DISSERTATIONES KINESIOLOGIAE UNIVERSITATIS TARTUENSIS
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TRIIN POMERANTS

Ghrelin concentration in boys at different pubertal stages: relationships with growth factors, bone mineral density and physical activity

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CONTENTS

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BMAD	volumetric BMD L2-L4
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CV	coefficient of variation
DXA	dual-energy X-ray absorptiometry
FIRI	fasting insulin resistance index
GH	growth hormone
GHS-R	growth hormone secretagogue receptor
HR	heart rate
IGF-1	insulin-like growth factor 1
IGFBP-3	IGF binding protein-3
IVT	individual ventilatory threshold
LBM	lean body mass
SD	standard deviation
SDS	standard deviation score
VO_2max	maximal oxygen consumption

LIST OF ORIGINAL PUBLICATIONS

I **Pomerants T, Tillmann V, Jürimäe J, Jürimäe T.** Relationship between ghrelin and anthropometrical, body composition parameters and testosterone levels in boys at different stages of puberty. *Journal of Endocrinological Investigation*, 2006, 29, 962–967.

II **Pomerants T, Tillmann V, Jürimäe J, Jürimäe T.** The influence of serum ghrelin, IGF axis and testosterone on bone mineral density in boys at different stages of sexual maturity. *Journal of Bone and Mineral Metabolism*, 2007, 25, 193–197.

III **Pomerants T, Tillmann V, Karelson K, Jürimäe J, Jürimäe T.** Ghrelin response to acute aerobic exercise in boys at different stages of puberty. *Hormone and Metabolic Research*, 2006, 38, 752–757.

Triin Pomerants had primary responsibility for protocol development, patient enrollment, outcome assessment, data analysis, and writing the manuscripts.

1. INTRODUCTION

Ghrelin is a peptide hormone that was discovered in year 1999 as an endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) [Kojima et al. 1999]. There has been a vigorous discussion over the importance of ghrelin concentration in overall energy expenditure and a wish to know more about different aspects of how ghrelin reacts in different situations and how it affects the hormonal system and organism in all. One of the obvious reasons for this great interest was of course the hope to find a new miracle substance which would help to solve the growing amount of problems connected to obesity and within last years the information gained from different studies has certainly given us all a better knowledge about ghrelin.

In short, ghrelin is a 28-amino-acid peptide, which possesses a unique fatty acid modification, an *n*-octanoylation at Ser 3. It has two circulating forms: acylated and unacylated (desacyl). The acylated form is thought to be essential for ghrelin's biological activity though GHS-R. However, decacyl ghrelin has also been reported to influence both cell proliferation and adipogenesis through an unknown receptor different from GHS-R [Korbonits et al. 2001; Akamizu & Kangawa 2006]. Ghrelin is mainly produced in the stomach and circulates in the blood at a conciderable plasma concentration. Expression of ghrelin is also detectable in the hypothalamus, intestine, pituary, placenta and other tissues [Kojima et al. 1999; Korbonits et al. 2001]. The level of ghrelin concentration is known to play a role in a number of different physiological processes [Korbonits et al. 2004].

Based on the intriguing information known to us already and the fact that there were obviously quite a few unanswered questions, the general aim of this study was to gather additional information about the possible role of ghrelin in growth and pubertal development boys at different stages of biological maturation and how does acute physical exercise influence these processes.

2. REVIEW OF THE LITERATURE

2.1. General overview of ghrelin

Ghrelin is a regulator of a large array of endocrine and non-endocrine functions, including the influence on GH secretion, food intake and energy balance [Korbonits et al. 2004]. In fact, accumulating evidence suggests that ghrelin is a key contributor to the short- and long-term regulation of body weight as an important element of a complex central signaling network that regulates food intake and energy expenditure [Wren et al. 2001; Murray et al. 2003; Zigman et al. 2003; Cummings et al. 2003]. Human plasma ghrelin concentration level rises and fall over the course of the day in relation to food intake and the elevation in circulating ghrelin concentration before meals has often been cited as evidence supporting the hypothesis that rise of ghrelin concentration serves as a hunger signal in humans. First observations in that area were made by fixed meal schedule [Cummings et al. 2001], but a later study by Cummings et al. [2004] revealed that ghrelin concentration level also peaks before freely requested meal. Ghrelin concentration stays constant during sleep [Kraemer & Castracane 2007].

Ghrelin also increases blood glucose concentration by inhibiting insulin secretion [Broglio et al. 2001] and stimulates gastric acid secretion and motility [Date et al. 2001]. Fasting ghrelin concentration has been found to be negatively correlated with body height and mass [Bunt et al. 2003], as well as with body mass index (BMI) [Haqq et al 2003; Chanoine 2005]. In addition, fasting ghrelin concentration is negatively associated with body fat percent [Bunt et al. 2003].

An overview of physiological actions of ghrelin is given in Table 1 [Hosoda 2006].

Table 1. Physiological actions of ghrelin

Hormone release
GH release ↑
Adrenocorticotrophic hormone release ↑
Cortisol release ↑
Prolactin release ↑
Thyroid stimulating hormone release ↓ *?* →
Luteinzing hormone release ↑ *?* →
Follicle-stimulating hormone release →
Insulin release ↑ *?* ↓
Anabolic effects
Appetite ↑
Adiposity ↑
Cardiovascular functions
Cardiac output ↑
Blood pressure ↓
Apoptosis of cardiomyocytes in vitro ↓
Gastric functions
Gastric acid secretion ↑
Gastric motility ↑

2.2. Ghrelin concentration through childhood and puberty

Cross-sectional studies conducted in healthy children and adolescents have revealed that level of ghrelin concentration peak in early postnatal life (until age of 2 years) [Soriano-Guillen et al. 2004] and then gradually decreases during childhood and adolescence with advancing pubertal stage [Lebenthal et al. 2006; Whatmore 2003]. Serum ghrelin concentration levels are 30–50% lower in postpubertal compared to pubertal subjects [Chanoine 2005]. There are some data that the changes in serum ghrelin concentration is more pronounced in boys than girls. The results of study on a group of healthy male and female children (age 5–18 years) by Whatmore et al. [2003] showed a statistically greater fall in circulating ghrelin concentration over puberty in boys, consistent with the greater magnitude of their growth spurt. However, as the authors also stated, the wide range of results in both sexes needs to be confirmed in a larger cohort. The precise mechanisms underlying these changes have not been fully elucidated yet.

2.3. Ghrelin interaction with other hormones

Since ghrelin has been suggested to have such a large array of different physiological roles, the interactions of ghrelin with other hormones have also been a subject of interest in a number of studies.

One of the most intriguing study objects in a point of view of developmental physiology has been the interrelationships of ghrelin and GH concentrations. It has been suggested that ghrelin concentration might have a physiological role on pulsatile GH secretion, but further studies are necessary to clarify its precise role [Lengyel 2006], because the results, again, are controversial. First study showing evidence that ghrelin strongly stimulates GH release in humans was presented by Takaya et al. [2000]; however the authors also indicated that the influence of ghrelin concentration is not completely specific for GH release.

Circulating levels of IGF-I (insulin-like growth factor 1) and its principal carrier protein, IGFBP-3 (IGF binding protein-3), reflect both growth, GH and nutrient status, while IGFBP-I, as a small IGF binding protein, acts to facilitate transport of IGF-I into tissues and is rapidly modulated by insulin levels. Thus the relationship between ghrelin concentration and the IGF-axis may help to clarify its role in growth [Whatmore et al. 2003]. A study by Liu et al. [2002] demonstrated that ghrelin gene expression is age dependent and is influenced by the level of circulating IGF-I concentration. Also, Whatmore et al. [2003] have assumed that a negative relationship of ghrelin concentration with IGF-I would suggest that a decrease in ghrelin facilitates growth acceleration in puberty. It could be suggested that the beginning of puberty stimulates IGF-I secretion [Kanbur-Oksuz et al. 2004], and IGF-I may suppress ghrelin secretion via negative feedback. The results of study by Jürimäe et al. [2007] have supported idea of a negative feedback mechanism between the levels of ghrelin and IGF-I concentration in healthy normal-weight adolescent girls at puberty.

Circulating plasma concentration of ghrelin is influenced principally by changes in energy balance. It has been suggested that insulin concentration may play an important role in the decrease of ghrelin concentration after meals [Anderwald et al. 2003; Murdolo et al. 2003]. Most studies have reported a negative relationship between fasting ghrelin and insulin concentration [Tschöp et al. 2001; Bunt et al. 2003; Soriano-Guillen et al. 2004], including studies in children [Park et al. 2005].

Little is known about the relationship between the ghrelin and sex hormones concentration in children. Recently study by Lebenthal et al. [2006] did not find significant correlation between ghrelin and testosterone concentration in either peripubertal (aged 8 to 12 yrs) boys or girls. There is a link between energy homeostasis and fertility [Tena-Sempere 2005], but the potential role of ghrelin in the control of puberty onset and gonadal function has been not so well studied. Expression of the ghrelin and the functional ghrelin receptor, the GHS-R type 1a has been demonstrated in Leydig cells of rat and human testis [Tena-

Sempere 2005]. Significant decrease observed in plasma ghrelin concentration of pregnant rats [Shibata et al. 2004] and normal pregnant women [Makino et al 2002] suggests that increased estrogen levels directly induce a down-regulation of ghrelin expression. Hence, the increase in sex hormones during puberty may modify ghrelin secretion. The results of study by Lebenthal et al. [2006] stated that a pharmacological increase in sex hormones is associated with a marked decline in circulating levels of ghrelin in boys but not girls.

Next to ghrelin exists another hormone that reflects peripheral nutritional status – leptin [Whatmore et al. 2003]. It has been well established that leptin is an adipocyte-derived hormone that acts directly on the hypothalamus, where it regulates a large number of molecules that are involved in the regulation of energy homeostasis and food intake [Shinatani et al. 2001; Foster-Schubert et al. 2004; Wren et al. 2000; De Vos 1996]. Leptin may also have a role in the control of growth hormone release [Zieba et al. 2003; Luque et al. 2007] and has been suggested to be involved also in the pubertal activation [Clayton et al. 1997]. Normally, after onset of puberty (pubertal stage 2), serum leptin concentration in boys starts to decline and reaches a nadir in stage 5 [Clayton et al. 1997]. Serum leptin concentration is positively related to fat mass and inversely to lean body mass in boys [Ong et al. 1999]. The interaction between ghrelin and leptin concentration in blood has been subject of interest and so far the results have been rather contradictory. Some studies have suggested that leptin concentration apparently does not affect ghrelin secretion in a mixed group of obese boys and girls [Haqq et al. 2003; Ikezaki et al. 2002]. However, next to that is also data about an inverse association between ghrelin and leptin concentration in healthy boys and girls [Park et al. 2005; Whatmore et al. 2003].

2.4 Ghrelin concentration and bone health

The growing skeleton has to adjust to the large increase in height and body mass that occurs during puberty as the result of rapid hormonal changes. Bone mineralization increases with age, height, and weight throughout childhood, with a significant gain during pubertal development [Saggese et al. 2002]. It has been estimated that about 40% of peak bone mass is achieved during pubertal development [De Schepper et al. 1991; Gordon et al. 1991]. The factors that contribute to the pubertal bone turnover are still not fully known, but it has been stated that important role belongs to the sex steroids [Saggese et al. 1997; Yilmas et al. 2005] and also GH-IGF-axis [Ohlsson et al. 1998].

The effects of sex steroids on bone are mediated mainly by GH and IGF-I [Clark & Rogol 1996], but they also exert a direct effect on bone metabolism. Both estrogens and androgens have been shown to stimulate the proliferation of osteoblasts *in vivo* [Kasperk et al. 1989]. Most of GH anabolic actions are mediated through IGF-I [Patel et al. 2005]. IGF-I stimulates endochonandral

bone formation and rapidly activates bone turnover [Blum et al. 1993; Kanbur-Oksuz et al. 2005]. IGFBP-3 as the major binding protein of IGF-I has also a direct effect on bone metabolism [Kanbur et al. 2005] by stimulating osteocalcin synthesis through osteoblasts and preosteoblasts [Canalis & Lian 1988] and collagen synthesis [Jones & Clemmons 1995]. Additionally to sex hormones and GH-IGF-axis, ghrelin has also been recently shown to stimulate bone formation in rats by increasing the number of osteoblasts [Fukushima 2005].

Ghrelin secretion has been shown to predict BMD independently from body composition, GH-IGF-I axis, or estradiol in adolescent girls [Misra et al. 2005]. The study by Misra et al. [2005] found that ghrelin secretion predicted BMD in healthy adolescents strongly. Secretory burst mass was the strongest predictor of lumbar spine bone mineral apparent density (BMAD) (r=0.66, *P*=0.003), lumbar spine BMAD z-scores (BMAD-z) (r=0.59, *P*=0.01), hip BMD (r=0.55, *P*=0.02), and hip BMD-z (r=0.52, *P*=0.03). When body composition measures (lean and fat mass, body mass index), and hormonal predictors (GH, IGF-I, cortisol, leptin, and estradiol) were entered into a regression model with ghrelin secretion to determine independent BMD predictors, ghrelin concentration was the strongest predictor of lumbar BMAD, BMAD-z, hip BMD, and hip BMD-z, contributing to 43, 30, 26, and 19% of the variability, respectively, independent of GH or cortisol concentration effects.

To our knowledge there have been no studies looking for the relationship between BMD and ghrelin concentration in healthy boys.

2.5. The effect of acute physical activity on levels of ghrelin, leptin and GH-IGF-axis

The noticeable effects of a long-term physical exercise have been observed in many anthropometrical and biochemical characteristics. In case of ghrelin it has been suggested that regular physical activity increases plasma ghrelin concentration to stimulate appetite and food intake to cover the higher energy expenditure [Cummings et al. 2001; Horvath et al. 2001]. According to this theory ghrelin concentration gives signals for a need of energy consumption to counter a further deficit of energy storage by helping to maintain body mass [Horvath et al. 2001; St-Pierre et al 2004].

There is a number of studies including adult subjects that have examined the influence of an acute bout of aerobic exercise on total plasma ghrelin concentration [Dall et al. 2002; Kraemer et al. 2004; Schmidt et al. 2004; Burns et al. 2007; Jürimäe et al. 2007]. Kraemer et al. [2004] completed a progressively intense intermittent exercise trial with well trained males on a treadmill on four exercise intensities: 10 minutes at 60%, 10 minutes at 75%, 5 minutes at 90%, and 2 minutes at 100% of the level of maximal oxygen consumption (VO_{2max}).

The study demonstrated no changes in ghrelin concentrations. In middle-aged males, acute exercise for 45 minutes at the level of lactate threshold also did not alter circulating plasma ghrelin concentration [Dall et al. 2002]. In a study by Schmidt et al. [2004], plasma ghrelin concentrations remained unchanged at different workloads (50%, 70% and 90% of VO_{2max}) in young males. Burns et al. [2007] found that plasma total ghrelin concentration is not responsive to acute exercise (1-h bout of high-intensity treadmill running) induced alterations in metabolism in young adults.

However, the recent study by Jürimäe et al. [2007] with elite male rowers demonstrated a significant increase in ghrelin levels immediately after maximal short-term exercise (124.4%; $p<0.05$). These results suggest that possibly the exercise bouts used in previous studies [Dall et al. 2002; Kraemer et al 2004; Schmidt et al. 2004; Burns et al. 2007] generated a limited amount of negative energy balance and using protocols that require relatively high percent of total muscle mass are more energy costly would induce significant changes.

There is no information about the influence of acute physical exercise on ghrelin concentration of the children to best of our knowledge and there are also only very few studies looking for the response of leptin concentration to acute exercise in children. It is known that as in case of ghrelin, serum leptin concentrations are also reduced only in the presence of a negative energy balance using prolonged exercise [Zaccaria et al. 2001; Souza et al. 2004].

Study results [Jürimäe and Jürimäe 2005] in elite male rowers showed that a 30-minute all-out rowing ergometer test caused an immediate decrease in plasma leptin level, probably by the involvement of all major muscle groups and therefore also high amount of energy expenditure.

The GH-IGF-1-axis modulates growth in many tissues and is also known to play a role in the adaptation to exercise [Adams 2002; Nemet et al. 2004]. Acute exercises increase plasma GH concentration, with a threshold level of approximately 30% of VO_{2max} level and the response is more pronounced in pubertal than prepubertal children [Jenkins 1999]. The data about the influence of acute exercise on the IGF-system are contradictory. The response of IGF-I and IGFBP-3 concentration to acute endurance exercise is fast and concentrations tend to peak within 10 minutes [Cappon et al. 1994; Schwarz et al. 1996]. Schwarz et al. [1996] also found that IGF-I concentration increased similarly after both low- and high-intensity exercise while IGFBP-3 concentration demonstrated a significantly greater increase following the high-intensity compared to low-intensity exercise.

The influence of physical activity on circulating ghrelin concentration in the blood has been a subject of interest in a number of studies, but since the results have been contradictory and further information about different physical exercise types is needed to state the patterns more clearly. To our knowledge, the influence of concurrence of acute exercise and ghrelin concentration at different stages of pubertal maturation has not been investigated so far. There have also

been no studies investigating the response of serum IGF-I and IGFBP-3 levels to different acute exercises in boys at different pubertal stages.

2.6. Summary

Circulating ghrelin concentration is responsive to acute and chronic energy imbalance, increased by food deprivation and energy restriction, and decreased by food consumption and obesity. However, it is still not fully clear what factors are involved in the regulation of ghrelin secretion [Hosoda et al. 2006]. To our knowledge, there have been no studies investigating the relationship between fasting plasma ghrelin concentration and specific anthropometrical and body composition parameters in boys at different pubertal stages.

There are some data about the interrelations of ghrelin concentration and BMD in adolescent girls [Misra et al. 2005], but there is a lack of discussion over the same subject in healthy boys.

In addition, the influence of leptin, insulin and testosterone to ghrelin concentration needs more attention. New data about the relationships between serum ghrelin concentration and changes in anthropometry, body composition and sex hormone levels at different stages of puberty, may give us additional information about the possible role of ghrelin concentration in human pubertal development.

3. AIM AND PURPOSES OF THE STUDY

The general aim of this study was to clarify the role of serum ghrelin concentration in growth and pubertal development in boys at different stages of biological maturation and how does acute physical exercise influence these processes.

The specific purposes of this study were to:

- investigate the impact of anthropometrical and body composition characteristics, and different hormones on fasting blood ghrelin concentration in boys at different pubertal stages;
- study the impact of ghrelin, IGF-1, IGFBP-3, and testosterone concentration levels on BMD in boys at different stages of puberty;
- investigate the changes in serum ghrelin and leptin concentrations during and after 30 minute cycle ergometer load at ~95% of the individual ventilatory threshold (IVT) in boys at different pubertal stages.

4. MATERIAL AND METHODS

4.1 . Subjects

The study included 60 healthy non-obese Estonian schoolboys from Tartu and Lähte at the age of 10 to 18 years. The subjects were recruited from physical education classes that they had twice a week. None of the subjects was receiving any medications during or prior to the study or had a history of bone or renal diseases. They were on their ordinary everyday diet.

This study was approved by the Medical Ethics Committee of the University of Tartu, Estonia. The purpose, risks, and benefits were explained to the children and their parents who signed the consent form.

4.2 . Body composition assessment

Body height was measured to the nearest 0.1 cm using the Martin's metal anthropometer and body mass (kg) of participants dressed in light clothing was measured to the nearest 0.05 kg using medical scales (A&D Instruments Ltd, UK). Height and body mass data were used for calculating BMI (kg/m^2). Additionally, the body height and body mass were both also converted to standard deviation scores (SDS), using the age- and sex-specific data based on Estonian population [Grünberg et al. 1998].

Body fat mass, body fat percentage and LBM, total body and lumbar spine (L2-L4) BMD (g/cm^2) were determined by dual-energy X-ray absorptiometry (DXA) (DPX-IQ, Lunar Corporation, Madison, WI, USA). To reduce the effect of body size on BMD values, apparent volumetric mineral bone density (BMAD) of the lumbar spine was calculated using a formula by Kröger et al. [1995]: BMAD= BMDx[4/(π*width of L2 to L4)].

4.3. Sexual maturity assessment

The subjects, based on results of self-assessment of genitalia and pubic hair stage using illustrated questionnaire of pubertal stage according to Tanner classification [1962], formed three groups (20 subjects per group). Pubertal development assessment according to the method of Tanner using self-assessment of genitalia and pubic hair stage in boys has been validated previously [Duke et al. 1980]. The subjects were given photographs, figures and descriptions and asked to choose from these descriptions the one that most accurately reflected their appearance. In case of discrepancies between the two variables (genitalia and pubic hair stage), greater emphasis was for determination of

Tanner stage placed on the degree of genital development. We also had a skilled observer with many years of practical experience always nearby in case the subjects had some questions.

The subjects were grouped as Group I (prepubertal), Group II (included pubertal stages 2 and 3), and the Group III (pubertal stages 4 and 5). In addition, all subjects were also analyzed as a total group.

4.4. Exercise testing protocols

The level of maximal oxygen consumption and IVT were measured using a continuous incremental protocol until volitional exhaustion on an electronically braked cycle ergometer (Tunturi T8, Finland). A standardized 2-minute warm-up at 60 W was completed prior testing. The multistage test was performed by a mean pedaling rate at 70 rpm. In group I, increments of 20 W were imposed at the end of each 2 minute stage, starting at 80 W. In groups II and III, the testing started at 100 W and the load was increased by 30 W after every 2 minutes. At the end of the testing, participants were required to sprint as fast as possible for 1 minute at the last reached work load. The participants were verbally encouraged throughout the test. The expired gas during cycle ergometer test was sampled continuously breath-by-breath for the measurement of oxygen consumption (TrueMax 2400 Metabolic Measurement System, Parvo Medics, USA).

VO_2 values were considered maximal, when two of the following three criteria were met:

1. VO_2 plateau defined as a failure of oxygen uptake to increase by greater than 2.0 $ml \cdot kg^{-1} \cdot min^{-1}$ with increase of test load;
2. HR≥95% from the predicted individual maximum (formula 220-age); and/or
3. respiratory exchange ratio ≥1.05 [Pettersen et al. 2001].

In addition the test-administrator subjectively evaluated as the characteristics of maximal performance (especially in case of younger subjects) if:

1. the subject expressed the exhaustion orally or with body language [Pettersen et al. 2001], and/or
2. the subject could not continue to pedal properly and safely at required pedaling rate [Bloxham et al. 2005].

The second exercise test was performed 2–3 days after the VO_2max test and consisted of a 30 minute exercise on the same cycle ergometer at the level of ~95% of IVT calculated by Reybrouck et al. [1985]. Tests were performed in the afternoon after school between 3:00 PM and 5:00 PM about 2–3 hrs after standardized light lunch. Heart rate (HR) was recorded every 5 seconds (Sporttester Polar Vantage NV, Kempele, Finland) during tests.

4.5. Blood analysis

10-ml blood samples to determine the concentration of ghrelin, leptin, insulin, testosterone, IGF-I, IGFBP-3 and glucose were obtained after an overnight fast from an antecubital vein with the participant in the seated position between 8.00 am and 10.00 am. The blood samples were also obtained right before, immediately after and 30 minutes after cycle ergometer exercise for the measurement of ghrelin, leptin, insulin, testosterone, GH, IGF-I, IGFBP-3 and glucose concentration. The blood serum was separated and frozen at –20°C for later analysis. Samples from one individual were run in the same assay.

Ghrelin concentration was determined in duplicate by radioimmunoassay (Linco Research, USA). The sensitivity was 93 $pg \cdot mL^{-1}$, the intra-assay and inter-assay coefficients of variation (CV) were <10% and <14.7%, respectively. Testosterone, insulin, GH, IGF-1 and IGFBP-3 concentration were analyzed in duplicate on IMMULITE 2000 (DPC, Los Angeles, USA). The inter- and intra-assay CV for testosterone and GH were less than 5%. The inter- and intra-assay CV for IGF-1 and IGFBP-3 were less than 7%. Glucose concentration was measured by means of the hexokinase/glucose 6-phosphate-dehydrogenase method using a commercial kit (Boehringer, Mannheim, Germany). All samples were administered on the same assay. Aliquots of whole blood were also analyzed in quadruplicate for packed cell volume at 12 000 rpm for five minutes and for hemoglobin using a Lange microanalyser. Post-exercise changes in plasma volume were calculated by using the formula of Dill and Costill [1974] and reported hormone values have been corrected for plasma volume changes.

In addition to evaluating IGF-1 and IGFBP-3 concentration as dependent outcomes, we assessed the molar ratio of IGF-1/IGFBP-3 concentrations. The molar ratio was obtained as follows:

IGF-1:IGFBP-3 = [IGF-1(ng/ml)x0.130]/[IGFBP-3 (ng/ml) x 0.036] as suggested by Morimoto et al. [2005].

4.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences 13.0 (SPSS 13.0). Outcome measurements between different pubertal groups were compared by analysis of variance (ANOVA) and independent samples t-test. Spearman correlation coefficient was used to express bivariate relationships. In addition, partial correlation analysis corrected for age and pubertal stage was used. Stepwise multiple regression analysis was performed to determine the independent effect of the different body composition and biochemical variables on BMD. The level of significance was $p<0.05$.

5. RESULTS

5.1. Relationships between ghrelin concentration and anthropometrical, body composition parameters and testosterone concentration

The differences between groups, based on sexual maturation, were significant in age, height, body mass and LBM (Table 2 and Table 3). Group III had significantly higher BMI compared to other groups. Differences in body fat % and body mass were not significant during puberty compared with group I (Table 2 and Table 3).

Table 2. Mean values ($\overline{X}$ ± SD) of anthropometrical parameters in different study groups.

Group	**I (n=20)**	**II (n=20)**	**III (n=20)**	**Total (n=60)**
Age (y)	11.0±0.8[a]	13.4±0.8	16.0±1.9	13.4±2.5
Height (cm)	148.4±14.4[a]	165.3±8.9	178.7±6.3	164.1±14.4
Height SDS	0.1±0.9	0.3±1.0	0.5±0.8	0.3±0.9
Body mass (kg)	40.2±8.8[a]	53.1±9.6	70.7±12.0	54.7±16.0
Body mass SDS	0.5±1.6	0.3±1.1	0.9±0.9	0.5±1.2
BMI (kg/m^2)	18.1±3.0 [b]	19.3±2.0	22.0±2.0	19.8±3.1

[a] significant difference between groups I, II and III ($p< 0.001$)
[b] significant difference between group III and groups I and II ($p<0.001$)

Table 3. Mean values ($\overline{X}$ ± SD) of measured DXA parameters in different study groups.

Group	**I n=20**	**II n=20**	**III n=20**	**Total n=60**
Fat (%)	16.2±7.0	15.5±7.2	12.3±5.6	14.5±6.7
Fat mass (kg)	6.4±3.8	7.9±4.7	8.6±5.6	7.8±4.8
Lean Body Mass (kg)	30.1±4.6[a]	41.4±7.5	57.5±7.3	44.7±12.7
Total body BMD (g/cm^2)	0.940±0.005[b]	1.027±0.008	1.207±0.114	1.072±0.141
BMD L2-L4 (g/cm^2)	0.788±0.007[b]	0.898±0.150	1.252±0.201	0.989±0.250
BMAD L2-L4 (g/cm^3)	0.284±0.003[c]	0.293±0.004	0.378±0.005	0.320±0.005
BMC (g)	1550.9±274.8[a]	2152.6±414.7	3144.6±575.2	2368.0±792.4

BMD – bone mineral density; BMAD – volumetric BMD L2-L4; BMC – bone mineral content;
[a] significant difference between groups I, II and III ($p< 0.001$)
[b] significant difference between group I vs. groups II and III ($p<0.05$)
[c] significant difference between group III vs. groups I and II ($p<0.001$)

Ghrelin concentration in different study groups decreased significantly by the end of the puberty (Table 4). As expected, testosterone concentration increased significantly during puberty. Mean plasma leptin and insulin concentration were not different between the groups. FIRI was higher in group III compared to the group I.

Table 4. Mean values ($\overline{X}$ ± SD) of fasting state concentration of biochemical parameters.

Group	**I n=20**	**II n=20**	**III n=20**	**Total n=60**
Ghrelin (pg/mL)	1327.1±389.8[a]	964.9±321.0	829.9±265.0	1040.6±386.7
Leptin (ng/mL)	2.7±2.9	2.7±2.7	1.7±1.8	2.4±2.5
Insulin (mU/L)	8.9±5.0	11.8±7.0	11.3±4.7	10.7±5.7
Testosterone (nmol/L)	1.1±1.3[b]	10.2±5.9	19.8±4.0	10.4±8.7
IGF-1	163.2±47.0[a]	364.9±168.4	370.4±91.8	299.5±148.4
IGFBP-3	4.1±0.7[a]	5.1±0.8	5.4±0.6	4.9±1.0
IGF-1/ IGFBP-3 molar ratio	0.13±0.02[c]	0.22±0.08	0.21±0.04	0.19±0.07
Glucose (mmol/L)	4.5±0.6	4.8±0.7	4.9±0.4	4.7±0.6
FIRI	1.6±0.9[d]	2.3±1.4	2.2±1.0	2.0±1.1

FIRI – insulin resistance index

[a] significant difference between group I and groups II and III ($p<0.05$)

[b] significant difference between groups I, II and III ($p< 0.001$)

[c] significant difference between group I and groups II and III ($p<0.001$)

[d] significant difference between group I and group III ($p<0.05$)

Serum ghrelin concentration was inversely related to body height, body mass and LBM in pubertal group II as well as in the total group (Table 5). BMI correlated negatively with ghrelin concentration only in the total group. Body fat % and body mass did not correlate with blood ghrelin concentration in any of groups. There was a significant negative correlation between serum ghrelin and testosterone concentration in the group II ($r = –0.51$; $p<0.05$) as well as in the total group ($r = –0.59$; $p<0.001$) (Figure 1).

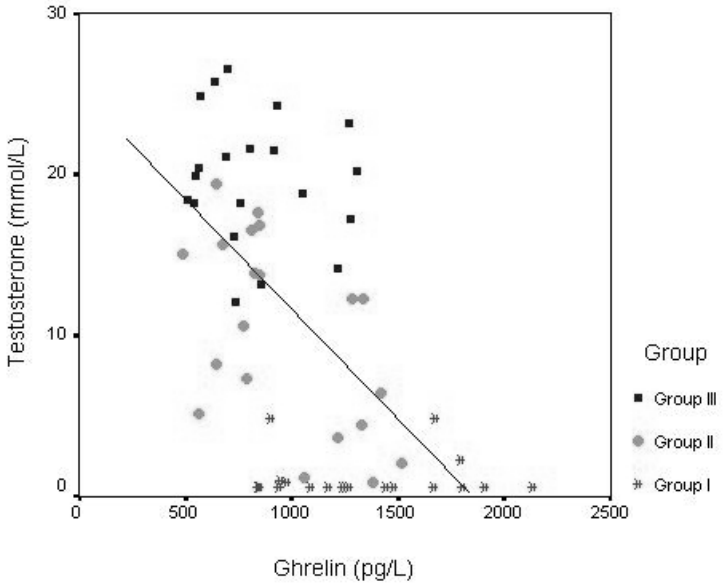

Figure 1. Relationships between fasting concentration of serum ghrelin and testosterone in total group ($r=-0.59$; $p<0.001$).

Serum ghrelin concentration correlated negatively also to plasma insulin concentration. Ghrelin concentration correlated significantly with FIRI in the total group. Blood leptin concentration correlated with body mass, BMI, body fat %, insulin and glucose concentration, and FIRI (Table 5). After controlling for age, all these relationships remained significant, except the correlation between ghrelin and glucose concentration.

The results of stepwise multiple regression analysis where ghrelin concentration was dependent variable and independent variables were separately age with anthropometrical data (body height, mass and BMI), age with body composition data (fat %, fat mass and LBM) and age with biochemical parameters (glucose, insulin and testosterone concentration) are presented in Table 6. From the anthropometrical data, the body height and from the body composition data, the LBM, were the two most important determinants of the ghrelin concentration variability (Table 6). From the biochemical data, testosterone concentration explained 33.7% (R^2x100) of the ghrelin concentration variability in the total group.

Table 5. Correlation coefficients of the relationships between serum ghrelin and leptin concentration and anthropometrical, body composition and biochemical data. Correlation coefficients of partial correlation after controlling for age are shown in brackets.

	Ghrelin				Leptin			
Group	I (n=20)	II (n=20)	III (n=20)	Total (n=60)	I (n=20)	II (n=20)	III (n=20)	Total (n=60)
Age	0.31	0.25	0.05	–0.40**	–0.24	–0.19	–0.04	–0.18
Height	–0.09 (–0.22)	–0.63** (–0.62**)	–0.25 (–0.43)	–0.63*** (–0.56***)	0.46* (0.31)	–0.00 (0.07)	–0.09 (0.06)	–0.04 (0.07)
Height SDS	–0.28	–0.68***	–0.29	–0.43***	0.54*	0.11	–0.00	0.21
Body mass	–0.21 (–0.05)	–0.58** (–0.56*)	–0.16 (–0.34)	–0.60*** (–0.43**)	0.74*** (0.49)	0.28 (0.42)	0.45* (0.68**)	0.15 (0.48***)
Body mass SDS	–0.25	–0.61**	–0.36	–0.35**	0.82***	0.40	0.69***	0.60***
BMI	–0.14 (0.01)	–0.36 (–0.34)	–0.20 (–0.22)	–0.45*** (–0.22)	0.77*** (0.57*)	0.34 (0.67**)	0.61** (0.69**)	0.42*** (0.62***)
LBM	–0.06 (–0.04)	–0.71*** (–0.67**)	–0.12 (–0.30)	–0.62*** (–0.48***)	0.45 (0.33)	–0.07 (0.03)	0.24 (0.33)	–0.18 (0.11)
Fat %	–0.19 (–0.13)	0.17 (0.17)	–0.21 (–0.31)	0.09 (–0.03)	0.68* (0.70**)	0.85*** (0.88***)	0.66** (0.83***)	0.80*** (0.85***)
Fat mass	–0.32 (–0.08)	–0.09 (–0.07)	–0.25 (–0.30)	–0.24 (–0.14)	0.84*** (0.63**)	0.73*** (0.90***)	0.63** (0.87***)	0.65*** (0.85***)
Leptin	–0.32 (–0.46)	0.19 (–0.02)	–0.06 (–0.20)	0.03 (–0.21)	–	–	–	–
Insulin	–0.23 (–0.32)	–0.34 (0.32)	–0.32 (–0.04)	–0.34** (–0.30*)	0.25 (0.50)	–0.34 (0.30)	0.68*** (0.60**)	0.40** (0.45***)
Testoste-rone	–0.10 (–0.05)	–0.51* (–0.65**)	–0.12 (–0.14)	–0.59*** (–0.48***)	–0.15 (–0.30)	–0.51* (0.01)	–0.01 (–0.02)	–0.13 (–0.06)
Glucose	0.24 (0.08)	0.18 (0.35)	–0.03 (0.05)	–0.01 (0.09)	0.25 (–0.03)	0.18 (0.36)	0.24 (0.11)	0.32** (0.14)
FIRI	–0.13 (–0.33)	–0.23 (–0.24)	–0.00 (–0.04)	0.27* (–0.28*)	0.44 (0.55*)	0.23* (0.42)	0.64** (0.57*)	0.45*** (0.49***)

Statistical significance is shown by: * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$

Table 6. Stepwise multiple regression analysis where blood ghrelin concentration was dependent variable and age with anthropometrical data (body height, mass and BMI), age with body composition data (fat %, fat mass and LBM) and age with biochemical data (glucose, insulin, testosterone concentration) were independent variables.

Group	Independent variable	R^2 x 100	F	p
Age and anthropometry				
I	–	–	–	–
II	Body height	34.0	9.3	0.007
III	–	–	–	–
Total	Body height	38.1	35.70	< 0.001
Age and body composition				
I	–	–	–	–
II	LBM	41.7	12.2	0.003
III	–	–	–	–
Total	LBM	36.1	28.3	<0.001
Age and biochemical data				
I	–	–	–	–
II	Testosterone	31.9	8.4	0.009
III	–	–	–	–
Total	Testosterone	33.7	29.5	<0.001

5.2. The influence of serum ghrelin, IGF-axis and testosterone concentration on bone mineral density

The analysis showed that total BMD, lumbar BMD and BMAD all increased along the pubertal groups (Table 3). The mean serum ghrelin concentration decreased along the study groups. As expected, mean serum testosterone, IGF-I and IGFBP-3 concentration increased along the study groups. Mean serum IGF-I, IGFBP-3 concentrations and IGF-I/IGFBP-3 molar ratio were significantly lower in group I compared to the other groups (Table 4).

In the total group total body BMD was significantly related to serum ghrelin (r=–0.47), testosterone (r=0.73), IGF-1 (r=0.51) and IGFBP-3 (r=0.50) concentration ($p<0.001$ in all cases) (Table 7). However, the significance of these relationships was lost after adjustment for age. The relationships between lumbar BMD and BMAD and the concentrations of ghrelin, testosterone, IGF-1 and IGFBP-3 were similar to the associations seen in total body BMD (Table 7). IGF-1/IGFBP-3 molar ratio was strongly correlated with testosterone (r=0.76; $p<0.001$) and ghrelin concentration (r=–0.61; $p<0.001$), and also with total BMD (r=0.49; $p<0.001$), lumbar BMD (r=0.56; $p<0.001$), BMAD (r=0.39; $p<0.01$) and total BMC (r=0.63; $p<0.001$). These relationships remained significant after adjustment for age for total BMC, ghrelin and testosterone concentration but not for BMD parameters.

Table 7. Relationships between total BMD and lumbar apparent BMD (BMAD L2-L4), anthropometrical data and biochemical parameters.

	Total BMD				**BMD L2-L4**				**BMAD L2-L4**			
Group	I n=20	II n=20	III n=20	Total n=60	I n=20	II n=20	III n=20	Total n=60	I n=20	II n=20	III n=20	Total n=60
Height	0.66*	0.38	0.63**	0.84***	0.44	0.41	0.55*	0.82***	–0.38	0.25	0.56**	0.65***
Body mass	0.54*	0.39	0.91***	0.87***	0.19	0.50*	0.89***	0.82***	–0.56*	0.36	0.79***	0.65***
Ghrelin	0.13	–0.24	–0.02	–0.47***	0.29	–0.25	0.07	–0.44**	–0.38	–0.13	0.06	–0.34**
Testosterone	0.24	0.19	0.08	0.73***	0.27	0.30	0.10	0.76***	0.02	0.15	0.27	0.64***
IGF-I	0.21	0.25	–0.31	0.51***	0.26	0.43	–0.17	0.58***	–0.05	0.27	–0.09	0.41**
IGFBP-3	0.16	0.20	–0.16	0.50***	0.12	0.38	–0.09	0.56***	–0.04	0.26	–0.09	0.43***
IGF-I/IGFBP-3 ratio	0.27	0.30	–0.27	0.49***	0.28	0.47*	–0.13	0.56***	–0.01	0.33	–0.04	0.39***

Statistical significance is shown by: * $p<0.05$, ** $p<0.01$ *** $p<0.001$

Correlation analysis within different study groups showed significant relationships only in group II, where lumbar BMD was related to IGF-1/IGFBP-3 molar ratio (r=0.47) and total body BMC was correlated with ghrelin (r=–0.50), testosterone (r=0.50), IGF-1 (r=0.62) and IGF-1/IGFBP-3 (r=0.64) concentration. After adjusting these parameters for age, the relationship remained significant only for testosterone, IGF-1 and IGF-I/IGFBP-3 concentration molar ratio.

In stepwise multiple regression analysis was weight the most significant predictor of bone mineral characteristics explaining 45.3–77.5% of variability in total group and 19.3–69.1% of variability in smaller study groups (see Table 8). When the influence of age, weight and height was excluded serum testosterone concentration was the most important predictor of BMD in the total group explaining 48.8% of the variability in total body BMD, 51.4% in lumbar BMD and 36.8% in lumbar BMAD (see Table 8). Within the different study groups stepwise regression analysis revealed a significant association only in group II where serum IGF-1 concentration explained 16.7% of the variability in lumbar BMD (see Table 8).

Table 8. The results of stepwise multiple regression analysis where total body mineral density, lumbar mineral density and volumetric mineral density were dependent variables and age, body weight, body height and hormonal data were independent variables (R^2x100).

Group		**I**	**II**	**III**	**Total**
Total BMD	Body weight	39.7	19.3	69.1	77.5
	Testosterone*	–	–	–	48.8
BMD L2-L4	Body weight	–	27.1	62.4	71.7
	Testosterone*	–	–	–	51.4
	IGF-1*	–	16.7	–	–
BMAD L2-L4	Body weight	25.0	–	42.9	45.3
	Testosterone*	–	–	–	36.8

* after excluding the influence of age, body weight and body height

5.3. Ghrelin, leptin and GH-IGF-axis concentration response to acute aerobic exercise

The results of exercise testing (VO_{2max}, HR) in different groups are shown in Table 9.

Table 9. Mean values ($\overline{X}$ ± SD) of exercise testing parameters in different study groups.

Group	I (n=20)	II (n=20)	III (n=20)	Total (n=60)
VO_2max (L·min^{-1})	2.10±0.40[a]	2.29±0.70	3.40±0.60	2.40±1.00
VO_2max (mL·kg^{-1}·min^{-1})	44.8±10.5	42.2±7.9	48.3±5.8	43.9±9.8
HR_{max} (bpm) during VO_2max test	199±6	196±5	195±4	196±8
Mean HR (bpm) during 30' test	174±5[b]	168±6.0	165±4	169±6

[a] significant difference between results of group I and III ($p<0.01$)

[b] significant difference between results of group I with groups II ($p<0.01$) and III ($p<0.001$) and between groups II and III ($p<0.05$)

Before testing, serum ghrelin concentration was in group I significantly higher and serum testosterone concentration significantly lower compared to the groups II and III ($p<0.001$) (Table 10). Ghrelin, leptin and testosterone concentration did not change significantly by acute exercise. Insulin concentration decreased significantly after exercise testing in groups II and III and in total group. The increase in insulin concentration during 30 minute recovery was significant in group III and total group. Ghrelin concentration increased more than 10% in 13 and decreased more than 10% in 25 children after exercise, but mean changes were statistically not significant.

Table 10. Hormone concentrations in blood before exercise testing, right after and after 30 minutes of recovery.

	Group	**Before**	**After**	**Recovery 30'**
Ghrelin (pg/mL)	I	1374.3±404.6	1294.3±465.4	1297.8±432.4
	II	928.6±301.7	927.2±310.5	913.3±333.2
	III	779.8±323.7	780.5±343.3	828.6±349.1
	Total	1005.5±418.8	981.6±424.6	995.8±415.5
Leptin (ng/mL)	I	2.9±3.4	3.1±3.9	2.9±3.7
	II	2.1±2.9	2.3±3.1	1.9±2.7
	III	1.4±2.0	1.5±2.0	1.4±1.9
	Total	2.1±2.8	2.2±3.1	2.1±2.8

	Group	Before	After	Recovery 30'
Insulin (mU/L)	I	12.2±11.5	6.1±3.7	9.7±7.6
	II	22.8±26.2[a]	8.3±8.4	20.4±26.4
	III	28.9±17.2[b]	7.5±4.6	18.2±17.8
	Total	21.9±20.3[b]	7.4±5.9	16.3±19.2
Testosterone (nmol/L)	I	0.6±0.1	0.7±0.2	0.7±0.1
	II	4.1±3.2	5.6±5.1	3.9±3.6
	III	11.8±14.3	14.7±6.2	11.7±5.4
	Total	5.8±5.7	7.4±7.5	5.7±6.0
GH (mU/L)	I	5.1±7.7[b]	20.7±15.4	7.2±6.2
	II	8.1±13.4[c]	49.9±24.7	26.2±22.4
	III	5.9±22.4[c]	68.2±34.2	37.4±22.2
	Total	6.4±16.0[c]	48.0±32.6	24.7±22.4
IGF-I (µg/L)	I	160.0±63.5	157.1±62.5	148.5±50.5
	II	310.4±136.0	332.8±143.5	308.6±133.4
	III	337.9±83.3	372.1±92.6	337.4±85.6
	Total	276.0±124.4	295.3±138.8	271.1±125.2
IGFBP-3 (mg/L)	I	4.1±0.7	4.1±0.7	4.1±0.7
	II	5.1±0.8	5.1±1.0	5.0±0.9
	III	5.2±0.7	5.6±0.7	5.2±0.7
	Total	4.9±0.9	5.0±1.0	4.8±0.9

[a] significant difference ($p<0.05$) in group between results measured before and after testing

[b] significant difference ($p<0.05$) in group between results measured before and after testing and after testing and recovery

[c] significant differences ($p<0.01$) in results measured pre-testing, post-testing and post-30

The mean baseline concentrations of GH concentration were significantly ($p<0.05$) higher in group II (pubertal stages 2 and 3) compared to the group I (prepubertal) or group III (pubertal stages 4 and 5) (Table 10). As expected, serum IGF-I, IGFBP-3 and testosterone concentration increased according to the pubertal development (Table 10). Acute exercise did not affect significantly IGF-I or IGFBP-3 concentration in any study groups (Table 10). The concentrations of testosterone, IGF-1 and IGFBP-3 in the total group were slightly elevated immediately after acute exercise, but recovered with 30 minutes to the initial level.

GH concentration increased during acute exercise in all groups and was highest in group III (62.3±41.7 mU/L) compared to the mean increment of 41.8±20.0 mU/l in group II and 15.5±11.4 mU/l in group I (group I vs group III $p<0.001$). GH levels remained elevated in recovery phase in all groups except in group I. The increment in GH concentration during exercise was positively correlated ($r=0.64$; $p<0.001$) to basal testosterone concentration (Figure 2).

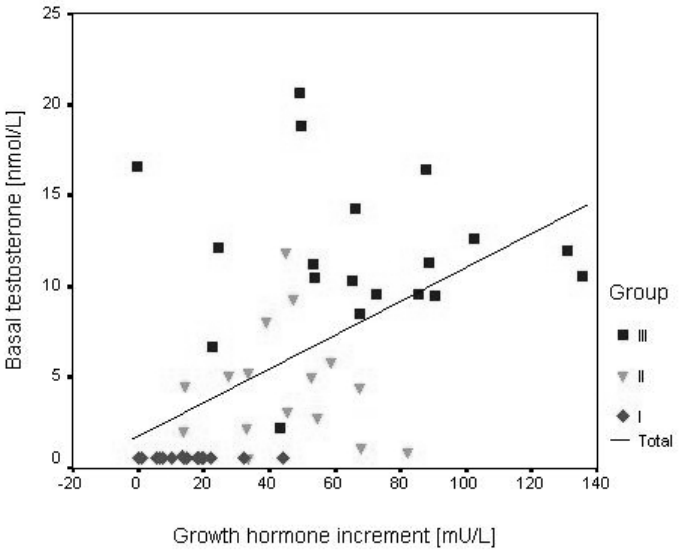

Figure 2. The relationships between increment in growth hormone and basal testosterone concentration.

The level of VO_{2max} was negatively correlated to ghrelin concentration measured before ergometer test (r=–0.61) and positively to testosterone (r=0.90) and insulin concentration (r=0.52) in the total group (all $p<0.001$). The correlations in total group between the relative level of maximal oxygen consumption (VO_{2max}/kg) and the concentration of the same metabolic characteristics at baseline were also significant – with ghrelin r=–0.35 ($p<0.05$), testosterone r=0.60 ($p<0.001$) and insulin r=0.34 ($p<0.05$). In pubertal boys the level of VO_{2max}/kg was positively correlated to level testosterone concentration (r=0.39; $p<0.05$). Additionally, in group II, the level of VO_{2max} was negatively correlated with ghrelin concentration (r=–0.57; $p<0.05$).

Negative correlation was found in total group between basal leptin concentration and the level of VO_{2max}/kg (r=–0.32; $p<0.05$). Stepwise multiple regression analysis showed that from age, body height, body mass, testosterone and ghrelin concentration, serum testosterone concentration was the most important determinant of VO_{2max}/kg level, explaining 32% (R^2x100) of the variability.

6. DISCUSSION

6.1. Relationships between ghrelin concentration and anthropometrical, body composition parameters and testosterone concentration

The results of this study demonstrated that body height from the anthropometrical parameters, LBM from body composition parameters and serum testosterone concentration are the main predictors of blood ghrelin concentration in boys at different stages of puberty. As expected, there were significant differences between study groups in body height, body mass, LMB and testosterone levels, indicating normal development of our subjects.

Serum ghrelin concentration declined with advancing pubertal stage. The mean ghrelin concentration in group III (Tanner stages 4 and 5) was about 40% lower than in prepubertal group I. This is similar to the previous studies by Whatmore et al. [2003], Soriano-Guillen et al. [2004] and by Chanoine et al. [2005], who also reported 30 to 50% fall in ghrelin concentration during puberty. In the total group, the pubertal stage was more important determinant of ghrelin concentration than chronological age. A negative correlation between ghrelin concentration and age is similar to studies by Whatmore et al. [2003] and Soriano-Guillen et al. [2004].

It has been shown previously that ghrelin concentrations decrease during puberty with advancing pubertal stage [Whatmore 2003; Chanoine et al. 2005; Vivenza et al. 2004], but none of these studies have looked the relationship between testosterone and ghrelin concentration. To our knowledge this is the first study showing the direct correlation between serum testosterone and ghrelin concentration in normal healthy boys around puberty. Recent study by Lebenthal [2006] showed that testosterone administration was associated with a marked decline in circulating levels of ghrelin in prepubetal boys, but not girls. However, a study by Pagotto et al. [2003] in 7 adult hypogonadal men on testosterone replacement therapy showed a positive correlation between ghrelin and testosterone concentration. This difference between those studies indicates that the effect of testosterone administration on ghrelin levels may be also age dependent. This may explain why the correlation between ghrelin and testosterone concentration in our study was significant only in the group II i.e. at the beginning of puberty. The expression of ghrelin and its receptor has been shown in Leydig cells of the testis both in rats and humans [Tena-Sempere 2005]. There are some animal data indicating that persistently elevated ghrelin levels, as a putative signal for energy insufficiency, may operate as a negative modifier of puberty onset in male rats [Fernandez-Fernadez et al. 2005]. The increase of testosterone levels at the beginning of puberty stimulates GH and IGF-I secretion and thus via negative feedback may suppress the ghrelin

secretion. However, there are not data supporting the negative feedback mechanism between ghrelin and GH or IGF-I.

All anthropometrical parameters correlated highly with ghrelin concentration in total group (except for BMI after controlling for age) and group II (except for BMI). Significant relationships between ghrelin and body height, weight and BMI have been reported by Tschöp et al. [2001] and Park et al. [2005]. Not only absolute values of height and weight, but also their standard deviation scores (SDS) were correlated to the ghrelin levels. We did not have the growth data, but the average age of 13.4 years in the group II suggests that boys in this subgroup were in the phase of rapid growth. From the Tanner and Whitehouse [1976] growth velocity curves we could estimate their average growth rate of 8.5 cm/year comparison of 5.0 cm/year (mean growth velocity at the age of 11.0 years) in subgroup I or 2.5 cm (mean growth velocity at the age of 16.0 years) in group III. The rapid growth and huge variability in growth rate in group II explains why majority of correlations were significant only in this subgroup, and not group I or III.

During puberty, with rapid increase in body height and body mass, body fat % as a rule decreases. In our study the decrease in body fat % was not significant. Many studies have found a negative correlation between body fat % and ghrelin [Bunt et al. 2003; Park et al. 2005; Tschöp et al. 2001], but our study did not find such correlation. Surprisingly, from the body composition data the most important parameter for ghrelin concentration was LBM (Table 5). This is a new finding that has been not previously observed and the mechanism underlying the observed association is unknown. One possible explanation may be through increasing testosterone levels which is known to enhance both muscle and bone mass [Mauras et al. 1996], the major components of LBM.

Studies on the effects of leptin on circulating ghrelin concentration in humans have produced conflicting results [Klok et al. 2007]. On one hand, it is possible that ghrelin concentration is inversely regulated by leptin concentration [Šhintani et al. 2001], but there are also data which suggest that leptin has no control over ghrelin [Haqq et al. 2003]. The results of our study did not indicate a significant relationship between concentrations of ghrelin and leptin, similar to the study by Haqq et al [2003]. Therefore we would agree with the theory that leptin has no direct regulatory control on ghrelin secretion.

In conclusion, body height, LBM and testosterone concentration are the major determinants of serum ghrelin concentration. Negative correlation between serum ghrelin and testosterone concentrations indicates that ghrelin may also have a role in male pubertal development. Longitudinal studies through puberty are needed to elucidate the physiological interaction between sex hormones and ghrelin.

6.2. The influence of serum ghrelin, IGF-axis and testosterone concentration on bone mineral density

Our study showed that the major biochemical determinants of bone mineral parameters (whole body BMD, lumbar BMD and BMAD) were testosterone and also IGF-1 concentration, whereas body ghrelin concentration had not as important role in boys at different stages of puberty.

The results of our study showed an increase in BMD and BMC of total body and lumbar spine with advancing age and pubertal stage as seen in previous studies [Yilmaz et al. 2005; Lu et al. 1996; Zanchetta et al. 1995; Boot et al. 1997]. In group I mean lumbar BMD was 16.7% lower than the total BMD, whereas in group III the latter was 3.3% higher than the lumbar BMD indicating more rapid increment of BMD in trabecular bone compared to the cortical. The data about dynamics of volumetric BMD during puberty have so far been controversial [Boot et al. 1997]. Our study demonstrated significantly higher BMAD in group III (Tanner stages 4 and 5) compared to the younger pubertal groups. This is similar to the study by Boot et al. [1997] where BMAD increased first also significantly at the Tanner stage 4 and not earlier.

Serum testosterone concentration increased throughout pubertal stages and was positively correlated to all three BMD parameters (total BMD, lumbar BMD and BMAD). Serum testosterone concentration explained half of the variability in total body and lumbar BMD. It remains unclear whether this association is due to the effect of testosterone itself or due to estrogens aromatised from testosterone. Male patients with either estrogen receptor deficiency or aromatase deficiency have reduced BMD and absence of a pubertal growth spurt [Smith et al. 1994; Morishima et al. 1995]. Our strong link between serum testosterone levels and BMD parameters, however, indicates that testosterone concentration is an important determinant of bone mineralization in boys.

Serum ghrelin concentration was inversely related to all three BMD parameters, but those correlations were not as strong as seen between BMD and testosterone or IGF markers. Our previous results showed significant negative correlation ($r= -0.59$) between serum ghrelin and testosterone concentration [Study I]. Thus, not surprisingly, in multiple regression analysis ghrelin concentration was an important determinant of BMD parameters only after removal of testosterone concentration, explaining 15.6, 14.3 and 6% of the variability on total BMD, lumbar BMD and BMAD in total group, respectively. Based on these results we suggest that the role of ghrelin concentration in bone development is largely mediated through testosterone concentration and explains independently only a small fraction of the variability in bone mineral parameters. Our results in boys differ from those seen in adolescent girls in whom ghrelin secretion was a strong predictor of BMD [Misra et al. 2005]. In that study total ghrelin secretion over 12 h period was measured in 18 adolescent girls (mean age 15.4±1.8 years) who were mostly at pubertal stages 4

and 5 (only 3 girls were premenarcheal). There was a significant correlation between ghrelin total secretion and lumbar BMAD [Misra et al. 2005], whereas no such correlation was seen in our study group III. One explanation for this discrepancy could be technical: it is obvious, that total ghrelin secretion over 12-h period reflects more accurately the role of ghrelin in the body than just the concentration in a single fasting blood sample. However, there are data indicating that the impact of ghrelin concentration on metabolism differs between boys and girls [Park et al. 2005]. It is also known that the decrease in ghrelin concentration with advancing pubertal stage or age is more marked in boys than girls [Whatmore et al. 2003]. Therefore in boys ghrelin concentration is more age and puberty dependent. In Study I we found a significant negative correlation ($r= –0.59$; $p<0.001$) between serum ghrelin and testosterone concentration in boys at pubertal stages 4 and 5, therefore, it is likely that the impact of ghrelin on metabolism, including that on bones, in boys is more sex hormone dependent than in girls.

As previously shown [Kanbur-Oksuz et al. 2005; Juul et al. 1994], serum IGF-1 and IGFBP-3 concentration increased with advancing puberty also in our study, whereas the prepubertal group had significantly ($p<0.05$) lower levels. The IGF-1 and IGFBP-3 concentration were related to all BMD parameters and BMC in the total group, but within the study groups the relationship was significant only between IGF-1 and BMC in group II ($r=0.62$; $p<0.01$). The role of IGF-1 concentration in bone mass accumulation has been shown to be particularly important during pubertal growth spurt [Kanbur-Oksuz et al. 2005], which usually occurs around the age of 14 years i.e. close to the mean age of 13.4 in group II. In this group we also find a correlation between IGF-1/IGFBP-3 molar ratio and BMC. In blood circulation IGF-I is bound to its binding proteins, mostly IGFBP-3. Biologically active is free IGF-I, but it is difficult to measure. Therefore IGF-1/IGFBP-3 molar ratio has been suggested as an indirect indicator of serum free IGF-I. Thus our findings support the concept that the role of IGF-I in bone mineralization in boys is particularly important around the age of 13–14 years when the growth velocity should reach the peak.

We conclude that serum testosterone concentration and serum IGF-1/IGFBP-3 molar ratio are the major determinants of bone mineral density in boys at different pubertal stages. Serum ghrelin concentration did not appear to have a direct independent affect on BMD. If present, the association may be mediated through the concentration of sex hormones and GH-IGF-I axis.

6.3. Ghrelin, leptin and GH-IGF-axis concentration response to acute aerobic exercise

In this study we assessed the effect of 30-minute cycle ergometer test to serum ghrelin and leptin, testosterone and insulin concentration at the intensity slightly lower than IVT in boys at different pubertal stages. This moderate acute exercise did not change serum levels of ghrelin or leptin in any pubertal group. This is similar to the findings from different studies in adults [Wallace et al. 1999; Dall et al. 2002; Schmidt et al. 2004]. The results of our study also showed that acute aerobic exercise did not alter the concentration of testosterone, IGF-I or IGFBP-3 in none of the studied groups. Acute exercise increased serum GH concentration in all pubertal groups and the increment was highly testosterone dependent. We found that the level of VO_{2max} was related to basal serum ghrelin, leptin and testosterone concentration.

Ghrelin and leptin both are directly linked to energy expenditure [Wilding 2002]. Ghrelin and leptin concentration have been found to be related in different studies with children [Whatmore et al. 2003]. In contrast, no relationships between ghrelin and leptin concentration have been found in other studies [Haqq et al. 2003]. Basal levels of ghrelin and leptin concentration were not related in our study groups. Therefore our results support the possibility that ghrelin and leptin act independently in boys with different maturational status.

Different studies in adults have found no changes in ghrelin concentration in blood after acute exercise [Dall et al. 2002; Kraemer et al. 2004]. Although we were expecting because of higher initial level of ghrelin in blood during puberty [Whatmore et al. 2003: Dall et al. 2002], that acute exercise will alter ghrelin concentration when measured immediately after testing or in recovery phase, no changes were found. It is now widely accepted that fit adults, such as marathon athletes, display significantly diminished serum leptin concentrations during recovery phase after moderate intensity aerobic exercise [Leal-Cerro et al. 1998]. There have been only very few studies investigating plasma leptin concentration response to physical exercise in children. The study of Souza et al. [2004] showed no changes in leptin concentrations in prepubertal children, similar to our findings (see Table 10). One possible explanation why we did not see any significant change in leptin or ghrelin, and also in testosterone concentrations may be that the energy expenditure and the intensity during our 30-minute exercise test were not high enough to elicit significant changes in blood. Also, we assume that maybe ghrelin and leptin respond to acute exercise slower than for example insulin. Therefore it would be interesting to know the variations in ghrelin and leptin concentrations in blood at different stages of puberty during more intense exercise or longer recovery period.

Insulin concentration response to exercise has been shown to be dependent on pubertal stage. Specifically, Wirth et al. [1978] showed that steady 15-minutes cycle ergometer test at 70% the level of VO_{2max} increased insulin

concentration in prepubertal, kept stable in mid-pubertal and decreased in postpubertal group. In our study, exercise did not induce significant changes only in prepubertal group, in other groups was insulin concentration elevated when measured immediately after testing. In or study insulin concentration decreased immediately after exercise similar to the study by Oseid and Hermansen [1971].

Basal serum ghrelin concentration in our study was negatively correlated with VO_{2max} in total group as well as in group II. Contrary, St Pierre et al. [2004] did not find significant relationship between VO_{2max} and ghrelin in young healthy women. These results indicate that different sex hormones may have different impact on the interaction between ghrelin and the level of VO_{2max}, and also on physical exercise. However, since little is known about the relationship between ghrelin and testosterone concentration in children [Lebenthal et al. 2006] and the physiological role of ghrelin to the adaptation to the exercise has not yet been clarified also, so further investigations are still needed.

The mechanisms involved in provoking GH secretion remain controversial since different studies have used various exercise durations and intensities; additionally influence the results also individual hormonal and nutritional status of the subjects [Jenkins 1999]. It is assumed that adrenergic mechanism plays a role in these relationships, since exercise-induced GH release can be enhanced by β-receptor antagonists and diminished by an α-receptor antagonist. In our study the biggest increment in GH concentration after exercise was highest in group III. Roemmich et al. [2005] showed that exercise-induced GH secretion was greatest during adolescent growth spurt. By Marshall and Tanner [1970] the peak height velocity in boys corresponds to the beginning of pubic hair stage 4 i.e. for the boys in group III in our study. We also found that the increment in GH level during exercise was strongly related to basal testosterone concentration ($r=0.64$; $p<0.001$). The GH increment was the highest in group III.

Plasma IGF-1 concentration response to physical exercise has not been demonstrated consistently – some researchers have observed increments, while others showed in adult subjects no change [Di Luigi et al. 1997; Nguyen et al. 1998; Wallace et al. 1999]. Although IGF-1 concentration is known to mediate GH action, their reaction to exercise has not been confirmed to be always similar [Roemmich 2005]. Controversial results are also presented in the changes of IGFBP-3 concentration to the physical exercise [Cohich and Clemmons 1993; Schwartz et al. 1996]. In our study, exercise did not change either IGF-1 or IGFBP-3 concentration in boys at any pubertal stages (see Table 9). Since negative energy balance plays a major role in the IGF-1 concentration and related growth mediators response to exercise [Nemet et al. 2004], it can be suggested that in our study the energy expenditure during exercise was not sufficient for significant changes, although the intensity was high enough to elicit changes in GH concentration. Acute exercise above the certain intensity

level is one of the most potent stimulators of GH secretion. It has also been shown that the magnitude of the GH concentration response is closely related to the peak intensity, rather than total work output [Ehrnborg et al. 2003].

In our study, probably one of the limitations was the fact that we did not study our children at the fasting state, because ghrelin concentration is very sensitive to the food intake [Cummings et al. 2001; Wren et al. 2000; Chanoine 2005]. It is well known that feeding suppresses ghrelin production and fasting stimulates ghrelin release, however, the underlying mechanisms controlling these processes remain unclear [Tschöp et al. 2000].

In summary, this study demonstrated that acute ergometer exercise at moderate intensity level did not change blood ghrelin or leptin concentrations in boys at different pubertal stages. We also conclude that GH concentration response to exercise in boys at different pubertal stages was directly dependent on serum testosterone concentration. Acute exercise did not affect significantly serum testosterone, IGF-I, IGFBP-3 concentration.

7. CONCLUSIONS

In conclusion:

1. Body height, LBM and serum testosterone concentration are the major determinants of serum ghrelin concentration among anthropometrical and biochemical parameters studied. Negative correlation between serum ghrelin and testosterone concentrations indicates that ghrelin concentration may also have a role in male pubertal development.

2. Serum testosterone concentration and serum IGF-1/IGFBP-3 molar ratio are the major determinants of bone mineral density in boys at different pubertal stages. Serum ghrelin concentration did not appear to have a direct independent effect on BMD. If present, the association may be mediated through the concentration of sex hormones and the GH-IGF-I axis.

3. Moderate acute aerobic exercise does not change serum ghrelin or leptin concentration in boys at different pubertal stages. The increment in GH concentration during exercise is dependent on testosterone concentration.

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SUMMARY IN ESTONIAN

Greliini kontsentratsioon erinevas suguküpsusastmes poistel: seosed kasvufaktorite kontsentratsiooni, luutiheduse ja kehalise aktiivsusega

Sissejuhatus

Greliin on organismis seotud paljude endokriinsete ja mitteendokriinsete funktsioonidega. Muuhulgas seostatakse greliini kasvuhormooni sekretsiooni, söögiisu tekkimise ja energia tasakaaluga. Järjest enam räägitakse kehakaalu lühi- ja pikaajalise regulatsiooni puhul greliini kontsentratsioonist kui võtmefaktorist söögiisu ja energiakulu kompleksse süsteemi keskmes. Täiendavate andmete kogumine greliini kontsentratsiooni seostest teiste hormoonide kontsentratsiooni ja erinevate kehakoostise parameetritega ning dünaamikast erinevates olukordades annab väärtuslikku teavet, mille põhjal on võimalik paremini mõista kogu energiatasakaalu süsteemi.

Uurimustöö eesmärk ja ülesanded

Käesoleva töö eesmärgiks oli täpsustada greliini võimalikku rolli kasvamises ja puberteedis erinevates bioloogilises arenguastmetes poistel ning samuti selgitada akuutse kehalise aktiivsuse mõju neile protsessidele.

Käesoleva töö ülesanneteks oli:

– uurida erinevate antropomeetriliste ja kehakoostise karakteristikute ja erinevate hormoonide mõju greliini kontsentratsioonile veres
– uurida greliini, IGF-I, IGFBP-3 ja testosteroonitaseme mõju luutihedusele
– uurida muutusi greliini- ja leptiinitasemes 30 minuti pikkuse veloergomeetritesti (koormus ~95% individuaalsest anaeroobsest lävest) kestel ja järgselt.

Uuritavad ja metoodika

Uuringus osales 60 tervet normaalkaalus poissi Tartust ja Lähtelt vanuses 10–18 eluaastat. Uuritavad jagunesid Tanneri suguküpsuse skaala põhjal kolme 20-liikmelisse uuringugruppi: grupp I (puberteedieelsed), grupp II (suguküpsusastmed II ja III) ja grupp III (astmed IV ja V). Vaatlusalustel mõõdeti kehapikkus ja kehakaal, lisaks kalkuleeriti kehamassiindeks ning kehapikkuse ja kehakaalu standardhälbe skoorid. Kehakoostise parameetrid (keha rasvamass ja -protsent, üldine ja lumbaarpiirkonna L2-L4 luutihedus) määrati DXA meetodil.

Uuritavad läbisid järgmised testid:

1. hommikune puhkeoleku vereproov kella 8 ja 10 vahel, milles määrati greliini, leptiini, testosterooni, IGF-I, IGFBP-3 ja insuliini kontsentratsioon;
2. maksimaalne hapnikutarbimise veloergomeetri test kasvavate koormustega suutlikkuseni;

3. 2–3 päeva peale esimest testi viidi läbi 30 minuti pikkune velotest koormusel ~95% individuaalsest anaeroobsest lävest. Vahetult enne, vahetult peale ja 30 minutit peale testi lõppu määrati uuritavate vere biokeemiliste näitajate kontsentratsioon (greliin, leptiin, testosteroon, kasvuhormoon, IGF-I, IGFBP-3 ja insuliin).

Järeldused

1. Uuritud antropomeetriliste ja biokeemiliste parameetrite hulgas on peamised greliini kontsentratsiooni determinandid kehapikkus, rasvavaba mass ja testosterooni kontsentratsioon. Greliini ja testosterooni kontsentratsiooni vaheline negatiivne seos viitab greliini võimalikule rollile meeste puberteedis.
2. Testosterooni kontsentratsioon ja IGF-I/IGFBP-3 molaarsuhe on mõõdetud karakteristikute hulgas erinevas suguküpsusastmes poiste luutiheduse peamised determinandid. Greliini kontsentratsiooni otsest mõju luutihedusele ei ilmnenud. Võimalik seos võib esineda läbi suguhormoonide ja kasvuhormooni-IGF-I-telje.
3. Mõõdukas akuutne aeroobne koormus ei muuda greliini ega leptiini kontsentratsiooni erineval suguküpsusastmel poistel. Kasvuhormooni kontsentratsiooni tõus on seotud testosterooni kontsentratsiooniga veres.

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PUBLICATIONS

I

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Relationship between ghrelin and anthropometrical, body composition parameters and testosterone levels in boys at different stages of puberty. *Journal of Endocrinological Investigation*, 2006, 29, 962–967.

II

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III

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Ghrelin response to acute aerobic exercise in boys at different stages of puberty.
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