Abstract

Objective: IGF1 plays an important role in growth and metabolism during puberty. IGF1 levels are increased in girls with central precocious puberty (CPP). However, the relationship with insulin before and during gonadal suppression is unknown. In addition, the influence of the exon 3-deleted GH receptor gene (GHRd3) on IGF1 levels was evaluated.

Design: Nine hundred and eleven healthy and 23 early pubertal girls (15 with CPP) participated and were evaluated by dual-energy X-ray absorptiometry (DXA) scans, fasting and oral glucose-stimulated insulin levels, IGF1 levels, and GHR genotyping. Fifteen girls with early puberty (13 with CPP) were treated with GNRH agonists and reevaluated after 3 and 12 months.

Results: IGF1 and insulin levels were higher in girls with CPP compared with healthy controls after adjustment for age, bone age, and breast development (all \( P < 0.02 \)). IGF1 levels were only significantly positively correlated with insulin levels in girls with CPP at baseline (\( P = 0.03 \)). During gonadal suppression, changes in IGF1 levels were inversely associated with changes in insulin levels (\( P = 0.04 \)). The GHRd3/d3 genotype was associated with significantly higher IGF1 levels (\( P = 0.01 \)) but not with earlier pubertal timing in healthy girls. The distribution of the GHRd3 genotypes among girls with CPP did not differ significantly from healthy girls (\( P = 0.2 \)).

Conclusion: The increased IGF1 and insulin levels in girls with CPP may be causally interrelated. In addition, the GHRd3 allele positively influences IGF1 levels in a copy number–response relationship but not pubertal timing in healthy girls.

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Introduction

The role of the GH/insulin-like growth factor 1 (IGF1) axis as a modulator of maturation-related timing is increasingly recognized (1, 2, 3). In healthy girls, higher childhood IGF1 levels predict earlier age at menarche (4), and girls with central precocious puberty (CPP) have higher IGF1 levels than accounted for by the advancement in bone age (BA) at time of diagnosis (5). In female rodents, central and peripheral IGF1 treatment advances age at vaginal opening (6, 7, 8), possibly through direct stimulatory effects on hypothalamic GNRH (7, 9) and kisspeptin neurons (10). Accordingly, GNRH neuron-specific IGF1 receptor knockout mice have delayed vaginal opening (7). Thus, IGF1 seems permissive for normal female pubertal timing and may even be able to advance time of puberty if prepubertal levels are elevated. Similar to IGF1, higher prepubertal insulin levels predict earlier age at menarche (11). Accordingly, we have recently shown that insulin sensitivity is decreased in girls with CPP at diagnosis and is aggravated further during gonadal suppression (12). However, to what extent this may be due to changes in IGF1 levels during gonadal suppression is unresolved.

The GH/IGF1 axis has a major influence on glucose homeostasis (13). In healthy girls, insulin sensitivity decreases approaching mid-puberty after which a partial recovery is generally seen (14, 15). This curvilinear pattern is associated with the increased activity of the GH/IGF1 axis (14). However, GH and IGF1 have opposing influences on insulin sensitivity (16). While GH antagonizes the hepatic and peripheral effects of insulin, IGF1 may partly counteract these effects through downregulation of GH secretion as well as through direct insulin-like effects (17). In accordance with the latter, IGF1 treatment decreases insulin levels in patients with GH deficiency (16) and insensitivity (18).

Another vital component of the GH/IGF1 axis is the GH receptor (GHR). In rodents as well as in humans, defective GH signaling due to loss-of-function mutations in the GHR gene is associated with GH resistance and IGF1 deficiency leading to enhanced insulin sensitivity as well as delayed onset and slower progression of puberty (19, 20). In contrast, a common gain-of-function GHR variant (21) differing from the wild-type gene (full length, GHRfl)
by exclusion (deleted). GHRd3 of exon 3 has recently been associated with increased IGF1 levels in childhood and with early onset of clinical and biochemical signs of puberty in boys (22). In addition, the presence of the GHRd3 allele has been associated with increased insulin secretion in a mixed cohort of healthy pubertal children (15). To our knowledge, no previous studies have evaluated the potential role of this genetic polymorphism for timing of puberty in girls. In addition, the influence of the GHRd3 allele on IGF1 levels in healthy girls has been discordant (15, 22, 23) despite the fact that short-term GH stimulation seems to enhance IGF1 levels more in the presence of the GHRd3 allele compared with GHRfl homozygotes (24). Thus, the higher IGF1 levels observed in girls with CPP may be related to a higher prevalence of this genetic polymorphism.

Thus, we hypothesize that girls with early puberty have higher IGF1 levels compared with normally timed pubertal girls and that these differences may be associated with differences in glucose homeostasis and/or with the presence of the GHRd3 allele.

Our aim was to evaluate: i) serum IGF1 and insulin levels in normal-timed pubertal girls and in girls with CPP before and during gonadal suppression and ii) the influence of the GHRd3 allele on serum IGF1 and pubertal timing.

Materials and methods

Participants

The study population consisted of two cohorts. All healthy subjects were recruited as a part of The Copenhagen Puberty Study from primary schools in the Copenhagen Community. Nine hundred and eleven girls aged 5.6–20.2 years volunteered. The influence of the GHRd3 allele on IGF1 levels and pubertal timing as well as differences in IGF1 levels between healthy girls and girls with early puberty were evaluated in this cohort. Other aspects of this study have previously been published (25). In brief, clinical evaluations included auxology (height and weight), and pubertal staging according to Tanner’s classification was performed (26). In 115 of the 911 healthy girls, body composition was additionally evaluated by dual-energy X-ray absorptiometry (DXA) scans (Hologic CDR 1000/W densitometer; Hologic, Inc., Bedford, MA, USA). The detection limit was 18 pmol/l; the intra- and interassay CV were 8% and 13% respectively. Insulin (MPI) and mean plasma glucose (MPG) levels during the OGTT calculated from the area under the curve. HbA1c was evaluated using Tosoh G7 (Tosoh bioscience, Tokyo, Japan).

Genomic DNA was extracted from blood lymphocytes. The frequency of GHR transcript variants with retention (GHRfl) or exclusion (GHRd3) of exon 3 was tested by

Analyses

Blood samples were drawn from the antecubital vein into standard vacuum tubes. From the majority of controls (n = 796), nonfasting blood samples were available, whereas fasting blood samples were obtained from the 115 girls with supplementary DEXA scans. In addition, standard 120 min OGTT (1.75 g of glucose/kg body-weight, max. 75 g) with 30 min sampling intervals for determination of blood glucose and insulin was performed in these 115 girls. Fasting blood samples were available from all girls with EP and CPP at all visits.

In addition, OGTTs were performed in 18 patients (n = 12 with CPP) at baseline. Of the 15 patients initiated on GNRHa, OGTTs were performed in 11 at the 3-month follow-up and 9 at the 1-year follow-up visit.

IGF1 and IGFBP3 levels were determined by Immulite 2000 IGF1/IGFBP3 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) on automated Immulite 2000 (Siemens Healthcare Diagnostics). For IGF1 and IGFBP3, the intra- and interassay coefficients of variation (CV) were below 4 and 9% respectively. Estradiol (E2) was measured using Pantex E2 (Pantex, Santa Monica, CA, USA). The detection limit was 18 pmol/l; the intra- and interassay CV were < 8 and 13% respectively. Insulin and glucose were determined on automated Roche Modular Analytics (SWA) Modules with Eclesys insulin and GLU assays (Roche Diagnostics). Insulin and glucose are expressed as fasting plasma insulin (FPI) and fasting plasma glucose (FPG) levels as well as mean plasma insulin (MPI) and mean plasma glucose (MPG) levels during the OGTT calculated from the area under the curve. HbA1c was evaluated using Tosoh G7 (Tosoh bioscience, Tokyo, Japan).

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the multiplex PCR assay as described by Pantel et al. (27). Further details have previously been published (15, 22). Genotyping for the \(\text{GHRd3}\) allele was obtained in 906 of the healthy girls and all the EP and CCP patients. Genotyping could not be obtained in five of the healthy girls due to missing or degraded DNA. The distribution of the \(\text{GHR}\) genotypes did not deviate significantly from the Hardy–Weinberg equilibrium (\(P > 0.25\)).

Statistical analysis

Results are presented as mean and 95% confidence intervals unless otherwise stated. Group comparisons were made with Students \(t\)-tests. Univariate analysis of variance (ANOVAs) were used to analyze IGF1 and insulin levels between groups adjusted for age, bone age pubertal stage respectively. IGF1 and insulin levels were log-transformed before analyses in order to obtain approximate normal distribution of the residuals as well as a residual variation that did not depend on the level. Owing to the complex biphasic relationship between IGF1 and age, the statistical model included age as both continuous and group variable (below 10.0, 10.0–13.9 and above 14.0 years) as well as the interaction between the two (age*age group). Similar for the relationship between IGF1 and pubertal stage, both age, pubertal stage, and the interaction between the two was included in models when adjusting for both age and pubertal stage (age*pubertal stage). Longitudinal changes during GNRHa treatment were evaluated by paired Student’s \(t\)-tests. Interrelated changes in main outcome variables during gonadal suppression were evaluated by Pearson’s correlation. Mean ages at entry into B2 and PH2 and age at menarche were estimated by Probit analysis. The Hardy–Weinberg equilibrium and prevalence of \(\text{GHR}\) genotypes were assessed with \(\chi^2\) tests.

Ethics

The study (clinicaltrials.gov #NCT01411527) was conducted in accordance with the ethical principles of the Helsinki II declaration. The study protocol was approved by the Local Ethics Committee (ref no. KF 01 282214 and KF 11 2006-2033). All children and parents gave their written informed consent.

Results

IGF1 and insulin levels in girls with normal and precocious puberty

In healthy girls, IGF1 levels increased significantly between each breast and pubic hair stage from stage I (prepuberty) to stage IV after which levels decreased between stages IV and V (all \(P < 0.004\); Fig. 1). A similar curvilinear pattern was seen with age (\(P < 0.001\); Fig. 2). Age was positively associated with IGF1 levels in breast stages I and III and negatively associated with age in breast stages IV and V (all \(P < 0.03\)) respectively. No association was found between age and IGF1 levels in breast stage II. IGFBP3 levels were significantly associated with age and pubertal stage in healthy controls in a similar pattern to IGF1 (both \(P < 0.001\)).

At the time of diagnosis, girls with EP and CPP had significantly higher IGF1 levels compared with age-matched healthy girls (\(P = 0.002\) and < 0.001 respectively; Fig. 2). BA and stage of breast development, but not chronological age (CA), were positively associated with IGF1 levels in girls with EP and CPP (both \(P \leq 0.001\)).

Figure 1

Levels of IGF1, FPI, and oral glucose-stimulated insulin (MPI during oral glucose tolerance test) in relation to pubertal development (breast stage I–V) in 911 (IGF1) and 115 (FPI and MPI) healthy girls. Data are shown as mean ± S.E.M.

Figure 2

IGF1 and MPI in relation to CA and BA in 23 girls with EP or CPP compared with healthy girls. Data are presented in green circles for girls with EP and in red squares for girls with CPP. Solid circles/squares represent the level according to CA and open circles/squares connect CA with BA in the individual patients and represent the BA advancement.
After adjustment for BA, girls with EP and CPP still had higher IGF1 levels at baseline than the healthy girls \( (P = 0.029 \) and \( < 0.001 \) respectively; Fig. 2). In addition, girls with CPP had higher IGF1 levels than breast stage-matched healthy girls \( (P = 0.027) \). IGF1 levels were not associated with BF\% in neither healthy girls nor girls with CPP. Accordingly, the abovementioned differences in IGF1 levels between groups remained significant after adjustment for BF\%. Girls with CPP had significantly higher IGFBP3 levels adjusted for age \( (10.5 (0.6–21.4) \% , P = 0.04) \), but not adjusted for BA or breast development, compared with the healthy girls. No differences in IGFBP3 levels were found between girls with EP and controls.

**Insulin levels and relation to IGF1 in girls with normal and preocious puberty**

In healthy girls, FPI and MPI levels paralleled the pattern of IGF1 levels during puberty (Fig. 1). Accordingly, IGF1 levels were positively associated with FPI \( (P = 0.001) \) and MPI \( (P = 0.003) \) after adjustment for age and pubertal stage. Within each breast stage, IGF1 levels were strongly correlated with FPI and MPI in breast stages III and IV respectively \( (r = 0.32, P = 0.048, \) and \( r = 0.43, P = 0.036 \) respectively). FPI and MPI levels (Fig. 2) were significantly higher in girls with EP and CPP compared with healthy girls matched for CA and BA \( (all P \leq 0.004) \). The girls with CPP showed significantly higher MPI levels than pubertal stage-matched healthy controls \( (P = 0.04) \). All the abovementioned differences in insulin levels between girls with CPP and healthy controls remained significant after additional adjustment for BF\% \( (P \leq 0.05) \). Similar to the finding in healthy girls, IGF1 was positively associated with FPI \( (P = 0.03) \) and MPI \( (P = 0.02) \) after adjustment for pubertal stage in girls with CPP at baseline. FPG, MPG, or HbA1c levels were not associated with IGF1 in neither healthy girls nor girls with EP and CPP. No differences were found in FPG, MPG, or HbA1c between healthy girls and girls with EP and CPP.

**Influence of gonadal suppression on IGF1 and insulin levels**

Longitudinal changes in BF\%, IGF1, IGFBP3, \( E_2 \), MPI, and HbA1c levels before and during gonadal suppression are shown in Fig. 3. Higher baseline IGF1 and \( E_2 \) levels as well as more advanced breast development and BA predicted a greater decline in IGF1 and a greater increase in MPI levels during the first 3 months of GNRHa treatment \( (all P \leq 0.02) \). Pretreatment levels of IGF1 and MPI positively predicted IGF1 and insulin levels at 3 and 12 months respectively \( (all P \leq 0.05) \). Interrelated changes in \( E_2 \), IGF1, and MPI levels during gonadal suppression are shown in Fig. 4. The decline in IGF1 was borderline significantly associated with the increase in HbA1c levels in the first 3 months of GNRHa treatment

![Graphs showing longitudinal changes in IGF1, IGFBP3, body fat percentage (BF\%), MPI, HbA1c, and \( E_2 \) levels during gonadal suppression with GNRHa in 15 girls with EP (13 with CPP). Baseline levels of all the above outcomes are shown in all 23 girls (15 girls with CPP) with EP. Twenty-four prepubertal age-matched controls are shown for reference. Longitudinal changes were evaluated by paired Student’s t-tests. a, \( P < 0.05 \) vs baseline; b, \( P < 0.05 \) vs 3-month visit; c, \( P < 0.05 \) vs 12-month visit.](https://www.eje-online.org)

\( (R^2 = 0.22, P = 0.09) \). The positive association between IGF1 and MPI and FPI levels at baseline \( (P \leq 0.03) \) was no longer present after initiation of GNRHa treatment \( (P > 0.5) \). Significant positive associations between BF\% and MPI were present at all time points \( (P \leq 0.05) \). No significant changes in FPG or MPG were observed during GNRHa treatment.

**Influence of GHRd3 genotype on IGF1 levels and pubertal timing**

The distribution of the three genotypes in the healthy controls was as follows: GHRfl/fl, 488 (53.9%); GHRfl/d3, 350 (38.6%); and GHRd3/d3, 68 (7.5%). The distribution of genotypes in girls with EP was GHRfl/fl: 4 (50.0%) and GHRfl/d3: 4 (50.0%) and in girls with CPP GHRfl/fl: 5 (33.3%) and GHRfl/d3: 10 (66.6%). None of the patients had the GHRfl/d3/d3 genotype.

The GHRd3 allele was significantly positively associated with IGF1 levels in a copy number–response relationship (Fig. 5). After adjustment for pubertal stage and age, the GHR3/d3 group had significantly higher IGF1 levels compared with the GHRfl/d3 \( (8.8 (0.6–17.5) \% , P = 0.035) \) and the GHRfl/fl groups \( (10.8 (2.7–19.6) \% , P = 0.008) \) respectively. IGF1 levels did not differ
the GHRd3 allele was found on pubertal timing in neither normal nor early pubertal girls.

Increasing evidence support that IGF1 may be a positive modulator of pubertal timing in both rodents and humans (4, 7, 8, 9). In accordance with previous studies (5, 28), we found that girls with CPP had increased IGF1 levels compared with both age- and BA-matched healthy girls. In addition, girls with CPP had higher IGF1 levels than healthy girls even for

**Discussion**

In this study, we found that girls with CPP had increased IGF1 as well as increased fasting and oral glucose-stimulated insulin levels compared with healthy girls after adjustment for age, BA, and pubertal stage respectively. IGF1 levels were significantly positively associated with insulin levels in both healthy girls and in girls with CPP at baseline. Surprisingly, neither IGF1 nor insulin levels were reversed to pretreatment levels during gonadal suppression. During gonadal suppression, an inverse relationship was found between changes in insulin and IGF1 levels. In addition, age- and puberty-adjusted IGF1 levels were moderately positively influenced by the GHRd3 allele in a copy number–response relationship in healthy girls. No influence of
a given stage of breast development. In girls with onset of puberty between 8 and 9 years, no differences were found in IGF1 levels compared with puberty-matched controls, indicating that these girls may represent an early but normal pubertal variant with a lesser distinct phenotype than girls with overt CPP. Owing to the cross-sectional design, the cause and effect relationship between pubertal timing and IGF1 levels could not be determined. Thus, the higher IGF1 levels in girls with CPP could be both causal as well as a consequence of early pubertal onset. In favor of a direct effect of IGF1 on pubertal timing, high prepubertal IGF1 predicts earlier age at menarche in healthy girls (4) and central and peripheral IGF1 infusion advances age at vaginal opening in female rodents (8, 9).

Similar to IGF1 levels, insulin levels were increased in girls with CPP at baseline compared with puberty-matched controls. In accordance with the previous studies on healthy children (14), IGF1 and insulin levels were strongly correlated in girls with CPP at the time of diagnosis. The major influence of GH on both IGF1 levels and glucose homeostasis is well documented (1). While GH antagonizes the insulin-mediated increase in peripheral glucose uptake by promoting lipolysis and free fatty acid oxidation (13), IGF1 may partly counteract these effects through negative feedback on GH secretion (29) as well as through direct insulin-like effects on glucose uptake (18). Although high GH secretion is most certainly involved in the increased IGF1 and insulin levels during normal puberty, previous studies have found normal to subnormal GH responses during GH stimulation tests in girls with CPP (5, 30), indicating that increased GH secretion may not be the cause of the increased IGF1 and insulin levels found in the girls with CPP. Adiposity has a strong influence on glucose homeostasis. Accordingly, we found that higher adiposity was associated with higher insulin but not higher IGF1 levels after adjustment for breast stage in both healthy girls and girls with CPP. However, the higher adiposity found in girls with CPP did not solely explain the increased insulin levels in these girls.

In previous studies, IGF1 levels have shown diverging changes during GNRHa treatment (5, 28). In our CPP cohort, IGF1 levels were significantly reduced during the first 3 months of GNRHa treatment but tended to rebound toward pretreatment levels after 1 year of treatment. In contrast, insulin levels and adiposity increased significantly from the pretreatment visit to the 1-year evaluation without significant changes observed at the 3-month visit. Accordingly, short-term gonadal suppression in young adult women is not associated with changes in glucose homeostasis (31). Surprisingly, the persistent gonadal suppression and concomitant regression of sexual characteristics induced by the GNRHa treatment neither reversed IGF1 nor insulin to prepubertal levels. In addition, the individual pretreatment level of IGF1 and insulin persisted throughout the 1-year treatment period. For insulin but not IGF1 levels this seems to be related to the degree of adiposity.

Pretreatment markers of advanced pubertal development such as advanced breast development and BA and higher baseline E2 levels predicted a greater decline in IGF1 levels and a greater increase in insulin levels during the first 3 months but not at later time points during treatment. Although this may indicate a direct effect of sex steroid withdrawal, GH secretion seems to be suppressed in parallel with the decrease in the activity of the hypothalamic-pituitary-gonadal (HPG) axis during GNRHa treatment (32). Accordingly, the positive influence of pubertal progression and E2 priming on secretion of GH during stimulation testing is well documented (33). Thus, these changes may be secondary to a possible concomitant suppression of GH secretion rather than primarily related to E2 withdrawal.

The positive association between IGF1 and insulin levels at baseline was not present during GNRHa treatment. In fact, changes in IGF1 were inversely associated with changes in insulin levels during the 1-year follow-up period. Although this period represents both withdrawal of and continuous suppression of sex steroid levels, the inverse relationship, albeit insignificant, were found during all these distinct periods, which could indicate lack of statistical power rather than lack of true association. Thus, sex steroid withdrawal or the possible concomitant GH suppression induced by GNRHa treatment seems to change the relationship between these hormones. Accordingly, the positive association between IGF1 and insulin was strongest during mid-puberty in the healthy controls corresponding to the peak in GH secretion. Although a true causal relationship cannot be determined from these data, previous studies on adults with GH deficiency or GH insensitivity have shown decrease in insulin levels during IGF1 treatment (16), in accordance with direct insulin-like effects of IGF1. Thus, the relative hyperinsulinemia during GNRHa treatment seems to be associated with changes in IGF1. In addition, the relative insulin dominance over HPG and GH/IGF1 axis activation during GNRHa treatment may be implicated in the adverse changes in body composition previously reported in this and other cohorts of girls with CPP (12, 34, 35). However, due to the small cohort size in our study, further studies are needed on the hormonal and body compositional changes induced by GNRHa treatment – as well as the reversibility upon discontinuation of treatment.

The unexplained findings of increased IGF1 levels in girls with CPP could be related to genetic effects. In the classical work by Dos Santos et al. (21), cells transfected with GHR dimers containing exon 3-deleted monomers had ~30% augmented intracellular STAT activation after GH binding compared with full-length GHR dimers. The presence of the GHRd3 allele has previously been associated with higher insulin levels in normal children (15). In addition, homozygosity of the GHRd3 allele has been associated with higher childhood IGF1 levels and earlier pubertal onset evaluated by both clinical and
biochemical markers in healthy boys (22). Accordingly, IGF1 levels in healthy girls were significantly higher in the GHRd3 homozygotes compared with both homo- and heterozygotes of the GHRfl allele in this study. However, no differences were found in age at onset of pubertal markers between the GHR genotypes in healthy girls. Although a previous genome-wide association study (GWAS) found that menarcheal age was associated with SNPs in the IGF1 locus (2), a recent GWAS meta-analysis did not support SNPs in genes related to the GH/IGF1 axis as significant contributors to the variation in timing of menarche (36). However, only very few case reports in girls with CPP have confirmed the same genetic variations in the genes associated with menarcheal timing in population-based studies (37), indicating that other genes may be involved in the development of CPP. However, the distribution of the GHRd3 genotypes was not significantly different between the healthy girls and the girls with CPP in this study, indicating that this genetic polymorphism was not involved in the pathogenesis of CPP nor the increased IGF1 levels seen in these girls. In fact, none of the 23 girls with early puberty was GHRd3 homozygous.

In summary, we present novel data on the interrelationship between IGF1 and insulin levels before and during gonadal suppression in girls with CPP. Girls with CPP had significantly higher IGF1 and insulin levels than age- and puberty-matched healthy girls. Surprisingly, these differences were neither reversed during gonadal suppression nor explained by the GHRd3 allele. Changes in IGF1 levels were inversely associated with changes in insulin levels during GNRH-a treatment, which is in accordance with a positive effect of IGF1 on insulin sensitivity. Homozygosity of the GHRd3 allele was associated with significantly higher IGF1 levels during childhood and adolescence but not with earlier pubertal timing compared with GHRfl hetero- and homozygous healthy girls. Thus, the influence of IGF1 levels on pubertal timing still needs further elucidation.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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