CLINICAL STUDY

Leptin levels show diurnal variation throughout puberty in healthy children, and follow a gender-specific pattern

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Abstract

Objective: To investigate the levels and diurnal rhythm of serum leptin in healthy children, and to investigate the association between leptin levels and sex steroids.

Methods: Four girls and four boys, all healthy volunteers, were followed longitudinally throughout puberty. Their chronological ages ranged from 8.7 to 19.5 years, and body composition, expressed as weight-for-height standard deviation scores (SDS), ranged between 4.1.7 and +2.4. Serum leptin, oestradiol and testosterone concentrations were measured by radioimmunoassay at 1000, 1400, 1800, 2200, 0200 and 0600 h.

Results: In all girls and boys, both prepubertally and during pubertal development, serum leptin levels increased during the night, with no difference in relative peak amplitude. In boys, the leptin concentrations increased until the initiation of puberty and then declined, whereas in girls, the concentrations increased throughout puberty. The inter-individual variation in mean leptin levels among girls decreased to 11% at the time of menarche. A positive correlation was found for both oestradiol and testosterone versus leptin in girls throughout puberty $r = 0.64$ and $r = 0.71$ respectively, $P < 0.001$. A negative correlation was found between leptin and testosterone in boys in mid- and late puberty $r = -0.66$, $P < 0.01$. No correlation was found between oestradiol and leptin in boys or between testosterone and leptin in pre- and early pubertal boys.

Conclusion: Serum leptin concentrations show diurnal variation throughout pubertal development in both girls and boys. The changes in leptin levels during puberty follow a gender-specific pattern, probably due to an influence of sex steroids on leptin production.

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Introduction

The connection between nutrition – reflected by the amount of adipose tissue – and the onset of gonadarche, menarche and maintenance of fertility is well established in human females (1–3), whereas it is not so clear in male humans. The adipocyte-derived peptide hormone leptin (4) is a candidate for mediating the interaction between adipose tissue and the hypothalamic–pituitary–gonadal axis. In animal studies, it has been reported that female ob/ob mice that lack bioactive leptin remain prepubertal, and that administration of recombinant leptin induces puberty and fertility. On the other hand, male ob/ob mice can occasionally reproduce if maintained on a restricted diet (5). Clément et al. reported a mutation in the human leptin receptor. In addition to early-onset morbid obesity, patients homozygous for this mutation have no pubertal development (6). These results indicate that leptin is an important physiological regulator of, or a prerequisite for, several endocrine functions in humans.

The connection between adipose tissue, leptin and reproduction indicates that there is a relationship between sex steroids and leptin production. Several studies have shown that oestradiol increases, whereas testosterone decreases, leptin production in vivo (7–10). All these findings were independent of body fat changes. Thus, body fat can no longer be considered the only reason for the gender difference in leptin concentrations.

Measurements of leptin concentrations during puberty are consistent with the reports of sex-steroid influences on leptin. For example, serum leptin concentrations are higher in girls than in boys during puberty (11–14). The gender differences in leptin during puberty may be related to both the size of the fat depot and sex-steroid changes. As these parameters
vary between individuals, it is easier to make conclusions concerning changes in leptin during pubertal development in longitudinal studies.

Leptin is secreted in a diurnal rhythm in both adults (15–17) and children (18–20), but there are no reports on the diurnal rhythm in children throughout pubertal development.

The aim of the present longitudinal study was to investigate the changes in serum leptin concentrations in healthy children during puberty and to relate these to changes in sex steroids.

Subjects and methods

Study subjects

The study group consisted of four girls and four boys, who were all healthy volunteers at the Queen Silvia Children’s Hospital, Göteborg, Sweden. The children visited the clinic at approximately yearly intervals, from no sign of pubertal development (testicular volume 1 to 2, breast stage 1, pubic hair stage 1) until full pubertal development (testicular volume 20 to 25 ml, breast stage 5, pubic hair stage 5). Each girl’s puberty was classified into six stages according to breast development and time to menarche: pre (breast stage 1), early (breast stage 2), midpre (breast stage 3–4, pre-menarche), midpost (breast stage 4, 0–1 year post-menarche), late1 (breast stage 4–5, 1–2 years postmenarche) and late2 (breast stage 4–5, 2–5 years postmenarche). For boys, puberty was classified into five stages according to testicular volume: pre1 (testis 1–2 ml), pre2 (testis 3 ml), early (testis 4–8 ml), mid (testis 10–15 ml) and late (testis ≥ 20 ml). Breast and pubic hair development were assessed according to Tanner (21) and testicular volume according to Prader (22). Further detailed information regarding pubertal development, chronological age, height standard deviation score (SDS), weight SDS, weight/height SDS and body mass index (BMI) is shown in Tables 1 and 2.

The study was approved by the Ethical Committee of the Medical Faculty, University of Göteborg. Informed consent was obtained from all the children and their parents.

Heights and weights were converted into SDS using the Swedish Growth Reference Values for healthy children (23).

Study protocol

The children stayed at the hospital for at least 2 days. A heparinized needle was inserted during the first evening or morning. The children received a normal diet and were allowed normal activity. They all fell asleep between 2230 and 2400 h and woke between 0600 and 0800 h, except for two boys (boys 1 and 2) in the late pubertal stage who fell asleep about 2 h after midnight. Serum samples were taken at 1000, 1400, 1800, 2200, 0200 and 0600 h. The samples were stored at room temperature, centrifuged within 15 h

Table 1 Pubertal maturation, age and body composition of the girls.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Breast stage</th>
<th>Pubic hair development</th>
<th>Time from menarche (yr)</th>
<th>Pubertal stage</th>
<th>Age (yr)</th>
<th>Height SD score</th>
<th>Weight SD score</th>
<th>Weight/Height SD score</th>
<th>BMI (kg/m²)</th>
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<td>–3.3</td>
<td>Pre</td>
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<tr>
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<td>Early</td>
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<td>Midpre</td>
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</table>
and were then frozen until measurement of sex steroid and leptin concentrations.

On six occasions, samples were also taken every 20 min. A constant-withdrawal pump (Swemed, Göteborg, Sweden) with a non-thrombogenic catheter was used (24). The heparinized tubes were changed every 20 min for 24 h. The samples were stored at room temperature and centrifuged within 25 h. After centrifugation, the plasma samples were frozen until assayed.

**Measurement of serum leptin**

Serum leptin concentrations were determined by radioimmunoassay (RIA) (Human Leptin RIA Kit, Linco Research, Inc., St Charles, MO, USA) as described by Ma et al. (25). The lower limit of detection was 0.2 μg/l as defined by Rodbard (26). All samples from one child were measured in the same assay and determined in duplicate. The intra-assay coefficients of variation (CV) were 7.0% at 2.4 μg/l and 4.9% at 14.0 μg/l. The corresponding interassay values were 9.6% and 6.7% respectively.

**Measurement of serum 17β-oestradiol**

Serum 17β-oestradiol concentrations were determined in duplicate by a modified RIA using coated tube technology (Spectria estradiol; Orion Diagnostica, Espoo, Finland) after diethyl ether extraction, as previously described (27). The detection limit for the RIA is 7.8 pmol/l. The interassay CV was 13.8% for a mean value of 36.7 pmol/l and the intra-assay CV was 17.2% for a mean value of 10.0 pmol/l calculated from 20 diethyl ether extracted replicates. The interassay CV in unextracted serum was below 11% and the intra-assay CV was below 7% for concentrations between 50 and 200 pmol/l and below 3% for concentrations above 200 pmol/l.

**Measurement of serum testosterone**

Serum testosterone concentrations were determined in duplicate by RIA using coated tube technology (Spectria testosterone; Orion Diagnostica) as previously described (28). The detection limit was 0.03 nmol/l for 3 S.D. below the counts at maximum binding. The intra-assay CV for the RIA was 10.6% for 0.2 nmol/l and below 7% for concentrations above 0.4 nmol/l, calculated from 20 replicates. The interassay CV for the RIA was 15.5% for 0.2 nmol/l and below 10% for concentrations above 0.8 nmol/l.

**Statistical analysis**

The Wilcoxon signed rank sum test was used for evaluation of changes over time (29). When mean or
S.D. values are given, leptin concentrations were log transformed. A value of \( P < 0.05 \) was considered significant. As serum leptin and oestradiol concentrations were not normally distributed, linear regression analyses were performed on log-transformed data.

**Results**

**The diurnal rhythm of leptin**

In order to determine whether a profile obtained from six serum samples represents the 24-h rhythm, on six occasions serum samples taken every 4 h were analysed in parallel with plasma samples taken every 20 min (Fig. 1).

![Figure 1](image1)

**Figure 1** Representative comparison between serum concentrations of leptin in samples taken every 4 h (□) and samples taken every 20 min (●) in two subjects (A, girl 3; B, girl 1). The shaded area and error bars represent the interassay interval, assumed to be 10%.

![Figure 2](image2)

**Figure 2** The relative diurnal rhythm in serum leptin levels in girls and boys. The mean serum leptin concentration over 24 h is calculated on logarithmic values for each study subject. The percentage difference from the mean value over 24 h is calculated every 4 h between 1000–0600 h. Data is shown as the mean value ± s.d. for each clock time for all children and pubertal stages, in girls and boys respectively. The first measurements were used if a child participated more than once in one pubertal stage.

![Figure 3](image3)

**Figure 3** Individual 24-h serum leptin profiles throughout puberty in healthy girls. The first measurements were used when a girl participated more than once in a pubertal stage.
All children displayed a diurnal rhythm of serum leptin concentrations, both before and during pubertal development. Characteristically, they had significantly increased serum leptin levels during the night (2200 to 0600 h) compared with those during the day (1000 to 1800 h) ($P < 0.001$). Serum leptin levels in girls ranged from 2.7 to 20.3 mg/l during the day and from 3.4 to 34.8 mg/l during the night. In boys, the corresponding levels ranged from 1.5 to 9.8 and from 1.6 to 18.5 mg/l. No difference in peak amplitude was seen between the pubertal stages or between boys and girls. Figure 2 shows the average leptin rhythm during 24 h, including all profiles and pubertal stages, in girls and boys respectively.

**Changes in serum leptin levels throughout pubertal development**

**Girls** Serum levels of leptin increased in girls during pubertal development. The lowest concentrations were found during prepuberty and the highest levels 2 to 5 years after menarche in all four girls. Serum leptin concentrations increased from prepuberty to early puberty in the three girls with samples taken in early puberty. However, there was no concordant pattern in leptin changes during pubertal progression to the midpubertal stages (Fig. 3). Statistical analyses showed no difference in serum leptin concentrations between girls in pre and early or between early and midpre pubertal stages. On the other hand, girls had significantly higher leptin levels during mid-puberty premenarche compared with levels during prepuberty ($P < 0.001$).

Figure 4 shows the individual mean serum leptin concentrations for girls in relation to menarche. In general, mean leptin concentrations increased with time from menarche, with a more evident increase in the years after menarche. Before menarche, there were wide inter-individual variations in leptin concentrations, amounting to 43% about 2 years before menarche. However, at the time of menarche (~0.4 to 0 years) the inter-individual variation had decreased to 11%.

The girls had significantly higher serum leptin levels 1–2 years after menarche and between 2 and 5 years after menarche compared with those in girls pre ($P < 0.001$), early ($P < 0.001$) and midpre ($P < 0.001$) puberty. The serum leptin levels 2–5 years postmenarche were significantly higher than those in girls up to 2 years after menarche ($P < 0.001$).

**Boys** Figure 5 shows changes in individual serum leptin levels in boys during pubertal development. One of the boys (boy 4) had his serum leptin profile measured at all pubertal stages. In this boy, serum leptin levels increased until the start of puberty, and then declined. In the other three boys, samples from
either pre2 or early pubertal stages are missing. Even in these three boys, serum leptin levels increased up to the initiation of puberty (pre2 or early) and then declined. Serum leptin values were significantly higher in pre2 than in pre1 ($P < 0.001$), mid ($P < 0.001$) and late puberty ($P < 0.02$). Serum leptin values were also significantly higher in early than in pre1 ($P < 0.001$), mid ($P < 0.001$) and late puberty ($P < 0.001$). No difference was seen between pre2 and early, pre1 and mid, pre1 and late, or mid- and late puberty.

Figure 6 shows the mean serum leptin concentrations in relation to testicular volume. In all the boys, the mean serum leptin concentration increased as testicular volume increased from 2 ml to 3–5 ml and then declined to the end of puberty.

**Sex steroids in relation to leptin**

Sex steroid concentrations for healthy children throughout puberty have been published previously (27, 28, 30). Testosterone concentrations in the pre- and early pubertal boys in the present study were all below 3 nmol/l and oestradiol concentrations were below 8 pmol/l, whereas those in mid- and late pubertal boys were above 6 nmol/l and 8 pmol/l respectively (maximum level during 24 h). None of the girls had testosterone concentrations above 3 nmol/l, but they all had oestradiol concentrations above 8 pmol/l (maximum level during 24 h).

A positive correlation was found between oestradiol and leptin concentrations in girls ($r = 0.64$, $P < 0.001$), but not in boys (Fig. 7). A positive correlation was also found between testosterone and leptin concentrations in girls ($r = 0.71$, $P < 0.001$), whereas a negative correlation was found in boys in mid- and late puberty ($r = -0.66$, $P < 0.01$). No correlation was found between testosterone and leptin concentrations in pre- and early pubertal boys (Fig. 8). On the other hand, a positive correlation was found ($r = 0.63$, $P < 0.001$) when prepubertal boys and boys in early puberty were included in the regression analyses for the group of girls (testosterone concentrations in the same range).

**Discussion**

The present study is the first in which the diurnal rhythm of leptin has been investigated longitudinally throughout pubertal development in healthy children. We found that both girls and boys have a diurnal rhythm in serum leptin secretion both prepubertally and during pubertal development, with the peak value at around 0200 h and the nadir at 1000–1400 h.
There was no difference in relative rhythm between the sexes during puberty, although the levels were higher in girls than in boys during puberty. Leptin levels increased throughout puberty in girls, while those in boys increased until the initiation of puberty and then declined.

In the present study, we had a 4-h sampling period for detection of the 24-h leptin profile. A prerequisite for using this 4-h sampling period was that it mirrored the 20-min sampling profile. Apart from some extremely high peaks that appear between the 4-h serum samples, and for which we have no explanation, the six serum samples represented a simplified profile of the diurnal rhythm. Therefore, conclusions from this study concerning the diurnal variation are in concordance with more detailed profiles.

Palmert et al. and Pombo et al. have shown similar results in children (19, 20). In addition, Licinio et al. also demonstrated that there is no difference in the diurnal rhythm of leptin between adult women and men (16). It is not clear what produces the rhythm in serum leptin secretion. Simon et al. demonstrated that plasma leptin levels are modulated by both a slight circadian component and sleep (17). In the present study, the children had normal nocturnal sleep and the peak leptin concentrations appeared 2–3.5 h after the onset of sleep.

Serum leptin levels fluctuate during the menstrual cycle, with significantly higher levels in the luteal phase than in the follicular phase (10, 31, 32). In the present study, menstrual cycles were not included in the analyses, but the phase of the cycle should be taken into consideration when studying leptin levels post-menarche.

The present results demonstrate sexual dimorphism in serum leptin levels during puberty. In girls, concentrations increased in parallel with pubertal development, whereas those in boys increased until the initiation of puberty and then declined.

Mantzoros et al., who evaluated eight prepubertal boys longitudinally for 2.5–5.1 years, reported similar results for boys. Compared with baseline prepubertal levels, leptin levels rose by approximately 50% just before the onset of puberty and decreased to approximately baseline values after the initiation of puberty (33). The same results were also seen in two cross-sectional studies, one related to pubertal stage (12) and one related to age (13). In contrast, Ahmed et al. did not find any significant rise in serum leptin levels in boys at the onset of puberty, even though it was a longitudinal study (34), neither did our group in a previously published cross-sectional study (11). The reason for these differences may be due to the differences in pubertal classification. In this study, we divided boys with a testicular volume of 1–2 ml and those with a testicular volume of 3 ml into different groups. We know from previous results that boys with a testicular volume of 3 ml have increased nocturnal levels of luteinizing hormone and testosterone compared with those in boys with a testicular volume of 1–2 ml (30, 35).

The inter-individual variation in leptin concentrations was most evident in the boys in this study. Differences in basal levels in boys could be explained by variations in fat mass, as the highest leptin concentrations were found in the boys with the highest BMI and weight/height SDS (boys 2 and 4).

This is the first reported study in which puberty in girls is classified by considering both breast development and the time to menarche (pre, early, midpre, midpost, late1 and late2). It is interesting that even though the levels varied markedly before the time of menarche, mean leptin concentrations tended to reach the same level at menarche. Whether this finding is of any significance or is just a coincidence in this small number of girls is still to be determined. However, studies have shown that all girls have the same percentage of their body weight as fat at the time of menarche (2). This could explain our results, as the leptin concentration mirrors the amount of body fat (36).

Remarkably, the two girls with the highest BMI and weight/height SDS (girls 1 and 4) had a decreased leptin level at the time of menarche compared with the samples taken both before and after menarche, while the two leaner girls had a more constant increase of leptin throughout puberty. Furthermore, two of the girls had a surge in leptin concentrations in early puberty and one in pre-puberty (Fig. 4, Table 1). Perhaps, girls also have a surge in leptin concentrations just before the onset of puberty, although it is not as easy to detect as in boys, due to increasing levels throughout puberty. A recent study in male monkeys showed that the increase in nocturnal leptin precedes the increase in nocturnal luteinizing hormone (37). Furthermore, a study in vitro showed that leptin is involved in the acceleration of pulsatile gonadotrophin releasing hormone secretion that precedes the onset of puberty in the male rat (38). All these data indicate that leptin concentrations are permissive to puberty in both girls and boys.

Another interesting finding is that leptin levels, along with oestradiol and testosterone levels (28), continued to increase for a couple of years after menarche. Whether this increase is caused only by a parallel increase in body fat, and/or whether it is of importance for fertility and reproduction is still to be determined.

It is interesting to note the sexual dimorphism in leptin concentrations during pubertal development, but the mechanism behind this is not clear. As revealed in other studies, girls have higher 24-h mean leptin levels than boys, even after adjusting for adiposity (39). One tempting explanation could be a testosterone-feedback effect on leptin and/or a stimulatory effect of oestradiol. Previous studies have shown that administration of testosterone to humans has a suppressing effect on...
leptin (7–9), and suppression of testosterone increases leptin levels (8, 19). In vitro experiments using human adipocytes showed that testosterone is able to reduce leptin secretion (40), while oestradiol induces a notable increase in leptin in women, but does not alter leptin secretion in males (41). On the other hand, oestrogen substitution or suppression does not appear to increase leptin levels in normal-weight humans (19, 42). Nevertheless, oestradiol does appear to be involved in the regulation of leptin levels.

The present study points towards a suppressing effect of testosterone and a stimulatory effect of oestradiol above certain levels. In linear regression analysis, we found that leptin correlates positively with both oestradiol and testosterone, as long as the oestradiol levels were detectable (>8 pmol/l) and the testosterone concentrations were below 3 nmol/l, as are those in girls. On the other hand, no correlation, or a negative correlation, was found between leptin and sex steroids when either the testosterone levels were above 6 nmol/l (as in mid- and late pubertal boys) or the oestradiol levels were below the detection limit (as in pre- and early pubertal boys). It is interesting that when we included all children with testosterone levels below 3 nmol/l in a regression analysis, a positive correlation was found between leptin and testosterone. It is possible that the absence of a correlation in pre- and early pubertal boys was due to the small sample size. With testosterone concentrations above 6 nmol/l, as in mid- and late pubertal boys, the correlation between leptin and testosterone becomes negative, possibly due to an inhibitory effect.

The sexual dimorphism in leptin concentrations during puberty may be a result of negative feedback by testosterone. The boys with a testicular volume below 6 ml had testosterone concentrations in the same range as the girls throughout puberty. The boys with a testicular volume above 8 ml, all had testosterone concentrations above 6 nmol/l, a level that is not found in healthy girls (28). This could be one explanation why the leptin concentrations in girls increased in parallel with pubertal development, whereas in boys the concentrations increased until the initiation of puberty and then declined. In some of the boys the leptin levels started to increase after puberty was completed, probably as a result of increasing body fat.

In addition to previous studies, these new data show that there is a complex interplay between leptin and the hypothalamic–pituitary–gonadal axis.

We conclude that both girls and boys have a diurnal rhythm in serum leptin secretion, both prepubertally and during pubertal development, with the peak value occurring during the night and the nadir around noon. There is no difference in relative rhythm between the sexes during puberty, even though the levels in girls are higher than those in boys. The leptin levels increase throughout puberty in girls and are unified at the same level at the time of menarche. In boys, leptin concentrations increase until the initiation of puberty and then decline. The combination of non-inhibitory low testosterone levels and an increase in oestradiol levels in mid- and late pubertal girls possibly explains the increase in leptin levels in girls. In addition to low fat mass, inhibitory high levels of testosterone, in combination with low oestradiol levels, may explain the decreasing leptin levels after the initiation of puberty in boys.

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