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## **Normal Growth despite Combined Pituitary Hormone Deficiency**

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## 26 1. Established Facts and Novel Insights

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### Established Facts

. In some case reports, growth during childhood in the absence of growth hormone has been described

. In such cases, hyperinsulinemia, hyperprolactinemia, GH/IGF variants, and mutations of genes such as *HESX1* have been proposed as explanations for the observed normal linear growth. However, the mode of action remains unclear. Moreover, in most cases no cause has been identified and the involvement of as yet unidentified growth-promoting factors has been proposed.

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### Novel Insights

. Normal growth can be observed in teenagers with hypopituitarism and an absence of hyperinsulinemia, hyperprolactinemia or circulating growth-promoting factors.

. Extensive hormonal and genetic analyses suggested a *HESX1* mutation, probably associated with mutations of other genes involved in the regulation of intracellular signaling pathways, potentially accounting for the maintenance of normal growth.

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## 31 2. Abstract

32 **Background** The paradox of normal growth despite a lack of GH is an unexplained phenomenon  
33 described in some pathological (sellar, suprasellar and hypothalamic disorders) and overgrowth  
34 syndromes. It has been suggested that the paradoxical growth is due to other GH variants, GH-like  
35 moieties, prolactin, insulin, IGFs and unidentified serum factors or growth mechanisms. The objective  
36 of this study was to determine the mechanism underlying this normal growth without GH.

37 **Case description.** We describe here growth, hormonal and genetic analyses for an adolescent boy  
38 with panhypopituitarism who achieved an adult height above his genetic potential.

39 **Results.** Normal growth was observed despite low serum GH, IGF-I, IGF-II, IGFBP-3 and ALS  
40 concentrations, but IGF-II/IGFBP3 molar ratio was slightly high. Panhypopituitarism was associated  
41 with a heterozygous missense mutation of *HESX1*, with variable penetrance in heterozygous  
42 relatives. Exome analysis detected heterozygous missense mutations of various genes involved in  
43 intracellular signaling pathways. The growth-promoting activity of the patient's serum was unable to  
44 induce AKT phosphorylation in the MCF 7 cell line.

45 **Conclusion.** The high IGF-II/IGFBP3 molar ratio was not the cause of the sustained high growth  
46 velocity, due to the low affinity of IGF-II for IGF-1R. The key finding was the *HESX1* mutation, as  
47 similar cases have been described before, suggesting a common mechanism for growth without  
48 growth hormone. However, the variable penetrance of this variant in heterozygous relatives suggests  
49 that modifier genes or mechanisms involving combinations with mutations of other genes involved in  
50 intracellular signaling pathways might be responsible.

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### 56 **3. Introduction**

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58 Normal linear growth in children is a complex process dependent on genetic background, nutrition,  
59 environment and hormonal status, including the GH/IGF I axis in particular, but also thyroid  
60 hormones, gonadal steroids, insulin, and probably many other unknown factors [1,2] .

61 Postnatal growth in the absence of GH has been described in patients with organic combined  
62 pituitary hormone deficiency, following the resection of craniopharyngiomas and hypothalamic  
63 tumors and in septo-optic dysplasia [3-13]. Hyperinsulinemia [5,7,9,10,14], hyperprolactinemia  
64 [1,9,14], hyperleptinemia [8,10,15], normal or high levels of IGF, bioavailable GH/IGF variants or sex  
65 steroid sensitivity and local aromatase activity have been put forward as explanations for linear  
66 growth [11,16]. In some cases, unexplained normal growth has been reported despite GH/IGF I  
67 deficiency, suggesting a role for other unidentified growth-promoting factors [3,6,8,12,17]. Some  
68 patients with clinical signs, such as acromegaly, gigantism or overgrowth syndrome, but without  
69 excess GH have been described, and genetic abnormalities, such as *NSD1* mutation, have been  
70 reported in these patients [18].

71 We describe here the growth pattern, and the results of numerous hormonal and genetic analyses  
72 for an adolescent boy with panhypopituitarism diagnosed and followed in our pediatrics department  
73 between 2005 and 2013, who achieved an adult height beyond his genetic potential despite GH/IGF-I  
74 deficiency.

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#### 76 **4. Case report**

77 The patient was referred to the pediatric endocrinology unit of Ain Shams University Hospital, Cairo,  
78 at the age of 18.5 years, for evaluation due to excessive enlargement of his hands and feet, already  
79 noted by his family 2.5 years earlier. The patient was born at term. His birth was complicated by Erb's  
80 palsy, due to breech delivery. He weighed 2.5 kg at birth, but no information was available  
81 concerning his length and head circumference at birth or his growth during childhood before  
82 consultation. However, he and his parents said that he was always as tall as his classmates at school  
83 and that he first noticed at the age of 16 years that he was becoming taller than his friends and that  
84 his hands and feet were excessively large. He was taller than all but one of the boys in his secondary  
85 school class. On admission, physical examination (at the age of 18.5 years) revealed mild acromegalic

86 facial features (prominent supra-orbital ridges in frontal view but not in profile, a long face,  
87 prognathism and large ears) associated with particularly large measurements for both his hands and  
88 feet, exceeding the 97th percentile (fig. 1). The patient was 176.3 cm tall (SDS = + 0.25; midparental  
89 target height = 173.8 cm). His BMI was 22.5 kg/m<sup>2</sup> (SDS = + 0.3 SDS), his head circumference was 58.6  
90 cm (+2 SD). His body proportions were normal (upper segment/lower segment = 0.98), but the  
91 measurements for both his hands and feet were above the 97<sup>th</sup> percentile. The patient was still  
92 prepubertal and, at initial presentation (at 18.5 years), both testes had a volume of 3 ml.  
93 Orchidopexy was performed one month before this first presentation. Three years later, the left  
94 testis had a volume of 1 ml, subsequently becoming impalpable, indicating testicular atrophy. The  
95 penis was of normal size (6 cm). Bone age assessment according to Greulich and Pyle standards  
96 revealed a delay in bone maturation (bone age of 13.5 years). Whole-body bone mineral density Z  
97 score was -3.7. The only other abnormality noted was mild scoliosis. No delay in mental development  
98 was observed, and the patient had a normal IQ. Clinical cardiovascular examination and  
99 electrocardiogram results were normal. There was no history of consanguinity. Biochemical tests  
100 (Table 1) and extensive hormonal investigations were carried out, and karyotyping was performed.  
101 These tests revealed the existence of several pituitary deficiencies (Table 2). MRI revealed a  
102 hypoplastic anterior pituitary, stalk interruption and an ectopic posterior pituitary. Over the six  
103 months between the ages of 18.5 and 19 years, the patient grew by 3.7 cm. Remarkably, this patient  
104 had an almost normal life, suffering only from effort intolerance before the initiation of treatment,  
105 but not after hormonal substitution. Thyroid, adrenal and testosterone deficiencies were discovered  
106 during his first medical consultation (18.5 years ). Hormonal replacement therapy was initiated at the  
107 age of 19 years, with L-thyroxine, hydrocortisone and testosterone. The patient's insulin sensitivity  
108 was assessed before sex steroid replacement and followed during treatment, due to the strong  
109 family history of type 2 diabetes (father, uncles, aunts and grandmothers) (fig. 2).

110 Written informed consent was obtained from the patient for hormonal and genetic studies, and for  
111 photography. A DNA sample was stored for subsequent genetic analysis. The Research Ethics  
112 Committee at the Faculty of Medicine, Ain Shams University, reviewed and approved the study  
113 protocol.

114 **4.A. Hormonal evaluation** (see details in supplemental Table 1)

115 *GH secretion status* was evaluated by stimulation with GHRH, arginine, insulin and L-dopa (GenWay,  
116 Inc. hGH ELISA kit). *Serum IGF-I concentration* was determined after separating IGF from binding  
117 proteins by ultrafiltration, as previously described [19]. Another radioimmunoassay for IGF-I  
118 (Biosource, Nivelles, Belgium) was also carried out.

119 *Serum IGFBP-3 concentration* was determined by immunoassay (BioChem Immuno Syst, Bologna,  
120 Italy). IGFBP-3 levels were also determined with the Nichols Advantage IGFBP3 assay (Nichols  
121 Institute Diagnostics, San Clemente, California). This assay systematically gives values much lower  
122 than those obtained with other kits (personal data) and was therefore not used for determination of  
123 the IGF/IGFBP3 molar ratio.

124 *GHP concentration* was determined with the DSL kit (Webster, Texas).

125 *Serum IGF-II concentration* was determined by the IGF-II IRMA DSL-9100 with extraction kit (DSL,  
126 Webster, Texas).

127 *IGF-II immunoblotting* was performed as previously described [20,21].

128 *Serum ALS concentration* was determined with the total ALS DSL 10-8200 ELISA kit (Diagnostic  
129 System Laboratories, Webster, Texas).

130 *Serum leptin concentration* was determined with the human leptin RIA HL-81HK kit (Linco Research,  
131 St Charles, Missouri).

132 *Serum insulin concentration* was determined after overnight fasting, in basal conditions and following  
133 oral glucose loading, with the AxSYM Insulin MEIA assay (Axis-Shield Diagnostics, Ltd., Dundee, UK for  
134 Abbott Diagnostics Division).

135 *Serum concentrations of LH, FSH, free T4, cortisol, prolactin, and ACTH* were determined with an  
136 Abbott AxSYM analyzer.

#### 137 *Growth-promoting activity of the patient's serum*

138 GH-mediated growth-promoting activity was assessed in the GH biactivityNb2 [22] and AKT  
139 phosphorylation assays, as follows: MCF-7 breast cancer cells were cultured in DMEM supplemented  
140 with 10% fetal bovine serum. The cells were plated in complete medium, in 96-well plates (5000 cells  
141 per well), and were left to expand overnight. The medium was removed and the cells were placed in  
142 phenol red-free and serum-free DMEM supplemented with 10 nM fulvestrant, a competitive  
143 estrogen receptor (ER) antagonist, for 24 hours. The cells were then exposed to a 2% dilution of  
144 serum from the patient or an age-matched control in DMEM for 15 minutes. We then assessed pAKT  
145 levels in quadruplicate wells, after lysis, by homogeneous time-resolved energy transfer (HTRF®),  
146 with a Phospho AKT (ser 473) HTRF kit (Cis Bio France, 30200 Codolet).

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#### 148 **4. B. Genetic analysis**

##### 149 *Molecular diagnosis of overgrowth syndromes*

150 Methylation of the H19/IGF2 and KCNQ10T1 genes on chromosome 11p15 was analyzed, as  
151 previously described [23] for the diagnosis of Beckwith-Wiedemann syndrome. The 23 coding exons  
152 and intron-exon junctions of the NSD1 gene were subjected to direct bidirectional sequencing to test  
153 for Sotos syndrome [24].

##### 154 *Array platform*

155 Oligonucleotide SNP array analysis was performed with the Human CytoSNP-12 BeadChip (Illumina).  
156 This array contains ~300,000 probes, including ~200,000 tagged SNPs and has a mean resolution of  
157 approximately 10 kb. SNP copy numbers (log ratio) and allelic composition (B allele frequencies) were  
158 assessed with Genome Studio Version 2010. 2 (Illumina, Inc) and CNV partition Version 2.4.4  
159 software. The IGF-1R region was analyzed from SNP 96,978,785 to SNP 97,356,782 (hg18). This area

160 covers approximately 378 kb with 57 SNPs, including 52 specific to the 315 kb of IGF-1R (the mean  
161 distance between SNPs in IGF-1R is about 6 kb).

162 *Sequencing and/or MLPA analysis of the IGF-I, IGFBP3, ALS and IGF type 1 receptor genes*

163 The sequences of the five coding exons and intron-exon junctions of the *IGF 1* gene and its cDNA  
164 were determined as previously described [25], together with the sequences of the 21 coding exons  
165 and intron-exon junctions of the IGF type 1 receptor *gene*. MLPA analysis (MRC-Holland, Amsterdam,  
166 the Netherlands) was also performed for the IGF type 1 receptor, IGFBP3 and ALS genes.

167

168 *Whole-exome sequencing*

169 Library preparation, exome capture, sequencing and data analysis were carried out by IntegraGen SA  
170 (Evry, France). Sequence capture, enrichment and elution were performed according to the kit  
171 manufacturer's instructions (SureSelect, Agilent), except for library preparation, for which we used  
172 the NEBNext® Ultra kit (New England Biolabs®). Each DNA sample was then sequenced on an Illumina  
173 HiSeq4000 as paired-end 75 bp reads. Image analysis and base calling were performed with Illumina  
174 Real-Time Analysis, using the default parameters.

175 *Bioinformatics analysis of sequencing data based on the Illumina pipeline (CASAVA1.8.2).*

176 CASAVA was used to align sequencing runs to a reference genome (hg19), to call the SNPs based on  
177 allele calls and read depth, and to detect variants (SNPs & indels). All targets were sequenced at  
178 greater than 20X coverage. The alignment algorithm used was ELANDv2e (multiseed and gapped  
179 alignments). Only the positions included in the bait coordinates were conserved. Genetic variants  
180 were annotated with the IntegraGen in-house pipeline. We annotated genes (RefSeq) and known  
181 polymorphisms (dbSNP 132, 1000 Genomes (1000G), EVS), and characterized the mutations detected  
182 (exonic, intronic, silent, nonsense). For each position, exomic frequencies (Homo & HTZ) were  
183 determined from all the exomes already sequenced at Integragen, and the exome results provided by

184 1000G, EVS and HapMap. We required variants to be non-synonymous and not present in dnSNP132,  
185 1000G, EVS, and HapMap, with most bases scoring Q30 or above, to meet our filtering requirements.

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## 188 **5. Results**

### 189 **5.A. Growth follow-up**

190 From diagnosis, when the patient was still prepubertal, to the start of sex steroid replacement  
191 therapy, the patient had a spontaneous growth velocity of 3.7 cm in six months. After the initiation  
192 of hormonal treatment, the patient attended regular follow-up visits including clinical and biological  
193 examinations, and his final height at the age of 22 years and nine months was 187 cm (SDS +1.85),  
194 with a reduced growth spurt and complete fusion of the epiphyses (fig. 3). BMD was recently  
195 evaluated at 32 y and completely normal Z scores were obtained for the spine, femur and forearm.

### 196 **5.B. Laboratory and hormonal evaluations**

197 Blood biochemical parameters (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, creatinine) were normal, except for lipid profile (table 1).  
198 Endocrine evaluation (table 2) before replacement therapy revealed severe GH and cortisol (without  
199 high ACTH levels) deficiencies, slightly low FT4 levels and LH/FSH/testosterone deficiencies. Estradiol  
200 concentration was very low (<10 pg/ml), but rose to 28 pg/ml after six months of testosterone  
201 replacement. Basal prolactin and leptin concentrations were moderately high. The GH/IGF I axis was  
202 evaluated in various assays and the three pituitary stimulation tests showed severe GH deficiency.  
203 IGF I, IGF II, IGFBP3 and ALS levels were very low, even though GHBP levels were in the normal range.  
204 Western blot analyses excluded the possibility of high-molecular weight forms of IGF II precursors  
205 and confirmed the low levels of the mature 7 kDa IGF II form detected by IRMA (fig. 4). Serum GH  
206 biological activity, as assessed in the GH-NB2 assay, was surprisingly normal, but growth promotion  
207 activity, as assessed by evaluating AKT phosphorylation in the MCF 7 cell line, was barely detectable  
208 (fig. 5).

209 The molar ratio of IGF-I/IGFBP3 was low, regardless of the IGF-I kit used: 0.06 and 0.10 [reference  
210 values, mean (range): prepubertal stage = 0.16 (0.10-0.20), pubertal stage = 0.27 (0.19-0.39), and  
211 young adults = 0.28 (0.14-0.46)], whereas the IGF-II/IGFBP3 ratio was moderately high: 1.24 (in-  
212 house reference values according to age= 0.84 ± 0.16).

213 Before replacement therapy, the patient had normal insulin sensitivity (fasting serum glucose  
214 concentration = 0.89 g/L and insulin level = 16.5 mIU/L). However, in the six months following cortisol  
215 and testosterone replacement treatment, he developed glucose intolerance (fasting glucose  
216 concentration of 1.22 g/L, a two-hour OGTT value of 1.50 g/L, with insulin levels of 38 mIU/L and 161  
217 mIU/L, respectively). Subsequent analyses during follow-up revealed a normalization of fasting and  
218 two-hour post-prandial glucose concentrations after OGTT.

### 219 **5.C. Genetic analysis**

#### 220 ***Overgrowth syndromes***

221 The patient did not have the classical phenotype of overgrowth syndromes, such as Beckwith-  
222 Wiedemann syndrome or Sotos syndrome, but genetic and epigenetic analyses were carried out.  
223 These analyses revealed an absence of centromeric (*KCNQ1OT1*) and telomeric abnormalities  
224 (*H19/IGF2*) in the 11p15 region and no paternal isodisomy or 11p15 imprinting errors. Tests for Sotos  
225 syndrome based on analysis of the *NSD1* gene revealed no deletions or mutations. No nucleotide  
226 polymorphisms, mutations or deletions were detected on exome analysis for the following genes:  
227 *NSD1*, *CUL4B*, *EZH2*, *NFIX*, *PHF6*, *PTEN*, *UPF3B*, *IGF-1 receptor*, *ACTH receptor* and *ERα* or *ERβ*.  
228 However, two very frequent heterozygous missense polymorphisms were found for *GLI3* (rs929387  
229 and rs846266), together with one frequent heterozygous missense for *MED12* (rs372765), and these  
230 polymorphisms were also detected in other members of the family. A very rare variant (0.0002%), a  
231 heterozygous missense *PTCH2* mutation, was present only in the patient (rs138324984). Polyphen2  
232 predicted this variant to be benign, and SIFT predicted that it would be tolerated.

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234 **IGF system analysis** by gene sequencing, MLPA, SNP array and exome analyses showed the IGF1  
235 gene sequence to be normal, ruling out the presence of an activating mutation and revealed  
236 numerous polymorphisms of the IGF2, IGFBP3 and ALS genes, which were also found to be present in  
237 other members of the family. Analyses of the IGF type 1 receptor (IGF-1R) gene ruled out deletion-  
238 duplication of the IGF-1R region. The nucleotide sequence of the 21 exons and exon-intron junctions  
239 was normal, with several frequent polymorphisms in heterozygous state. However, these nucleotide  
240 variants are also found in children with growth retardation. These variants were subsequently  
241 confirmed by exome analysis, which identified three variants in the proband: rs45461797 (also  
242 present in the patient's brother, sister, and mother), rs2229765 (also present in the brother and  
243 sister) and rs17847203 (also present in the brother). The exonic mutations were synonymous. Finally,  
244 the R1353H activation mutation associated with extreme tallness [26] was not found in our patient.

#### 245 **Whole-exome sequencing**

246 Whole-exome sequencing (WES) was performed for the proband, his mother, one of his brothers  
247 (second male sibling, the third child of the family in birth order, see pedigree) and his sister.  
248 Unfortunately, no DNA was available for the patient's father, who had already died, making the list of  
249 candidate genes longer. We therefore excluded some genes with heterozygous missense variants  
250 and unknown functions.

251 No significant gene function was identified for the *homozygous mutations present only in the*  
252 *proband* (the mother and/or siblings were heterozygous) or *for the compound heterozygous*  
253 *mutations found only in the proband* (the mother carried one heterozygous variant and the second  
254 was probably inherited from the father).

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256 No nonsense mutations relating to pathological growth were found among the rare heterozygous  
257 mutations present only in the proband (which were either inherited from the father or were *de novo*  
258 mutations).

259 - Only one heterozygous exonic nucleotide deletion was found, for *AKAP12* (NM\_144497)  
260 (Chr6: 151673006 T/-),

261 - Some heterozygous missense mutations of interest were also detected:

262 (i) *ARAP1* (NM\_001040118) Chr11: 72437737 G>T, A146D amino-acid substitution (ii) *HS6ST1*  
263 (NM\_004807), Chr12: 129025782 C>T, R397H amino-acid substitution (iii) *SH2B3* (NM\_005475),  
264 Chr12: 111856572 A>G, E208G amino-acid substitution; this gene encodes a member of the SH2B  
265 adaptor family of proteins (iv) an insulin-like growth factor 2 mRNA binding protein 3 (rs150697725;  
266 frequency < 0.2%) missense mutation (NM\_006547:p.N359D) was found in the heterozygous state in  
267 the proband, but not in the other three family members tested. However this mutation is not  
268 considered to be deleterious and has been predicted to be benign (Polyphen) or tolerated (SIFT).

269 - Other heterozygous missense mutations identified in the proband were also found in the  
270 other three members of the family tested: *CDKN2A* (rs116150891, LP\_001182061.1:p.R144C), *GPR98*  
271 (NP\_115495.3:p.G3346R; new variant not previously reported in any database) and *PIK3C2G* (a 4 bp  
272 deletion leading to a truncated protein NP\_004561.3:p.Gln448LeufsTer5, described in Exac but not in  
273 any dbSNP).

274 - Finally, one of the key genetic alterations detected was the *HESX1* heterozygous missense  
275 mutation (rs 121909173) resulting in a non-conservative Q6H substitution. This mutation was found  
276 in the proband and in other heterozygous family members, but its penetrance was variable, as the  
277 same mutation was detected in the patient's sister and brother (second male sibling, third in the  
278 birth order, see pedigree).

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## 283 **6. Discussion/Conclusion**

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285 We describe here the growth pattern of an adolescent boy with mild acromegaloid features,  
286 panhypopituitarism, a small anterior pituitary associated with a truncated stalk and an ectopic  
287 posterior pituitary. The plasma concentrations of the components of the somatotrophic axis, GH, IGF-I,  
288 IGF-II, IGFBP-3 and ALS, were very low, contrasting with the lack of growth failure. The patient  
289 achieved an adult height above his genetic potential without GH replacement therapy.

290 Many similar cases of normal linear growth without GH have been reported in patients with organic  
291 combined pituitary hormone deficiency or septo-optic dysplasia, and after the resection of  
292 craniopharyngiomas or hypothalamic tumors [5-8,11-13,27-30]. As previously discussed [11], it is  
293 remarkable that these patients are able to lead an almost normal problem-free life.

294

295 Several hypotheses have been put forward to account for the normal linear growth of patients with  
296 similar conditions, including hyperleptinemia, hyperinsulinemia, normal IGF-I levels despite GH  
297 deficiency, and hyperprolactinemia, but as yet unidentified growth mechanisms have also been  
298 evoked in some cases [3,6,8-10,12,14,15,17].

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300 However, our patient had leptin levels that were only slightly high, just above the upper limit of the  
301 reference range, and there was no evidence of hyperinsulinemia before replacement therapy. Our  
302 patient had moderately high prolactin levels. Serum GH bioactivity with Nb2 was normal (probably  
303 due to the effect of prolactin on the GH/prolactin receptor in the rat cell assay) [22,31], but was not  
304 associated with IGF I stimulation in the serum of our patient, so prolactin cannot account for the  
305 growth of this patient. Our findings thus indicate that leptin, insulin and prolactin are unlikely to be  
306 responsible for the stimulation of linear growth in this patient.

307 Our patient had very low levels of GH, and normal GHBP levels in a context of GH deficiency, as  
308 described in several previous patients [17,32], together with low levels of IGF-I, IGFBP-3, ALS and IGF-  
309 II in the absence of high-molecular weight forms of IGF-II. Trisomy of 15q26qter and activating  
310 mutations of the IGF type 1 receptor gene, which have been shown to cause tall stature [26,33],  
311 were excluded in our patient by karyotyping, MLPA, SNP array analysis, sequencing and exome  
312 analyses.

313 Despite low total IGF-I and IGF-II levels, the IGF-I/IGFBP3 molar ratio did not confirm enhanced  
314 bioavailability, and the IGF-II/IGFBP3 molar ratio was higher than the reference values for  
315 prepubertal boys or young adults. However, this higher IGF-II/IGFBP3 molar ratio could not have  
316 triggered an IGF-I-like effect, given the lower affinity of IGF-II for the IGF type 1 receptor. The  
317 bioavailable IGF-II may instead have activated the signaling pathway involving isoform A of the insulin  
318 receptor, with proliferative effects [34]. However, clinical and biological arguments (absence of  
319 hypoglycemia and AKT phosphorylation, respectively) do not support the implication of this high  
320 ratio in the maintenance of growth.

321 The patient's serum did not have a stimulatory effect in our cellular AKT phosphorylation assay. This  
322 finding contrasts with those of previous studies reporting an increase in the proliferation of erythroid  
323 progenitor cells in response to serum from patients [3,6,17], and a response in human osteoblast  
324 cells similar to that obtained with control serum [8] for cell counting and <sup>3</sup>H-thymidine  
325 incorporation. There, therefore, appears to be another, as yet unidentified growth mechanism at  
326 work, stimulating growth and proliferation via another signaling pathway. Alternatively, the low level  
327 of activity of our patient's serum in the AKT phosphorylation assay may be due to inhibition by other  
328 overstimulated pathways, as observed, for example, in some patients with *SHP2* mutants inducing  
329 RAS/ERK1/2 hyperactivation associated with alterations to the JAK 2/STAT 5 or PI3K/AKT pathways  
330 [35-38].

331 Heterozygous missense mutations of *HESX* (encoding a promoter-specific transcriptional repressor)  
332 may be associated with mild clinical manifestations, with incomplete penetrance in heterozygous  
333 family members [13,39,40]. This situation applied to this family, which presented a heterozygous  
334 *HESX1* mutation (causing a Q6H substitution) in exon 1 previously described by Thomas *et al.* and  
335 shown to be associated with GH, TSH and LH/FSH deficits [40]. Similar cases, in which *HESX1*  
336 mutations were associated with CPHD, suggest a possible common mechanism for growth without  
337 growth hormone [13], although the mechanism of action remains unresolved. The point mutation  
338 causing the Q6H substitution in our patient has not been identified as a relatively common sequence  
339 variant in the global population. If this mutation is deleterious, its inheritance by unaffected  
340 members of the family would confirm the variable penetrance of *HESX1* mutations. This variable  
341 penetrance suggests that there must be another cause of hypopituitarism, acting in combination  
342 with the *HESX1* variant, and it remains possible that other modifier genes affect *HESX1* function.

343  
344 Some of the heterozygous exonic nucleotide deletions or heterozygous missense mutations revealed  
345 by the exome analysis concerned genes with activities related to G-protein signaling pathways and  
346 the activation of cAMP-dependent PKA (AKAP12), GPCR signaling, Rho GTPase signaling pathways  
347 (ARAP1), a range of signaling activities involving growth factor and cytokine receptors (SH2B3),  
348 inhibition of the G1 phase of the cell cycle (CDKN2A), inhibition of cell growth (GPR98) or regulation  
349 of the translation and production of insulin-like growth factor II (insulin-like growth factor 2 mRNA  
350 binding protein 3), suggesting a role in normal growth and development [41-43]. Interestingly, SH2B3  
351 (associated with hypogonadotropic hypogonadism in particular) mutations may have contributed to  
352 the hypopituitary phenotype of our patient, and PIK3C2G mutations (involved in diabetes type 2)  
353 may have contributed to the susceptibility of this family to diabetes.

354 Taken together all these abnormalities associated with *HESX1* mutation may account for the  
355 phenotype of our patient.

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**In conclusion,**  
The normal growth despite CHPD was not mediated by GH, IGF-I, IGF-II , insulin, prolactin or leptin and the high IGF-II/IGFBP3 molar ratio was probably not responsible for the sustained high growth velocity of this patient. The lack of growth-promoting activity of the patient’s serum suggests that stimulation by an intracellular mechanism is more likely than an effect of circulating growth-promoting factors. The *HESX1* mutation detected in this patient may be one of the most important alterations, because several mutations of this gene have already been reported to be associated with CHPD. However, the penetrance of these mutations is variable, because it was also found in other healthy members of this family. This incomplete penetrance suggests a possible effect of modifier genes or of other associated genetic abnormalities. The exome analysis performed on this patient highlighted multiples variants of genes involved in intracellular signaling pathways that might act in combination with the *HESX1* mutation, accounting for the observed phenotype. However, the mechanisms of linear growth in this patient remain unclear, despite the large panel of genetic and laboratory investigations performed.

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**7. Statements**

**7.1. Acknowledgment**

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## 382 **7.2. Ethics statement**

383 The authors have no ethical conflicts to disclose.

## 384 **7.3. Disclosure Statement**

385 The authors have no conflicts of interest to declare relevant to this study.

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## 392 **7.5. Author Contributions**

393 MEKM, HE, provided the familial cases and recorded their clinical and radiological data, carried out  
394 the stimulation tests, determined serum levels of GH, insulin, LH , FSH and FT4 and participated in  
395 discussions concerning the manuscript.

396 LP, YLB performed hormonal evaluations: serum concentrations of IGF-I, IGFBP-3, GHBP, IGF-II, acid-  
397 labile subunit and IGF II immunoblot analysis

398 SR, NT, IN, YLB performed and analyzed BWS molecular diagnosis at the 11p15 level, *IGF 1* and *IGF*  
399 *type 1 receptor* sequencing and MLPA analysis.

400 WAH, and YLB analyzed the whole-exome sequencing data

401 MB, assessed GH bioactivity

402 PL, FG performed AKT phosphorylation assays

403 SCB, performed and interpreted the SNP microarray analysis

404 YLB designed and analyzed data, coordinated and supervised the study and drafted the manuscript.

405 All the authors have approved the final version

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## 9. Figure Legends

Fig. 1. Photographs of the patient from childhood under the age of 19 years:

a) 2 years old ; b) 10 years old; c) 16 years old; d) whole-body and facial features at 19 years of age.

Fig. 2. Pedigree of the patient (arrows indicate the height data available for these members of the family).

Fig. 3. Patient's growth chart from first visit until fusion of the epiphyses.

The open circles show bone ages; the arrows indicate the ages at which the patient underwent endocrine treatment. Hormone replacement: thyroxine, cortisol and testosterone. MPH = mid-parental height F = father, M = mother

Fig. 4. IGF II immunoblotting

Plasma samples were incubated in 2 mL 0.01 N HC1 to separate IGF from IGFBP. The ultrafiltrate (containing the normal 7 kDa mature IGF-II) and the retentate (IGF binding protein) were lyophilized, then dissolved and subjected to electrophoresis in a 15% polyacrylamide gel under nonreducing conditions. The resulting bands were electrotransferred onto a membrane. IGF-II was identified with a monoclonal anti-IGF-II antibody and visualized by chemiluminescence with a second anti-immunoglobulin G antibody coupled to horseradish peroxidase. The pattern obtained for the patient was compared with that of normal serum (mature IGF-II) and serum from a non-islet cell hypoglycemia tumor (NICTH) containing high-molecular weight forms of IGF-II (big IGF II).

Fig. 5. Growth-promoting activity of the patient's serum

AKT phosphorylation was evaluated in MCF-7 breast cancer cells, as described in the methods. Cells were exposed to a 2% dilution of the patient's serum or serum from an age-matched normal subject for 15 min. The cells were then lysed, pAKT was added and homogeneous time resolved energy transfer (HTRF®) analyses were performed in quadruplicate cells with a kit. P2, P4 and P5 = control sera from individuals at Tanner stages 2, 4 and 5, respectively.

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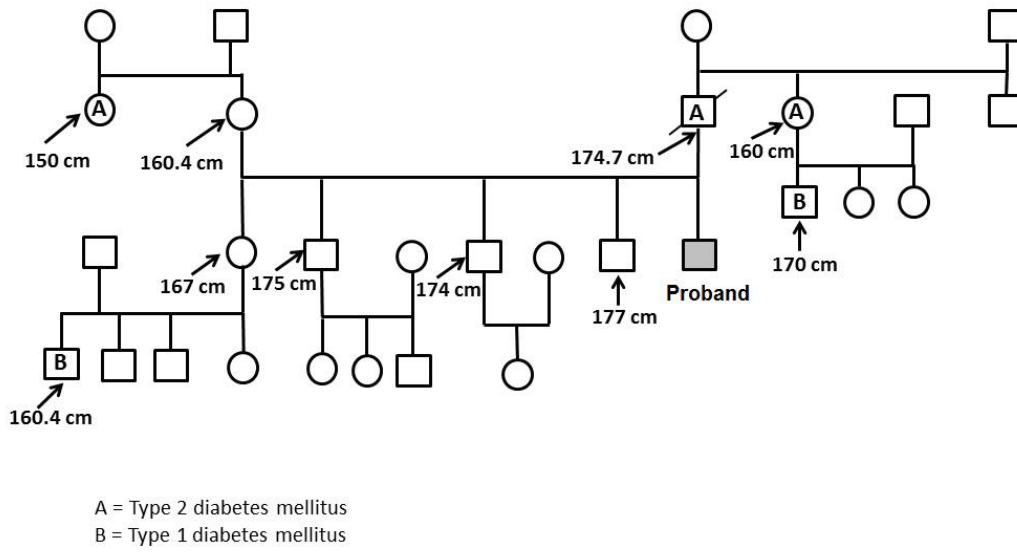
**Fig 1**



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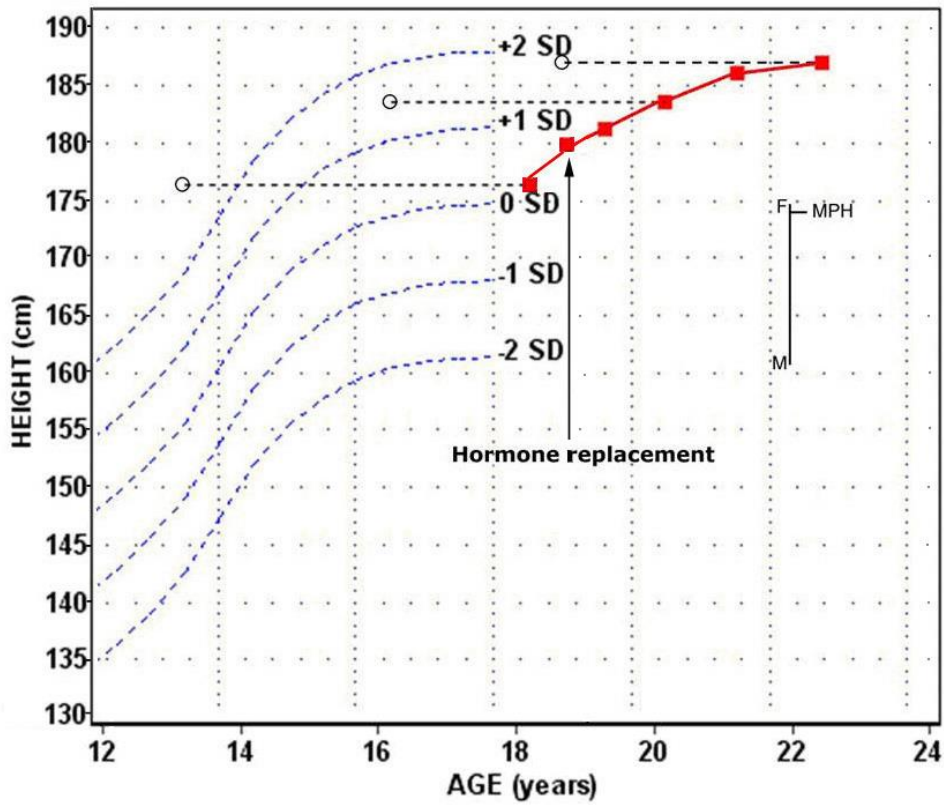
544 Fig 2.

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546 **Fig 3**

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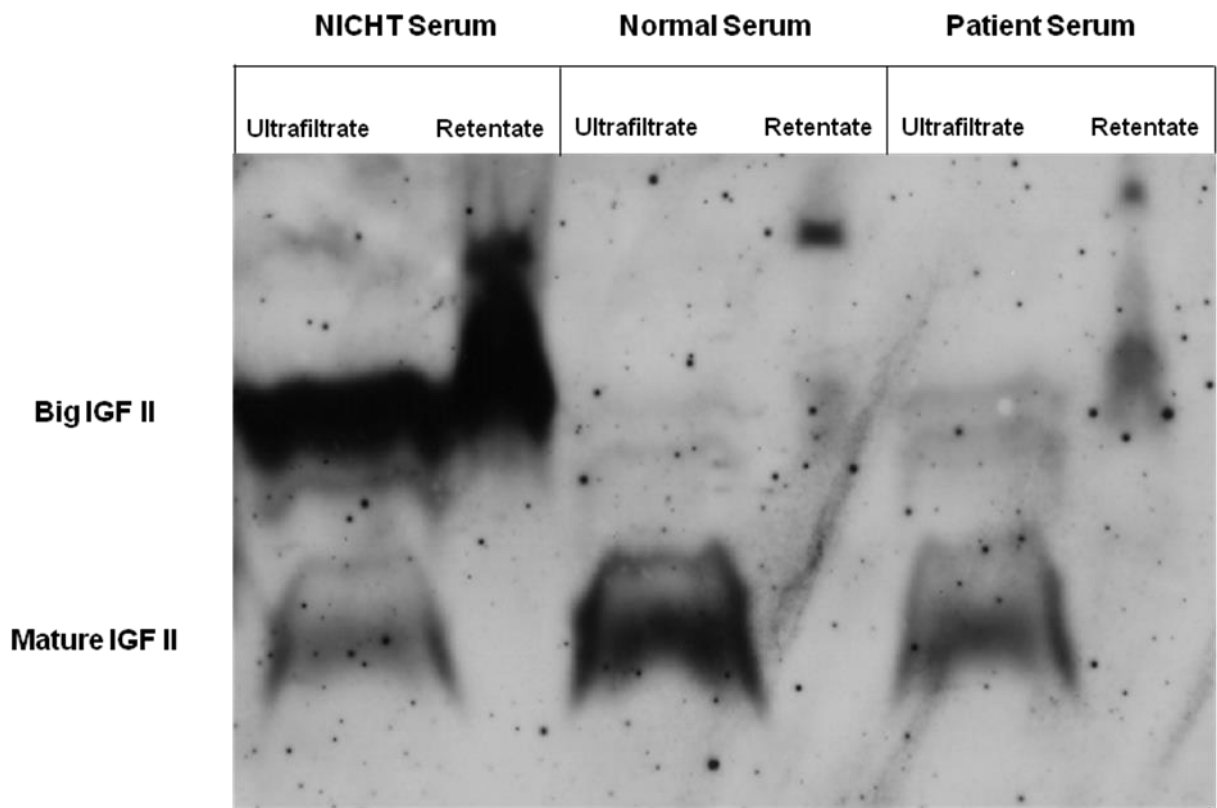
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553 **Fig 4**



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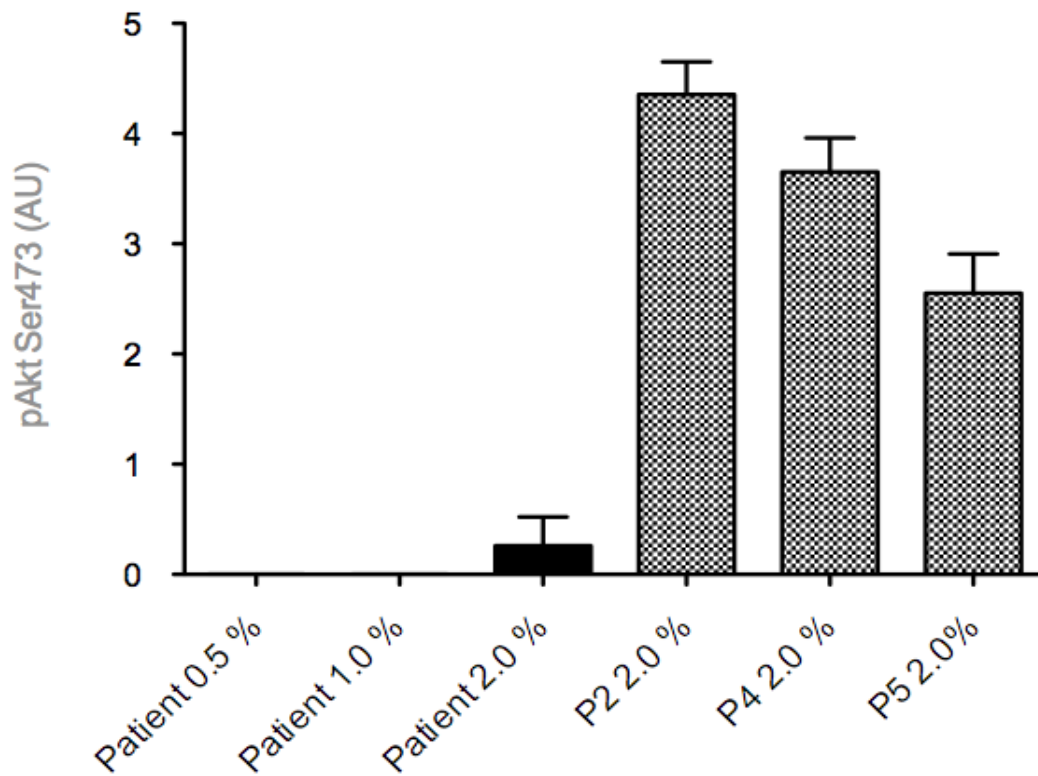
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569 **Fig 5**



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**Table 1:** Serum biochemistry results (normal values)

<i>Lipid profile</i>	
Cholesterol	2.63 g/L (< 2)
LDL-Chol	2.04 g/L (< 1.6)
HDL-Chol	0.35 g/L (> 0.6)
Triglycerides	1.17 g/L (< 1.5)
<i>Liver functions</i>	
ASAT	39 UI/L (5-34)
ALAT U/L (up to 55)	54 UI/L (< 55)
<i>Kidney functions</i>	
Creatinine	12.2 mg/L (7.2-12.5)
Electrolytes	
Na <sup>+</sup>	139 mmol/L (136-145)
K <sup>+</sup>	4.0 mmol/L (3.5-5.1)
Cl <sup>-</sup>	101 mmol/L (97-111)

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**Table 2:** Patient hormonal determinations (and normal values according to age and pubertal stage). All serum samples were evaluated before starting replacement therapy

TSH	2.3 mIU/mL (0.35-5.5)
FT4	8.1 pmol/L (11-21)
Peak GH (ITT)	0.2 ng/ml (> 7 ng/ml)
Peak GH (GHRH + arginine)	2.7 ng/ml (> 7 ng/ml)
Peak GH (L Dopa)	0.1 ng/ml (> 7 ng/ml)
Peak cortisol (ITT)	2 ng/ml (40 -380 am)
Peak LH (LHRH Test)	0.1 mIU/ml (Tanner I: 0.04-3.6)
Peak FSH (LHRH Test)	0.2 mIU/ml (Tanner I: 1.4-5.9)
Basal Prolactin	37.5 ng/ml (2.1-17.7 ng/ml )
ACTH	21 pg/ml (up to 65)
Testosterone	0.1 ng/ml (prepubertal: <0.03-0.1)

Estradiol *	<10 pg/ml (prepubertal <20)
GH-Nb2**	8.3 ng/ml (> 6.7 ng/ml)
IGF I in house method (polyclonal antibody)	25 ng/ml (200-520 ng/ml according to age; and 110-350 ng/ml according to prepubertal stage)
IGF I RIA Biosource Kit	44 ng/ml (normal values : 100-500)
IGFBP3 immunoassay (BioChem Immuno Syst)	1.6 µg/ml (1.5-6 µg/ml)
IGFBP3 (Nichols Advantage automate) ***	0.85 µg/ml (1.67 -3.25 µg/ml according to age and to prepubertal stage)
ALS	7 µg/ml (8 -35 µg/ml)
GHBP	2959 pmol/l (350-3435 pmol/l)
IGF II (kit IRMA DSL)	522 ng/ml (582-1034 ng/ml)
Leptin RIA Kit (Linco)	6.8 ng/ml (0.7-5.3 ng/ml)

604 \* E2: 28 pg /ml 6 months after testosterone replacement

605 \*\* Growth hormone biological activity

606 \*\*\*This assay systematically gives values much lower than those obtained with other kits  
607 (personal data) and then was not used for the determination of the IGF/IGFBP3 molar ratio

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617 Supplemental Table 1: characteristics of hormonal assays

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Hormonal Assay	Manufacturer	sensitivity	Intra -assay CV	Inter-assay Cv
GH	Genway	0.2ng/ml	<6%	<8.9%
IGF-I (in house)	(ref 19)	0.2ng/ml	<3.7%	<4.5%
IGF-I	Biosource	0.2nmol/L	<6%	<7.5%
IGFBP3	Biochem Immuno Syst	0.04 nmol/L	<3.3%	<5.6%
IGFBP3	Nichols Avantage	0.02µg/ml	<8.8%	<16.7%
GHBP	DSL	1.7pmol/L	<5.6%	<8.4%
IGF-II	DSL	0.1ng/ml	<7.2%	<10.4%
ALS	DSL	0.07 µg/ml	<7.5%	<8.9%
Leptin	Linco	0.5ng/ml	<3.4%	<8.3%
Insulin	AxSym	2µIU/ml	<6.4%	<8%
LH	Abbott Axsym analyser	<0.5mIU/ml	<3.6%	<3.9%
FSH	Abbott Axsym analyser	<0.5mIU/ml	<4.2%	<4.6%
f.T4	Abbott Axsym analyser	<3 pmol/L	<5.3%	<7.8
Cortisol	Abbott Axsym analyser	<10ng/ml	<5.9%	<7.7%
Prolactin	Abbott Axsym analyser	0.6ng/ml	<3.8%	<4.7%
ACTH	Abbott Axsym analyser	5pg/ml	<9;5%	<10%

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