first signs of onset of puberty, but the presence of sperm

in the urine is periodic and therefore not a reliable indicator of puberty for all boys. Paradoxically, spermaturia is more common in early than in late puberty, which favors the argument that there may be a continuous flow of sperm through the urethra in early puberty, but that ejaculation is a prerequisite for sperm to appear in the urine in late puberty (Pedersen, Nysom, Jorgensen, Nielsen, Muller, Keiding, & Skakkebaek, 1993). Spermaturia in a FMV urine specimen appears at a median chronologic age of 14.1 years, and on average during pubic hair stage (Tanner PH) 2 to 3 (Schaefer, Marr, Seidel, Tilgen, & Scharer, 1990), but it has also been reported that spermaturia may occur in healthy, clinically prepubertal boys with bilateral testicular volumes of only 3 mL (Schaefer et al., 1990; Nysom, Pedersen, Jorgensen, Nielsen, Muller, Keiding, & Skakkebaek, 1994).

Testicular growth is the first sign of puberty. This occurs on average 6 months later than initiation of breast development in girls (Bordini et al., 2009). Pubertal testicular enlargement is clinically evident when the length of the longitudinal axis of a testis is greater than 2.5 cm (excluding the epididymal tissue) or the testicular volume is greater than 3 mL (Van Vliet, 1991). In 1974, Zachmann et al. further developed the Prader orchidometer, a practical, rosary-like tool consisting of ellipsoids of increasing standardized volumes which correlate with the stage of puberty. The testicular volume index ([length × width of right testis + length × width of left testis]/2) and testicular volume are measured by comparing the testes with the ellipsoids (Zachmann, Prader, Kind, Hafliger, & Budliger, 1974; Taskinen, Taavitsainen, & Wikstrom, 1996).

A longitudinal study by Biro et al. shows that puberty progresses within 6 months in 82% of boys with a testis volume of 3 mL, supporting the earlier finding by Stafford et al. (Stafford, Weir, Pearl, Imai, Schydlower, & Gregory, 1989; Biro, Lucky, Huster, & Morrison, 1995). Ultrasonography is a reliable method to determine testicular volume. However, testicular volumes measured by ultrasound are only about half of those determined by orchidometry, but the correlation between these measurements is quite strong (Goede, Hack, Sijstermans, van der Voort-Doedens, Van der Ploeg, Meij-de Vries, & Delemarre-van de Waal, 2011).

S-LH concentrations keep rising as adult-type Leydig cells emerge and proliferate in the testes as pubertal development progresses. Leydig cell maturation seems to occur mainly during the three phases of increasing testosterone production, i.e., 14 to 18 weeks of fetal life, 2 to 3 months after birth, and puberty (Prince, 2001).

Most boys attain Tanner stage 3 level of pubic hair maturity within 1.0 to 1.5 years of the start of testicular growth (Tanner & Davies, 1985). Boys at Tanner genital stage 4 present with further masculinization and secondary sexual characteristics: facial hair and male type voice change. Asymmetric testicular development and gynecomastia (breast tissue development in boys) are normal variations of pubertal progression. Half of the boys have varying degrees of gynecomastia during pubertal development. It presents after Tanner stage G2 (predominantly during Tanner stage PH3 to 4) and persists rarely for more than 1 year (Biro, Lucky, Huster, & Morrison, 1990).

Pubarche occurs as a result of increased production of androgens, which may be of adrenal or gonadal origin. Therefore pubarche alone cannot be regarded as a definitive indicator of gonadal functionality. Tanner stage PH2 may be a sign of early sexual hair growth, but may also be caused by generalized hypertrichosis. Thus, Tanner stage PH3, i.e., the appearance of frank sexual hair, should be regarded as an accurate sign of pubertal development, rather than Tanner stage PH2. The pubertal changes of Tanner stage PH3 usually result from gonadal androgen production (Rosenfield et al., 2009), although the changes of Tanner stages PH2 through PH4 may also result exclusively from adrenal androgens. This is the case for both sexes, although the development of the male escutcheon (Tanner stage PH6), i.e., sexual hair extending along the linea alba from the pubic area, is explicitly related to an androgenic effect.

A gradual deceleration of growth velocity occurs slightly before the onset of puberty (Tanner & Davies, 1985). With puberty the production of estrogens and androgens stimulates statural growth by facilitating the production of growth hormone via direct and indirect pathways.

The pubertal growth spurt is primarily related to pubertal stage, not chronological age. The female pubertal growth spurt occurs at Tanner breast stage 3, about 1 year before menarche and in males at Tanner genital stage 3 or 4, during which spermarche takes place (Marshall & Tanner, 1970; Nielsen, Skakkebaek, Richardson, Darling, Hunter, Jorgensen, Nielsen, Ingerslev, Keiding, & Muller, 1986). Thus, the pubertal growth spurt starts about two years later in boys than girls. Boys grow taller than girls because boys have a longer duration of prepubertal growth and a greater pubertal peak height velocity.

1.2.4 Disorders of puberty

Delayed puberty and sexual infantilism

If a girl is prepubertal still at age 13 and a boy at age 14, it is prudent to evaluate clinically whether this constitutes normal variation of maturation or whether examinations are needed. A hypothalamic, pituitary or gonadal disorder is possible, if sexual maturation has not proceeded to its completion by 4.5 years after the onset of puberty in boys or if there is no menstruation 5 years after the onset of puberty in girls.

LH and FSH concentrations are typically elevated in hypergonadotropic hypogonadism. On the other hand, differentiating between hypogonadotropic hypogonadism and constitutional delay of growth and puberty (CDGP) is challenging, because laboratory findings, clinical signs and symptoms overlap. The concentrations of serum gonadotropins are low in both conditions. Most boys with pubertal delay are late variants in the onset of puberty and growth pace (CDGP) and the condition is transient. The laboratory studies required for the differential diagnosis are sensitive serum gonadotropin assays, a GnRH stimulation test to assess the degree of rise of the S-LH concentration and serum testosterone or estradiol.

Idiopathic or constitutional delay in growth and puberty

CDGP is the most common form of delayed puberty, particularly in boys. CDGP refers to onset of puberty after age 13 in otherwise healthy girls and 14 in boys. Subjects with CDGP are often short in stature (≥2 SD below the mean height for age) and present with a typical history of short stature for several years, although their growth velocity and height are usually in line with the expected bone age. Most of these adolescents have a family history of a mother or sister with delayed menarche or a father (or brother) with delayed puberty and a prolonged growth period extending beyond that of peers. This suggests that CDGP follows a familial pattern of genetic dominant inheritance with incomplete penetrance (Sedlmeyer, Hirschhorn, & Palmert, 2002; Sedlmeyer & Palmert, 2002). CDGP refers to physiologic immaturity and a slow pace of maturation; full sexual maturity will eventually be reached, but progression is slower than among peers. Since these children have a temporal lag in reactivation of their GnRH pulse generator and the HPG axis, they experience delays in pubertal maturation with reference to their chronologic age but not their physical growth. Children with CDGP present often with a lag in both adrenarche and gonadarche (Sedlmeyer & Palmert, 2002), whereas adrenarche usually occurs at the expected age in patients with isolated gonadotropin deficiency (Sklar et al., 1980). In CDGP bone age is usually delayed at presentation and during pubertal development.

Sexual precocity

Sexual precocity is the appearance of any clinical sign of puberty before 8 years in girls and 9 years in boys. These are the lower reference age limits for the appearance of the first clinical signs of puberty. Children with signs or symptoms of precocious puberty should, regardless of age, be evaluated with regard to central nervous system disorders and other serious diseases. The etiology is often idiopathic in girls and organic in boys (Sorensen, Mouritsen, Aksglaede, Hagen, Mogensen, & Juul, 2012).

1.3 Clinical use of urinary gonadotropin measurements

General considerations

Urine is a convenient medium for the assessment of many biomarkers, including the gonadotropins. Kidney function develops from birth throughout childhood in terms of the number of nephrons, the glomerular filtration rate (GFR) and tubular secretion and reabsorption. GFR values increase significantly during the neonatal period and infancy (Chen, Aleksa, Woodland, Rieder, & Koren, 2006): the glomerular filtration rate (GFR) is 2-4 ml per min per 1.73 m^2 in term neonates, and it doubles within 1 week. This increase is due to an increased cardiac output and decreased renal vascular resistance. By the end of 1st year of life, GFR reaches adult values. However, it should be kept in mind that the GFR in relation to body surface area or body weight, even at this stage, is much higher in 1 yr old infants than in adults (Chen et al., 2006). GFR continues to increase also after it has reached adult values until prepubescent age, and this results in a higher clearance rate of overall renal excretion in children compared to adults (Chen et al., 2006). Our findings in regard to gonadotropin concentrations from serum and urine confirm this observation: the median concentrations of LH in the urine were several fold compared to those in the serum

(I-III). This provides an additional advantage for the determination of gonadotropins which circulating in the blood at very low levels before the onset of biochemical puberty.

Therefore, urine has been used as an alternative body fluid for gonadotropin studies in children and adults for many years (Raiti, Light, & Blizzard, 1969; Baghdassarian, Guyda, Johanson, Migeon, & Blizzard, 1970; Girard & Hadziselimovic, 1987). Assays of urinary gonadotropins for pediatric studies and studies related to puberty were first reported more than 60 years ago (Dorff, Appelman, & Liveson, 1948). These assays were very laborious bioassays which required repeated urine collections and pretest processing procedures (Fitschen & Clayton, 1965; Rifkind et al., 1967). When urine rather than serum is used, invasive procedures such as venipuncture are eliminated. This improves patient cooperation and compliance in the clinical setting as well as volunteer availability for research studies. Initially, pooled urine was collected for urinary gonadotropin analysis. Several samples from the same patient and from groups of several patients were pooled together (Kulin et al., 1967; Kulin et al., 1968). In some studies, pooling was performed by pubertal status (Reiter, Kulin, & Hamwood, 1973).

Timed urine collection was previously the most common form of urine sample collection for urinary gonadotropin measurements, and the duration of urine collection varied greatly as shown in Table 1.

Single, non-timed urine samples (e.g., morning-time spot samples) were also used in several studies (Faiman & Ryan, 1967), but they did not reflect the night-time hormonal activity (despite being taken in the morning). FMV urine samples based on accumulated urine from the time of retiring to that of waking reflect the sleep-time HPG-axis activity and are the most commonly used method (Hansen & Ross, 1975; Kulin, Moore, & Santner, 1976; Penny, Goldstein, & Frasier, 1976; Saketos et al., 1994; Maesaka, Tachibana, Adachi, & Okada, 1996). Random samples have again been used in some recent studies (McNeilly, Mason, Khanna, Galloway, & Ahmed, 2012; Singh, Jimenez, Newman, & Handelsman, 2013).

Table 1. Various forms of timed urine collections for urinary gonadotropin measurements.

VARIOUS TIMED URINARY SAMPLE COLLECTIONS	STUDY (Author, Year)
3-hour urine collections before and after naloxone infusion	Veldhuis, 1982
3-6 hour collections	Kulin, 1975; Kulin, 1979; Santner, 1981; Kulin, 1985; Kulin, 1994
3 to 9 hour collections	Kulin, 1984
3 to 13 hour collections	Kulin, 1976
3 to 24 hour collections	Kulin, 1977
8-hour collections	Beitins, 1976
3 times 3-hour collections (before, during and after GnRH infusion)	Reiter, 1977
12-hour collections (some during the day, some during the night)	Reiter, 1977
Wake-time (0800 h – retiring) and sleep-time (retiring - 0800 h) collections	Chipman, 1981
Timed overnight collection	Penny, 1978
3-hour urine samples collected for 24 hours and pooled	Beitins, 1976
4-hour urine samples collected for 24 hours and pooled	Bourguignon, 1980
The largest single pool: 24 hours	Kulin, 1967; Kulin, 1969; Stevens, 1969; Baghdassarian, 1970; Rifkind, 1970; Sciarra, 1970; Kulin, 1972; Reiter, 1973; Kulin, 1975; Beitins, 1976; Bourguignon, 1977; Bourguignon, 1980; Rettig, 1981; Chipman, 1981; Witchel, 1996

Urinary gonadotropins have been assayed directly after sampling in some studies (Kulin et al., 1968), but often urine samples are stored under various conditions before assay (Table 2).

Table 2. Various conditions of storing urine samples before gonadotropin assays.

VARIOUS STORAGE CONDITIONS for urinary samples	**STUDY Author, Year**
4 °C for "short term"	Saketos, 1994
4 °C during collection period	Rifkind, 1969
4 °C for two weeks	Stevens, 1969
4 °C for 3 days	Sciarra, 1970
4 °C for 1 to 8 weeks	Bourguignon, 1977
4 °C for 1 month	Maesaka, 1996
-16 °C for two weeks	Rifkind, 1969
-16 °C for 12 to 14 months	Rifkind, 1970
-20 °C for up to 1 week	Rettig, 1981
-20 °C for 4 weeks	Saketos, 1994
-20 °C for 3 to 6 months	McNeilly, 2012
-20 °C for 4 years	Singh, 2013
-20 °C simply indefinitely until assayed	Stevens, 1969; Stevens, 1969; Faiman, 1967; Rettig, 1981; Bourguignon, 1980; Kulin, 1984; McNeilly, 2012; Buckler, 1970; Beitins, 1976; Beitins, 1976

In several studies on gonadotropins adjustment for the urinary flow rate has been made by correcting for the serum creatinine concentration (Hansen & Ross, 1975; Girard & Hadziselimovic, 1987; Maesaka et al., 1990a; Saketos et al., 1994; McNeilly et al., 2012). However, with the advent of highly sensitive assays, creatinine adjustment neither reduces day-to-day variability regardless of the gonadotropin concentrations nor improves the intrasubject correlation between concentrations in the urine and serum (Boeniger, Lowry, & Rosenberg, 1993). Singh et al. corrected the gonadotropin concentrations in urine to correspond to the specific gravity of 1.020 for gonadotropin assays in adult men (Singh et al., 2013).

Variable proportions of FSH and LH are lost when urine is stored frozen at -20 °C and this can be prevented by adding glycerol to a concentration of 70 mL/L. This retains full gonadotropin immunoreactivity for almost one year (Saketos et al., 1994). In some studies, urine samples have been treated with urea (e.g., 6 mol/L) to dissociate the subunits (Saketos et al., 1994). Although the assays described in the study by Saketos et al. appear to measure intact glycoprotein hormones, some FSH immunoreactivity is retained after urea treatment (Saketos et al., 1994). (Saketos et al., 1994). This finding supports previous work and confirmed the protective role of glycerol, which stabilizes protein conformation by decreasing peptide-reaction sites for urea (Gekko & Timasheff, 1981).

Addition of albumin has also been used to preserve immunoreactivity (Livesey, Roud, Metcalf, & Donald, 1983; Saketos et al., 1994; Kesner, Knecht, & Krieg, 1995). Recovery was similar in glycerol and after acetone precipitation (Saketos et al., 1994). Preservation of urine samples with 70 mL/L glycerol and storage at -20 °C compares favorably with the more time-consuming method of acetone extraction (Livesey et al., 1983). Storage of urine at -20 °C alone causes loss of gonadotropin (hCG) immunoreactivity (Lempiainen, Hotakainen, Alfthan, & Stenman, 2012). Because of the loss of LH and FSH in frozen and unpreserved urine, studies involving gonadotropin measurements in unpreserved specimens should be evaluated with caution. In some studies, urine samples have been preserved using merthiolate or chloroform (Buckler & Clayton, 1970), whereas no additives before storage at -20°C have been used in some other studies (McNeilly et al., 2012; Singh et al., 2013). No loss of gonadotropins was reported in these studies.

Most of the early studies on urinary gonadotropins have used some form of sample pretreatment before assay. Kulin et al. concluded that radioimmunoassays of unprocessed urine do not provide adequate sensitivity, unlike immunoassay or bioassay of urine extracts (Kulin et al., 1968). Different pre-treatments have been used to concentrate the urine of prepubertal children enough to obtain detectable levels of gonadotropins. Many different concentration methods have been used, e.g., vacuum dialysis (Beitins, Derfel, O'Loughlin, & McArthur, 1976), kaolin-acetone extraction (Kulin et al., 1967; Rifkind et al., 1967; Kulin et al., 1968; Stevens, 1969), and acetone precipitation (Raiti & Blizzard, 1968; Baghdassarian et al., 1970; Santner, Santen, Kulin, & Demers, 1981; Kulin, Demers, Chinchilli, Martel, & Stevens, 1994; Saketos et al., 1994). In a comparative study, acetone was favored over kaolin-acetone extraction (Reiter et al., 1973).

Acidification of the urine improves the efficiency of the precipitation. In some studies the pH was adjusted to 7 (Rettig, Duckett, Sweetland, Reiter, & Root, 1981). Further concentration of the protein precipitate may be achieved by lyophilization (Rifkind et al., 1967; Sciarra & Leone, 1970) or air drying (Baghdassarian et al., 1970; Santner et al., 1981; Saketos et al., 1994). In most studies, the attained ratio of concentration was 20-fold compared to the unconcentrated urine sample (Kulin et al., 1976; Santner et al., 1981).

In early bioassay studies, urinary samples had to be concentrated to compensate for poor gonadotropin assay sensitivity. Nor were RIA methods for urine samples sensitive enough to detect the sleep/wake differences in gonadotropin secretion during the late prepubertal period or the sleep-augmented LH surge during later stages of pubertal development (Chipman, Moore, Marks, Fevre, Segel, Ramsey, & Boyar, 1981). Recent studies have demonstrated that critical S-LH peak concentrations either during sleep or in response to GnRHag predict the onset of biochemical puberty in both sexes, more reliably in boys (Rosenfield, Bordini, & Yu, 2012; 2013).

OBJECTIVES OF THE STUDY

1. To study if invasive serum determinations can be replaced by highly sensitive IFMAs for urinary FSH and LH measurements in children.
2. To determine the age-related course of gonadotropin concentrations from birth to post-puberty and define age-specific reference values.
3. To evaluate FMV gonadotropin determinations as markers of imminent pubertal development and the transition from prepuberty to puberty.
4. To analyze the agreement between FMV urinary gonadotropin concentrations and GnRH stimulation test results for assessing pubertal development.

SUBJECTS AND METHODS

2.1 Subjects, inclusion criteria and samples

The subjects, inclusion criteria and samples for studies I-IV are summarized in Table 3.

Table 3. Subjects, inclusion criteria and samples.

DESCRIPTION	STUDY I	STUDY II	STUDY III	STUDY IV
Number of subjects (male/ female)	65 (43/22)	184 (102/82)	297 (145/152)	274 (196/78)
Age range (yr)	0-15 and adults	0 - 18	5 - 17	5 - 17
Origin	Finnish	Finnish	Danish	Swedish
Health status	No disorders or medication affecting endocrine and/or kidney function	No disorders or medication affecting endocrine and/or kidney function	No disorders or medication affecting endocrine and/ or kidney function	Referred for investigation of growth or pubertal disorders
Sample	Paired urine and serum	Urine	Paired urine and serum	Paired urine and serum, GnRH test
Collection time and period	FMV early morning from newborns	FMV early morning from newborns	FMV	FMV
Preservatives	None, sodium azide or tymol	None	None	None
Storage	20 °C, 4 °C and -20 °C	4 °C	-20 °C	4 °C
Notes	No urine sample taken during menstruation	No urine sample taken during menstruation	No urine sample taken during menstruation; for both sexes: blood samples in the same morning as urine	No urine sample taken during menstruation; for both sexes: blood samples in the same morning as urine

The time for sample collection and storage was approximately one week before assaying (II-IV), except for the studies on the effect of storage temperature and time on the recovery of urinary gonadotropins (I).

2.2 Assay procedures

Polypropylene tubes were used for storage of urine in order to reduce adsorption of gonadotropins onto the tube wall. The additional value of coating the tube walls with bovine serum albumin (BSA) to reduce sticking of gonadotropins onto the tube walls was also studied. 1/3 filled tubes with 2 or 20 g/L of BSA in 0.9 % NaCl in phosphate buffer, pH 7.4 (Boehringer Ingelheim's Fraction 5) were rotated overnight. The effects of sodium azide and thymol as preservatives were studied using concentrations of 1 g/L and 20 g/L, respectively.

FSH and LH concentrations in paired urine and serum samples were measured in duplicate by the DELFIA® IFMA (Stenman et al., 1985; Soini & Lövgren, 1987; Pettersson & Soderholm, 1990; Stenman, Alfthan, & Turpeinen, 1991) using diagnostic kit reagents obtained from Wallac (Turku, Finland); slight alterations to the assay protocols were made. The assays are solid phase, two-site immunofluorometric assays using two monoclonal antibodies directed against different epitopes. The walls of microtiter wells were coated by immobilization of antibodies to the solid phase. Europium chelate was the tracer of choice for labeling the indicator antibody, specific for the alpha subunit.

The assays were calibrated against the WHO Second International Standard for pituitary LH for immunoassay (80/552) and the Second International Reference Preparation of pituitary FSH/LH (78/549), respectively. The sample (25 μL) was diluted with 0.2 mL of assay buffer.

Each assay followed a two-step procedure. The sample was brought into reaction with the immobilized capture antibody for 2 h (LH) or 3 h (FSH). Following the first washing step, europium-labeled indicator antibody was added into the wells. After incubation for 1 h and a subsequent (second) wash, enhancement solution was added to dissociate europium from the labeled antibody into the mixture, in which highly fluorescent chelates were formed due to content of the enhancement solution. The quantity of gonadotropin in the sample was calculated in proportion to the intensity of the fluorescence light, which was measured for 1 s/sample in an Arcus 1230 fluorometer (Wallac).

The effect of correction for variations in urinary excretion on gonadotropin concentrations was studied using the following formulae [I]:

Concentration corrected for creatinine = concentration/creatinine [IU/mol], or

Concentration corrected for density = concentration x (0.02/(density-1) [IU/L].

U-FSH and U-LH concentrations were not corrected for variations in urine excretion rate (such as urinary density or creatinine), because their correlation with the respective serum levels did not improve. On the contrary, this correlation became poorer due to overcorrection with very dilute urine samples in some cases [I].

The detection limit of the test was defined as the concentration indicating a mean fluorescence of 12 zero standard repeats plus two standard deviations. The intra-assay and inter-assay coefficients of variation (CV) were determined by assaying 20 aliquots of 3 samples of varying concentrations. A CV <10% was regarded as acceptable. CVs were not calculated separately for low, medium and high concentrations, as the CV was recorded as a function of zero standard repeats, which have been taken as a reference point because especially the low concentration range was of clinical interest.

In study III, we determined S-estradiol and S-testosterone by RIA (Diagnostic Products Corp., Los Angeles, CA, USA and Immunodiagnostic System Ltd., Boldon, UK, respectively). The detection limit of the testosterone assay was 0.23 nmol/L and that of the estradiol assay 0.018 nmol/L (data from kit inserts).

Two different IFMAs were used to assay U-LH. The LH Delfia detects only intact LH, whereas the LHspec Delfia detects also ß-subunits and certain fragments of LH. The detection limit of the LHspec assay was 0.012 IU/L. The intra-assay and inter-assay CVs were 5.7 and 6.4%, respectively.

2.3 Ethical considerations

The research protocol was approved by the Ethics Committee of the Children's Hospital, Helsinki University Central Hospital. Where applicable, informed consent was obtained from the parents. For study III, the research protocol was also approved by the ethics committee of the Department of Growth and Reproduction, Copenhagen University. As for study IV, approval regarding the research protocol was obtained from the Ethics Committee at Sahlgrenska University Hospital, University of Gothenburg.

2.4 Statistical analysis

The detection limit was based on the mean concentration of 12 repeat assays of the zero-standard plus two standard deviations. Samples with concentrations of LH and FSH below the detection limit were assigned a value of 0.01 IU/L. Correlation and regression analyses were used to analyze the relationships between serum and urinary gonadotropin concentrations. Fisher's r to z test was used to examine the statistical significance of the correlation. The effects of sample volume (25 to 200 µL, keeping the total incubation volume at 225 µL) and incubation time (1 h to overnight) were studied. Statistical significance was defined as a P value < 0.05.

Analysis of variance (ANOVA) was used to compare concentrations by age groups and the Student's t test to compare the means between adjacent age groups and between the boys and girls in each age group.

The non-parametric Kruskal-Wallis and the Mann-Whitney U Test were used to analyze differences in hormone concentrations between different pubertal stages.

Paired comparisons were performed using the Wilcoxon signed rank test. The increase in gonadotropin and steroid levels from early childhood (median level at 5 years) to late adolescence (median level at 15 years) and the basic pubertal increase from genital/breast stage 1 (median level at stage 1) to genital/breast stage 5 (median level at stage 5) were calculated.

The GnRH stimulation test result was classified as pubertal if the maximal S-LH concentration (S-LHmax) was above 5 IU/L and the S-LHmax/S-FSHmax ratio above 1.0. This follows clinical practice at the Gothenburg Pediatric Growth Research Center (GP-GRC). Regression and correlation analyses (Spearman's test) were used to establish the correlation between gonadotropin concentrations and their ratios in FMV urine (U-FSH, U-LH, U-LH/U-FSH) and serum during the GnRH stimulation test (basal FSH = S-FSH, basal LH = S-LH, maximal FSH = FSHmax, maximal LH = LHmax, S-LHmax/S-FSHmax). Also, the correlations between pubertal stages (Tanner breast stage B, genital stage G, pubic hair stage PH, testicular volume) and the gonadotropin concentrations were studied. Receiver operating characteristics (ROC) curve analysis (Zweig & Campbell, 1993) was used to calculate the area under the curve (AUC), sensitivity and specificity levels at various cut-off values for the differentiation of prepubertal (Tanner B/G stage 1) from pubertal stages (Tanner B/G $\geq$2) and also separately to predict the results of the GnRH stimulation test.

RESULTS

3.1 Characterization of (urinary) gonadotropin assays

Detection limits

The detection limits of the FSH and LH assays were 0.018 IU/L and 0.015 IU/L, respectively. The intra-assay and inter-assay CVs ranged from 2.3% to 7.8% and 5.2% to 8.7%, respectively.

Effect of incubation time

The detection limits of 2-h, 3-h and overnight LH incubations were 0.015 IU/L, 0.018 IU/L and 0.023 IU/L, respectively, and the corresponding values for FSH were 0.020 IU/L, 0.018 IU/L and 0.018 IU/L. An incubation time of 2 h for LH and 3 h for FSH provided the best level of sensitivity. Extending the incubation time overnight did not improve the sensitivity.

Effect of sample volume

When the urinary sample volume was increased from 25 µL to 225 µL, results were lower than expected (Figure 5).

Effect of storage temperature

The gonadotropin concentrations were stable when urinary samples was stored at 4 °C. By the end of seven weeks, 93.3% of the FSH and 95.1% of the LH was recovered. Storing of urine at -20 °C, at room temperature and performing 3 freeze-thaw cycles caused a significant decrease in FSH (down to 76.9, 71.0, and 48.7%, respectively) and LH concentrations (down to 77.4, 43.8, and 47.3%, respectively) by the end of the study period for this experiment (7 weeks) (Figure 5).

Effect of coating sample tubes

Coating the inner walls of the tubes with various concentrations of BSA had no effect on recovery of FSH or LH from urine samples.

Effect of additives

Recovery of FSH or LH in urine stored at 4 °C was not influenced by addition of sodium azide or thymol as preservatives and storage for up to 7 weeks.

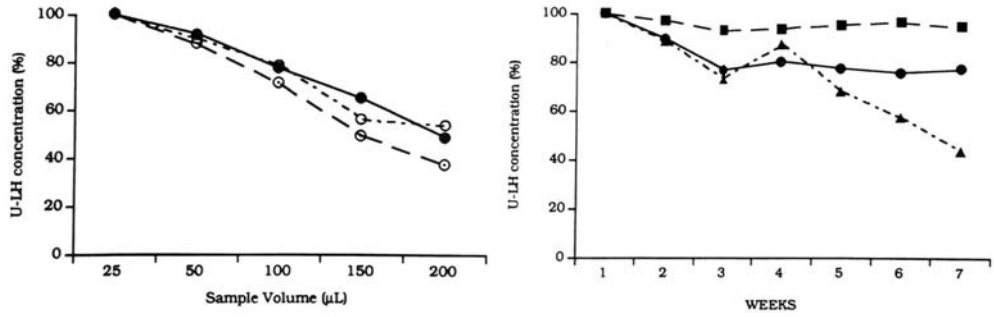

Figure 5. Effect of storage time and sample volume on three different samples (left), and storage time and temperature (right: -20 °C [●], 4 °C [■] and 20 °C [▲]) on urinary LH concentrations. *Modified from study I. © Pediatric Research, Copyright Clearance Center, License ID: 3624381266914.*

3.2 Correlation between urinary and serum gonadotropin concentrations

Gonadotropin concentrations measured in paired urine and serum samples correlated very well. The correlation did not improve after corrections for variations in urinary flow rate. If the urine was very dilute (density ≤ 1.007), correction for urinary density and especially for creatinine impaired the correlation (Figure 6 and I). Gonadotropin concentrations in urine and serum of two girls with Turner syndrome correlated well also when gonadotropin concentrations were high (I). On the other hand, the concentrations of intact LH were invariably very low in all once frozen and thawed urine samples, and here the correlation between U-LH and S-LH was low, suggesting degradation of LH in the frozen and thawed urine samples (Figure 5, I and III).

The serum LH concentration correlated well with the FMV U-LH concentrations determined by the LHspec assay ($r = 0.64$, $P<0.0001$) (III). The median concentrations of LH in the urine were 1.1- to 5-fold compared to the concentrations in serum (III). The correlation between U-LH and S-LH levels was lower in children in early puberty ($r = 0.44$, $P = 0.001$ for boys and $r = 0.44$, $P<0.0001$ for girls) than in prepuberty and late puberty ($r = 0.83$, $P<0.0001$ for boys and $r = 0.78$, $P<0.0001$ for girls) (III).

The FMV U-LH and U-FSH concentrations correlated well with the corresponding basal and GnRH-stimulated serum levels ($P<0.001$ for all) (IV). The ratios of FMV U-LH/U-FSH and S-LHmax/S-FSHmax correlated strongly ($P<0.001$ for both) (IV). U-LH and S-LH correlated strongly with pubertal stage (Tanner B, G) and testicular volume ($P<0.001$) (IV).

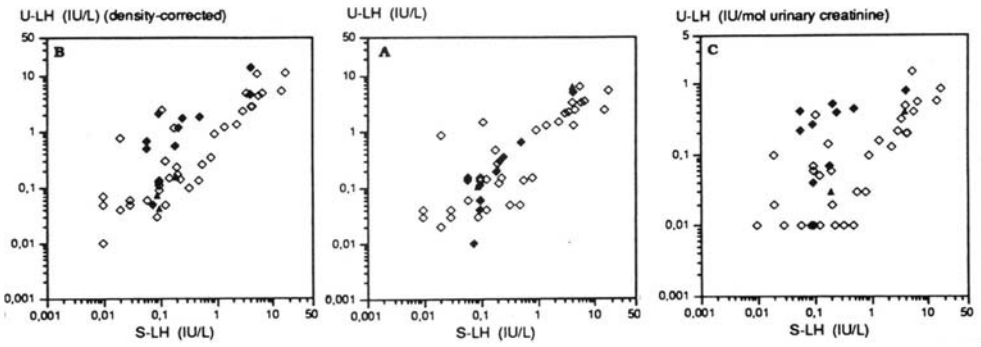

Figure 6. Effect of correcting or not correcting urinary LH concentrations for urinary density and creatinine concentration. Left: Density-corrected, Center: Uncorrected, Right: Creatinine-corrected. The symbols refer to urine samples of different densities (normal ◊ density = 1.008-1.024, dilute u density ≤ 1.007 and concentrated s density ≥ 1.025). *Modified from study I.* © Pediatric Research, Copyright Clearance Center, *License ID: 3624381266914.*

3.3 Gonadotropin concentrations in relation to age and pubertal stage

The age-related course of urinary gonadotropin concentrations is shown in Figures 7 through 9 (detailed in II). The course of gonadotropin concentrations measured in paired urine and serum samples by age (during clinical prepuberty) and pubertal stage are shown depicted in Figure 8 (detailed in III).

Boys

The mean concentration of U-LH (about 1 IU/L) in newborn boys (less than 4 weeks old) was much higher than in any of the other prepubertal age groups (about 0.1 IU/L) (II). The relatively high concentrations encountered during infancy were followed by very low levels of U-LH at age 2-8 years ($P<0.01$ between 0-2 and 2-8 years) (II). Among Finnish boys aged 5–8 years had very low U-LH concentrations (median 0.06-0.07 IU/L) (III). The U-LH concentrations in boys started to increase from about 0.1 IU/L before age 8 years and reached a mean level of 1.0 IU/L by age 11 and 3.0 IU/L by age 12 years ($P<0.01$ between 2-8 and 8-12 years) (II). In study III, the U-LH concentrations of Danish boys followed a very similar course: it started to increase from a median of 0.06-0.07 IU/L before age 9 years and reached a median level of 0.6 IU/L by age 11, 2.4 IU/L by age 12 and 3.0 IU/L by age 13-14 years (III). By the end of pubertal development of Finnish boys, the mean U-LH concentration reached 5.0 IU/L (II), whereas the median U-LH concentration reached 4.64 IU/L by the end of puberty in Danish boys (III). The increase in U-LH concentrations was statistically significant already between age groups 7-8 and 9-10 years (III).

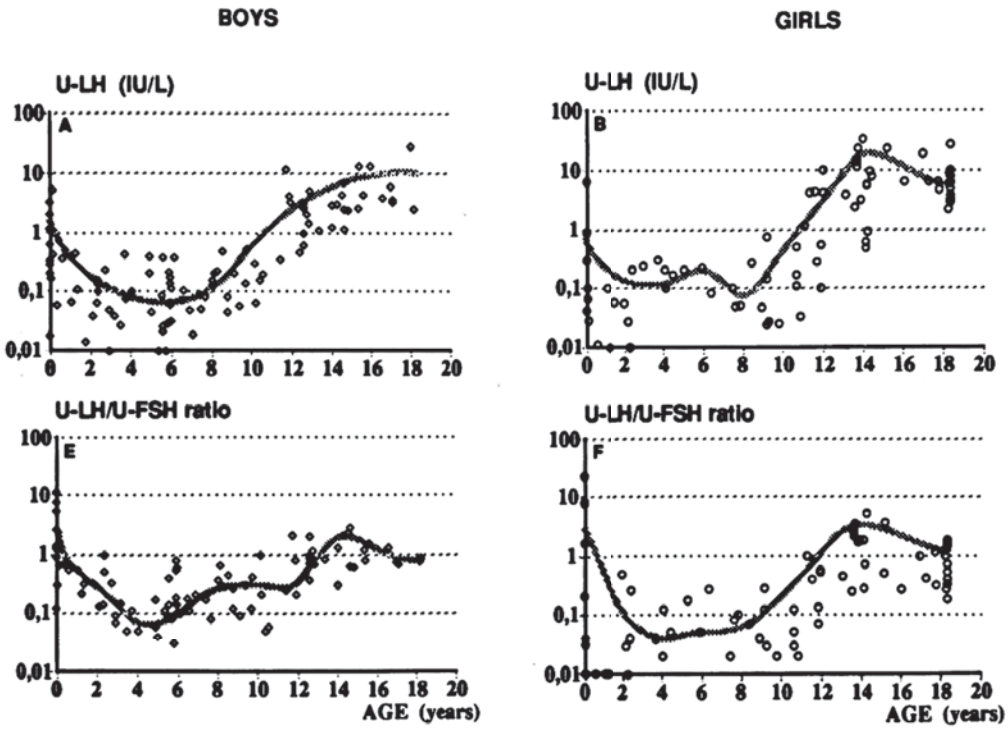

Figure 7. Age-related course of U-LH (upper left and upper right) and of the U-LH/U-FSH ratio (lower left and lower right) in 184 healthy children. *Modified from study II.* © Journal of Clinical Endocrinology and Metabolism, Copyright Clearance Center, *License ID: 3624380909793.*

The increase in gonadotropin concentrations during puberty was steep: there was 75-fold increase in U-LH from age 5 to 15 years, and the overall increase in S-LH levels was 50-fold from early childhood to late adolescence. The difference between the median U-LH values during genital stages 1 and 5 was approximately 35-fold (III).

The increase in U-LH preceded the increase in S-LH. The median S-LH concentrations reached 1.0 IU/L at around 12 years and 2.0 IU/L at 13-14 years. The initial rise in the U-LH concentrations by the age 9–10 was accompanied two years later by an increase in S-LH concentrations, as the increase in S-LH of boys between age 11 and age 12 years became prominent and statistically significant (III). Both the U-LH and the S-LH concentrations correlated with those of serum testosterone ($r = 0.77$, $P<0.0001$ and $r = 0.85$, $P<0.0001$, respectively) (III). However, the increase in serum testosterone levels became significant only one year later, i.e., after age 12 years. Thus, the increase in serum testosterone did not occur among clinically prepubertal children, not until transition to pubertal stage 2.

The U-FSH concentrations of newborn boys were relatively high, the mean value was slightly above 1.0 IU/L. The U-FSH decreased to about 0.3 IU/L by the time the boys reached an age of 3 years. A gradual increase in U-FSH concentrations emerged after age 5 years. The mean U-FSH concentrations increased about 5-fold during puberty ($P<0.01$ from age 2-8 to age 8-12 years; $P<0.05$ from 8-12 to 12-18 years) (II).

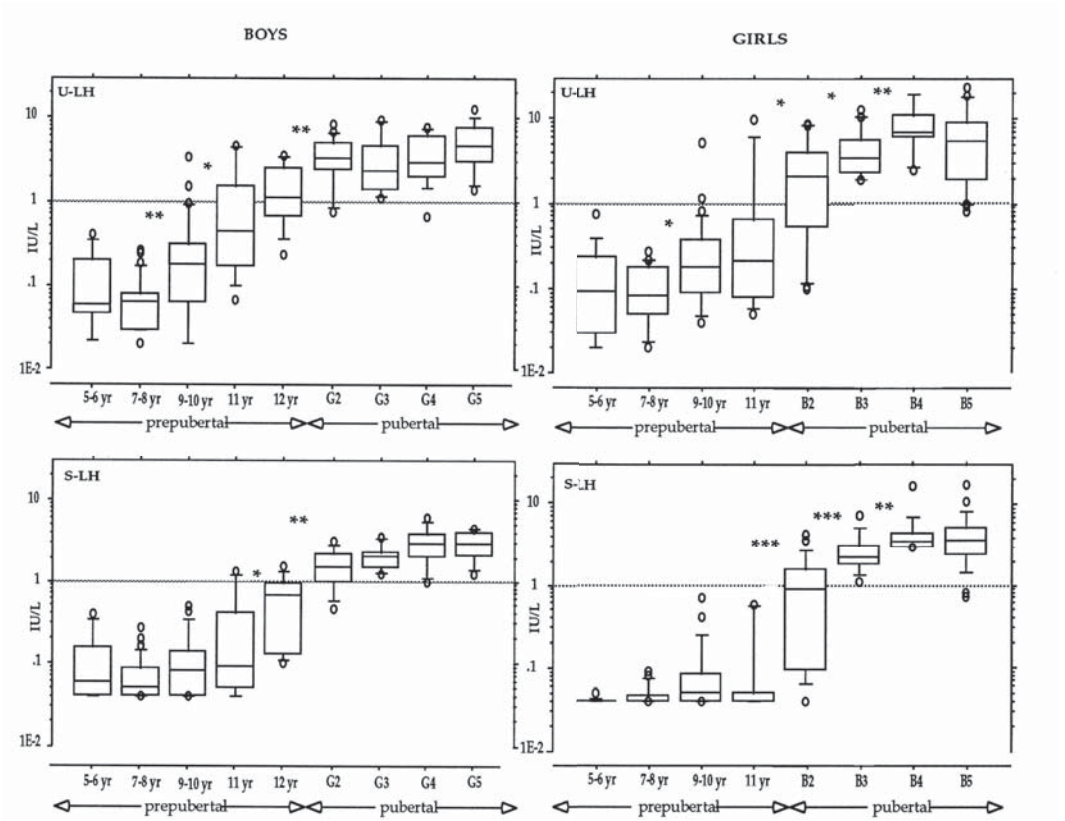

Figure 8. Distribution pattern of urinary and serum LH levels in children, all pubertal stages. Stage 1 subjects (prepubertal) were divided into 5 age groups. The lines in the boxes are median values (50th percentiles). The lower and upper limits of the boxes correspond to the 25th and 75th percentiles and the notches show the 10th and 90th percentiles. Outliers are shown separately. Asterisks denote statistically significant differences between hormone levels in consecutive age groups or pubertal stages. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, blank: not significant. *Modified from study III. © Journal of Clinical Endocrinology and Metabolism, Copyright Clearance Center, License ID: 3624380909793.*

Girls

During the neonatal period and infancy, the U-LH levels were similar for girls and boys. After a significant decrease following the neonatal period, the U-LH concentrations of Finnish girls remained below 0.5 IU/L until age 8 years (II). This low U-LH level was confirmed in our subsequent study on Danish children, in which U-LH concentrations remained below 0.5 IU/L in girls until 8 years of age, with the exception of one case with a level of 0.8 IU/L (III).

The mean U-LH concentration of 0.1 IU/L during prepuberty starts to increase after age 8 years and reaches the mean pubertal concentration of 9 IU/L at age 14 ($P<0.05$ between 2-8 and 8-12 years; $P<0.0001$ between 8-12 and 12-18 years), which corresponds to a 90-fold increase in U-LH during female pubertal development (II).

U-LH and serum estradiol concentrations started to increase at around age 9 years, preceding the rise in S-LH levels which start from age 11 years (III). The age-related course of U-LH during pubertal maturation followed an almost identical pattern in studies II and III; the median U-LH concentration increased approximately 90-fold from a prepubertal median level of 0.07 IU/L to 2.0 at 11 years, 5.0 IU/L at 12 years and 6.3 IU/L at 14 years (regardless of pubertal stage) (III). The increase in median U-LH from breast stage 1 to 5 was only 40-fold (III).

The U-LH concentrations of girls were slightly higher than the corresponding S-LH concentrations and increased statistically significantly at an earlier age than the S-LH. Among prepubertal girls, there was no significant difference in S-LH concentrations by age group. The increase in S-LH levels from the early prepubertal median of 0.04 IU/L to the late pubertal median of 3.7 IU/L was approximately 90-fold. The increase in S-LH levels was also 90-fold from breast stage 1 to 5.

The serum estradiol concentrations correlated strongly with the LH concentrations in both serum (r = 0.70, $P<0.0001$) and urine (r = 0.65, $P<0.0001$) samples. The rise in U-LH and serum estradiol concentrations, which took place at age 9–10 years, preceded the increase in S-LH levels by approximately 2 years (Fig. 1 in III). Unlike the boys, the S-LH levels of the girls rose significantly only after the clinical signs of puberty were seen, not in any prepubertal age group (III).

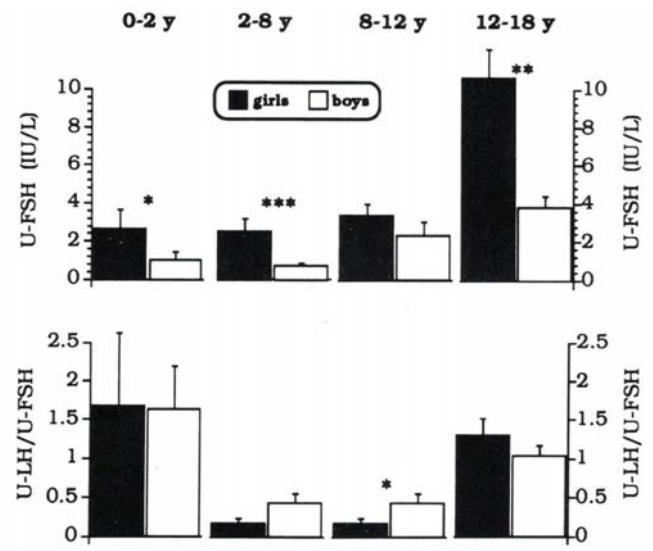

Figure 9. Comparison of U-FSH concentrations and the U-LH/U-FSH ratio in both sexes at different age groups. Data is shown as the mean ± SEM. * p<0.05, ** p<0.001, *** p<0.0001 (sex difference within the age group). *Modified from study II. © Journal of Clinical Endocrinology and Metabolism, Copyright Clearance Center, License ID: 3624380909793.*

The mean U-FSH levels of the girls followed an almost identical course as of the boys during childhood and puberty, but the girls did have somewhat higher U-FSH concentrations than the boys before age 8 years (II). Like the boys, the U-FSH concentrations of the girls increased 5-fold from prepubertal age to full puberty ($P<0.001$ between 8-12 and 12-18 years) (II).

LH/FSH ratio in both sexes

The U-LH/U-FSH ratio of newborns varied markedly. The mean was about 3.0 for newborn boys and decreased to the mean level of 0.1-0.3 by age 1-8 years, started to rise steeply at about age 12 years, reached temporarily a level of 2.0, and settled at about 1.0 in later puberty (II).

After the newborn period, the U-LH/U-FSH ratio of prepubertal girls remained, on average, below 0.1 (lower than for the boys) (Figure 9). The ratio started to increase sooner than among the boys, at age 9 years onwards, attained a level of 2.0 by age 14 years, and settled later at about 1.0, as for the boys (II).

3.4 Urinary gonadotropins as an alternative to basal and GnRH-stimulated gonadotropins

The FMV U-LH and U-FSH concentrations correlated strongly with the corresponding basal and GnRH-stimulated S-LH and S-FSH levels ($P<0.001$). The FMV U-LH/U-FSH ratios also correlated strongly with the corresponding S-LHmax/S-FSHmax ratios ($P<0.001$). U-LH and S-LH correlated strongly with the pubertal stage (Tanner B, G) and testicular volume. The increase in FMV U-LH concentrations and U-LH/U-FSH ratios from prepuberty (Tanner stage B/G1) to the earliest pubertal stage (Tanner B/G2) was highly significant ($P<0.001$ in boys and<0.01 in girls). These increases were at least equally significant as the corresponding increases in S-LH or S-LHmax concentrations and the S-LHmax/S-FSHmax ratios at this developmental stage (IV).

ROC curve analyses showed that for differentiation of clinically classified pubertal and prepubertal stages the U-LH concentrations and U-LH/U-FSH ratios were at least as good as the S-LH and S-LHmax concentrations or the S-LH/S-FSH and S-LHmax/S-FSHmax ratios (Table 1, IV). The AUC-values were about 0.9 for all these parameters.

Among boys, the sensitivity to detect early puberty (Tanner G stage 2) by the GnRH test was 92% at a specificity of 66%. With cut-off values of 1.48 IU/L for U-LH and 0.05 IU/L for S-LH, equal sensitivity (92%) and specificity (67%) were obtained. Among girls, the selected cut-off limits for a pubertal GnRH-test result (detecting puberty Tanner B stage 2) gave a specificity of 66% but a sensitivity of only 59%. At the same level of sensitivity (59%), a cut-off level of 2.60 IU/L for U-LH and 0.17 for the U-LH/U-FSH ratio provided higher levels of specificity (96% and 79%, respectively). Suitable cut-off values to identify puberty with U-LH were 1.5 IU/L for boys and 1.2 IU/L for girls, and with S-LH 0.04 IU/L for boys and 0.02 IU/L for girls (IV).

DISCUSSION

4.1 Methodological considerations of urinary gonadotropin determinations

The choice of sampling for diagnostic tests is often challenging in pediatric practice, since invasive methods are particularly problematic. Urinary samples can be obtained by non-invasive, simple and inexpensive methods but not all analytes of clinical interest are excreted into urine. However, gonadotropins are present in urine at levels similar to those in the plasma. Interestingly, the first gonadotropin bioassays were performed on urine samples (Rosemberg, Smith, & Dorfman, 1957; Rosemberg, 1960). Those studies showed that gonadotropin concentrations increase strongly during pubertal development, but the methods were too complicated for routine diagnostic use. Determination of gonadotropins for clinical diagnostics became feasible with the advent of RIA methods. The sensitivity of RIA made it possible to evaluate HPG function in adults, but the concentrations in the plasma of prepubertal children were below the detection limit of RIA methods.

The development of sandwich assays in combination with monoclonal antibodies and highly sensitive labeling techniques made it possible to measure much lower hormone concentrations than with conventional RIAs. The combination of time-resolved fluorometry and lanthanide labels made it possible to develop highly sensitive IFMA. These methods for assessment of the concentrations of LH and FSH were about 100 times more sensitive than conventional RIA, and now the detection of the low prepubertal gonadotropin levels in serum and urine was substantially facilitated. The crucial step in eliminating nonspecific interference caused by variations in the composition of urine was the emergence of an assay which could be run on small sample volumes. IFMAs provided sufficient sensitivity with sample volumes of only 25 µL in a total volume of 225 µl per assay well (Stenman et al., 1991).

Conventional RIA methods overestimate LH in low levels, which is due to nonspecific interference caused by serum components (Stenman et al., 1991). Thus, ratios of bioactive to immunoreactive gonadotropin concentrations from earlier RIA-based studies had to be revised by further studies (Apter et al., 1989; Chappel, 1990; Haavisto et al., 1990; Jaakkola et al., 1990; Huhtaniemi et al., 1992). Two-site immunometric assays with high specificity and sensitivity are currently considered the methods of choice for determination of very low concentrations of LH and FSH in the serum (Apter et al., 1989; Dunkel et al., 1990a; Dunkel et al., 1990b; Haavisto et al., 1990; Dunkel et al., 1992; Apter, 1993; Apter et al., 1993; Kuiri-Hanninen et al., 2014, I-IV).

Another challenge presented by urinary gonadotropin measurements is the possible underestimation of true concentrations due to degradation of gonadotropin molecules during excretion into urine, which leads to the formation of fragments such as LHß and LHcβf. Depending on the assay, these fragments may or may not be immunoreactive (Franchimont, Gaspard, Reuter, & Heynen, 1972; Young et al., 1975; Beitins et al., 1976; Neven, Iles, Howes, Sharma, Shepherd, Edwards, Collins, & Chard, 1993). We measured only intact LH and FSH but not their fragments, since the main methodology employed

in all our studies requires the presence of both alpha and beta subunits. However, in one of our earlier studies (III), we found that the U-LH concentrations determined from fresh samples by the LHspec kit which detects β-subunits and its fragments (in addition to intact LH molecules), were not statistically different from those obtained with the LH assay measuring only intact LH (unpublished data). Thus, in our experience, fragmentation of LH does not seem to be a problem in children.

We studied modifications of the assay methods to further improve sensitivity of the standard method. Increasing the incubation time in the first step from 1 to 2 or 3 h was useful (I). This is in line with an earlier study by Apter et al., who found that the detection limit for the LH assay improved slightly when an incubation time of 2 instead of 1 h was used (0.019 IU/L) (Apter et al., 1989). The detection limits for 2-h, 3-h and overnight incubations for U-LH were 0.015, 0.018 and 0.023 IU/L, respectively. The optimal detection limit was thus obtained with an incubation time of 2 h. However, increasing the sample volume from 25 to 50 µL or larger reduced recovery, apparently due to nonspecific interference by the sample matrix (Stenman et al., 1991) (I).

Sample handling and storage turned out to be important. Urinary gonadotropins were found to be stable for several weeks when stored at refrigerator temperature, but freezing at -20 °C decreased gonadotropin concentrations in many samples. In addition, repeated freezing and thawing of urine samples caused sharp losses in the FSH and LH concentrations measured in the fresh samples (I). However, in previous studies using RIAs (Landy et al., 1990) and immunoenzymometric assays (Brindle, Miller, Shofer, Klein, Soules, & O'Connor, 2006), LH and FSH remained stable and measureable even after freezing and thawing. This may be explained by variable recognition of degradation products formed during freezing (and thawing) by different assays.

Livesey et al. also reported that LH and FSH are unstable at -20 C. They showed that glycerol can be used as a preservative during storage at -20 to -25 °C (Livesey et al., 1983). After addition of glycerol (70 mL/L), urine samples can be stored at -20 °C (Saketos et al., 1994) for long periods of time. This is useful for clinical follow-up as well as for longitudinal studies. However, glycerol is on the WADA (World Anti-Doping Agency) list of prohibited substances, which may prohibit its use for storage of urine samples for doping testing (Singh et al., 2013).

We have routinely stored urine samples without preservatives at +4 °C. Adding preservatives, such as sodium azide or thymol, did not bring any benefit to the storage of urine samples at 4 °C for up to 7 weeks in attempts to conserve the original LH and FSH concentrations in freshly assayed samples for even longer periods of time (I).

Coating of inner walls of the tubes with BSA prevents loss of growth hormone (GH) in urinary samples. This has been found useful for measuring GH in urine (Moreira-Andrés, Cañizo, & Hawkins, 1993), because the GH concentrations in urine are only about 0.01% of those in plasma (Baumann & Abramson, 1983). Coating of collection tubes with BSA did not improve the recovery of gonadotropins in urine (I), which may at least partly be explained by the relatively high gonadotropin concentrations in urine (comparable to those

in plasma). However, coating the inner walls of the tubes with different dilutions of BSA did not affect the recovery of urinary gonadotropins disadvantageously (I). Therefore, urine samples collected for any purpose can also be used for U-LH or U-FSH measurements, which should be kept in mind particularly for initial evaluation of growth and pubertal disorders.

The effect of urinary flow rate on urinary hormone concentrations is a potential confounder. This is often corrected by adjusting the measured hormone concentrations for urinary density or urinary creatinine concentration. However, in children, this may lead to overcorrection, which has been substantiated for urinary hCG in very dilute samples (Alfthan, Haglund, Dabek, & Stenman, 1992). The correlation between urinary and serum levels could not be improved by employing various methods of correction on the U-FSH and U-LH levels obtained by IFMA, such as adjusting for urinary density or urinary creatinine concentration (I). Interestingly, correction of urinary GH concentrations with creatinine did not improve the correlation between urinary and serum GH concentrations, either (Main, Philips, Jorgensen, & Skakkebaek, 1991). Until the optimal method for correction of urine concentrations has been established, it may be advisable not to perform any correction.

In spite of the strong correlation between the gonadotropin concentrations measured in paired urine and serum samples, discrepancies between these occur occasionally (I). High urinary but low serum LH values in the morning may be explained by the fact that morning urine actually represents the night-time urine collection, in other words, 'the filtered picture of the night-time blood circulation'.

4.2 Urinary gonadotropins during pubertal development

By RIA and (Kulin, 1993) and immunoradiometric methods (Bridges, Matthews, Hindmarsh, & Brook, 1994), the age-related changes in urinary gonadotropins have been shown to be congruent with those in serum. These findings indicate that determinations of U-FSH and U-LH levels by sensitive assays may well be used for evaluation of gonadotropin secretion in children.

Urinary gonadotropin concentrations are generally low (except for the newborn period); however, they are almost always detectable throughout prepuberty (Figure 7 and 8). During the immediate prepubertal period, U-LH and U-FSH levels were slightly higher than those of the respective serum levels. The age-dependent changes in U-FSH and U-LH concentrations were similar to those reported for serum (Apter et al., 1989; Dunkel et al., 1990b; Dunkel et al., 1992; Kletter, Padmanabhan, Brown, Reiter, Sizonenko, & Beitins, 1993a; Bridges, Hindmarsh, Matthews, & Brook, 1994). Because we assayed FMV urine, which reflects the integrated secretion of gonadotropins during night-time, the discrepancy between urinary and serum gonadotropin concentrations during the late prepubertal period can be attributed to increased pulsatile frequency and amplitude in nocturnal gonadotropin secretion (Kletter et al., 1993b), not, of course, to the type of sample.

The U-FSH concentrations were significantly higher in girls than boys aged 2 to 8 years (II). By serum assays, the pulsatile secretion of FSH is stronger in girls than boys (Wu et al., 1991). The biological basis for this difference is unknown. During puberty, U-FSH concentrations increase approximately 5-fold while those of LH increase 50- to 100-fold (III). A similar increase throughout puberty was reported earlier by Kulin and Santner, who measured LH by RIA in concentrated extracts of 3 to 24-h urine collections (Kulin & Santner, 1977), and more recently by Apter et al., who determined LH and FSH by IFMA in serum samples (Apter et al., 1989).

Fluctuations of serum gonadotropin concentrations in children are problematic in the case of single serum determinations (Johanson, 1974). This drawback be overcome by serial determinations or by a GnRH stimulation test. These require, however, invasive sampling and multiple assays. As urine accumulating in the bladder during night reflects the night-time plasma concentrations, the levels of LH and FSH in FMV urine may be more informative than random serum samples for detection of early pubertal increases in gonadotropin secretion (Bourguignon et al., 1980). On the other hand, gonadotropin levels in urine display broader variations than gonadotropin levels in serum, for any given age group or pubertal stage (II). Despite this, the age-related changes in U-LH levels are clinically meaningful. The U-LH concentrations did not overlap at all in the age subgroups 1-8 and 14-18 years.

4.3 Urinary gonadotropins for assessing onset of puberty

The urinary gonadotropin concentrations measured during sleep and wake periods at the onset of puberty presented with a distinct difference between these periods, which was reflected even more strongly for LH than FSH levels (Chipman et al., 1981). Serum FSH levels remain relatively constant during pubertal development, while the levels of testosterone or estradiol levels increase more sharply (Beitins, Padmanabhan, Kasa-Vubu, Kletter, & Sizonenko, 1990). In agreement with this, we observed that the increase in the U-LH/U-FSH ratio was a biochemical marker for the onset of central puberty for both boys and girls (III). The early morning plasma testosterone concentration among boys heralded the onset of puberty (Wu, Brown, Butler, Stirling, & Kelnar, 1993), but a corresponding increase in estradiol in girls had never previously been reported to predict an approaching onset of puberty. Study III was a large-scale cross-sectional study on the FMV U-LH levels and sex steroids in both sexes (III). Since this study was the first to show that FMV urine assays reveal the nocturnal increase in LH secretion before clinical signs of puberty (III), a longitudinal follow-up of nocturnal urinary gonadotropin concentrations (measured from FMV urine samples) combined with pubertal staging is a clinically viable method for predicting the onset of female puberty and evaluating the progress of HPG activation.

The HPG axis becomes initially activated only during part of the night in relation to the onset of deep sleep and occurs a few years before the clinical onset of puberty (Boyar et al., 1972). Amplification of the pre-existing pulsatile pattern of hypothalamic GnRH secretion and an approximately two-fold increase in GnRH pulse frequency initiate the pubertal

process, possibly by enhancing sensitivity of the gonadotropic cells. As a result of this, the amount of LH produced per burst increases (Wu et al., 1996). This short-lived night-time activity at the initial phase is not detectable by morning S-LH determinations, but it can be identified by determination of LH in FMV urine (II, III). An increase in the amplitude of nocturnal LH and FSH secretion bursts is observed for the first time in prepubertal boys and girls aged 4–5 years (Mitamura et al., 1999; 2000). The amplitude and frequency of the gonadotropin secretion peaks increase, and daytime secretion increases as puberty advances. In boys, a striking rise in the amplitude of LH secretion bursts is observed in serum samples at least 1 year before the clinical signs of puberty are seen (testicular volume of at least 3 mL) (Mitamura et al., 1999; 2000; Styne & Grumbach, 2002), whereas the rise in FSH levels emerges as a consistently increasing pulse amplitude pattern throughout puberty (Spratt & Crowley, 1988; Beitins & Padmanabhan, 1991; Dunkel et al., 1992; Wu et al., 1996; Albertsson-Wikland et al., 1997; Styne & Grumbach, 2002).

The introduction of ultrasensitive assays made it possible to demonstrate the rapid increase in S-LH concentrations during puberty (Stenman et al., 1985; Apter et al., 1989). Nonetheless, studies involving a large number of individuals who provided a single daytime sample showed that there are changes in mean serum gonadotropin concentrations between prepuberty and puberty (Burr et al., 1970; Sizonenko, Burr, Kaplan, & Grumbach, 1970; August, Grumbach, & Kaplan, 1972; Jenner, Kelch, Kaplan, & Grumbach, 1972; Apter et al., 1989). However, single S-LH measurements cannot be relied upon for establishing the time when puberty starts. Neely et al. suggested that assessment of the pubertal development by random S-LH levels requires very sensitive immunometric assays (Neely, Hintz, Wilson, Lee, Gautier, Argente, & Stene, 1995a). The short-lived night-time activity during the initial phase of activation of the pubertal HPG axis is not detectable by morning S-LH determinations, but it can be detected and quantified by LH determinations in FMV urine (II, III). We found that there is a strong correlation between S-LH and FMV U-LH concentrations measured by the Delfia assay (I, II). However, 1 to 2 years before the onset of puberty, the correlation was weaker, which is apparently due to the night-time increase in LH secretion reflected in FMV urine but not yet in daytime serum samples (III).

The median U-LH concentration increased approximately 75- to 90-fold from the early prepubertal to late pubertal age range, while the increase in median U-LH from Tanner stage 1 to 5 was 35-fold in boys and 40-fold in girls (II). These results indicate that a remarkable increase in U-LH concentrations occurs already in prepubertal boys and girls before any clinical signs of puberty. A similar increase in S-LH concentrations occurs later in parallel with the development of the clinical signs of puberty.

Our results also show that the nocturnal increase in LH secretion during late prepuberty, or in other words, at the very beginning of puberty were indeed detectable in FMV urinary samples up to 4 years before the onset of physical puberty and the increase in S-LH concentrations (III). These results conform with those of a previous study, in which a gradual prepubertal increase in the nighttime S-LH concentrations of boys correlated with an increase in the prepubertal testis volume (Manasco et al., 1995).

The increase in FMV U-LH preceded the rise in morning S-LH levels and the appearance of clinical signs of puberty by 1 to 2 years (III). Therefore, the early pubertal nighttime increase in S-LH levels (Boyar et al., 1972; Boyar et al., 1974; Boyar et al., 1976; Wennink et al., 1988; Delemarre-van de Waal, Wennink, & Odink, 1989; Apter et al., 1993) can be detected by FMV U-LH determinations and the prepubertal development of pituitary gonadotrope function can be assessed already before the clinical onset of puberty. This finding may facilitate early differential diagnosis of CDGP from gonadotropin deficiency. Apter et al. also reported a gradual prepubertal enhancement of gonadotropin secretion (Apter et al., 1993), which is in agreement with our findings (III). The increase in FMV U-LH implies that the hypothalamic GnRH pulse generator which initiates biochemical puberty is activated at about the same time for boys and girls (III), although the first physical signs of puberty are usually detectable earlier in girls than in boys.

A considerable part (about 15-fold) of the overall increase in U-LH concentrations during biochemical puberty in boys occurs before clinical signs of puberty between ages 7 and 12 years. The use of a testicular volume above 3 mL as a definite clinical sign of puberty may cause a misclassification of some boys as prepubertal, although they in fact have 'subclinical puberty'.

In girls, the rise in FMV U-LH concentrations (between 7-8 and 9-10 years) was not reflected in clinical signs of puberty, as all girls in this age range in our third study were classified as prepubertal (III). Clinical puberty was observed only after a 10-fold increase in FMV U-LH and this took place after age 11 years (III).

The fact that variations in bone age have not been taken into account in assessing the course of urinary gonadotropins related to chronological age or pubertal stage can be regarded as a limitation of our study design. However, no subject with any disorder of growth or puberty was admitted into studies II and III, where the main focus was on the course of urinary gonadotropins by chronological age or pubertal stage. Besides, studying the nature of sleep while investigating the course of urinary gonadotropins along with pubertal development can be an additional improvement over the study design presented here; and again, it is quite uncommon that children would have sleeping disorders that would hinder the occurrence of deep sleep.

4.4 Clinical use of urinary gonadotropin measurements

FMV U-LH concentrations in the serum or urine of subjects being studied for growth or pubertal disorders (mainly short or tall stature and suspected precocious or delayed puberty) (IV) also correlated strongly with the basal S-LH levels. This finding agrees with our studies on children and adolescents with no growth or pubertal disorders (I-III).

The correlations between FMV U-LH and S-LHmax as well as between the FMV U-LH/U-FSH ratio and the S-LHmax/S-FSHmax ratio were both very strong (IV). These findings are in conformity with those reported by Witchel et al., who reported a strong correlation between 24-h U-LH and S-LHmax in 18 girls on GnRH analog treatment for precocious

puberty (Witchel, Baens-Bailon, & Lee, 1996) and by Zung et al. who reported a strong correlation between FMV U-LH and S-LHmax and the S-LHmax/S-FSHmax ratio in 47 girls being studied for precocious puberty (Zung, Burundukov, Ulman, Glaser, Rosenberg, Chen, & Zadik, 2014).

U-LH concentrations and the U-LH/U-FSH ratio correlated at least equally well as S-LHmax and the S-LHmax/S-FSHmax ratio with the pubertal developmental stage. The FMV U-LH and S-LHmax levels as well as the U-LH/U-FSH and S-LHmax/S-LHmax ratios increased significantly between the prepubertal and early pubertal stage in both sexes (G1 vs. G2 and B1 vs. B2), but there was still significant overlapping between the pubertal stages. Thus, separation between the prepubertal and early pubertal stages was not complete. The results of study III were subsequently confirmed by Maqsood et al. (Maqsood, Trueman, Whatmore, Westwood, Price, Hall, & Clayton, 2007) and McNeilly et al. (McNeilly et al., 2012).

ROC curve analyses showed that the AUC values for FMV U-LH and U-LH/U-FSH predicted clinical puberty (Tanner B/G stage 2) slightly better than the GnRH stimulation test, particularly for girls. The sensitivity of the GnRH stimulation test to identify clinical puberty was rather low (59%) but at the same sensitivity level, U-LH and the U-LH/U-FSH ratio were more specific (96% and 79%, respectively) than the GnRH test (66%).

Of the boys 38% and of the girls 8% classified as prepubertal by Tanner staging had evidence of early puberty on the basis of the FMV U-LH concentration; the corresponding figures for the GnRH-test were 23% for the boys and 13% for girls (IV). This is in accordance with previous studies reporting activation of the HPG axis before clinical manifestations of puberty (Apter et al., 1993; II; III). However, the fairly high rate of apparently "false negative" U-LH and GnRH test results especially among the girls could also be caused by erroneous Tanner staging due to breast development initiated by other endocrine factors than gonadotropins and ovarian estrogens. Tanner staging is more subjective for girls than boys. A too high Tanner breast stage in girls may occasionally be due to a false interpretation of benign premature thelarche as a sign of puberty: there may be excessive palpable adipose tissue or precocious pseudopuberty. Precocious pseudopuberty may be due to congenital adrenal hyperplasia, tumors that secrete hCG, tumors of the adrenal gland or ovary, exposure to exogenous sex steroid hormones from oral contraceptives or cosmetic products, vitamins, or dietary supplements that may contain estrogenic substances, e.g., placental extracts, or to excess consumption of soy or similar plant-derived xenoestrogens (phytoestrogens). In general, U-LH measurements worked well for evaluation of pubertal development in both sexes. On the other hand, with the current cut-off values, the GnRH test does not seem to be as reliable in girls as in boys as a method to identify clinical puberty.

When determined by sufficiently sensitive methods, a random S-LH measurement is useful for separating pubertal stages more clearly and S-LH be suggested as a useful screening method for evaluating children with CPP and DP (Garibaldi, Picco, Magier, Chevli, & Aceto, 1991). The basal concentrations of serum LH diagnoses CPP in more than 90% of the girls (Houk, Kunselman, & Lee, 2009). The diagnostic accuracy of serum gonadotropin

measurements can be improved by using the GnRH stimulation test, the gold standard for determining HPG activity.

Setting cut-off values for a pubertal response for the GnRH test results has been quite demanding in girls, as reviewed recently by Zung et al. (Zung et al., 2014). Based on the 95th percentile of the S-LH levels of healthy prepubertal girls undergoing the GnRH test, Brito et al. (Brito, Batista, Borges, Latronico, Kohek, Thirone, Jorge, Arnhold, & Mendonca, 1999) suggested a S-LHmax cut-off level of 6.9 IU/L. Resende et al. (Resende, Lara, Reis, Ferreira, Pereira, & Borges, 2007) suggested a cut-off level of 3.3 IU/L (ICMA) or 4.2 IU/L (IFMA) for S-LHmax, and Neely et al. (Neely et al., 1995a) suggested 5 IU/L as the S-LHmax cutoff value (which is 2 SD above the mean in prepubertal girls) or alternatively 8 IU/L (4 SD above the mean). The 5 IU/L threshold has been adopted by many researchers (Oerter, Uriarte, Rose, Barnes, & Cutler, 1990; Neely et al., 1995a; Neely, Wilson, Lee, Stene, & Hintz, 1995b; de Vries, Horev, Schwartz, & Phillip, 2006; Mogensen, Aksglaede, Mouritsen, Sorensen, Main, Gideon, & Juul, 2011). A single S-LH concentration of ≥4 IU/L 30–60 min after injection of GnRH has been proposed to indicate the onset of puberty (Cavallo & Zhou, 1994). Likewise, a single S-LH concentration of ≧15 IU/L at 30 min after injection of GnRH may be as useful as the peak LH in the GnRH test for diagnosing CPP (Cavallo et al., 1995).

The results from our latest study (IV) suggest that the S-LHmax cut-off value for girls may be slightly lower than for boys in the GnRH test. This view is indirectly supported by the slightly lower cut-off values for U-LH in girls than boys based on ROC curve analyses (IV). Determination of basal S-LH concentrations is also suitable for evaluation of the pubertal stage if blood is to be drawn for other reasons. However, our results show that neither boys nor girls require measurement of S-LH nor a GnRH test for the initial evaluation of pubertal stage. Determination of S-LH in children is reliable only if the assay is sensitive enough to measure concentrations in the range 0.02-0.04 IU/L, which correspond to the cut-off values (IV). Because of the somewhat higher U-LH than S-LH concentrations, a single measurement of LH in FMV urine provides information about pubertal stage that is at least as reliable as measurement of S-LH or the GnRH stimulation test.

A possible limitation of the FMV U-LH assays is related to the fact that a large part of the LH immunoreactivity in urine is caused by the LH core fragment (LHβcf), a fragment of the β-subunit of LH (Birken, McChesney, Yershova, Gaughan, Pettersson, Rechenberg, Wu, & Taliadouros, 2007). Different assays recognize this fragment to variable extent, and the LH concentrations are therefore highly dependent on the assay (Singh et al., 2013). Poor stability of LH in urine is another problem if urine is stored frozen before assay, since much of LH may be degraded during storage at -20 ˚C (I; (Singh et al., 2013).

When interpreting the results of study IV, it should be noted that the findings are based on patients referred for investigation of various growth and pubertal disorders. This means that the concentrations of FMV U-LH and U-FSH in that study may not be appropriate reference values for healthy children. We have previously prepared reference values for healthy children for FMV U-LH and U-FSH (II) and U-LH with the LHspec assay using IFMA methodology (III). However, there are some upfront shortcomings of the above-

mentioned sets of 'normative' data: 1) the results are based on cross-sectional data, 2) considering different sex, age and pubertal stage groups, the number of subjects per any given age/pubertal stage group of either sex is rather limited and conclusions must be drawn cautiously, 3) the subjects were referred to an outpatient clinic because of some clinical matter; therefore there may be a slight disadvantage over a clinical study design with exclusively fully healthy subjects. Also, any other LH or FSH assay should be validated for use on urine samples and method-specific reference values have to be established.

In conclusion, we have shown that measurement of FMV U-LH and the U-LH/U-FSH ratio can be used as a non-invasive method for assessment of pubertal maturation and its disorders. This reduces the need for invasive GnRH stimulation tests and for drawing blood for serum assays. Determination of FMV U-LH should also be useful for monitoring the progression and treatment of precocious puberty (Zung et al., 2014).

FUTURE PERSPECTIVES

Clinical use of urinary gonadotropin measurements has not been studied in depth since our first study (I). This is probably due to the fact that collecting an additional urine sample has not been a favored method of choice in the clinical evaluation of pubertal development, since the prospects of obtaining additional information through urinary gonadotropin measurements have not been established. There are no solid reference values for urinary gonadotropins for subgroups of sex, age and pubertal stage, in particular in relation to various pathological states. Also, very few LH assays, apart from IFMA, with sufficient sensitivity have been available and the AutoDelfia analyzer for mass IFMA applications has not been widely used. Besides, the sensitivity of S-LH, S-FSH, S-testosterone and S-estrogen assays suitable for common autoanalyzers has improved substantially over the last two decades. Study IV showed that AUC values for S-LH and FMV U-LH present quite similar levels. There is, nevertheless, still room for using urinary gonadotropin measurements, but reference ranges for sufficiently large, healthy groups by sex, age and pubertal stage are needed, and the same goes for patients with a confirmed diagnosis of some pubertal disorders.

Different diagnostic subgroups should be studied with regard to HPG axis function in terms of (urinary) gonadotropins and sex steroids. A sufficient number of subjects is needed per disorder, sex, age and pubertal stage because observations may differ between children suspected for disorders of pubertal development and those suspected for only a somatic growth disorder.

Further studies should be designed to investigate the usefulness of repeated FMV gonadotropin determinations within a short time frame and over a long period of time.

Determination of FMV U-LH may be valuable for the differential diagnosis of pubertal disorders, which might reduce the need to initiate treatment in adolescents with CDGP having already clearly increasing U-LH concentrations.

FMV U-LH may also be valuable for following up the treatment outcome of patients with pubertal disorders, e.g., during follow-up of GnRH analogue treatment in patients with CPP.

Since urinary gonadotropin measurements are practical and easily repeated at regular intervals, it would be useful to examine the added value of repeated analyses of U-LH, U-FSH or the U-LH/U-FSH ratio for evaluating pubertal development and pubertal disorders. In addition to the studies in this book, we have collected clinical and laboratory data of 30 subjects for a period of over 10 years, the analyses of which should provide valuable data for our understanding of the true relationship between chemical and clinical onset of puberty in individual cases. This study should provide valuable information on the expected time to the onset of puberty is, i.e, on how to predict puberty, not merely identify it. It should also help clinicians re-evaluate the currently established criteria for confirming true puberty.

We have further cross-sectional data based on 3 repeated FMV urine samples from over 500 subjects. I hope that designing this study will also further benefit our understanding of the clinical utility of urinary gonadotropin measurements.

We have gathered yet another unpublished set of longitudinal data, which comes from 40 newborns or infants younger than 6 months. The analysis of that data will provide further valuable information on this topic.

CONCLUSIONS

Gonadotropin concentrations in FMV urine reveal activation of the HPG axis earlier than the first clinical manifestations of puberty or the concentrations of gonadotropins in day-time serum samples. This is due to the fact that gonadotropins measured in samples of urine accumulated in the bladder overnight express the integrated night-time secretion of gonadotropins, and thus the average night-time gonadotropin concentrations in the plasma.

The clinical use of urinary gonadotropin measurements should be facilitated, since the method provides a non-invasive estimate of gonadotropin secretion in healthy children and adolescents, and in subjects with pubertal development disorders. The appropriate use of urinary gonadotropin measurements should ultimately reduce the need for the GnRH stimulation test and for blood sampling for serum gonadotropin assessments.

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