Growth Hormone & IGF Research xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Association of serum components of the GH-IGFs-IGFBPs system with *GHR*-exon 3 polymorphism in normal and idiopathic short stature children

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ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 30 July 2013
Accepted 13 August 2013
Available online xxxx

Keywords:
GHBP
GHR-exon 3 polymorphism
GHR gene
Idiopathic short stature
IGF-I
IGFBP-3

ABSTRACT

Objective: To investigate the possible association of circulating components of GH-IGFs-IGFBPs system with the *GHR*-exon 3 genotype in normal and idiopathic short stature (ISS) children.
Design: Descriptive, cross-sectional study in normal and ISS children.
Subjects and methods: 192 normal and 81 ISS children (age: 5–17 years) were included. Serum IGF-I, IGFBP3, ALS and GHBP levels were measured. GHR-exon 3 polymorphism (GHRd3) was analyzed by multiplex PCR assay. Normal and ISS children were divided according to *GHR*-exon 3 genotype: homozygous for the full-length GHR isoform (GHRfl) and carriers of one or two copies of the GHRd3 allele.
Results: GHRd3 genotype distribution (fl:fl/fl:d3/d3:d3,%) in normal (60:34:6) and ISS (64:32:4) was similar and reached Hardy–Weinberg equilibrium. ISS children had significantly reduced levels of GHBP and GH-dependent factors as compared to controls ($p < 0.0001$). Within the normal group, homozygous carriers of the *GHR*fl allele had significantly higher GHBP serum levels than those with one or two copies of the *GHR*d3 allele (Mean ± SEM; GHRfl: 3.2 ± 0.2 vs GHRd3: 2.7 ± 0.2 nmol/L, $p = 0.04$). No other significant association with *GHR* exon 3 polymorphism was found in either the normal or the ISS groups.
Conclusions: GHR exon 3 polymorphism is distributed similarly in normal and ISS children, however only normal homozygous children for *GHR*fl allele showed higher GHBP levels. The lack of association between GHBP and GHR polymorphism in ISS children might be related to the heterogeneity of this group, where potential defects in GH receptor action may result in partial GH insensitivity.

1. Introduction

The growth hormone receptor (GHR) mediates the effect of GH on linear growth and metabolism [1]. The GHR protein consists of a large extracellular domain involved in GH binding and GHR dimerization, a single transmembrane domain that anchors the receptor to the cell surface, and an intracellular domain involved in GH signaling [1]. With respect to the extracellular domain-encoding region of the *GHR* gene, a common polymorphic variant in exon 3 has been reported in the general population [2]. This polymorphism leads to retention (full-length, fl; *GHR*fl) or deletion of exon 3 (d3; *GHR*d3), which encodes a 22-aminoacid residue sequence in the extracellular domain. At the protein level, this *GHR*d3 polymorphism does not affect GH binding to its receptor [3]. The high affinity GH-binding protein (GHBP) is produced by proteolytical cleavage from the extracellular domain of the GHR and is considered a circulating marker of the GHR [4,5]. Although the regulation of GHBP shedding at the molecular level is not fully understood, it has been suggested that the shedding process might be dependent on *GHR*-exon 3 polymorphic variants [3,6]. In a small cohort of normal adults, the type of GHBP present in serum corresponded to the type of *GHR*-exon 3 genotype, supporting that this protein isoform resulted from a genomic deletion as it had been previously stated [2].

Idiopathic Short Stature (ISS) describes a heterogeneous group of children consisting of many presently unidentified causes of short stature [7,8]. Abnormalities of the growth hormone-insulin-like growth factors system has been reported in ISS children [9–15], some of them reported in the *GHR* gene [9–14]. Nevertheless, the possible association of basal serum GHBP, IGF-I, IGFBP-3, and ALS with *GHR*-exon 3 genotype in ISS children as compared to normal children has not been investigated. The present study aimed to determine if *GHR*-exon 3 genotype is associated with auxological features and the abovementioned GH-dependent factors in children.

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http://dx.doi.org/10.1016/j.ghir.2013.08.003

2. Subjects and methods

2.1. Study design

We conducted a descriptive, cross-sectional study in a group of normal and ISS children at the Division of Endocrinology of the Ricardo Gutiérrez Children's Hospital, a tertiary pediatric public hospital in Buenos Aires, Argentina. The study was conducted in accordance with the ethical principles of Helsinki II declaration. The study protocol was approved by the Institutional Review Board and Ethics Committee of the Ricardo Gutiérrez Children's Hospital. All parents gave their informed written consent, and the children older than 8 years gave their written assent.

2.2. Subjects

2.2.1. Normal children

One hundred and ninety-two healthy children of normal stature (88 boys/104 girls) aged 4.9 to 17.5 years were recruited from children consulting at the Pediatric Department of the Ricardo Gutiérrez Children's Hospital for routine pediatric check-up. Participants were included provided that they fulfilled the following criteria: height higher than −2 SD score (SDS); weight for height between 90% and 110% of normal for the Argentinean population [16]. All children were born at term, had no history of low birth weight, have normal body proportions and good caloric intake. Body mass index (BMI) was calculated according to Cole et al. [17]. Pubertal staging was calculated according to Tanner's classification [18,19]. Systemic diseases were ruled out through a routine clinical and biochemical assessment. Endocrine diseases were ruled out by normal TSH, free thyroxine, anti-thyroperoxidase antibodies, prolactin, insulin and cortisol serum concentrations. To evaluate GH axis, IGF-I, IGFBP-3, ALS and GHBP serum levels were also measured.

2.2.2. ISS children

Eighty-one ISS children (61 boys/20 girls), aged 5.0 to 17.5 years were admitted to this study. All ISS children were included after an exhaustive auxological, clinical and biochemical assessment according to a Consensus Statement [7]. ISS children presented a height of more than 2 SDS below the corresponding mean height for a given age and sex for Argentinean population. All ISS children had no history of low birth weight for Argentinean standards [16], and were taking no medication. Systemic and endocrine abnormalities were ruled out as previously described for normal children. Absorption disorders were excluded by anti-transglutaminase antibodies measurement. Once excluding general causes of short stature, GH deficiency was ruled out in ISS children by maximal GH stimulated levels >6.0 ng/mL after pharmacological sequential arginine (0.5 g/kg body weight) and clonidine (100 μg/m^2 body surface) test as previously described [20].

2.3. Hormonal assays

Insulin, GH, IGF-I and IGFBP-3 concentrations were measured by a two-site chemiluminescent immunometric assays (IMMULITE® 2000 system, Siemens Healthcare Diagnostics Products Ltd. Gwynedd, UK). Intra- and interassay CV were < 5% for insulin, <4% for GH, <5.5% for IGF-I and < 7.2% for IGFBP-3 serum measurement. ALS was determined by RIA (Bioclone, New South Wales, Australia); intra- and interassay CVs% were <10%.

Total GHBP serum concentration was determined by an in house time-resolved fluoroimmunoassay (GHBP TR-FIA) modified from Fisker and colleagues [21,22]. The principle of the assay is to saturate serum GHBP with a GH solution and trap the complex with an immobilized antibody against GH. The complex is then detected by an Eu^{3+}-labeled antibody against human GHBP (Mab 263, American Diagnostica Inc., Greenwich, UK). The assay was performed using 96-well microtiter plates from the hGH kit (DELFIA®, Turku, Finland), coated with a monoclonal GH antibody. The antibody against human GHBP was labeled using a labeling commercial kit (DELFIA® Eu-Labelling kit, PerkinElmer, Boston, USA) according to the manufacturer's instructions. The standards used were recombinant human GHBP (ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) diluted in DELFIA multibuffer. The detection range of the assay was 0.43–12.8 nmol/L; intra- and interassay CVs% were <7.5% and <12.5%, respectively [22].

2.4. Molecular study

Genomic DNA from normal and ISS children was isolated from peripheral leukocytes based on the use of cetyltrimethylammonium bromide (CTAB) lysis buffer and isoamyl alcohol-chloroform extraction [23]. The genotype for the *GHR*-exon 3 polymorphism (retention—*GHR*fl- or exclusion -*GHR*d3- of exon 3) was determined by a simple multiplex PCR assay previously described by Pantel et al. using one sense primer (G1) and two anti-sense primers (G2 in intron 3 and G3 in exon 3) [2]. Amplification products were analyzed by electrophoresis on a 6% polyacrilamide gel stained with ethidium bromide. The *GHR*fl and the *GHR*d3 alleles are represented by a 935-bp and a 532-bp fragment, respectively. To avoid false homozygous GHRd3 genotyping, each GHRd3 isoform was confirmed by repeating individually a second PCR using only G1 and G3 primers only enabling the amplification of GHRfl allele according to Audi et al. [24].

Table 1
Clinical, auxological and biochemical features of normal and ISS children. Data are expressed as the Mean ± SEM.

Parameter/group	Pubertal and prepubertal children (n = 273)		Prepubertal children (n = 156)	
	Normal (n = 192)	ISS (n = 81)	Normal (n = 91)	ISS (n = 65)
CA (ys)	10.91 ± 0.24	9.99 ± 0.38[a]	8.11 ± 0.20	8.85 ± 0.31
Gender (females/males)	104/88	20/61[c]	23/68	18/47
Tanner stage (n): I-II-III-IV-V	91-15-16-43-27	65-9-3-1-3[d]	–	–
Height SDS	0.29 ± 0.08	−2.87 ± 0.06[c]	0.14 ± 0.10	−2.85 ± 0.06[c]
BMI SDS	0.27 ± 0.06	−0.63 ± 0.12[c]	0.20 ± 0.09	−0.56 ± 0.13[b]
Insulin (μIU/mL)	5.4 ± 0.2	5.4 ± 0.5	4.3 ± 0.3	4.8 ± 0.5
Maximal GH (ng/mL)	–	17.2 ± 0.9	–	17.0 ± 1.0
GHBP (nmol/L)	3.0 ± 0.1	2.7 ± 0.4[a]	3.1 ± 0.1	2.4 ± 0.2[b]
IGF-I (ng/mL)	237 ± 10	118 ± 9[c]	119 ± 7	91 ± 6[c]
IGFBP-3 (μg/mL)	4.0 ± 0.1	3.3 ± 0.1[c]	3.6 ± 0.1	3.0 ± 0.1[c]
ALS (nmol/L)	248 ± 5	173 ± 7[c]	213 ± 5	159 ± 6[c]

CA: chronological age; BMI: body mass index; SEM: standard error of the mean.
[a] $p < 0.05$ versus normal group.
[b] $p < 0.01$ versus normal group.
[c] $p < 0.001$ versus normal group.
[d] $p < 0.0001$ Proportion of prepubertal vs pubertal children in both groups of control and ISS children.

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2.5. Statistical analysis

To analyze the influence of early and late prepubertal ages on serum GH-dependent parameters (GHBP, IGF-I, IGFBP-3 and ALS) in normal children, the prepubertal group was subdivided into two subgroups according to median chronological age (8.5 years): subjects below the median, Group Tanner I-A [Girls: n = 23, median and range: 6.8 (5.0–7.7) years; Boys: n = 23, median and range: 6.4 (5.0–8.5) years], and subjects at the median or above, Group Tanner I-B [Girls: n = 22, median and range: 9.1 (7.9–11.9) years; Boys: n = 23, median and range: 10.1 (8.7–12.9) years].

Normal control data of GH-dependent parameters of this study were used to calculate normal reference intervals according to age, sex and pubertal development as appropriate. In the ISS group, GHBP, IGF-I, IGFBP-3 and ALS were expressed as Standard Deviation Score (SDS) according to normal control group values.

Inter-subject variability of circulating components of the GH system in normal children was calculated as follows: (SD/mean)*100; being SD the standard deviation and the mean of the data of normal children.

Data distribution of hormone serum levels were tested for normality using the Shapiro–Wilk test. All parameters followed a Gaussian distribution, except for serum GHBP, IGF-I and ALS concentrations that were log-transformed to reach normal distribution. Hardy–Weinberg equilibrium for genotype distribution was tested for normal and ISS cohorts by X^2 test < 3.84. Fisher's exact test was used to assess the proportion of gender, pubertal stage and *GHR*-exon 3 genotype for each group of children. To test the influence of *GHR*-exon 3 genotype on auxological

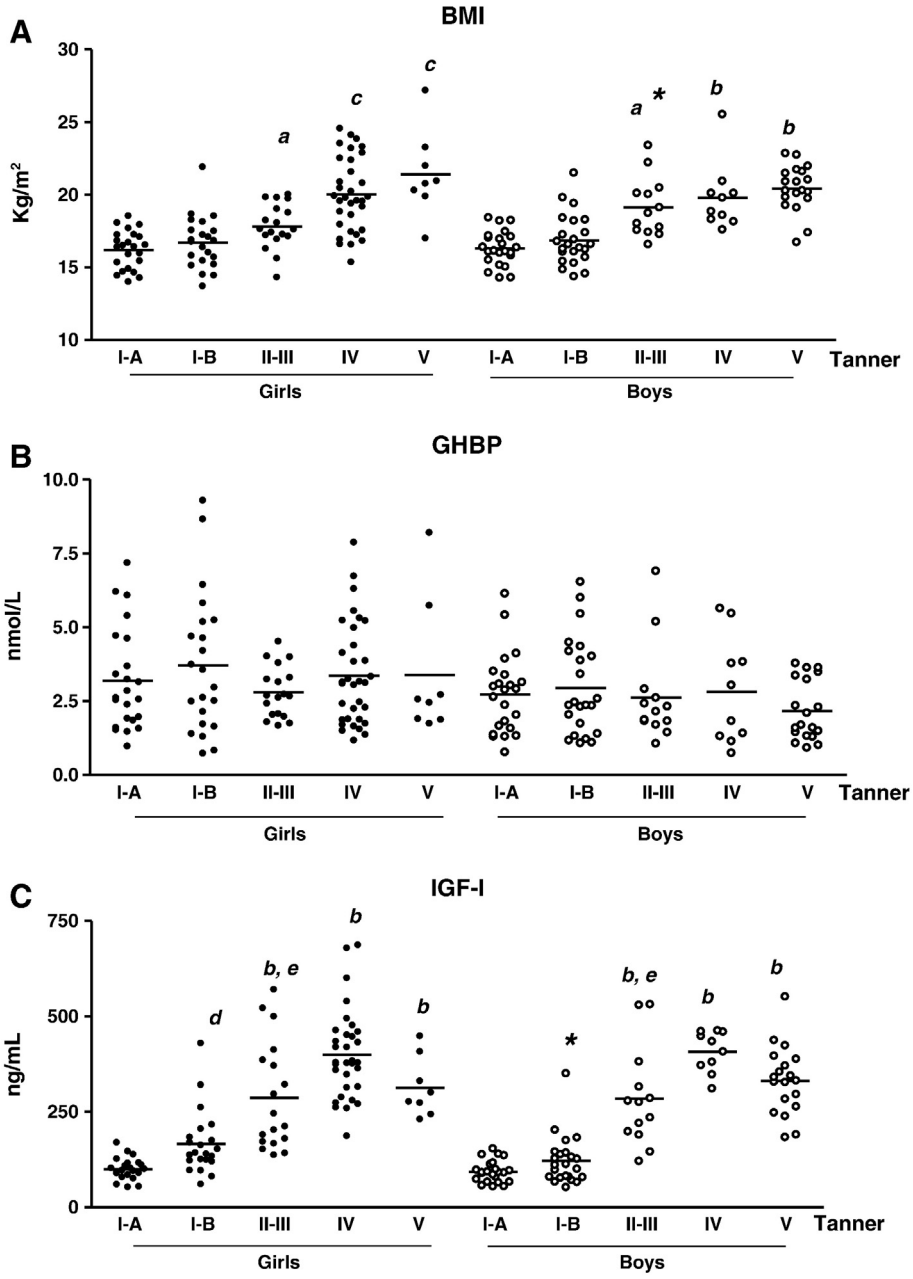

Fig. 1. BMI (A) values and serum GHBP (B), IGF-I (C), IGFBP-3 (D) and ALS (E) levels in normal children according to gender and sexual development. Prepubertal children were subdivided into T I-A and T I-B groups. *a* p < 0.05 vs Tanner I-A and Tanner I-B; *b* p < 0.01 vs Tanner I-A and Tanner I-B; *c* p < 0.001 vs T II–III and Tanner I-A and Tanner I-B; *d* p < 0.01 vs Tanner I-A; *e* p < 0.01 vs Tanner IV; * p < 0.05 vs girls with same Tanner; ** p < 0.01 vs girls with same Tanner.

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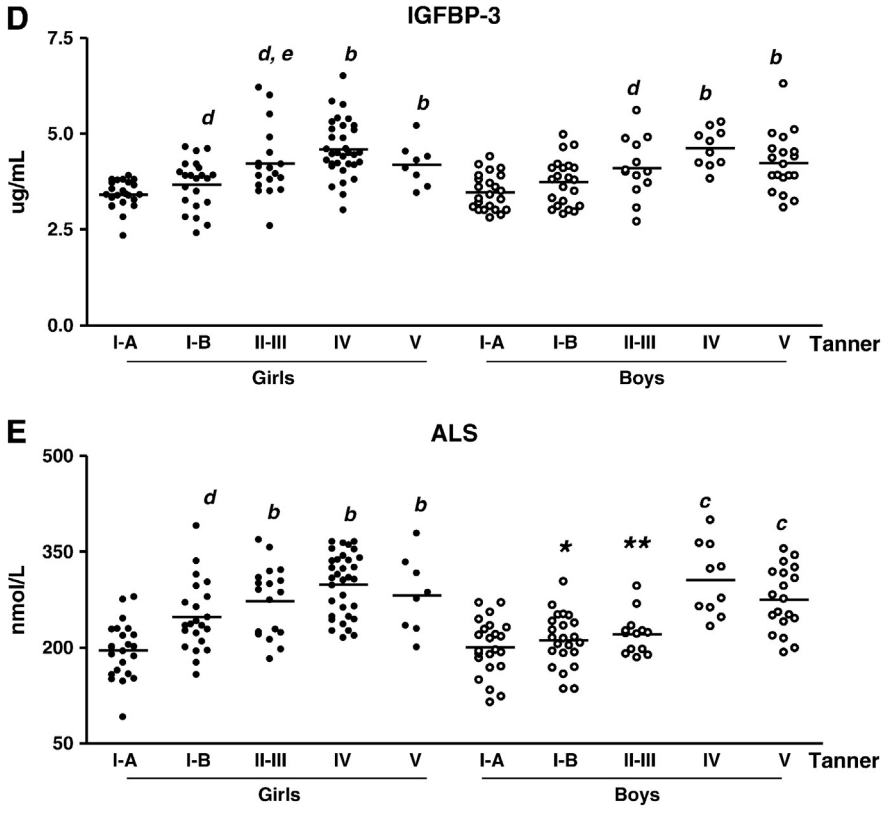

Fig. 1 (*continued*).

and biochemical parameters, children were grouped as follows: *GHR*fl (children homozygous for the full-length allele) or *GHR*d3 (children carrying at least one copy of the d3 allele). Two-way analysis of variance was used to evaluate differences in auxological and biochemical parameters (dependent variables) in relation to *GHR*-exon 3 genotype and the groups of normal and ISS children (independent variables). This analysis was also performed by including BMI SDS as covariable.

The level of significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Normal group

Clinical, auxological and biochemical features of normal children are presented in Table 1. Fig. 1 shows the pubertal changes in BMI and components of GH-IGF-I-IGFBPs system in normal children. GHBP levels (panel B) varied greatly among normal children (Inter-subject CV% for girls: 54.9%, boys: 55%, both sexes: 55%) with no age-related differences. While inter-subject variability (CV%) for serum IGF-I levels was on average 63% (girls: 59%, boys: 66%) those of IGFBP-3 (girls: 20%, boys: 18%, both sexes: 19%) and ALS (girls: 24%, boys: 25%, both sexes: 26%) were almost one third of IGF-I variability in normal children. Puberty, CA, and BMI were significantly associated to IGF-I levels ($r^2 = 0.64$, $p < 0.0001$) and ALS ($r^2 = 0.41$, $p < 0.0001$) and IGFBP-3 concentration ($r^2 = 0.32$, $p < 0.001$). Prepubertal girls at T I-B presented significantly higher IGF-I (Mean ± SEM: 164 ± 18 vs 99 ± 6 ng/mL, $p < 0.01$), IGFBP-3 (Mean ± SEM: 3.7 ± 0.1 vs 3.4 ± 0.1 ug/mL, $p < 0.01$) and ALS levels (Mean ± SEM: 247 ± 11 vs 195 ± 9 nmol/L, $p < 0.01$) than prepubertal girls at T I-A. IGF-I, IGFBP-3 and ALS concentration increased with age and pubertal stage in normal children, achieving peak values at Tanner IV in girls and boys (Fig. 1). The whole group of normal girls presented significantly higher GHBP serum concentrations (Mean ± SEM: 3.3 ± 0.2 nmol/L) than normal boys (2.6 ± 0.2 nmol/L, $p < 0.01$); higher IGF-I (Mean ± SEM, girls: 257 ± 15 ng/mL vs boys: 214 ± 15 ng/mL, $p < 0.01$) and ALS (Mean ± SEM, girls: 260 ± 6 nmol/L vs boys: 234 ± 6 nmol/L, $p < 0.01$) levels than boys.

3.2. ISS children

The group of ISS children had significantly lower CA, height SDS and BMI SDS than normal children (Table 1). We found that 6/81 ISS children had BMI < −2.0 SDS. The proportion of males and prepubertal children in this group was significantly higher than in control group (Table 1). Therefore, as most ISS children were at Tanner stage I, prepubertal children from control and ISS groups were also analyzed separately (Table 1). Both groups of prepubertal normal and ISS children had similar CA, but ISS children showed significantly lower height and BMI and higher proportion of boys than normal prepubertal group. Since significant differences in BMI SDS were obtained between normal and ISS children, either for the whole group of children or for the prepubertal one, BMI SDS was included as a covariable in the analysis. In this context, ISS patients had significantly lower serum GHBP, IGF-I, IGFBP-3 and ALS levels compared to normal children while no differences were obtained for insulin concentration (Table 1).

ISS children presented a left-biased distribution of serum GHBP (panel A), IGF-I (panel B), IGFBP-3 (panel C) and ALS (panel D) levels expressed as SDS (Fig. 2), being the percentage of values <0 SDS of 67%, 84%, 80% and 86% for serum GHBP, IGF-I, IGFBP-3 and ALS, respectively. Considering a cut-off value ≤−2.0 SDS, we found that 2.5% of ISS children presented low GHBP, while 30.8%, 19.7% and 28% of children had low IGF-I, IGFBP-3 and ALS values, respectively. Serum GHBP SDS did not correlate with maximal GH response to provocative test. Nevertheless, a significant inverse relationship between these two biochemical parameters was obtained when GHBP SDS below zero were analyzed ($r = -0.37$, $p < 0.05$).

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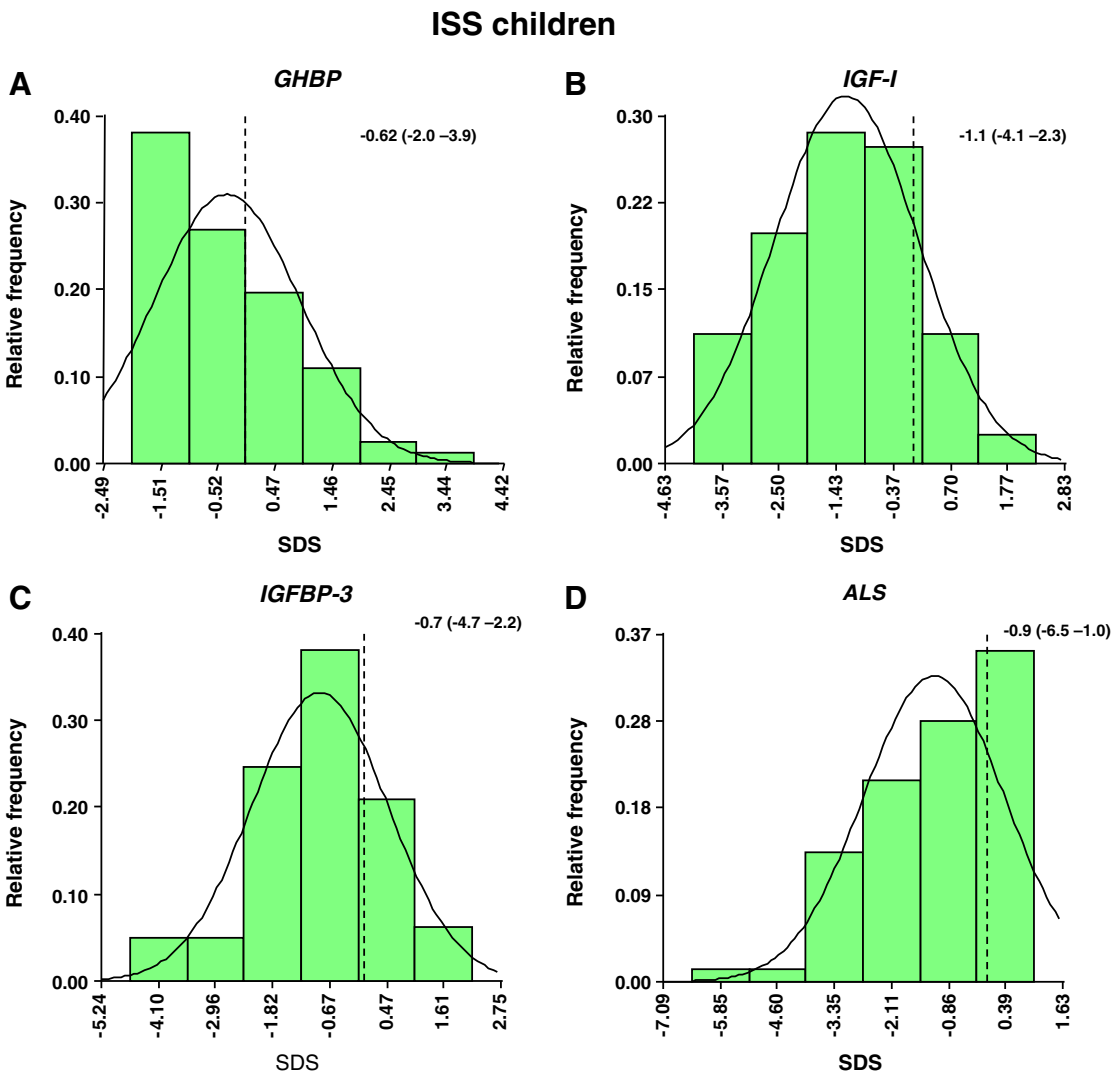

Fig. 2. Histogram of the distribution of: (A) GHBP, (B) IGF-I, (C) IGFBP-3, (D) ALS expressed in standard deviation score (SDS) for the whole group of ISS children. The dotted vertical line denotes the zero SDS. The median and range expressed in SDS is indicated for each GH-dependent parameter.

3.3. *GHR*-exon 3 genotype in normal and ISS children

The possible association of height, BMI, insulin, IGF-I, IGFBP-3, and ALS with *GHR*-exon 3 genotype was investigated. The percentage of ISS children with each genotype (fl:fl/fl:d3/d3:d3) was similar to control group (ISS: 64:32:4 vs Normal children: 60:34:6), reaching Hardy–Weinberg Equilibrium (ISS: X^2 test = 0.02 and Normal children: X^2 test = 0.222). The proportion of children having either none versus one or two copies of *GHR*d3 allele was not different between normal and ISS ($p = 0.496$). This result was also obtained for the prepubertal group ($p = 0.434$). Table 2 shows the auxological and biochemical characteristics of normal and ISS children regarding *GHR*-exon 3 genotype. Normal children homozygous for the *GHR*fl allele presented similar values regarding height SDS, BMI SDS, insulin, IGF-1, IGFBP3 and ALS levels as normal *GHR*d3 group (Table 2) while significantly higher serum GHBP levels were obtained for the *GHR*fl group as compared to children carrying one or two copies of *GHR*d3 allele (Table 2). This difference was also maintained for GHBP SDS ($p = 0.04$, Table 2 and Fig. 3) including BMI SDS as covariable in the analysis. The subanalysis of the group of normal prepubertal children also showed a tendency to higher circulating GHBP levels for homozygous carriers of the *GHR*fl allele expressed both in nmol/L (Mean ± SEM, *GHR*fl: 3.47 ± 0.29 vs *GHR*d3: 2.78 ± 0.24 nmol/L, $p = 0.06$), and in SDS (Mean ± SEM, *GHR*fl: 0.22 ± 0.16 SDS vs *GHR*d3: −0.18 ± 0.15 SDS, $p = 0.06$).

In the ISS group, we did not find any significant differences in height SDS, BMI SDS, IGF-1, IGFBP3, ALS or GHBP levels according to *GHR*-exon 3 polymorphism genotype (Table 2). Moreover, GH-dependent parameters were also reanalyzed after excluding ISS children with BMI SDS < −2.0 SDS ($n = 6$) and similar results were obtained for IGF-I (Mean ± SEM, *GHR*fl: −1.25 ± 0.20 SDS vs *GHR*d3: −1.38 ± 0.23 SDS, $p = 0.66$), IGFBP-3 (*GHR*fl: −0.79 ± 0.21 SDS vs *GHR*d3: −1.00 ± 0.22 SDS, $p = 0.40$), ALS (*GHR*fl: −1.32 ± 0.24 SDS vs *GHR*d3: −1.43 ± 0.28 SDS, $p = 0.60$) and GHBP (*GHR*fl: −0.27 ± 0.18 SDS vs *GHR*d3: −0.53 ± 0.22 SDS, $p = 0.44$).

4. Discussion

In the present study we found that normal homozygous children carrying the *GHR*fl allele of the *GHR*-exon 3 genotype have significantly higher GHBP serum levels than those carrying one or two copies of the *GHR*d3 polymorphism. On the other hand, we observed reduced serum GHBP, IGF-I, IGFBP-3, and ALS levels in a selected cohort of children with idiopathic short stature as compared to a control group of the

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Table 2
Auxological and biochemical characteristics of normal and ISS children regarding growth hormone receptor *GHR*-exon 3 genotype. Normal and ISS children were grouped into *GHR*fl (children homozygous for the full-length allele) or *GHR*d3 (children carrying one or two copies of the d3 allele). Data are expressed as the median and the 2.5 centile and 97.5 centile range.

Parameter/Group	Normal		ISS	
GHR exon 3 genotype (n)	GHRfl (115)	GHRd3 (77)	GHRfl (52)	GHRd3 (29)
CA (ys)	11.6 (5.4–16.3)	10.4 (5.6–16.4)	9.4 (5.1–16.5)	9.0 (5.0–16.2)
Gender (females/males)	65/47	36/41	15/37	5/24
Tanner stage (n): I-II-III-IV-V	46-7-14-30-18	45-8-2-13-9	39-7-2-1-3	26-2-1-0-0
Height SDS	0.58 (−1.92 to 1.94)	0.13 (−1.62 to 1.90)	−2.81 (−4.20 to −2.05)	−2.79 (−4.15 to −2.02)
BMI SDS	0.38 (−1.49 to 1.72)	0.27 (−1.40 to 1.91)	−0.71 (−2.53 to 1.62)	−0.61 (−2.42 to 1.00)
Insulin	5.1 (1.9–13.6)	4.7 (2.0–12.8)	3.6 (2.0–13.3)	4.5 (2.0–21.9)
μUI/mL				
GHBP	2.9 (1.0–8.2)	2.3 (0.7–6.2)[a]	1.9 (0.9–7.5)	1.8 (0.9–9.1)
nmol/L	0.18 (−1.75 to 2.10)	−0.20 (−2.3 to 1.55)[a]	−0.58 (−1.94 to 2.37)	−0.70 (−1.98 to 2.11)
SDS				
IGF-I	215 (54–600)	153 (54–521)	88 (25–347)	63 (25–267)
ng/mL	−0.23 (−1.83 to 2.15)	−0.10 (−1.98 to 1.60)	−1.10 (−4.0 to 1.3)	−1.40 (−3.60 to 0.70)
SDS				
IGFBP-3	3.9 (2.6–6.0)	4.0 (2.7–5.1)	3.3 (1.3–5.2)	3.1 (1.6–4.4)
μg/mL	−0.19 (−1.98 to 2.02)	0.08 (−2.12 to 1.99)	−0.59 (−4.24 to 1.73)	−0.93 (−3.56 to 0.84)
SDS				
ALS	240 (147–378)	233 (123–363)	161 (76–312)	165 (82–276)
nmol/L	0.03 (−1.62 to 2.06)	0.18 (−2.24 to 1.67)	−1.29 (−4.65 to 1.24)	−1.00 (−4.32 to 0.84)
SDS				

[a] $p < 0.05$ versus normal homozygous GHRfl group.

same Argentinean origin. These findings were not associated to *GHR*d3 polymorphism.

The *GHR*-exon 3 polymorphism is a common polymorphism present in the extracellular domain of the GHR and it constitutes the first identified genetic factor that modulates the individual response to GH treatment. In vitro studies demonstrated a higher transcriptional activity of cells transfected with *GHR*d3 than those cells transfected with the *GHR*fl after treatment with hGH [25]. Pharmacogenetic studies have also addressed a possible role of GHR polymorphism on the response to recombinant human GH treatment in short children, finding a positive influence of the GHRd3 allele on the growth response to hGH [25,26]. Moreover, Toyoshima et al. showed that *GHR*d3 isoform positively influences the increase in IGF-I levels at IGF-I generation test in ISS children, supporting the hypothesis of higher GH sensitivity in children carrying at least one copy of the *GHR*d3 polymorphism [27].

In the present study, we evaluated the possible association of *GHR*-exon 3 genotype with auxological variables and GH-dependant factors, under basal conditions, in normal and ISS children. We found that both groups had a similar distribution of hetero- or homozygous of *GHR*d3 genotype as previously reported by other authors [25,27]. Interestingly, we found that only GHBP serum levels were significantly associated with *GHR*-exon 3 genotype in normal children. Homozygous normal *GHR*fl carriers had significantly higher GHBP levels compared to children carrying at least one *GHR*d3 allele. This finding was maintained after GHBP correction for gender, pubertal stage (GHBP SDS) and BMI. The subanalysis performed on prepubertal children showed similar results. Recently, in line to our findings, Wan et al. showed that d3/d3 healthy adults had significantly lower serum levels of GHBP compared with fl/fl and d3/fl genotypes [28]. A functional different impact of *GHR*-exon 3 genotype on GHBP shedding mechanism possibly due to changes in the three-dimensional conformation of the GHR was previously suggested [3,6]. At least two studies demonstrated that serum GHBP isoforms (GHBP having or lacking the amino acids encoded by exon 3) directly correlated with *GHR*-exon 3 genotype in humans, thus suggesting an effect for the exon 3-possitive allele on GHBP shedding [3,6]. Nevertheless, one of these studies included a small and partly related group of adults, where Hardy–Weimberg law was not met [6]. To our knowledge, this is the first study conducted in a population of normal children that reports the association between circulating GHBP and the *GHR*-exon 3 genotype.

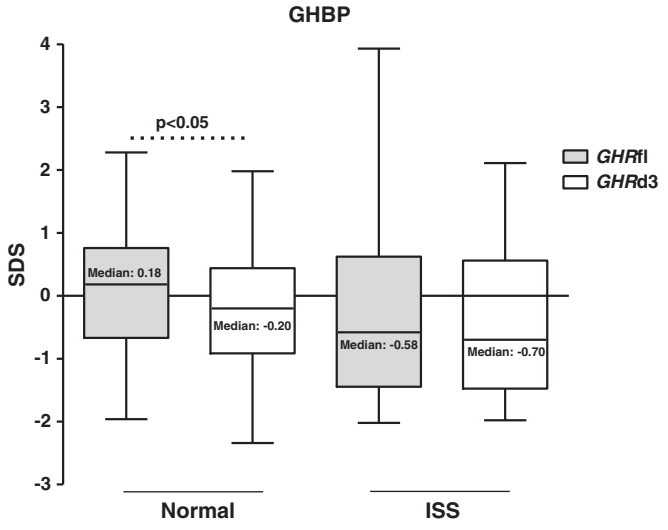

Fig. 3. GHBP SDS values in normal and ISS according to *GHR*-exon 3 genotype. The bottom and top of the box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), and the horizontal line near the middle of the box is the 50th percentile (the median). The whiskers indicate the upper and lower GHBP values. * $p < 0.05$ vs *GHR*fl.

The physiological variations of GHBP, IGF-I, IGFBP-3 and ALS levels related to age, gender, sexual development and BMI in normal children were confirmed in the present work. IGF-I, IGFBP-3 an ALS underwent the dramatic changes during puberty [29–34]. Inter-subject variability in normal children was approximately 60% for IGF-I and GHBP and nearly 20–25% for IGFBP-3 and ALS concentration, resulting higher than the ones previously reported for adolescent and adult populations [35]. Martha et al. showed that GHBP levels were regulated in individual children within much more narrow limits than inter-subject in a group of boys longitudinally evaluated as they matured through normal puberty [36]. We found that the most important determinants of GH-dependant inter-subject variability were Tanner stage and age. More previously seen by others, we found that IGF-I and ALS peaked earlier in girls than in boys during prepuberty, having their highest values at Tanner stage IV for both sexes [29–34]. Interestingly, the impact of age on IGF-I and ALS at prepuberty was only observed in girls, probably supporting the hypothesis of higher estradiol concentration in girls close to peripubertal ages [29]. Gender seemed not to significantly influence these GH-dependent factors. Gender-related differences were only

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observed in GHBP levels at complete puberty and in prepubertal girls at Tanner I-B in whom higher levels of IGF-I and ALS levels were found as compared to age- and Tanner-matched control boys. Besides gender, pubertal development and age, we also found that BMI significantly influenced GH-dependent markers thus, it is important to include BMI as a covariable when comparing populations where BMI differs. Moreover, it was demonstrated that BMI modulated the IGF-I response to GH administration in children with ISS, suggesting that GH sensitivity may be influenced by the nutritional status on them [37]. In normal children, the influence of the *GHR*-exon 3 genotype was not observed neither for GH, IGF-I, IGFBP-3, ALS or insulin levels, nor for auxological parameters such as height SDS and BMI SDS.

ISS children in this study had a significantly lower BMI SDS as compared to control children. Wudy et al. reported that ISS had reduced appetite and the lower BMI was suggested as a contributory factor to short stature [38]. Recently, low-normal BMI was reported in a 42% of ISS children [39]. ISS children included in this study had low GHBP levels as well as other GH-dependent factors and as compared to a control group of the same ethnic origin. These findings were not associated to *GHR*-exon 3 genotype, even after excluding ISS children with low BMI, thus supporting that this polymorphism is not primarily related to the genesis of short stature [25]. Carlsson et al. reported that 90% of ISS children had GHBP concentrations below the mean for a given age and sex [40]. Similarly, 63% of patients with ISS in this study had GHBP SDS below the mean. In accordance to previous studies in ISS [8], we found that 28% of ISS children had IGF-I values of less than −2 SDS by using a current chemiluminescent immunometric assay. Our present work also demonstrated low ALS SDS (27%) and IGFBP-3 (22%) SDS values in ISS children. GHBP, IGF-I, IGFBP-3 and ALS production may be disturbed in states of GH insensitivity due to heterozygosity for specific mutations affecting the GH-IGF axis in some children with ISS [9–14]. Therefore, our results may reflect potential defects in GH receptor action that may result in partial GH insensitivity.

The present study evaluated the possible association of GHBP, IGF-I, IGFBP-3 and ALS with *GHR*-exon 3 genotype in normal and ISS children. As GHBP constitutes the extracellular domain of GHR, the association of higher serum GHBP levels in normal homozygous carriers for the *GHR*fl allele may probably support the hypothesis of the influence of this polymorphism on GHBP shedding as it was demonstrated in vitro [3]. This study also shows that serum levels of GHBP, IGF-I, IGFBP-3 and ALS in ISS children are not associated to *GHR*-exon 3 genotype, probably reflecting that this is not a homogeneous population.

Acknowledgments

The authors wish to acknowledge Ana María Montese, Silvina Gonzalez, Dr. Liliana Karabatas and Perla Rossano for their technical assistance; Dr. Viviana Pipman and Dr. Viviana Bengolea from Hospital "E. Tornú" and Hospital "Juan Fernández", Buenos Aires, Argentina, respectively for the recruitment of normal children.

Disclosure

The present study was partially supported by grants PICT/2003 N 05-14354, of the Agencia Nacional de Promoción Científica, Argentina (to H.G.J.), Laboratorio Pfizer Global Pharmaceutical (to H.G.J.), Consejo de Investigación en Salud (CIS), Ministerio de Salud del Gobierno de la Ciudad de Buenos Aires, Argentina (to M.G.B.) and by a grant of Fundación Alberto J. Roemmers (to M.G.R.), Buenos Aires, Argentina.

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