HORMONE
RESEARCH IN
PÆDIATRICS

Horm Res Paediatr
DOI: 10.1159/000355412

Received: March 21, 2013
Accepted: August 16, 2013
Published online: ■■■

Heterozygous *IGFALS* Gene Variants in Idiopathic Short Stature and Normal Children: Impact on Height and the IGF System

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Key Words
Acid-labile subunit · *IGFALS* gene · Genetic endocrine disorders · IGFI · Idiopathic short stature · IGFBP-3

Abstract
Background: In acid-labile subunit (ALS)-deficient families, heterozygous carriers of *IGFALS* gene mutations are frequently shorter than their wild-type relatives, suggesting that *IGFALS* haploinsufficiency could result in short stature. We have characterized *IGFALS* gene variants in idiopathic short stature (ISS) and in normal children, determining their impact on height and the IGF system. ***Patients and Methods:*** In 188 normal and 79 ISS children levels of IGF-1, IGFBP-3, ALS, ternary complex formation (TCF) and *IGFALS* gene sequence were determined. ***Results:*** In sum, 9 nonsynonymous or frameshift *IGFALS* variants (E35Gfs*17, G83S, L97F, R277H, P287L, A330D, R493H, A546V and R548W) were found in 10 ISS children and 6 variants (G170S, V239M, N276S, R277H, G506R and R548W) were found in 7 normal children. If ISS children were classified according to the ability for TCF enhanced by the addition of rhIGFBP-3 (TCF+), carriers of pathogenic *IGFALS* gene variants were shorter and presented lower levels of IGF-1, IGFBP-3 and ALS in comparison to carriers of benign variants. In ISS families, subjects carrying pathogenic variants were shorter and presented lower IGF-1, IGFBP-3 and ALS levels than noncarriers. ***Conclusions:*** These findings suggest that heterozygous *IGFALS* gene variants could be responsible for short stature in a subset of ISS children with diminished levels of IGF-1, IGFBP-3 and ALS.

Introduction

Idiopathic short stature (ISS) is a clinical condition defined as height more than 2 standard deviation scores (SDS) below the corresponding mean for a given age, sex and population group in children without evidence of systemic, endocrine, nutritional or chromosomal abnormalities [1, 2]. This definition of ISS includes short chil-

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dren with constitutional delay of growth and puberty and familial short stature [2]. Noteworthy, ISS children have normal birth weight and are growth hormone (GH) sufficient (as defined by measurement of stimulated GH levels). This diagnosis is essentially one of exclusion made after a careful clinical history, physical examination and biochemical assessment ruled out other causes of short stature. Nevertheless, it is widely suspected that beneath ISS different etiologies are hiding.

Several molecular defects have already been characterized in ISS. Heterozygosity for GH receptor gene *(GHR)* mutations was first reported by Goddard et al. [3] and then confirmed by others [4–7]. Inactivating mutations in the *GH1* gene have been described in patients having a bioinactive GH [8, 9]. More recently, mutations and deletions in short stature homeobox gene *SHOX* [10], homozygous and heterozygous mutations in the GH secretagogue receptor gene *GHSR* [11–13] and heterozygous mutations in the natriuretic peptide receptor *NPR2* gene [14] have also been found in children with ISS. Except for most patients with mutations in the *SHOX* and *NPR2* genes, these conditions are characterized by low levels of IGF-1 in the face of normal GH secretion. These findings suggest partial GH insensitivity [15].

Human acid-labile subunit (ALS) deficiency is caused by homozygous or compound heterozygous inactivating mutations in the *IGFALS* gene [16]. This condition is characterized by moderate growth retardation, normal or even elevated GH secretion, and a marked reduction of IGF-1 and IGFBP-3 levels that remain low after GH treatment [16–25]. Delayed puberty has been reported in about 50% of the patients, most frequently in males [22]. It is of note that heterozygous carriers of *IGFALS* gene mutations are frequently shorter than their wild-type (WT) first-degree relatives [20, 26] and show levels of IGF-1, IGFBP-3 and ALS that are intermediate between ALS deficient and WT relatives [20]. In consequence, we hypothesize that milder cases of ALS deficiency (i.e. heterozygous carriers of *IGFALS* gene mutations or patients having less detrimental gene mutations in both *IGFALS* alleles) may be present in a subgroup of ISS children, particularly in those presenting low IGF-1 levels.

Subjects and Methods

The protocol was approved by the Ethics Committees of the respective hospitals. Written informed consent was obtained from the parents, and the participant children gave their assent. The study was conducted in accordance with the Declaration of Helsinki.

A total of 79 ISS children (aged 4.8–17.4 years) were admitted to this study, together with 188 normal children (aged 5.0–17.5 years), with stature between −2.0 and +2.0 SDS. The latter were recruited from the Clinical Pediatric Outpatient Clinics of the participating hospitals, where they were seen for routine pediatric check-up or for minor complaints and were subsequently found to be healthy. In addition, auxological, genetic and biochemical data were obtained from first-degree relatives of ISS children who were found to be carriers of an *IGFALS* gene variant: 14 siblings and 15 parents. All children were born at term, had normal birth weight for Argentinean standards, had no systemic diseases and were taking no medication. Normal and ISS children underwent a clinical, anthropometric (height, weight, body proportions and pubertal staging) and biochemical evaluation (RBC, WBC, glucose, urea, creatinine, GOT, GPT, cholesterol, triglycerides, TSH, thyroxine, free thyroxine, triiodothyronine, anti-thyroperoxidase and anti-transglutaminase antibodies, IGF-1, IGFBP-3, ALS, cortisol, and insulin levels). Additionally, a karyotype was obtained in ISS girls. The evaluation allowed ruling out most of the known causes of short stature, i.e. psychiatric problems (such as maternal deprivation), congenital bone diseases, malnutrition, celiac disease, hematological, liver, renal, thyroid and adrenal diseases and Turner syndrome. GH deficiency was excluded by GH maximal stimulated levels >6.0 ng/ml (chemiluminescence assay, IMMULITE-2000 system; Siemens Healthcare Diagnostics Products Ltd., Gwynedd, UK) after sequential arginine (0.5 g/kg body weight)/clonidine (100 μg/m^2 body surface) testing.

Serum levels of TSH, thyroxine, free thyroxine, triiodothyronine and prolactin were determined by electrochemiluminescence (Cobas e411 analyzer; Roche Diagnostics GmbH, Mannheim, Germany) and levels of anti-thyroperoxidase antibodies and insulin by chemiluminescence assay (IMMULITE-2000 system; Siemens Healthcare Diagnostics Products Ltd.). IGF-1 levels were determined by RIA after serum extraction by the acid/ethanol method followed by cryoprecipitation [27], IGFBP-3 by immunoradiometric assay (IRMA; DSL, Webster, Tex., USA), and ALS by RIA (Bioclone, Sydney, N.S.W., Australia).

Ternary complex formation (TCF) was evaluated in 72 ISS and in 131 normal children by neutral size exclusion chromatography [16]: 100 μl of serum was incubated overnight at 22°C with 3.5 × 10^6 counts per min of ^{125}I-IGF-1, and then cross-linked with the addition of disuccinimidyl suberate (Sigma-Aldrich). Samples were chromatographed on a HiPrep 16/60 Sephacryl S-200HR column; 500-μl aliquots were loaded onto the column and 1-ml fractions were collected and counted. Serum levels of IGF-1, IGFBP-3, ALS and TCF were expressed as SDS according to age and pubertal stage in relation to the 188 normal controls collected in this study. In a subset of 11 ISS children and 19 normal controls (10 prepubertal and 9 pubertal) TCF was performed twice, as previously described, and enhanced (TCF+) by the addition of 6 μg/ml rhIGFBP-3 (R&D Systems, Minneapolis, Minn., USA). All TCFs were calculated as follows: (area under the curve of ternary complex peak/total area) × 100, and expressed as SDS according to those obtained in 131 normal children (without the addition of rhIGFBP-3) and TCF+ in 19 normal children (with the addition of rhIGFBP-3).

Molecular Studies

Genomic DNA from controls, ISS patients and first-degree relatives of heterozygous carriers of *IGFALS* gene variants was isolated from peripheral leukocytes based on the use of cetyltrimethylammonium bromide lysis buffer and isoamyl alcohol-chloro-

Table 1. Clinical, auxological and biochemical data from normal control and ISS children

	Normal	ISS	t test
n	188	79	
Sex (M/F)	86/102	58/21	
Age, years	10.9±3.2 (5.0 to 17.5)	9.9±3.4 (4.8 to 17.4)	
Height, SDS	0.17±1.00 (–1.96 to +2.00)	–2.85±0.52 (–4.56 to –2.00)	p < 0.0001
BMI, SDS	0.23±0.83 (–2.19 to +2.17)	–0.62±1.07 (–4.27 to +2.41)	p < 0.0001
Pubertal stage, n	90/33/40/25	65/11/1/2	
IGF-1, SDS	–0.03±0.99	–1.47±1.30	p < 0.0001
IGFBP-3, SDS	–0.02±0.98	–0.87±1.19	p < 0.0001
ALS, SDS	–0.07±1.02	–1.30±1.44	p < 0.0001
TCF, SDS	–0.02±0.99	–0.92±1.23	p < 0.0001

Values are presented as mean ± SD and range, when appropriate. Pubertal stage: I, II + III, IV and V.

form extraction [28]. Exons 1 and 2 and contiguous intron sequences, corresponding to the *IGFALS* gene (GenBank accession number NM_004970.2), were amplified by PCR using oligonucleotide primers flanking both exons. PCR products were purified by using AccuPrep PCR purification columns (Bioneer, Seoul, South Korea), and sequenced in both directions in an ABI 3730xl automatic sequencer (Macrogen, Seoul, South Korea). Primer sequences are available on request.

We analyzed the effect of *IGFALS* gene variants in silico by using various bioinformatic predicting tools: PolyPhen2 [29], SIFT [30], Mutation Taster [31] and MutPred [32]. Human Splicing Finder [33] was used to analyze synonymous variants.

We also analyzed with these software programs the 18 mutations previously described in a homozygous or compound heterozygous state in ALS-deficient patients. The result of such analysis showed discordant results in predicting the pathogenic effect of missense variants. Only 8 out of 13 missense variants were predicted as pathogenic by the 4 programs, while the remaining variants were predicted as pathogenic by 3 programs in 3 cases and by only 2 in the remaining 2 cases. On the other hand, the 5 insertion/deletion mutations were predicted as pathogenic by the Mutation Taster, the only program capable of analyzing this kind of mutation. Due to the inconsistent results of the in silico analysis, *IGFALS* gene variants found in ISS patients were classified as pathogenic or benign based on the result of in vitro TCF enhanced by the addition of rhIGFBP-3 (TCF+), a functional assay to estimate the ability of ALS to bind to IGF-1/IGFBP-3 binary complexes: pathogenic with TCF+ below –2.0 SDS and benign with TCF+ above –2.0 SDS. We applied the in silico prediction tools for complementary analysis.

One-way ANOVA (followed by Tukey's test or linear trend as posttests), Wilcoxon signed rank test, and unpaired t test or Mann-Whitney and χ^2 test, were used for statistical analysis.

Results

Clinical and auxological data from normal control and ISS children, including sex distribution, age, height, body mass index (BMI) and pubertal stage are presented in table 1. Although BMI was significantly lower in ISS compared to normal children, only 6 out of 79 (7.5%) ISS children presented a BMI below –2.0 SDS. Bone age was delayed more than 2 years in 45 out of 69 ISS patients, with a bone age delay of 3.35 ± 0.81 (mean ± SD) years.

IGF-1, IGFBP-3, ALS and TCF Levels in ISS Children

In ISS children, levels of IGF-1, IGFBP-3, ALS and TCF were significantly lower than those found in normal children (table 1). Levels below –2.0 SDS were more frequent for IGF-1 (25/79, 31.6%) and ALS (23/79, 29.1%) than low levels of IGFBP-3 (10/79, 12.7%) (χ^2 test, p = 0.004 and p = 0.011, respectively); 8 out of 79 (10.1%) presented 3 parameters (IGF-1, IGFBP-3, ALS) below –2.0 SDS.

IGFALS Genetic Variants in ISS and Normal Children

Two common previously described single nucleotide polymorphisms (SNPs) were frequently found both in ISS and in normal children: D70D (CC 60.8%, CT 39.2% in ISS and CC 59.0%, CT 35.1% and TT 5.9% in normal children) and Y462Y (CC 83.5%, CT 16.5% in ISS and CC 84.6%, CT 15.4% in normal children). All other genetic variants were found in heterozygosis: 13 different *IGFALS* genetic variants were found in 11 ISS children (3 patients had 2 variants in one allele): 4 genetic variants were synonymous SNPs – a single base change that does not change the encoded amino acid – (P73P, L271L, L316L and T522T), 8 nonsynonymous SNPs – a single base change which does change the encoded amino acid – (G83S, L97F, R277H, P287L, A330D, R493H, A546V and R548W) and 1 frameshift point mutation (E35Gfs*17). *IGFALS* gene variants were also found in 16 normal children: 5 synonymous SNPs (N236N and

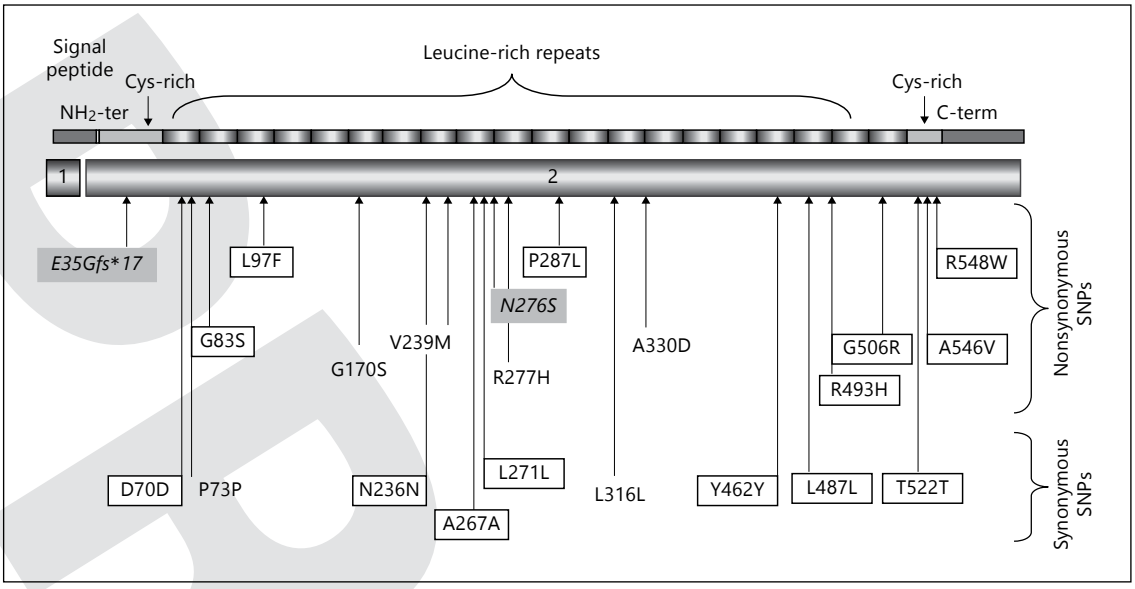

Fig. 1. Heterozygous *IGFALS* gene variants in ISS and normal children. Schematic representation of *IGFALS* gene showing allelic variants identified in the present study. Gray boxes: previously described inactivating mutations; open boxes: SNPs included in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp).

A267A in 1 subject each, L271L and L487L in 2 subjects and T522T in 3 subjects) and 6 nonsynonymous SNPs (G170S, V239M, N276S, R277H, G506R and R548W). While the frequency of synonymous SNPs was not different in ISS compared to normal children (3.8 vs. 4.8%), nonsynonymous SNPs were more frequently found in ISS (11.4 vs. 3.7%, p = 0.013), as shown in figure 1 and table 2.

Based on the results of the TCF+, we classified the following gene variants as pathogenic (E35Gfs*17, R277H, P287L and R548W) and benign (P73P, G83S, L97F, L271L, L316L, A330D, R493H and T522T).

In silico analysis found that the variants classified as pathogenic by TCF+ were predicted as pathogenic by 2/4 (R277H), 1/4 (P287L) and 0/4 (R548W) bioinformatic tools, while the Mutation Taster predicted the E35Gfs*17 variant as pathogenic. In the group of variants classified as benign only L97F was predicted as pathogenic by 1/4 of the prediction tools. None of the synonymous gene variants were predicted to result in severe splicing alterations by the Human Splicing Finder.

There were 5 other gene variants found in normal children (presenting normal IGF-1, IGFBP-3 and ALS serum levels) which were predicted as pathogenic by 4/4 (N276S), 2/4 (R277H), 1/4 (V239M and G506R) and 0/4 (G170S) bioinformatic tools. These variants were not evaluated by TCF+ analysis.

Height and Levels of IGF-1, IGFBP-3, ALS and TCF in ISS Children with and without IGFALS Gene Variants

There were no differences in height SDS, IGF-1, IGFBP-3, ALS and TCF levels between carriers and noncarriers of the common *IGFALS* gene variants D70D and Y462Y, both among normal control and ISS children. There were no differences in height SDS between heterozygous carriers of the other gene variants and WT ISS children (–2.85 ± 0.31 vs. –2.84 ± 0.56). Biochemically, levels of IGF-1 (–1.90 ± 1.69 vs. –1.40 ± 1.23 SDS, p = 0.2374) and TCF (–1.26 ± 0.96 vs. –0.99 ± 1.08 SDS, p = 0.4425) were not different in ISS heterozygous carriers compared with WT. However, IGFBP-3 (–1.90 ± 1.46 vs. –0.71 ± 1.08 SDS, p = 0.0016) and ALS levels (–2.34 ± 1.77 vs. –1.13 ± 1.32 SDS, p = 0.0090) were significantly lower in ISS heterozygous carriers of an *IGFALS* gene variant compared to WT (fig. 2).

Height, IGF-1, IGFBP-3, ALS and TCF+ Levels in ISS Children with Pathogenic and Benign IGFALS Gene Variants

By definition TCF+ levels were significantly lower in ISS children carrying a pathogenic variant compared to those in carriers of a benign variant (–5.04 ± 1.43 vs. 1.06 ± 2.18 SDS, p = 0.0005). In addition, ISS children carrying a pathogenic variant were shorter (–3.01 ± 0.32 vs. –2.64 ± 0.16, p = 0.0367) and presented significantly lower levels of IGF-1 (–3.43 ± 0.89 vs. –0.63 ± 0.89 SDS, p =

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Table 2. Allelic variants in the *IGFALS* gene found in ISS and normal children

Genetic variant	Nucleotide change	dbSNP identification	Allelic frequency		MAF
			ISS	controls	
p.E35GfsX17	c.103dupG		0.0063		
p.D70D	c.210C>T	rs3751893	0.1962	0.2340	0.2153
p.P73P	c.219G>A		0.0063		
p.G83S	c.247G>A	Rs375218525	0.0063		NA
p.L97F	c.289C>T	rs35947557	0.0063		0.0152
p.G170S	c.508G>A			0.0027	
p.N236N	c.708C>T	rs77490547		0.0027	0.0028
p.V239M	c.715G>A			0.0027	
p.A267A	c.801G>A	rs35632685		0.0027	0.0119
p.L271L	c.813G>C	Rs369679075	0.0063	0.0053	NA
p.N276S	c.827A>G			0.0027	
p.R277H	c.830G>A		0.0063	0.0027	
p.P287L	c.860C>T	rs35706152	0.0063		0.0055
p.L316L	c.948G>A		0.0063		
p.A330D	c.989C>A		0.0063		
p.Y462Y	c.1386C>T	rs17559	0.0825	0.0771	0.1892
p.L487L	c.1461G>T	rs113804102		0.0053	0.0051
p.R493H	c.1478G>A	rs200380381	0.0063		0.0005
p.G506R	c.1516G>A	rs141460735		0.0027	0.0014
p.T522T	c.1566G>A	rs2230053	0.0063	0.0079	0.0344
p.A546V	c.1637C>T	rs202125685	0.0063		0.0005
p.R548W	c.1642C>T	rs9282731	0.0127	0.0053	0.0569

Allelic frequencies were calculated as the ratio between the number of alleles presenting the gene variant and total number of alleles analyzed.

MAF: global minor allele frequency. NCBI curated dbSNP is reporting the minor allele frequency for each rs included in a default global population. The current default global population is 1,000 Genome phase 1 genotype data from 1,094 worldwide individuals, released in the May 2011 dataset (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?showRare=on&chooseRs=coding&locusId=3483&mrna=NM_004970.2&ctg=NT_010393.16&prot=NP_004961.1&orien=reverse&refresh=refresh).

NA = Not available.

0.0006), IGFBP-3 (–3.14 ± 0.69 vs. –0.86 ± 1.02 SDS, p = 0.0022) and ALS (–3.98 ± 0.58 vs. –0.98 ± 1.06 SDS, p = 0.0003) compared to carriers of benign variants. The 5 ISS children who are heterozygous carriers of pathogenic *IGFALS* gene variants also had levels of IGF-1, IGFBP-3 and ALS below –2.0 SDS, while none of the ISS children who are heterozygous carriers of benign gene variants had all 3 parameters below –2.0 SDS (χ^2 test, p = 0.0009; fig. 3).

TCF with (TCF+) and without (TCF) the Addition of rhIGFBP-3

Figure 4 shows TCF profiles with and without the addition of rhIGFBP-3 in normal children (prepubertal: fig. 4a; pubertal: fig. 4b) and ISS children carrying pathogenic or benign (fig. 4c, d) *IGFALS* gene variants. TCF was significantly higher in pubertal compared to prepubertal normal children both before (p < 0.0001) and after rhIGFBP-3 addition (p = 0.0049). After rhIGFBP-3 addition, ^{125}I-IGF-1 was preferentially found in the ternary peak both in prepubertal and pubertal normal children. There were no differences in TCF between the 2 groups of heterozygous carrier ISS children without rhIGFBP-3 (–1.00 ± 1.19 vs. –1.48 ± 0.77 SDS).

Only in ISS children who are heterozygous carriers of benign *IGFALS* variants was ^{125}I-IGF-1 predominantly found in the 150-kDa ternary complex peak after rhIGFBP-3 addition, indicating some degree of impairment for TCF in ISS children who are heterozygous carriers of pathogenic variants.

Height and IGF-1, IGFBP-3 and ALS Levels in ISS Children Who Are Heterozygous Carriers of IGFALS Gene Variants and Their First-Degree Relatives

Auxological, genetic and biochemical data were obtained from first-degree relatives of ISS children carrying an *IGFALS* gene variant: 14 siblings (2 heterozygous carriers) and 15 parents (7 heterozygous carriers). Height and IGF-1, IGFBP-3 and ALS levels were analyzed together in

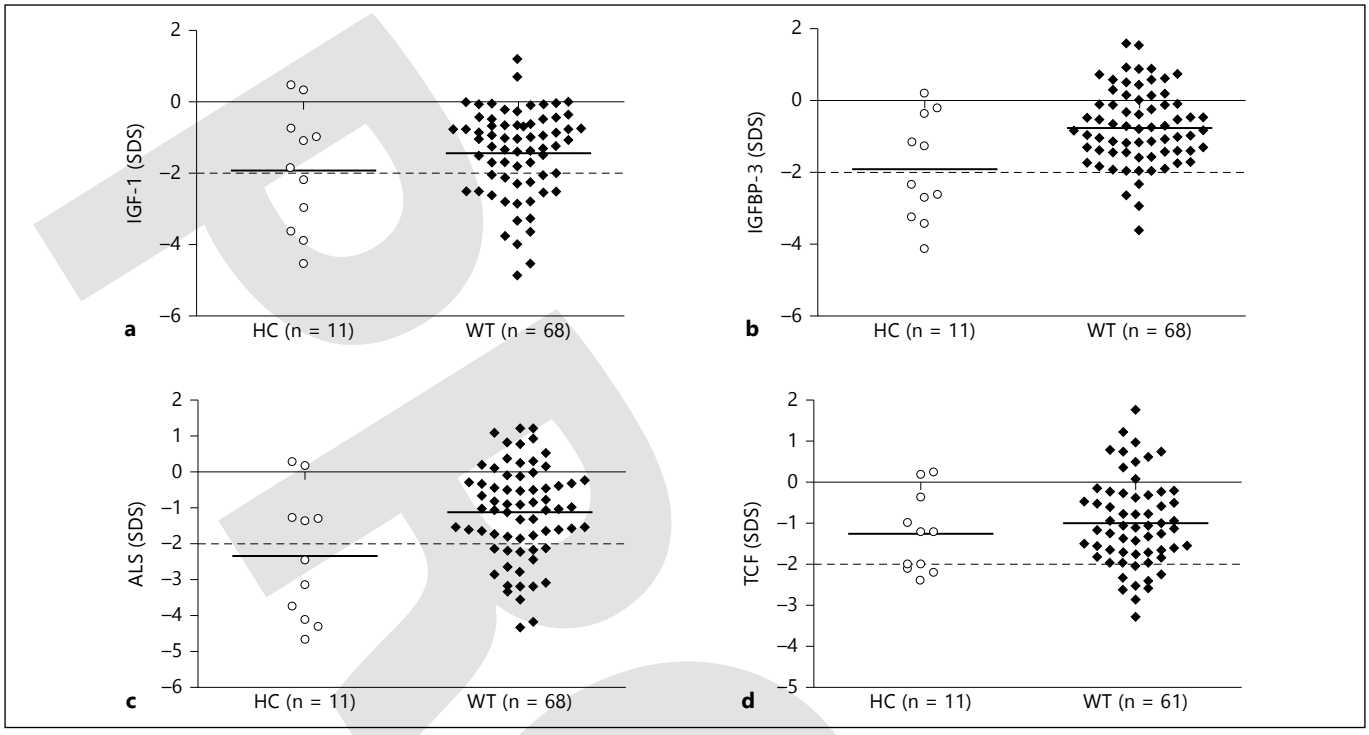

Fig. 2. IGF-1 (**a**), IGFBP-3 (**b**), ALS (**c**) and TCF (**d**) expressed as SDS in ISS children who are heterozygous carriers of an allelic variant in the *IGFALS* gene and in noncarriers (WT). HC = Heterozygous carriers.

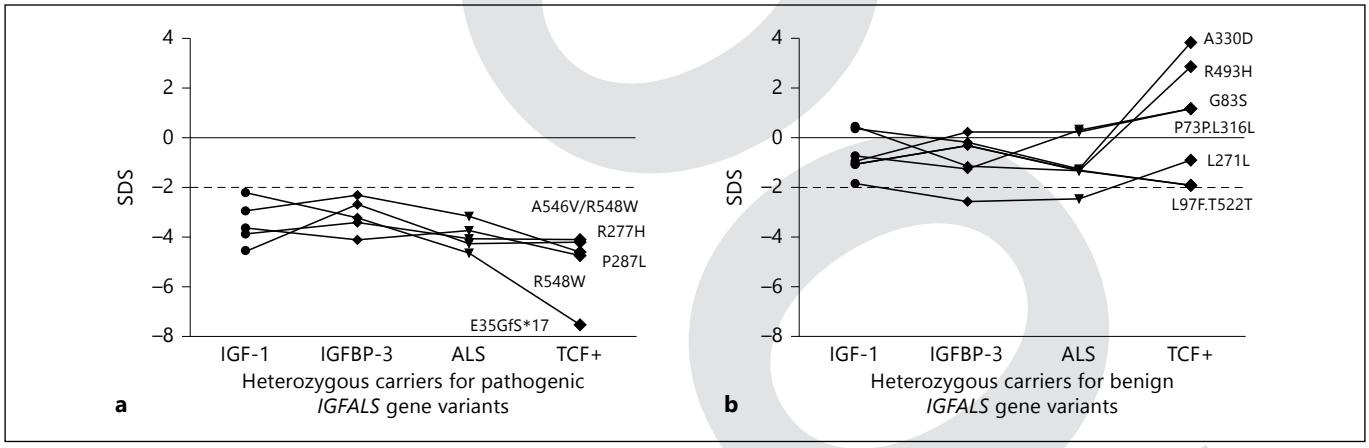

Fig. 3. IGF-1, IGFBP-3 and ALS levels and TCF+ (TCF enhanced by the addition of rhIGFBP-3) in ISS and normal children who are heterozygous carriers for pathogenic (**a**) and benign (**b**) *IGFALS* gene variants.

patients and family relatives divided into carriers of pathogenic (height: –2.43 ± 1.20, IGF-1: –2.93 ± 1.18, IGFBP-3: –2.46 ± 1.16, ALS: –3.2 ± 1.54, n = 8) and benign (height: –2.03 ± 0.93, IGF-1: –0.68 ± 0.85, IGFBP-3: –0.78 ± 0.87, ALS: –0.81 ± 0.80, n = 12) *IGFALS* gene variants or WT noncarriers (height: –0.38 ± 0.92, IGF-1: –0.46 ± 1.03, IGFBP-3: 0.19 ± 1.12, ALS: –0.65 ± 0.98, n = 20; fig. 5). Heterozygous carriers of pathogenic variants were shorter and presented lower IGF-1, IGFBP-3 and ALS levels in comparison to WT relatives (all parameters, $p < 0.001$) and lower IGF-1 ($p < 0.05$) and ALS ($p < 0.01$) levels compared to heterozygous carriers of benign variants (fig. 5).

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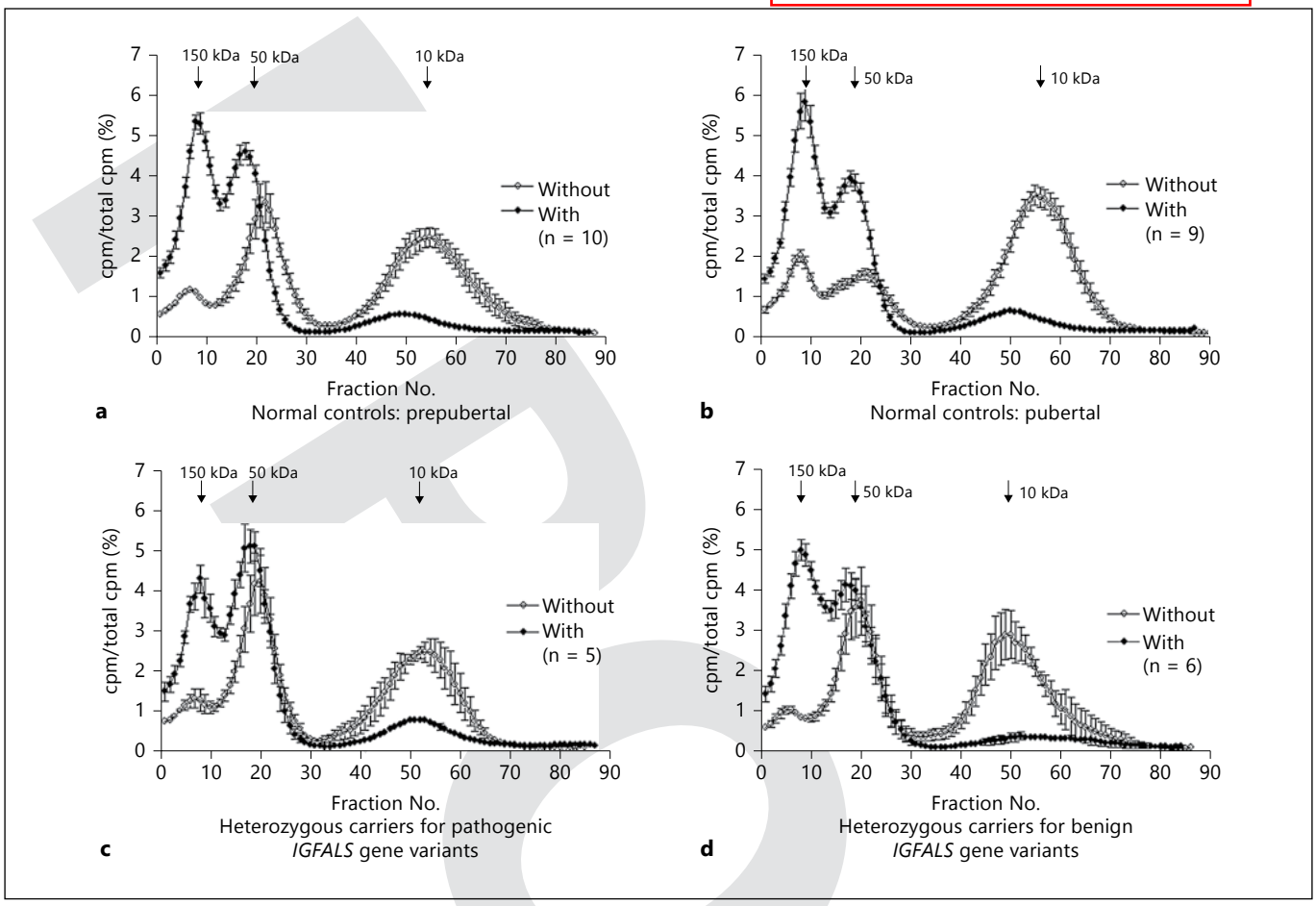

Fig. 4. TCF profiles (mean ± SEM) with and without the addition of rhIGFBP-3 in prepubertal (**a**), and pubertal (**b**) normal children and ISS children who are heterozygous carriers for pathogenic (**c**) or benign (**d**) *IGFALS* gene variants. HC = Heterozygous carriers; cpm = counts per min. The 150-kDa peak corresponds to ternary complex, the 50-kDa peak to binary complex and the 10-kDa peak to unbound ^{125}I-IGF-1.

Target Adjusted Height in ISS Children Carrying IGFALS Gene Variants in Comparison to WT Siblings

Within ISS families of heterozygous carriers of *IGFALS* variants, height data from 8 WT and 7 heterozygous carrier parents were available to calculate target-adjusted height SDS (TAH-SDS). In 9 heterozygous carriers and 8 WT siblings TAH-SDS was calculated as height SDS at diagnosis minus target height SDS, defined as follows: [paternal height (cm) + maternal height (cm)]/2 ± 6.5. TAH-SDS was lower in heterozygous carriers (median –1.51, range –2.86–0.34, n = 9) in comparison to WT siblings (median 0.68, range –0.17–1.42, n = 8) (Mann-Whitney test, p = 0.0003; fig. 6). We found no differences in height between WT parents and parents who are heterozygous carriers (height SDS –1.29 ± 1.14 for heterozygous carriers and –0.90 ± 0.79 for WT parents).

Discussion

We have found an 11.4% prevalence of heterozygous nonsynonymous *IGFALS* gene variants in ISS children. In addition, these genetic variants were particularly frequent (56%) in those children presenting low levels of IGF-1, IGFBP-3 and ALS. Interestingly, a similar prevalence of *IGFALS* gene variants has been reported by other groups [34, 35].

Although this cohort of ISS and control subjects originates from the same ethnic background, there were some differences between these groups other than height. For example, the sex ratio and distribution of pubertal stage distribution was different. This phenomenon, particularly the sex bias, is generally observed in ISS children referred to a pediatric endocrinologist for short stature evaluation.

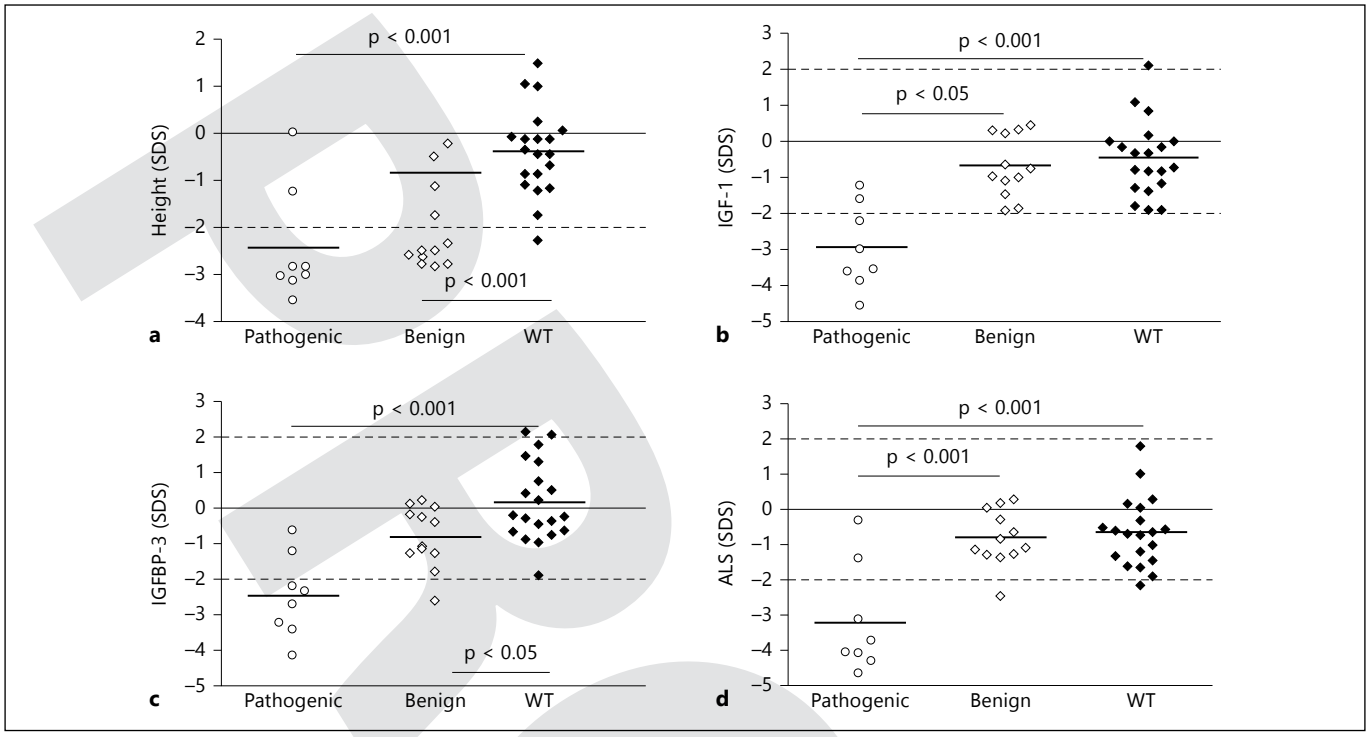

Fig. 5. Height (**a**), IGF-1 (**b**), IGFBP-3 (**c**) and ALS (**d**) levels in ISS children and first-degree relatives who are heterozygous carriers of pathogenic (n = 8) or benign (n = 12) *IGFALS* gene variants, or WT (n = 20). ANOVA, p > 0.0001; linear trend, p < 0.0001.

We have identified 22 variants, 16 of which have been already reported and 6 of which are novel. They include the following: 12 missense variants, 9 synonymous and 1 frameshift change. The *IGFALS* gene spans about 4.5 kb in chromosome 16p13.3. While exon 1 contains only 16 bases, coding for the 5 first amino acid residues and the first base of residue 6, exon 2 consists of 1,802 bases including the 2 remaining bases of the 6th codon and codes for the remaining 599 residues [36]. Thus, exon 2 constitutes 99% of the coding region. From a statistical point of view, it is not surprising that all previously reported mutations and all variants described here are located in exon 2, considering that it encodes more than 99% of the protein. Nevertheless, we have analyzed both exons, i.e. the whole coding region of the gene, and found no gene variants in exon 1 comparing with the reference gene sequence. As previously reported [37], the degree of sequence variation is relatively high in extracellular proteins, probably because these proteins are less constrained to mutational changes by selection mechanisms compared to genes encoding transcription factors, receptors and structural proteins.

In accordance with previous studies, we have found that about 30% of ISS children present IGF-1 levels below –2.0 SDS [38, 39]. This finding can be partially explained by the reduced BMI and delayed bone maturation frequently observed in ISS children. Nevertheless, the significant reductions of IGF-1, IGFBP-3 and ALS associated with normal GH response to provocative tests (observed in 11% of ISS

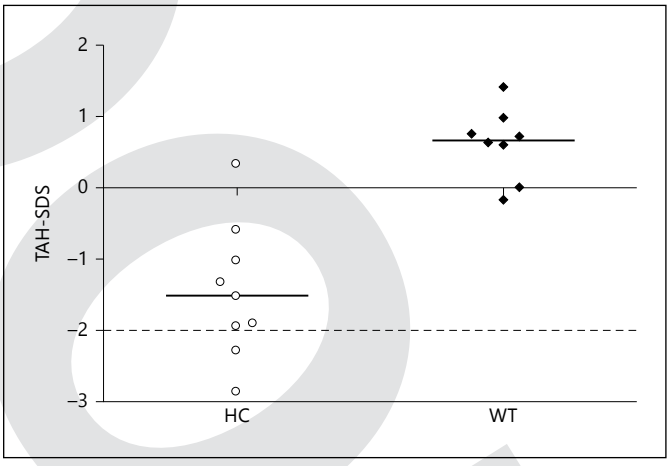

Fig. 6. Target-adjusted height in ISS children heterozygous carriers (n = 9) of *IGFALS* gene variants and their WT siblings (n = 8). HC = Heterozygous carriers. Mann-Whitney test, p = 0.0003.

children in this study) suggest that some degree of GH resistance could be present in these subjects. Further support for this hypothesis is the finding of heterozygous *GHR* gene mutations in about 5% of ISS children [3–7]. Although most of the heterozygous *GHR* gene mutations previously reported have not been fully characterized at a functional level, more recently partial GH insensitivity has been demonstrated in patients with heterozygous *GHR* gene defects causing either dominant negative effects (splicing defects causing exon 8 skipping) [40] or a premature stop codon resulting in nonsense-mediated mRNA decay [41].

Since the biochemical profile of complete ALS deficiency closely resembles complete GH insensitivity (normal or elevated GH secretion and marked reduction of IGF-1 and IGFBP-3 levels that remain low after GH stimulation), it is not unexpected that heterozygous genetic variants in the *IGFALS* gene, predicted to result in damage to the protein structure, are overrepresented in ISS compared to normal children from the same population.

The comparison with a normal control group from the same population resulted informative to obtain an adequate characterization of ISS children who presented an altered IGF system. The effect of these variants was further analyzed by comparing auxological measurements and IGF system data from ISS children who are heterozygous carriers of pathogenic and benign genetic variants and their WT first-degree relatives. The results confirm the gradual effect of benign and pathogenic *IGFALS* variants on IGF-1, IGFBP-3 and ALS levels. Heterozygous carriers were about 2.0 SDS shorter than WT siblings and presented lower levels of IGF-1, IGFBP-3 and ALS. This difference in height is larger than reported by Fofanova-Gambetti et al. [26], who reported that heterozygous carriers and WT relatives showed a 1.0 SDS difference in height. We speculate that the different result may be caused by the inclusion of pubertal and adult subjects in the study by Fofanova-Gambetti et al. [■■]. It is possible that, similar to that reported in some ALS-deficient children, height SDS of heterozygous carriers of pathogenic *IGFALS* genetic variants may be lower before puberty than after reaching adult height, due to delayed puberty, so that they may reach an adult height closer to the previously reported 1.0 SDS deficit [22]. Longitudinal follow-up data are needed to show whether the adult height difference is closer to 1 or 2 SDS. At any rate, our observations are in agreement with the concept that both *IGFALS* gene alleles are required to maintain a molar excess of circulating ALS in order to stabilize IGF-1/IGFBP-3 binary complexes, due to the relatively low affinity of ALS for these complexes [42], and that normal levels of circulating IGF-1 are required to fulfill growth potential. The finding that all ISS children classified as carriers of a pathogenic *IGFALS* variant presented reduced levels of IGF-1, IGFBP-3 and ALS, while no carrier of a benign variant presented reduction in all 3 markers, supports the hypothesis that partial ALS deficiency could result in limited TCF and, consequently, in reduced ability to maintain normal circulating levels of IGF-1 and IGFBP-3.

Although some of the genetic variants found have been previously described as polymorphisms in population studies, the lack of data comparing ALS levels precludes the evaluation of their effect on ALS expression. In the present study a clear association was revealed between heterozygous status and low levels of components of the circulating IGF system.

It is of note that some of these genetic variants were also present in normal children. It is particularly interesting that 2 inactivating *IGFALS* gene mutations: E35Gfs*17 [25] and N276S [19], previously described in complete ALS-deficient patients, have been found in heterozygosis in 1 ISS and 1 normal child, respectively. It is not completely understood why similar inactivating mutations predicted to disturb ALS structure may affect height and the IGF system differently in these children. It should be considered that since all genetic variants were found in heterozygous state, haploinsufficiency for the *IGFALS* gene may result in variable expression of the WT allele, leading to normal or reduced ALS levels. In previous studies, differential expression of the *PRPF31* gene has been reported in retinitis pigmentosa as the underlying mechanism responsible for incomplete penetrance [43]. In fact, in asymptomatic carriers expressing a high amount of functional *PRPF31* mRNA, this compensates for the mutation-induced loss of one allele and prevents the manifestation of symptoms [44]. Similarly, a compensatory increased expression of the WT allele might also occur in some subjects, preventing the consequences of *IGFALS* haploinsufficiency.

Another interesting point is that even *IGFALS* gene mutations that are expected to completely inactivate the *IGFALS* gene were present in normal children, indicating that these variants are not under a strong negative selection pressure, remaining in low frequency in the population. This fact may explain the appearance of complete ALS-deficient patients in nonconsanguineous families, frequently as compound heterozygous for 2 different *IGFALS* gene mutations [26].

In conclusion, we have shown that in a subset of ISS children presenting diminished levels of IGF-1, IGFBP-3 and ALS heterozygous *IGFALS* gene variants can be considered as the molecular mechanism underlying the etiol-

Please provide Reference number of Fofanova-Gambetti, or add in the Reference list, respectively.

ogy of short stature. The in vitro characterization of the effect of these and future variants on ALS intracellular trafficking, protein production, secretion and binding properties is required before the variants can be considered pathogenic.

Acknowledgments

We are grateful to the children and their parents who agreed to take part in this study. We would also like to thank Mrs. Perla Rossano and Mrs. Silvina González for their invaluable technical assistance and Mrs. Susana Mancini for her support in obtaining bibliographical material. We would also like to thank Dr. Sabina Domené for the language revision of this manuscript.

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (BID 1201/OC-AR PICT-2003 No. 05-14354) and an independent research grant from Pfizer Global Pharmaceuticals.

Disclosure Statement

J.J.H. is medical advisor of Sandoz Argentina. All other authors have nothing to declare.

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