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The consequences of growth hormone-releasing hormone receptor haploinsufficiency for bone quality and insulin resistance

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Summary

Objective Growth hormone (GH)/insulin-like growth factor (IGF) axis and insulin are key determinants of bone remodelling. Homozygous mutations in the GH-releasing hormone receptor (GHRHR) gene (GHRHR) are a frequent cause of genetic isolated GH deficiency (IGHD). Heterozygosity for GHRHR mutation causes changes in body composition and possibly an increase in insulin sensitivity, but its effects on bone quality are still unknown. The objective of this study was to assess the bone quality and metabolism and its correlation with insulin sensitivity in subjects heterozygous for a null mutation in the GHRHR.

Patients and methods A cross-sectional study was performed on 76 normal subjects (68.4% females) (N/N) and 64 individuals (64% females) heterozygous for a mutation in the GHRHR (MUT/N). Anthropometric features, quantitative ultrasound (QUS) of the heel, bone markers [osteocalcin (OC) and CrossLaps], IGF-I, glucose and insulin were measured, and homeostasis model assessment of insulin resistance (HOMAIR) was calculated.

Results There were no differences in age or height between the two groups, but weight (P = 0.007) and BMI (P = 0.001) were lower in MUT/N. There were no differences in serum levels of IGF-I, glucose, T-score or absolute values of stiffness and OC, but insulin (P = 0.001), HOMAIR (P = 0.001) and CrossLaps (P = 0.01) were lower in MUT/N. There was no correlation between OC and glucose, OC and HOMAIR in the 140 individuals as a whole or in the separate MUT/N or N/N groups.

Conclusions This study suggests that one allele mutation in the GHRHR gene has a greater impact on energy metabolism than on bone quality.
phenotype, by assessing bone quality and biochemical markers of bone formation and resorption.

**Subjects and methods**

**Subjects**

The study comprised adult individuals with normal stature, aged 25–75 years from the Itabaianinha community who were first-, second- or third-degree relatives of IGHD individuals, recruited by advertisement on a local bulletin board and by word of mouth. We collected buccal cells from 240 adult individuals, and DNA was extracted by alkaline lysis. Genotyping for the c.104+1G>A mutation was carried out as previously described. Subjects with a history of childhood disease known to influence bone density such as hepatic or renal failure or with a history of exposure to chronic steroids, medications for the treatment of osteoporosis, anticonvulsants, any use of alcohol and tobacco (current or previous), physical inactivity (bedridden or walking difficulties) and vigorous physical activity (athletes) were excluded. We identified 76 subjects heterozygous for the c.104+1G>A GHRHR mutation (MUT/N) and 77 sex-matched genotype-proven normal subjects (N/N), as previously reported. Twelve MUT/N subjects and one individual in the N/N group were excluded because of the lack of data regarding one or more variables of the protocol. Therefore, 64 MUT/N (46.1% female) and 75 N/N (68.4% female) individuals were included in the study. The protocol was approved by the Johns Hopkins University, CONEP and the Federal University of Sergipe Ethics Committees. All subjects gave written informed consent.

Height (m) and body weight (kg) were measured with a portable stadiometer and a portable scale. Body mass index (BMI) was calculated using the formula: weight in kg/height m².

**Laboratory assessment**

Insulin-like growth factor-I was measured in duplicate by DSL-5600 immunoradiometric assay (IRMA; Diagnostic Systems Laboratories, Webster, TX, USA). To pool IGF-I measurements in both genders and in different ages, results were normalized by standard deviation scores (SDS). SDS for serum IGF-I was calculated by subtracting the mean IGF-I level for age from the individual value and dividing this value by the standard deviation of the respective mean age given by the manufacturer. The normal SDS distribution has a mean of 0 and a standard deviation of ±1. Values of SDS above +2 or below −2 are by definition abnormal. Glucose was measured by Accu-Check Advantage® (Roche, San Francisco, CA, USA). Serum levels of insulin was measured by a solid-phase, two-site chemiluminescent assay DPC-5210 (Diagnostic Products Corporation, Los Angeles, CA, USA), and insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMAIR) with the formula: fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5. IGF-I and insulin assays were performed at the hormone laboratory of the University Hospital, Federal University of Sergipe. Osteocalcin (OC) was measured by DSL-7600 IRMA (Diagnostic Systems Laboratories, Inc., Webster, TX, USA), and serum levels of the C-terminal cross-linking telopeptide of type I collagen (CrossLaps) were measured by DK-2730 ELISA IRMA (Orion Diagnostica, Webster, TX, USA) in the Metabolism Laboratory of the General Hospital, School of Medicine of Ribeirão Preto, University of São Paulo. The intra-assay coefficients of variation (CVs) for the determination of IGF-I, insulin, OC and CrossLaps were 6.5%, 4.2%, 8.3% and 7.2%, respectively. All the assays were performed in a single run.

**Bone status measurements**

We determined the bone stiffness index by heel QUS. QUS is a water-based system that uses transmission of an ultrasound wave in a temperature-controlled water bath (37 °C) through the heel. This measurement was performed with rigorous standardization of subject positioning using the Achilles Insight device (Lunar/GE, Madison, WI, USA). QUS measures the stiffness index by the equation: stiffness = (0.67 × coefficient of ultrasound broadband attenuation in dB/MHz + 0.28 × speed of sound in m/s) − 420. Bone stiffness was expressed as the percentage of the values obtained by the manufacturer for a young adult population. For the determined bone stiffness index, we chose a normal South American population. The CV of bone stiffness measurement was 2.23%.

**Statistical analysis**

Values for continuous variables were expressed as mean ± standard deviation. Physical characteristics (weight, height and BMI), were analysed by the Student t-test. Insulin and HOMAIR data were transformed into decimal logarithm before analysis. A general linear model, encompassing both analysis of variance (ANOVA) and regression, using HOMAIR as a dependent variable and ANOVA with two factors (sex and group) adjusted for age and BMI was subsequently used. The regression model was used to examine possible associations between weight and BMI with bone markers, and between bone markers with glucose, insulin and HOMAIR by estimating their square R (R²). To test the hypothesis of any correlation between OC and glucose, insulin and HOMAIR, we used Spearman’s rank correlation. The Pearson correlation coefficient was used to analyse the correlation between stiffness and OC and between stiffness and CrossLaps. P values of 0.05 or less were considered to be statistically significant. SAS software (SAS 9.1, Cary, NC, USA) was used for the analysis.

**Results**

Anthropometric, biochemical and bone marker data (mean ± SD) for homozygous normal subjects (N/N) and individuals heterozygous for the GHRHR mutation (MUT/N) are shown in Table 1. The statistical analysis did not change when individuals aged more than 50 years (37%) were excluded. There were no differences in age or height between groups but weight (P = 0.007) and BMI P = 0.001 were lower in MUT/N.

There were no differences in IGF-I or glucose between groups, but insulin and HOMAIR (P < 0.01) were significantly lower in MUT/N. For the HOMAIR, the mean magnitude of difference was 0.23 ± 0.11, 95% confidence interval from 0.12 to 0.448, P = 0.03.
Table 1. Anthropometric data, SDS of serum IGF-I levels (SDS IGF-1), biochemical and heel quantitative ultrasound (mean ± SD) in 76 normal homozygous and 64 heterozygous (MUT/N) subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F</th>
<th>Sig.</th>
<th>Partial η²</th>
<th>Observed power*</th>
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</thead>
<tbody>
<tr>
<td>Corrected model</td>
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<td>5</td>
<td>2.76</td>
<td>7.90</td>
<td>0.0001</td>
<td>0.23</td>
<td>0.999</td>
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<td>2.07</td>
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<td>0.23</td>
<td>0.999</td>
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<td>Age</td>
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<td>0.03</td>
<td>0.09</td>
<td>0.92</td>
<td>0.001</td>
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<tr>
<td>BMI</td>
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<td>7.26</td>
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<td>0.0001</td>
<td>0.134</td>
<td>0.995</td>
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<td>1.53</td>
<td>4.37</td>
<td>0.03</td>
<td>0.03</td>
<td>0.55</td>
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<tr>
<td>Sex</td>
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<td>0.10</td>
<td>0.019</td>
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<tr>
<td>Group*sex</td>
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<td>0.44</td>
<td>1.25</td>
<td>0.26</td>
<td>0.009</td>
<td>0.199</td>
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<td>Error</td>
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<td>134</td>
<td>0.35</td>
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Partial η² squared = effect dimension; Group*sex: interaction group and sex.
*Computed using alpha = 0.05.
†R² = 0.23 (adjusted R² = 0.199).

Table 2 shows the data of the general linear model using HOMA IR as a dependent variable and ANOVA with two factors (sex and group) adjusted for age and BMI. The model was able to explain 23% of the variability of the HOMA IR, being in the model BMI, the principal determinant of this variability, with an adjusted R square of 0.134 (explaining 13.4% of the HOMA IR variability). The only other significant covariate was group, with an adjusted R square of 0.134 (explaining only 3% of HOMA IR variability). There was no effect of age, sex and interaction between group and sex in HOMA IR.

While serum OC did not differ between groups, CrossLaps levels were significantly lower in the MUT/N group. The regression model showed no association between weight with OC and CrossLaps in MUT/N (R² = 0.00001, P = 0.98 and R² = 0.0000001, P = 0.99, respectively) and N/N (R² = 0.00003, P = 0.96 and R² = 0.02, P = 0.18, respectively) or BMI with OC and CrossLaps in MUT/N (R² = 0.02, P = 0.3 and R² = 0.00001, P = 0.98, respectively) or N/N (R² = 0.02, P = 0.28 and R² = 0.02, P = 0.19, respectively). There was no correlation between OC and glucose (r = 0.12, P = 0.693), OC and insulin (r = −0.05; P = 0.673) and OC and HOMA IR (r = −0.06; P = 0.603), between stiffness and OC (r = 0.06; P = 0.83), or between stiffness and CrossLaps (r = 0.08; P = 0.59) when all 140 individuals were analysed as a whole or when the individual groups were analysed. Similarly, there was no correlation between age and any bone marker in all individuals, or when the groups were analysed separately (Fig. 1). The parameter values obtained by QUS T-Score (N/N = 0.09 ± 1.53 vs MUT/N = 0.07 ± 1.32) and stiffness (N/N = 96.63 ± 23.83 vs MUT/N = 96.32 ± 19.32%) did not differ significantly between groups.

Discussion

The GH-IGF-I axis plays a pivotal role in maintaining bone health, and GHD may predispose to loss of bone and to fracture. Additionally, previous studies have shown that a mild decline in the GH/IGF-I axis is associated with bone loss during ageing. Our results show that heterozygosity for a GHRHR mutation is associated with reduced body weight, BMI, serum insulin and HOMA IR, all factors that could potentially influence bone strength. The present study confirms data obtained previously when using infrared interactance, we showed that absolute lean mass was reduced in MUT/N, while there was significant difference in percentage of fat mass but a trend of reduction in absolute fat. It is necessary to take into account a critical period for fat mass development take place during childhood. Therefore, the trend in reduction in fat mass and BMI may reflect impairment of preadipocytes differentiation, which have high IGF-I receptor expression. Although both BMI and group are significantly associated with HOMA IR, the general linear model showed that BMI explains the reduction in HOMA IR more than the group effect in MUT/N shown in this protocol with a good power. However, the mechanism responsible for these alterations remains to be determined; one may hypothesize that they reflect a reduction in GH secretion caused by GHRHR haploinsufficiency, which may not be unveiled by a reduction in serum IGF-I. To test this hypothesis, it will be necessary to compare the 24-h GH secretion profile of MUT/N to N/N individuals. The lack of difference in serum IGF-I between WT/MT and N/N suggests that these effects in adulthood are not modulated by circulating serum IGF-I, although the expression of IGF-I receptor in muscle or fat tissue in MUT/N is unknown.

Particularly, the low BMI has been reported to influence the risk of osteoporosis. However, in our study, T-score and absolute value of stiffness as measured by heel QUS were similar in both
groups, demonstrating that bone quality is not impaired in heterozygous GHRHR individuals. However, while homozygosity for the mutation leads to decreased bone quality, heterozygosis has no impact on bone ultrasound parameters. This condition is not exclusive of GHRHR deficiency. The genetically engineered mouse knockout for the vitamin D receptor (VDR) shows bone disorder as well as lean phenotype. On the other hand, VDR heterozygote male mouse has a lean phenotype but normal bone structure.

The use of QUS rather than dual-emission X-ray absorptiometry (DXA) in this study was dictated by logistic reasons. Although the WHO has objectively defined osteoporosis based on BMD assessment (i.e. T-Score ≤−2.5 standard deviations below normal peak bone mass), osteoporotic fracture can occur across a wide spectrum of BMDs, suggesting an important role not only of bone mass, but also of bone quality. Additionally, in several conditions such as diabetes mellitus and obesity, increased bone fragility occurs in spite of normal or even increased BMD. Heel ultrasound has some advantages over other more expensive options such as computed tomography, quantitative peripheral tomography, magnetic resonance and DXA. Particularly, QUS is the only technique suitable for use in field studies and correlates with both fracture risk and bone quality.

MUT/N individuals exhibited a predominantly anabolic profile of biochemical markers of bone remodelling: while serum OC levels were similar to those of control individuals, the parameter of bone resorption was significantly decreased in MUT/N patients. Therefore, it can be hypothesized that the conserved bone formation associated with lower resorption activity is a mechanism for the maintenance of bone quality in MUT/N individuals in comparison with the previous MUT/MUT group. There are few studies dedicated to the investigation of BMI effect on bone remodelling markers. BMI has been shown to have a negative effect in both types of bone markers, that is formation and resorption with women after menopause exhibited the strongest negative association. In the present study, however, this association was not found.

We did not find any correlation between serum levels of OC and glucose, insulin and HOMAIR. Lee et al. have recently demonstrated that bone regulates the insulin/glucose axis and energy metabolism in mice. Osteoblast cells and uncarboxylated OC have a pivotal role in this network. In parallel, Pitta et al. showed that both carboxylated and undercarboxylated OC was significantly correlated with insulin and HOMAIR. In the BMI range of our patients (25–30 kg/m²), the reference HOMA IR of Brazilian adult individuals ranges between 0 and 4.3, with a mean of 1.7, and a threshold value for insulin resistance of 2.71. This value is above our HOMA IR values in both MUT/N and N/N. Therefore, the low values of both MUT/N and N/N might have extinguished a possible correlation between OC and HOMA IR. In addition, recently reduced serum total OC was associated with metabolic syndrome in older men as shown by waist circumference, hyperglycaemia and triglyceride levels. The associations between serum total OC and these parameters were most apparent at the levels of total OC below a threshold of 20 µg/l, while the mean OC value of our MUT/N individuals was 25.5 ± 11.32 µg/l. Differently from individuals with metabolic syndrome, the MUT/N individuals presented lower lean mass, waist and hip circumferences, and glucose and triglyceride levels.
similar to those of N/N. These data suggest that only reduced serum total OC can identify individuals at increased risk of metabolic syndrome.

Our study has some limitations. We did not measure serum levels of vitamin D, calcium and alkaline phosphatase, but both groups live in the same environmental condition (equatorial area, with abundant year-around sunlight exposure) and have similar diet habit. Additionally, the normal result obtained in bone evaluation suggests no major impairment on vitamin D supply. Another one is that only one-third of all individuals were older than 50 years. In control individuals, bone loss and decreased IGF-I production are coincident factors of the ageing process. It will be important to evaluate whether ageing in MUT/N individuals is associated with accelerated bone loss by studying a larger number of older individuals.

In conclusion, the present study shows that a monoallelic mutation in the GHRHR gene does not impact skeletal growth and bone ultrasonometry parameters in middle-aged individuals. However, these individuals exhibit a lean phenotype and an advantage in insulin sensitivity, assessed by homeostasis model assessment of insulin resistance.

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