Genetic Mutations in the GH/IGF Axis

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Abstract

The GH/IGF axis plays an important role in the control of pre and postnatal growth. At least 48 monogenic defects have been described affecting the production, secretion, and action of GH and IGFs.

*Molecular defects of the GH/IGF axis resulting in short stature were arbitrarily classified into 4 groups: **1**. Combined pituitary hormone deficiency (CPHD) (**a**. syndromic CPHD and **b**. non-syndromic CPHD), **2**. Isolated GH deficiency (IGHD), **3**. GH insensitivity, and **4**. IGF-I insensitivity.*

Genetic diagnosis is obtained in about 30-40% of children with growth retardation, severe IGHD, CPHD, apparent GH or IGF-I insensitivity, and small for gestational age. Increased accessibility to next generation sequencing (NGS) techniques resulted in a significant number of likely pathogenic variants in genes previously associated with short stature as well as in completely novel genes. Functional in vitro assays and in vivo animal models are required to determine the real contribution of these findings.

***Ref**: Ped. Endocrinol. Rev. 2018;16(Suppl1):000-000*
***doi**:*
***Key words**: Combined pituitary hormone deficiency, Isolated GH deficiency, GH insensitivity, IGF-I insensitivity, Genetic defects*

Introduction

The GH/IGF axis plays an important role in the control of pre and postnatal growth (1). While in the prenatal period, growth factors IGF-I and IGF-II are essential for longitudinal growth, mainly under the control of placental lactogen (PL) and nutritional resources (2), after birth pituitary GH becomes the predominant stimulator of IGF-I expression (3). From a historical point of view, in the 1920s and 1930s the growth promoting effect of pituitary extracts on rats (4) and the effect of the anterior pituitary on carbohydrate metabolism (5) were already recognized. However, much more research was still needed until human pituitary-extracted GH was available in 1958 to treat children diagnosed as GH deficient (GHD) based upon either clinical evaluation or bioassays measuring sulfation factor (6). It was not until the 1960s that a specific radioimmunoassay was available for the quantification of GH in serum samples (7) and the use of stimulation tests was a requirement to confirm the clinical diagnosis of GHD (8). The availability of recombinant human GH (rhGH) in 1985 opened up the possibility to potentially treat all patients diagnosed as GHD.

Although the pathogenic etiology of GHD is variable, and may result from different causes such as trauma, brain surgery, tumor, infection, radiation, and autoimmune diseases, it became clear that genetic defects in the GH/IGF axis also

could be involved in alterations in the production, secretion, and action of GH and IGFs. The first molecular diagnosis of complete GHD was reported in 1981 in three families with severe isolated GHD that developed anti GH-antibodies in high titer when treated with hGH (9). In this review, we describe the molecular defects of the GH/IGF axis resulting in short stature. We arbitrarily classified the genetic defects in the GH/IGF axis into 4 groups: **1.** CPHD (a. syndromic CPHD and b. non-syndromic CPHD), **2.** Isolated GH deficiency (IGHD), **3.** GH insensitivity, and **4.** IGF-I insensitivity (**figure 1**).

Because genetic causes of defects in the GH/IGF axis have been exhaustively reviewed elsewhere (10-15), we chose to focus mainly on the genetic aspects, such as mode of inheritance and type of mutations, as well as *in vivo* knockout models in both mice and zebrafish to compare the clinical human phenotype with other vertebrate models widely used to model human disease.

Generation of Animal Models for Endocrine Genetic Diseases using Gene-Targeting Techniques

Animal models of genetic diseases constitute an important tool to understand the function of individual genes, particularly when they are used to reproduce human inherited diseases (16). In the case of the GH-IGF axis, several mice strands presenting severe growth impairment were studied (17,18). Later, molecular studies demonstrated that they presented naturally occurring gene mutations in specific genes involved in the regulation of GH expression (19). The development of homologous recombination in embryonic stem cells (20,21) by using specific targets to disrupt gene sequences allowed the generation of null mutants, where a gene is disrupted by the introduction of a cassette carrying a positive selection marker, such as the neomycin resistance gene, under the control of a strong promoter (16,22). Soon it became clear that, even considering the anatomic and physiological differences between rodents and humans, single-gene-knockout (KO) mice may recapitulate some of the consequences of the lack of GH (GH-deficient mice) and the lack of GH action (GH-insensitivity mice) (23-26).

To further dissect the impact of the ablation of a gene in a specific tissue, Sauer & Henderson developed the Cre/loxP system (27), in which a targeted gene was flanked by two loxP sequences (a 34-base pair sequence) that are specifically recognized by Cre, a recombinase protein encoded by the coliphage P1. Recombination occurs specifically at the loxP sequences with loss of the sequence flanked by these two sites. While the gene of interest is flanked by loxP sites, a Cre protein is transfected by using a vector under the control of a tissue specific enhancer-promoter (for example, the albumin promoter to selectively disrupt gene expression in the liver). With this strategy, the expression of IGF-I was selectively disrupted in the liver by Cre-mediated site-specific recombination. This was a remarkable achievement that allowed the characterization of the impact of circulating IGF-I on postnatal growth (28).

More recently, a novel technique was developed that uses engineered nucleases such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (cas)9 that generates a DNA double-strand break at the targeted genomic locus. In the absence of a template, it results in an insertion and/or deletion that disrupts the targeted locus. In the presence of a donor template, carrying the mutation of interest, the repair results in the inclusion of the designed mutation (29,30). This technique proved much more efficient than homologous recombination and has demonstrated some therapeutic applications (31).

The zebrafish (Danio rerio) is a vertebrate animal model which has many advantages compared to the mouse and is becoming an important model in translational research (32). Among the most important advantages are the transparency of the embryos (making it an ideal model for developmental biology), external fertilization, rapid development, and the large number of embryos obtained from each couple (ideal for high throughput analysis). Genetic manipulation is also relatively easy. Transient knockdown is achieved using morpholinos, which are synthetic oligonucleotides that either block translation by being complementary to the translational start site or block splicing by complentarity to the splicing sequence of the target mRNA (33). More recent knockout techniques, also applied in zebrafish, involve the use of CRISPR/Cas9, useful for obtaining stable transgenic lines with deletions or insertions (34).

Combined Pituitary Hormone Deficiency (CPHD)

Combined pituitary hormone deficiency (CPHD) or panhypopituitarism is characterized by the absence of GH and one or more other pituitary hormones (LH, FSH, PRL, TSH, and ACTH). Although as many as 30 genes have been found to be associated with CPHD, eight of them are the most frequently studied: GLI2, HESX1, LHX3, LHX4, POU1F1, PROP1, OTX2, and SOX2 (12,13,35-37) (**table 1**).

Mutations in early transcription factors, such as LHX3, LHX4, HESX1, GLI2, OTX2, and SOX2 that participate in pituitary ontogenesis, lead to syndromic CPHD where pituitary dysfunction is associated with craniofacial anomalies such as septo-optic dysplasia or holoprosencephaly (HPE). On the other hand, mutations in later-acting transcription factors involved in pituitary cell differentiation, such as POUF1 and PROP1, lead to non-syndromic CPHD with a pituitary-specific phenotype and absence of craniofacial anomalies (13).

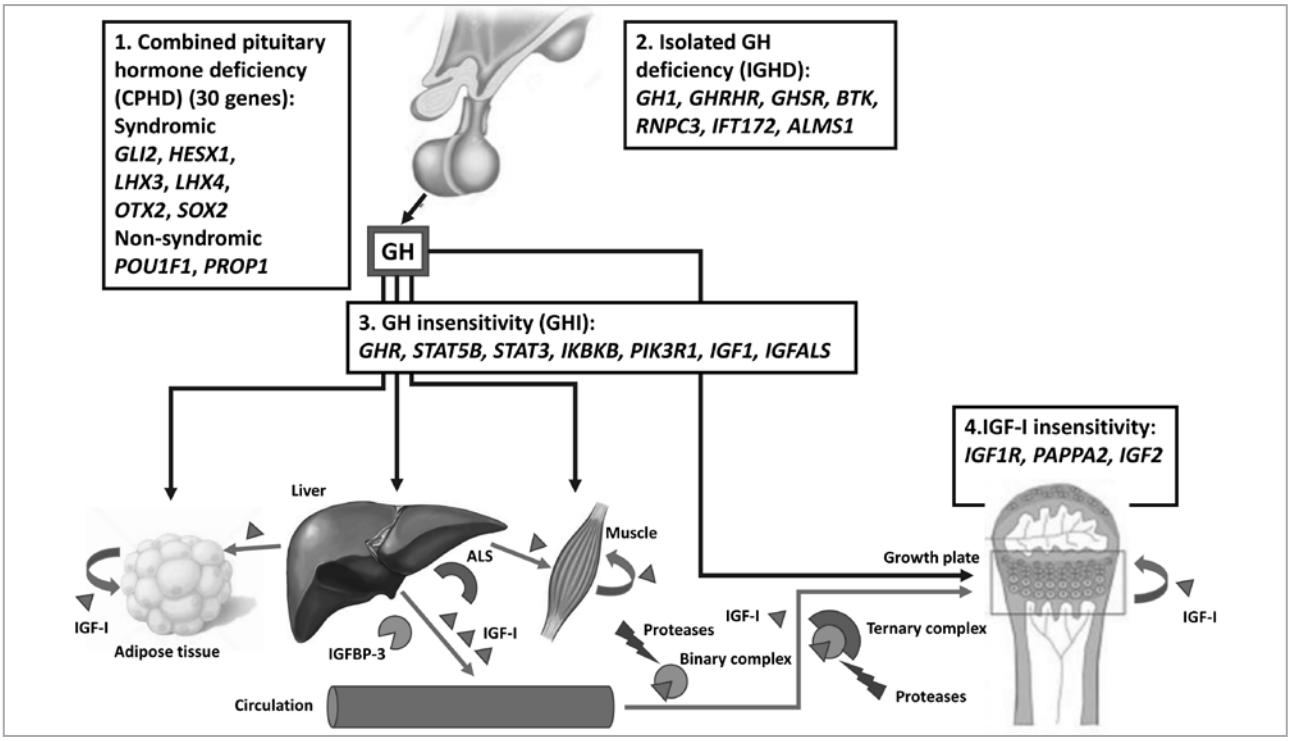

Figure 1. Monogenic defects in the GH/IGF axis

Genetic defects in early transcription factors that participate in the pituitary ontogenesis (LHX3, LHX4, HESX1, GLI2, OTX2, and SOX2, among others), result in syndromic combined pituitary hormone deficiency (CPHD), while gene mutations in those transcription factor that are expressed later during pituitary cell differentiation (POU1F1 and PROP1) result in non-syndromic CPHD.

Genetic causes of isolated GH deficiency (IGHD) include defects in the gene that encodes GH (GH1), and other genes that are involved in the synthesis and secretion of GH (GHRHR, GHSR, BTK, RNPC3, IFT172, ALSM1).

GH insensitivity can arise from molecular defects in the gene encoding the GH receptor (GHR) or in several other genes that participate in the signaling transmission of GH action (STAT5B, STAT3, IKBKB, IL2RG, PIK3R1), IGF-I synthesis (IGF1), or IGF-I transport (IGFALS).

Resistance to IGF-I occurs associated to gene defects in its own receptor (IGF1R), a specific protease (PAPPA2) or the gene encoding IGF-II (IGF2). Straight blue arrows indicate sites of GH action (liver, muscle, adipose tissue). Straight red arrows indicate the action of "endocrine IGF-I", mainly produced in the liver and circulating as free-IGF-I, forming binary complexes (associated to IGFBPs) or ternary complexes (associated to IGFBP-3, or -5 and ALS). Curved red arrows denote the action of "paracrine IGF-I" (acting near its site of production).

Appendix

Glossary of gene names

LHX3: Lim Homeobox Gene 3
LHX4: Lim Homeobox Gene 4
HESX1: Homeobox Gene Expressed in ES Cells
GLI2: Gli-Kruppel Family Member 2
OTX2: Orthodenticle, Drosophila, Homolog of, 2
SOX2: SRY-Box 2
POU1F1: Pou Domain, Class 1, Transcription Factor 1
PROP1: Paired-Like Homeobox 1
GH1: Growth Hormone 1
GHRHR: Growth Hormone-Releasing Hormone Receptor
GHSR: Growth Hormone Secretagogue Receptor
BTK: Bruton Agammaglobulinemia Tyrosine Kinase
IFT172: Intraflagellar Transport 172, Chlamydomonas Homolog of
ALMS1: Alms1 Gene
GHR: Growth Hormone Receptor
STAT5B: Signal Transducer and Activator of Transcription 5b
STAT3: Signal Transducer and Activator of Transcription 3
IKBKB: Inhibitor of Kappa Light Chain Gene Enhancer in B Cells, Kinase of, Beta
IL2RG: Interleukin 2 Receptor, Gamma
PIK3R1: Phosphatidylinositol 3-Kinase, Regulatory Subunit 1
IGF1: Insulin-Like Growth Factor I
IGFALS: Insulin-Like Growth Factor-Binding Protein, Acid-Labile Subunit
IGF1R: Insulin-Like Growth Factor I Receptor
IGF2: Insulin-Like Growth Factor II

Table 1. Combined pituitary hormone deficiency (CPHD)

Gene OMIM	Phenotype OMIM	Human phenotype	Mouse phenotype	Zebrafish phenotype
LHX3 600577	CPHD 3 221750	Absence of GH, PRL, TSH, LH, FSH, rigidity of the cervical spine	Die *in utero* or after 24 hours post birth, absence of GH, PRL, TSH, LH, FSH	Endocrine phenotype unknown
LHX4 602146	CPHD 4 262700	Variable GH, TSH, ACTH and LH, and FSH deficiencies, hypoplasia of the pituitary, poorly developed Sella-turcica	Absence of differentiation of the anterior pituitary cell lineages: GH, TSH, ACTH, LH, and FSH deficiencies	Endocrine phenotype unknown
HESX1 601802	GH deficiency with pituitary anomalies CPHD 5 Septo-optic dysplasia 182230	Optic nerve hypoplasia, pituitary hypoplasia, midline brain abnormalities	Anterior central nervous system defects and pituitary dysplasia	Wildtype
GLI2 165230	Culler-Jones syndrome 615849 Holoprosencephaly 9 610829	HPE, craniofacial abnormalities, hypopituitarism	Embryonically lethal	Reduced number of corticotrophs and increased number of lactotrophs
OTX2 600037	CPHD 6 613986	Anophthalmia or microphthalmia associated with pituitary hormone deficiency	Homozygous knockout mice die during midgestation. Heterozygous mutant mice present eye, pituitary and craniofacial defects	Mild microphthalmia and shortening of the pharyngeal skeleton
SOX2 184429	Optic nerve hypoplasia and abnormalities of the central nervous system 206900	Anophthalmia or microphthalmia and hypoplastic anterior pituitary	Heterozygous mutants present reduced somatotroph number, GH content and reduction of pituitary size. Adult mouse presents GH, PRL, and TSH deficiencies	Shorter anteroposterior axis, smaller eyes in and early lethality
POU1F1 173110	Pituitary hormone deficiency, combined, 613038	Pituitary hypoplasia and absence of GH, PRL and TSH	Snell mouse: growth insufficiency, infertility, hypothyroidism and deafness due to absence of GH, PRL, TSH, and gonadotropins	Severe dwarfism with absence of lactotrophs, somatotrophs, and thyrotrophs
PROP1 601538	Combined pituitary hormone deficiency 2 262600	Absence of GH, PRL, TSH, LH and FSH and pituitary hypoplasia	Ames mouse: absence of GH, PRL, TSH, LH and FSH and pituitary hypoplasia	Abnormal adenohypophysis and reduced expression of pou1f1, prl and gh

Syndromic CPHD

LHX3 gene (OMIM 600577), CPHD3 (OMIM 221750)

LHX3 is a member of the LIM homeodomain family of transcription factors which has a role in pituitary development and the organization of spinal cord neurons. Mutations in LHX3 cause less than 1% of cases of CPHD and are characterized by absence of all anterior pituitary hormones except for ACTH and rigidity of the cervical spine (13). Some patients have sensorineural hearing loss. It has an autosomal recessive mode of inheritance and although there are reports of complete deletions of the gene, most mutations are missense or nonsense. LHX3 gene mutations were first reported by Netchine *et al*. in two unrelated families where affected subjects presented severe growth retardation and GH, TSH, PRL, LH, and FSH deficiencies (38). Affected subjects also presented a rigid cervical spine leading to limited head rotation. These patients were homozygous for a missense mutation (p.Tyr116Cys). Homozygous mutant mice for Lhx3 die *in utero* or within 24 hours post birth. Although Rathke's pouch forms, it fails to grow and differentiate, resulting in the absence of the anterior and intermediate lobes of the pituitary and affecting the determination of all pituitary cell linages except the corticotrophs, similar to the human clinical phenotype (39-41).

Endocrine defects in zebrafish have not yet been characterized in lhx3 morphants. Nevertheless, lhx3 morphants zebrafish embryos show motoneuron alteration (42).

LHX4 gene (OMIM 602146), CPHD4 (OMIM 262700)

LHX4 also encodes a LIM homeodomain transcription factor implicated in pituitary development and the organization of spinal cord neurons. Mutations in LHX4 are responsible for less than 1% of CPHD cases which manifest with variable GH, TSH, ACTH and gonadotrophin deficiencies, hypoplasia of the pituitary and a poorly developed sella turcica (13). Mutations are mostly missense and deletions which are inherited in an autosomal dominant fashion with incomplete penetrance. One homozygous missense variant (p.Trh126Met) is associated with a lethal phenotype (43). A germline splice-site mutation in the LHX4 gene was first reported by Machinis *et al* in 2001 in a family where affected members presented short stature, pituitary and hindbrain defects, and abnormalities of the sella turcica. The intronic mutation has a dominant pattern of inheritance (44). Clinical characteristics of patients with LHX4 mutations have been reviewed recently (45). Mice deficient of Lhx4 do not undergo differentiation of the anterior pituitary cell lineages (40). As in lhx3, endocrine consequences of the absence of lhx4 have not been studied in zebrafish yet.

HESX1 gene (OMIM 601802), GHD with pituitary anomalies, CPHD5, and septo-optic dysplasia (OMIM 182230)

HESX1 is a member of the paired-like class of homeobox transcription factors with a crucial role in the formation of the pituitary and forebrain. HESX1 mutations are responsible for less than 1% of cases in which CPHD is associated with optic nerve hypoplasia, pituitary hypoplasia, and midline brain abnormalities (13). Dattani *et al*. reported two siblings with septo-optic dysplasia (SOD) homozygous for a p.Arg53Cys missense mutation within the HESX1 homeodomain which destroyed its ability to bind target DNA (46). However, HESX1 mutations are not a common finding in patients with SOD since sequencing of this gene in 228 patients presenting congenital pituitary defects (IGHD or SOD with panhypopituitarism) identified only three different heterozygous missense mutations in three patients with mild pituitary hypoplasia or SOD (47). Autosomal dominant with incomplete penetrance as well as autosomal recessive patterns of inheritance have been reported (48). Hesx1 null mice exhibited anterior central nervous system defects and pituitary dysplasia similar to the phenotype observed in humans (46). Although hesx1 knockdown had no effect on zebrafish, Andoniadou *et al*. showed that injecting a hesx1 morpholino into a 'sensitized' headless (*tcf3*) zebrafish mutant leads to severe forebrain and eye defects, suggesting an interaction between hesx1 and the wnt pathway in zebrafish (49).

GLI2 gene (OMIM 165230), Culler-Jones syndrome, (OMIM 615849) holoprosencephaly 9 (OMIM 610829)

The GLI2 gene is a transcription factor involved in the Sonic Hedgehog (SHH) pathway. GLI2 mutations were first identified in patients with holoprosencephaly (HPE) (50). HPE is characterized by defects in forebrain cleavage which include defective anterior pituitary formation and panhypopituitarism. HPE can also be caused by mutations in SHH, PTCH1, TGIF, SIX3, ZIC2, NODAL, FOXH1, CDON, FGF8, and DISP1 (51-54). GLI2 molecular defects are inherited in an autosomal dominant pattern with incomplete penetrance and variable phenotype, and the most common mutations are frameshift, nonsense and missense heterozygous mutations. By screening 390 unrelated patients, Roessler *et al* identified heterozygous truncating mutations in the GLI2 gene caused by non-sense (p.Trp113*; p.Arg168*), frameshift (c.2274del1), and splice-site (IVS5 + 1G>A) mutations in four families. Clinical features included defective anterior pituitary formation and panhypopituitarism, with or without overt forebrain cleavage abnormalities, and HPE-like midfacial hypoplasia (50). Mutations in GLI2 occur in 1.5 % of CPHD cases (13). Gli2 knockout mice die embryonically, and conditional knockout in Rathke's pouch showed that it is necessary for pituitary progenitor specification, proliferation, and differentiation (55). Zebrafish knockdown studies show important differences with mice and humans. While in all other vertebrates, Gli2 is the main activator of Shh signalling and Gli1 is a minor one, in zebrafish it is the opposite; Gli1 rather than Gli2 is the main activator of Shh signalling (56-58). Zebrafish have two gli2 genes (gli2a and gli2b) and knockdown of both using morpholinos affected the endocrine cell position in the pituitary, reduced the number of corticotrophs and increased the number of lactotrophs (59).

OTX2 gene (OMIM 600037), CPHD 6 (OMIM 613986)

OTX2 is a gene encoding a member of the homeobox transcription factor that is involved in the development of the brain and head structures (11). Heterozygous missense gene mutations were reported in 2 patients presenting GH, TSH, LH, FSH, and ACTH deficiencies. Magnetic resonance imaging revealed anterior pituitary hypoplasia with an ectopic posterior pituitary (60). Both patients presented the same missense mutation in the OTX2 gene (p.Asn233Ser). *In vitro* studies showed that while wildtype and mutant OTX2 protein bound equally well to two specific sites in the 5-prime flanking region of the HESX1 gene, mutant OTX2 revealed decreased transactivation, resulting in a dominant negative inhibitor of HESX1 gene expression. While homozyogous Otx-knockout mice die during midgestation, Otx heterozygous mutant mice present eye, pituitary and craniofacial defects (61,62). In zebrafish, morpholinos targeting otx2 result in mild microphthalmia and shortening of the pharyngeal skeleton at 5 days post fertilization (dpf) (63).

SOX2 gene (OMIM 184429), Optic nerve hypoplasia and abnormalities of the central nervous system (OMIM 206900)

SOX2 (together with SOX1 and SOX3) is a member of transcription factor related to SRY family that is expressed in many different embryonic tissues involved in the development of brain, pituitary, and otic and nasal placodes (64). Submicroscopic deletions and truncating mutations in the SOX2 gene were identified in a small number of individuals with anophthalmia (65). In humans, the clinical presentation of patients with SOX2 mutations includes hypogonadotrophic hypogonadism and CPHD. Most of the affected cases presented anophthalmia/microphthalmia, developmental delay, short stature, and male genital tract abnormalities. Patients presented heterozygous de novo or inherited SOX2 mutations. They also may present other anomalies like anterior pituitary hypoplasia, defects of the corpus callosum, learning difficulties, sensorineural hearing loss, and esophageal atresia. Some of the mutations result in truncated protein products, exhibiting partial or complete loss of function (DNA binding, nuclear translocation or transactivation). Heterozygous loss of function of Sox2 in the mouse is associated with a reduction in somatotroph number and GH content, as part of a general reduction in pituitary size. It was also shown that the adult mouse presents low circulating levels of GH, PRL, and TSH (66). Zebrafish sox2 morphants have a shorter anteroposterior axis and smaller eyes in addition to early lethality (5 dpf) compared to wildtype controls (67).

Non-Syndromic CPHD

POU1F1 gene (OMIM 173110), CPHD1 (OMIM 613038)

The transcription factor POU1F1 (previously known as Pit1) belongs to the POU family of transcription factors and is essential for the differentiation of somatotrophs, lactotrophs, and thyrotrophs. POU1F1 gene is specifically expressed in the developing pituitary before the differentiation of somatotrophs, lactotrophs, and thyrotrophs and plays an important role in the differentiation of these cell linages as well as in the transcriptionally regulated expression of GH, PRL, and TSH. POU1F1 mutations account for about 2.8 % of cases of CPHD with pituitary hypoplasia and absence of GH, PRL and TSH (13). In 1992, four independent groups (68-71) identified homozygous or heterozygous gene mutations in the POU1F1 gene (p.Arg172*, p.Arg271Trp, Ala158Pro) in patients with CPHD. In homozygous patients, POU1F1 mutations were loss-of function, while in patients presenting heterozygous mutations, the mutant protein retained the ability to bind DNA but lost its transcriptional activity, presenting a dominant-negative effect. Its inheritance is usually autosomal recessive, but it can be autosomal dominant for dominant negative mutations such as p.Arg271Trp. Missense, nonsense, splicing, and frameshift mutations have been reported. Interestingly, the Snell mouse has a mutation in the Pou1f1 gene ($Pou1f1^{dw/dw}$, p.Trp251Cys) and it exhibits growth insufficiency, infertility, hypothyroidism and deafness, with absence of GH, PRL, TSH, and gonadotropins (72-74). Zebrafish knockdown of pou1f1 shows severe dwarfism with absence of lactotrophs, somatotrophs and thyrotrophs (75).

PROP1 gene (OMIM 601538), CPHD2 (OMIM 262600)

Prop1 is a paired-like homeodomain transcription factor involved in the development of somatotrophs, lactotrophs, thyrotrophs, and gonadotrophs. Wu *et al* (76) identified homozygous or compound heterozygous mutations in the PROP1 gene in four CPHD families, in which affected patients presented GH, PRL, TSH, LH, and FSH deficiencies. Mutations in PROP1 are the most common cause of CPHD (representing up to 15 % of cases) and cause CPHD with pituitary hypoplasia and absence of GH, PRL, TSH, LH, and FSH (13). Its inheritance is autosomal recessive, and the most recurrent mutations are 301-302delAG and 150delA which have been found to be founder variants (77). The Ames dwarf mutant mouse is Prop1 deficient and shows the same phenotype as patients with PROP1 mutations (78,79). Knockdown of prop1 in zebrafish embryos using morpholinos showed abnormal adenohypophysis morphology with reduced expression of pou1f1, PRL and GH (80). Interestingly, expression of lhx3 was also diminished.

Many other genes have been found to cause CPHD, but they are well beyond the scope of this review (for a more detailed description see reference 13).

Isolated GH Deficiency (IGHD)

Growth hormone deficiency (GHD) is a relatively common disorder, occurring in 1 out of 4,000 to 10,000 live births (81). Most frequently, it occurs as a sporadic condition of unknown etiology (82) but severe forms of isolated GHD (IGHD) may have a genetic basis (83). The diagnosis of GHD in childhood is based on auxological assessment, radiological evaluation, and biochemical tests. The diagnosis of isolated GHD requires the characterization of normal function of other pituitary hormones including TSH, ACTH, PRL, LH, FSH, and ADH. However, since a progressive compromise of pituitary hormones has been reported in children previously diagnosed as GHD, this diagnosis is often a provisional one (84,85) and systematic follow-up of these patients is mandatory to identify those subjects that develop additional pituitary hormone deficiencies. Familial IGHD has been associated with four Mendelian disorders (86-88), including two autosomal recessive (Type IA and IB), one autosomal dominant (Type II), and one X-linked (Type III) form (**table 2**).

Table 2. Isolated GH deficiency (IGHD)

Gene OMIM	Phenotype OMIM	Inher.	Human phenotype	Mice phenotype	Zebrafish Phenotype
GH1 139250	Type IA complete GHD 262400	AR	Ab formation on GH treatment	Dwarf phenotype	vizzini mutant: severe growth retardation, small body size and increased accumulation of adipose tissue
GH1 139250	Type IB GHD 612781	AR	Immune tolerance to GH treatment. Low but detectable GH	Idem	Idem
GH1 139250	Type II GHD 173100	AD	Variable severity of GHD, potential evolved to MPHD	idem	Idem
GH1 139250	Kowarski syndrome 262650	AR	Bioinactive GH	Idem	Idem
GHRHR 139191	Type IB GHD 612781	AR	Immune tolerance to GH treatment. Low but detectable GH	Little mouse: reduced GH secretion and a dwarf phenotype	Unknown
GHSR 601898	Partial GHD 615925	AR AD	Partial GHD	Serum IGF-I levels and body weight are modestly reduced, less appetite and adiposity compared to wildtype.	Unknown
BTK 300300	Agammaglobulinemia and GHD 307200	XLR	Type III, hypogammaglobulinemia	Xid mouse: impairment of peripheral B cell maturation	Severe anterior truncation of embryos (dorsalization)
RNPC3 -	-	AR	Severe GHD, pituitary hypoplasia	Unknown	caliban mutant: arrested development of digestive organs (intestine, liver and pancreas)
IFT172 607386	Short-rib thoracic dysplasia 10 with or without polydactyly 615630	AR	Growth retardation, pituitary hypoplasia, and ectopic posterior pituitary	Wimple mouse: altered left-right patterning	Ventral body-axis curvature, formation of renal cysts and cartilage defects
ALMS1 606844	Alstrom syndrome 203800	AR	Reduced GH reserve	Obesity, hypogonadism, hyperinsulinemia, retinal dysfunction, and late-onset hearing loss	Reduced beta-cell production

GH1 gene (OMIM 139250), Type IA GHD (OMIM 262400)

Type IA IGHD was first described by Illig *et al*. (89) in 1970 in three Swiss siblings with severe short stature, early growth retardation, extreme dwarfism in adulthood, and a characteristic phenotype. These patients developed high titers of anti-GH antibodies, which arrested their growth response to pituitary-extracted GH treatment. However, it was not until 1981 that the etiology of this condition was resolved (9). Genomic DNA samples were enzymatically digested using endonucleases and DNA fragments characterized by Southern blot using ^{32}P-labeled hGH cDNA sequences as probes. A homozygous deletion of about 7.5 kb, including the GH1 gene, was found in four affected subjects from three different families. This work could be considered the first genetic characterization of a molecular defect in the GH-IGF axis. Although most of the patients presenting relatively large deletions (including the GH1 gene) develop anti-GH antibodies preventing a growth response when treated with hGH, preservation of a growth response has been reported in some patients despite their high titer of anti-GH antibodies (90). In

addition, some patients harboring GH1 gene deletions do not develop anti-GH antibodies (91).

To date, deletions of different sizes (6.7, 7.0, 7.6, 45 kb, double deletions) within the GH-gene cluster have been characterized as molecular defects in IGHD (92-95), with the 6.7 Kb deletion being the most frequent (70-80%). Although most of the patients are homozygous for a specific deletion, and the parents heterozygous for the same genetic defect, some compound heterozygous cases with one deleted and one mutated allele or two different GH1 gene deletions have been reported in non-consanguineous families (96-99). Small deletions and even a single amino acid substitution can be the cause of isolated Type 1 GH deficiency (100,101).

Mouse models of the disease include a dwarf phenotype observed when somatotrophs were genetically ablated (102). Finally, a zebrafish mutant with a stop codon mutation in the gh1 gene, called vizzini, was identified in 2013 which displayed severe growth retardation and small body size compared to wildtype fish (103). This mutant also had increased accumulation of adipose tissue which was expanded at maturity.

GH1 gene (OMIM 139250), GHRHR gene (OMIM 139191), Type IB IGHD (OMIM 612781)

Patients with Type 1B IGHD are characterized by low but detectable circulating GH levels and short stature. Because these patients do not develop neutralizing anti-GH antibodies, they retain the capability to display growth acceleration when treated with rhGH (104). They present an autosomal recessive pattern of inheritance and the clinical phenotype is more variable than that observed in Type 1A. Some patients resemble those with Type 1A, presenting early postnatal severe growth retardation, whereas in other cases growth failure is only evident later in childhood. All these observations suggest that more than a single gene could be responsible for this alteration. Indeed, defects in 2 genes have been reported to cause GHD Type 1B: GH1 and GHRHR. While mutations in the GH1 gene are usually splice site mutations (105), non-sense and missense mutations in the GHRHR gene also have been found in patients with Type IB IGHD (106). The little mouse, a spontaneous mutant mouse, presents severe growth retardation, an autosomal recessive pattern of inheritance, and diminished secretion of GH and IGF-I, which resembles the clinical phenotype of patients with Type IB IGHD. In 1993, analysis of the GHRHR gene in this mouse revealed a single substitution at codon 60 (p.Asp60Gly) that resulted in complete absence of binding of GHRHR for its ligand (107,108). Three years later, a non-sense mutation in the GHRHR gene was described in two cousins from a consanguineous Indian family (109). Two large kindreds presenting GHRHR gene defects have been described: **a.** eighteen IGHD subjects from the Pakistani province of Sindh all presented the same nonsense mutation (p.Glu72*) (110,111) and **b.** a cohort of 105 individuals from the rural county of Itabaianinha, in the northeastern Brazilian state of Sergipe, presented a transversion (c.57+1G>A) in the consensus GT of the 5´splice donor site of intron 1 of the GHRHR gene (112,113). This mutation leads to the retention of intron 1 and the appearance of a premature stop codon 213 bases downstream. These subjects exhibit reduced GH responsiveness to stimulatory tests, reduced levels of IGF-I, IGFBP-3 and ALS, and anterior pituitary hypoplasia (for a detailed review see reference 114). To date, no zebrafish knockdown studies have been performed in the GHRHR gene.

GH1 gene (OMIM 139250), Type II IGHD (OMIM 173100)

This autosomal dominant IGHD constitutes the more frequent genetic alteration in the GH1 gene (106). Most of the mutations affect the first six base-pairs of intervening sequence 3 (5´IVS-3) (106,115,116), resulting in misssplicing at the mRNA level and the subsequent loss of exon 3. This alteration results in the production of a 17.5 kb GH isoform (117). Mutations have also been reported in exon 3 splice enhancer 1 (ESE1) as well as ESE2 (118). These mutations also result in increased levels of exon 3 skipped transcripts (119,120). This GH isoform lacks amino acids 32-71 and exhibits a dominant-negative effect on the secretion of the 22-kDa isoforms. The 17.5 kDa GH isoform is initially retained in the endoplasmic reticulum, disrupting the Golgi apparatus, and thus impairing the normal trafficking of the 22-kDa GH (121). Transgenic mice overexpressing the 17.5 kDa isoform exhibit a defect in the maturation of the GH secretory vesicles and present anterior pituitary hypoplasia due to loss of the majority of somatotrophs (122,123). Patients affected with Type II IGHD present a variable degree of growth retardation, probably reflecting the variable degree of exon 3 skipping (82). *In vitro* experiments have demonstrated that in a pituitary cell line, the expression of the 17.5-kDa-mutant GH induced endoplasmic reticulum stress and apoptosis, contributing to the decrease in wildtype GH secretion (124). The relative amount of the 17.5 kDa to 22 kDa hGH isoform could determine the impact on pituitary size, the severity of GHD, and the appearance of other pituitary hormone deficiencies (125,126). For this reason, these patients should be carefully followed for the early detection and replacement of other hormonal deficiencies.

A recurrent missense mutation in the GH1 gene also results in IGHD Type II. The p.Arg183His (p.Arg209His according to the novel nomenclature) GH1 gene mutation, characterized in more than 50 subjects worldwide, results in large phenotypic variability, ranging from normal stature and GH secretion to severe GHD (127,128). Accordingly, in ten affected subjects from three unrelated families followed in our hospital, we have found a large variability in height SDS among untreated

affected individuals, with adult heights ranging from –5.41 to -2.28 SDS (unpublished data). The biological mechanism by which this heterozygous mutation results in a functional deficient state has not been completely elucidated. The mutant p.Arg209His-GH appears to fold properly and has full bioactivity, but after packaging into secretory granules it is poorly secreted, presenting a dominant-negative effect on the secretion of the WT-GH (127).

BTK gene (OMIM 300300), Type III IGHD (OMIM 307200)

Type III IGHD is an X-linked recessive condition in which affected patients present deficiency of both GH and immunoglobulin (129,130). Mutations and/or deletions in the long arm of chromosome X could be responsible for this alteration. In addition, an intronic point mutation (c.1882+5G>A), leading to exon-skipping and a premature stop codon in the BTK gene, has also been reported to be responsible for this disease (131). The xid or X-linked immunodeficiency mouse has a missense mutation in the Btk protein (132). Btk deficiency in the mouse is associated with an impairment of peripheral B cell maturation, without a major early B cell developmental block (133). In zebrafish, knockdown of btk gene using a splicing morpholino leads to severe anterior truncation of embryos (dorsalization) and this was shown to occur through an increase in wnt-beta-catenin signaling evidencing BTK as a negative regulator of this signaling pathway (134).

GH1 gene (OMIM 139250), Bioinactive GH (OMIM 262650)

Short stature associated to a bioinactive GH was first proposed by Kowarski *et al* (135) in two short boys presenting normal stimulated GH and low somatomedin/IGF-I levels. They responded normally to acute and chronic rhGH administration by increasing IGF-I levels and growth velocity. Several years later, patients with normal levels of GH and short stature were found to have heterozygous GH1 gene mutations. Arg77Cys-GH not only failed to stimulate tyrosine phosphorylation in IM-9 cells but also inhibited the ability of wildtype GH to stimulate phosphorylation, thus having a dominant negative action (136). The GH1 mutation p.Asp112Gly results in a protein that, when associated to GHBP, preferentially forms GH-GHBP complexes with a 1:1 ratio, instead of the normal 1:2 ratio produced by wild type GH. This mutant GH was less capable of phosphorylating tyrosine residues in GHR, JAK2, and STAT5 in IM-9 cells compared to wildtype GH (137). These two GH-mutant proteins with reduced or absent bioactivity, probably impaired the wildtype GH action and therefore could be responsible for the short stature observed in these patients.

GHSR gene (OMIM 601898), isolated partial GHD (OMIM 615925)

Isolated partial GHD (GHDP) can also be caused by heterozygous, compound heterozygous or homozygous mutations in the growth hormone secretagogue receptor gene (GHSR). In 2006, Pantel and colleagues (138) described homozygous and heterozygous p.Ala204Glu mutations in two probands from two unrelated Moroccan families. Short stature was present in some but not all heterozygous carriers' relatives, indicating incomplete penetrance and variable expressivity. Functional *in vitro* studies indicate that the mutant GHSR presented decreased cell surface expression and lacked constitutive activity of the receptor, while preserving its ability to respond to ghrelin, its natural ligand. Subjects carrying GHSR mutations present a clinical and biochemical phenotype of partial GHD or idiopathic short stature (139). Three different mouse models with GHSR deficiency have been reported in the literature. In two of them, GHSR gene has been removed by homologous recombination of mouse embryonic stem (ES) cells (140,141) while the other had the GHSR locus modified by the insertion of a loxP-flanked transcription blocking cassette (142). Although the Ghsr-null mice are not dwarf, serum IGF-I levels and body weight are modestly reduced compared to wildtype littermates (140,142). Despite this modest impact on postnatal growth, the major impact of GHSR deficiency seems to be related to some protection against diet-induced obesity (142). There is no known zebrafish knockdown of this gene yet.

RNPC3 gene

A novel monogenic defect resulting in severe IGHD has been reported (143) in three sisters born with normal length to normal statured and non-consanguineous parents. The patients showed severe postnatal growth retardation (height -5.0 to -6.6 SDS at diagnosis), typical physical features of GHD including delayed bone maturation, mild microcephaly and normal development. GH levels after standard stimuli and basal IGF-I and IGFBP-3 levels were almost undetectable. They presented a good response to therapeutic rhGH replacement. RT-PCR indicated normal amount and sequence of GH1 gene transcripts. Whole exome sequencing (WES) analysis of one proband revealed a missense (c.1320C>A, p.Pro474Thr) and a nonsense (c.1504C>T, p.Arg502*) mutation in the RNPC3 gene. Sanger sequencing validated that the three affected sisters are compound heterozygous for both mutations. This gene encodes a 65K protein that is a component of the U12-type spliceosome. Two types of spliceosomes catalyze splicing of pre-mRNAs. The major U2-type spliceosome is found in all eukaryotes and removes more than 99% of pre-mRNA introns. The minor U12-type spliceosome is found in some eukaryotes, is rare and has distinct splice consensus signals. The p.Pro747Thr mutation alters a highly conserved proline residue located in a turn position between ß-3-strand and α-2-helix. Such turn positions are typically non-replaceable by other amino acids. In addition to mRNA instability due to non-sense mediated RNA decay (NMD), the p.Arg502* mutation deletes the last 15 C-terminal residues that are highly conserved.

No mouse model for Rnpc3 gene knockout has been reported yet. Zebrafish rnpc3 mutant caliban, identified in an ethylnitrosourea (ENU) mutagenesis screen, shows arrested development of digestive organs, intestine, liver, and pancreas at 120 hours post fertilization (hpf) and these embryos die between 7- 10 dpf. These embryos also show delayed yolk resorption and smaller eyes (144).

IFT172 gene (OMIM 607386), short-rib thoracic dysplasia 10 with or without polydactyly (OMIM 615630)

A single case of functional GHD caused by compound heterozygous mutations in the IFT172 gene (a missense mutation p.Cys1727Arg and a splice site mutation c.337-2A>C) have been reported in a boy with growth retardation, pituitary hypoplasia, and ectopic posterior pituitary (145). Although mutations in this gene, important for ciliary function, have been previously described in retinitis pigmentosa and short-rib thoracic dysplasia, the interaction between the protein coded by the IFT172 gene with LHX3 and LHX4 could indicate a role for this gene in pituitary development (146). The mouse null mutant wimple has loss of motor neuron specification in the ventral neural tube and defects in left-right patterning. Both are due to a loss of hedgehog signaling (147). Zebrafish morphants have ciliopathy phenotypes, including ventral body-axis curvature, formation of renal cysts and cartilage defects which resemble the human phenotype (148). They also show hydrocephaly, an altered cranial structure and defects in photoreceptors of the retina (149).

ALMS1 gen (OMIM 606844), Alström syndrome (OMIM 203800)

Reduced GH reserve indicative of functional GHD has been reported in non-obese patients affected with Alström syndrome, a rare autosomal recessive monogenic disease classified as a ciliopathy disease (150). Alms1-/- mice, generated through an ES cell line with gene-trapped Alms1, developed obesity, hypogonadism, hyperinsulinemia, retinal dysfunction, and late-onset hearing loss, similar to the human phenotype of the disease (151). Zebrafish embryos depleted of alms1 using morpholinos showed beta-cell decrease in the pancreas (152). This was also validated using a CRISP/cas9 approach (152).

GH Insensitivity ("Primary IGF-I Deficiency")

Insensitivity to GH (GHI) is characterized by low IGF-I levels associated with normal or elevated GH levels and a lack of IGF-I response to GH treatment. Since GH synthesis and secretion are preserved in IGF-I insensitivity, some authors have suggested the term "primary IGF-I deficiency" to differentiate these patients from those with GHD in which IGF-I is low due to the lack of GH ("secondary IGF-I deficiency"). Several genetic defects are responsible for the impairment of GH action resulting in short stature that can affect intrauterine growth or be present in the postnatal period (14,153-155). These disorders involve at least eight different genes (**table 3**).

GHR gene (OMIM 600496), Laron syndrome (OMIM 262500), partial GH insensitivity (OMIM 604271)

Complete GH insensitivity (GHI) was first reported by Laron *et al*. in 1966 (156) in three siblings of Yemenite origin, presenting the classical clinical appearance of GHD but with GH levels that were markedly elevated. Although the possibility of an inactive GH molecule was first hypothesized, the finding that liver membranes prepared from biopsies of these patients were unable to bind iodinated GH, strongly suggested that the alteration resided in the target effector for GH. Cloning of the GHR gene opened up the possibility to characterize patients with this condition presenting a partial deletion of the GHR gene (157). In 30 patients described by Laron and his colleagues, adult height ranged from 108 to 136 cm (158). Years later, 20 patients with GHR deficiency were described among members of an inbred white population from the province of Loja in southern Ecuador (159,160). At least 70 different mutations affecting the GHR gene have been reported in more than 300 patients (161). The majority of cases were homozygous for GHR gene mutations, usually in consanguineous families (161). In most cases, the mutations affect the extracellular domain of the receptor, resulting in abnormal GH binding and low to undetectable GHBP levels. When the gene defect occurs in the transmembrane or cytoplasmatic domains, GHBP levels could be normal or even high. GHR gene mutations may result in defects in receptor dimerization, cell membrane anchorage, or signal transduction (161). Usually, GHI is inherited as an autosomal recessive condition, but a few cases have been reported where heterozygous GHR mutations exert a dominant negative effect (162-164). These last cases, as well as those caused by an intronic mutation and the activation of a pseudoexon (165), present less pronounced growth retardation and a milder clinical phenotype. In the most severe clinical cases of complete GHI, rhIGF-I is the only therapeutic option to increase linear growth. However, patients with less severe GHI, such as those presenting heterozygous GHR mutations, may benefit from rhGH or from a combination of rhGH and rhIGF-I (164). GHR-knockout mice showed severe postnatal growth retardation, undetectable GHR and GHBP, and very low levels of IGF-I, all findings similar to what was observed in patients with complete GHR deficiency (Laron syndrome) (25). In addition, this mouse has lower glucose and insulin levels, indicators of increased insulin sensitivity (166). In the liver, lack of GH receptor resulted in a higher abundance of insulin receptor (IR) and increased insulin-stimulated tyrosine phosphorylation of IR, likely mechanisms that could explain the increased insulin sensitivity (167). There is no known zebrafish mutant or morphant for this gene yet.

Table 3. GH insensitivity

Gene OMIM	Phenotype OMIM	Inher.	Human phenotype	Mice phenotype	Zebrafish Phenotype
GHR 600946	Laron dwarfism 262500 Partial GH insensitivity 604271	AR AD	Severe growth retardation, high GH and reduced IGF-I levels	Severe postnatal growth retardation, undetectable GHR and GHBP, and very low levels of IGF-I. Low glucose and insulin levels, indicators of increased insulin sensitivity	Unknown
STAT5B 604260	GH insensitivity with immune deficiency 245590	AR AD	Severe growth retardation, high GH, and reduced IGF-I levels. Moderate to severe immunodeficiency. Recurrent pulmonary infections and lymphocytic interstitial pneumonia	Fewer thymocytes and splenocytes and a SCID phenotype	Significant reduction of body weight and size in embryos and adults. Loss of sexual size dimorphism
STAT3 102582	Infantile-onset multisystem autoimmune disease 615952	AD	Variable degree of immune dysregulation and the early appearance of different autoimmune diseases. Partial GH insensitivity	75% perinatal mortality and growth retardation with increased apoptosis in thymocytes	Scoliosis, excessive inflammation and smaller than wildtype. Die at juvenile stages
IKBKB 603258	Immunodeficiency 15 615592	AR	Immune disorder, growth retardation and partial GH and IGF-I insensitivity	Defective induction of HIF-1α target genes including vascular endothelial growth factor. Alteration of innate immunity	Unknown
IL2RG 308380	Severe combined immunodeficiency, X-linked, T-cell-negative, B-cell-positive, NK cell-negative 300400	AR	Severe combined immune deficiency. Some patients present GH insensitivity	Hypoplastic thymuses and a reduced number of lymphocytes. Absence of NK cells	Reduced embryonic lymphopoiesis
PIK3R1 171833	SHORT syndrome 269880	AR	Some patients present low levels of IGF-I with insufficient response to rhGH	Increased insulin sensitivity and hypoglycemia	Angiogenesis defects
IGF1 147440	IGF-I deficiency 608747	AR AD	Growth retardation with deafness and mental retardation	Birth weight of about 60% compared to normal mice. Severe postnatal growth retardation. Increased GH levels	Unknown
IGFALS 601489	Acid-labile subunit deficiency 615961	AR	Severe IGF-I and IGFBP-3 deficiencies with mild growth retardation. Poor response to rhGH treatment	13% smaller at 10 weeks of age and marked reductions of circulating IGF-I and IGBP-3 levels	Unknown

STAT5B gene (OMIM 604260) GH insensitivity with immune deficiency (OMIM 245590)

The family of signal transducers and activators of transcription (STATs) includes seven members that act both as intracellular signaling mediators and transcription factors (168). They are activated by multiple growth factors and cytokines. Although GH activates four members of this family (STAT1, STAT3, STAT5a, and STAT5b), STAT-5b is the key mediator of GH promoting actions. In 2003, a homozygous mutation in STAT5B gene was described in a 16-year-old girl with severe postnatal

growth retardation and IGF-I deficiency (169). She had a history of recurrent pulmonary infections and lymphocytic interstitial pneumonia, presenting immunodeficiency characterized by a defect in T cell immunity. Since STAT5b is also required in the signaling of several cytokines such as interleukin-2 and γ-interferon, it seems likely that the growth failure and the immune defect are both due to its inactivation. Ten patients with STAT5b deficiency have been reported, all presenting severe growth failure, complete GHI and a moderate to severe immunodeficiency. GHI resulted in marked growth retardation which is always present, but with a more variable severity of the immune deficiency and pulmonary disease (170-174). Interestingly, haploinsufficiency for STAT5B gene appears to affect growth, since heterozygous carriers are shorter than their wildtype relatives (175). At the time this review was written, heterozygous STAT5B gene mutations with dominant-negative effect were described in three families in which affected members presented short stature associated with partial GH insensitivity but not severe immune alterations. These STAT5B missense mutations (p.Gln177Pro, p.ALa478Val, and p.Gln474Arg) are robustly phosphorylated upon stimulation but are not able to translocate to the nucleus or to bind STAT5B DNA response elements. In addition, these variants are able to dimerize to wildtype STAT5B disrupting the transcriptional function of wiltype STAT5B and exerting a dominant-negative effect (176).

Complete loss of Stat5 (a and b) in mice leads to Severe Combined Immunodeficiency (SCID). These mice die before or shortly after birth, presenting significantly fewer thymocytes and splenocytes than their wildtype littermates (177). Although there are 2 stat5b genes in zebrafish (stat5b.1 and stat5b.2), it appears that stat5b.1 is the corresponding homologue of mammalian STAT5B in fish (178). Stat5b.1 mutant fish generated using CRISPR/Cas9 results in a significant reduction of body weight and length in both embryos and adult zebrafish (178). Also, sexual size dimorphism was eliminated in these adult fish, where normally females are larger and heavier than males. Interestingly, there seems to be a positive feedback loop whereby stat5b positively regulates gh1 expression in zebrafish, which is absent in mammals (178).

STAT3 gene (OMIM 102582), infantile-onset multisystem autoimmune disease (OMIM 615952)

Heterozygous gain-of-function mutations in the STAT3 gene have been reported in patients presenting a variable degree of immune dysregulation and the early appearance of different autoimmune diseases (type-1 diabetes, autoimmune enteropathy, thyroid dysfunction, pulmonary disease, hemolytic anemia, thrombocytopenia, neutropenia, juvenile-onset arthritis, eczema) (179-182). Most of the affected patients present growth failure, normal GH levels, and low IGF-I levels, indicative of some degree of GHI. Constitutive activation of STAT3 is associated with increased expression of SOCS3 (suppressor of cytokines signaling) (179). Members of the SOCS family block STAT activation by turning off the initial signal (183). Epstein-Barr virus-transformed cell lines derived from patients carrying activating STAT3 mutations display reduced STAT5b phosphorylation in response to interleukin-2, a plausible explanation for the observed GHI (181). Patients carrying activating STAT3 mutations preserve some degree of responsiveness to rhGH treatment (181,182). The severity of the immune disorder and autoimmunity caused by germline STAT3 gain-of-function mutations results in a severe life-threatening condition. Although bone marrow transplantation and anti-IL6R monoclonal antibody have been used as therapeutic tools, the results were not always successful (179,180,182). Potential novel therapeutic approaches include small-molecule inhibitors of STAT3 (181).

$Stat3^{-/-}$ mice die around embryonic day 7 (184). Heterozygous mice carrying the mutation p.Ser727Ala (SA) in one allele and a deletion in the other Stat3 allele ($STAT3^{SA/-}$ mice) have normal amounts of Stat3 in all cells except fibroblasts which have a 25 % or less Stat3 transcriptional response. These mice had 75 % perinatal mortality and growth retardation with increased apoptosis in thymocytes in the surviving mice (185). There are many studies involving the zebrafish stat3 gene. Morpholinos to knockdown stat3 have implicated this transcription factor in heart, eye and hair cell regeneration in zebrafish (186-188). Finally, a recent study generated null mutations in zebrafish stat3 which resulted in mutants that die during juvenile stages exhibiting scoliosis and excessive inflammation (189). They also appeared smaller than wildtype fish. All these zebrafish mutants are models of STAT3 inactivation, but a gain-of-function stat3 zebrafish model has not yet been reported.

IKBKB gene (OMIM 603258), Immunodeficiency 15 (OMIM 615592)

The nuclear factor κB family of transcription factors modulates gene expression by binding to specific DNA regulatory elements as homo or heterodimers. In the unstimulated state, NF-κB dimers are bound to IκB preventing translocation to the nucleus (190), thereby maintaining NF-Kb in an inactive state. Heterozygous mutations in IKBKB gene, that encodes for the inhibitory IκBα protein, have been described in two patients with immune disorder, growth retardation and partial GH and IGF-I insensitivity (191).

In the mouse, IKK-β deficiency results in defective induction of HIF-1α target genes including vascular endothelial growth factor (VEGF). IKK-β is an important physiological contributor to the hypoxic response, linking it to innate immunity and inflammation (192). Zebrafish knockdown studies have not been performed in this gene yet.

IL2RG gene (OMIM 308380), Severe combined immunodeficiency, X-linked, T cell-negative, B-cell-positive, NK cell-negative, XSCID (OMIM 300400).

The IL-2 receptor γ common (IL-2Rγc) chain is the shared subunit of the receptors for the IL-2 family of cytokines. IL2RG associates with different interleukin receptor alpha chains to form heterodimers. Through the binding of cytokines, these receptors regulate homeostasis of the immune system. Mutations in the gene encoding the gamma subunit of the interleukin-2 receptor (IL2RG) are found in patients presenting this condition (193). Some patients with mutations in the IL2RG gene present a diminished or absent response to rhGH treatment both in terms of IGF-I increase as well as growth acceleration (194). In addition, GH stimulation of mutated B cells shows no phosphorylation of STAT5b and lack of nuclear translocation, indicative of a defect in GH signaling (195).

Knockout mice for Il2rg gene lack gamma chain expression and have hypoplastic thymuses. Splenic T cells were diminished at 3 weeks of age, and B cells were greatly diminished in contrast to the situation in patients with XSCID (196). There are 2 zebrafish IL-2Rγc paralogs, il-2rγc.a and il-2rγc.b, and knockdown of il-2rγc.a but not il-2rγc.b leads to reduced embryonic lymphopoiesis (197).

PIK3R1 gene (OMIM 171833) SHORT syndrome (OMIM 269880)

PIK3R1 codes for the regulatory subunits of the phosphatidyl inositol-3 kinase class IA (PI3K) and is involved in activation of the AKT/mTOR pathway to ensure proper growth and cell proliferation (198). SHORT syndrome historically has been defined by its acronym: short stature (S), hyperextensibility of joints and/or inguinal hernia (H), ocular depression (O), Rieger abnormality (R) and teething delay (T) (199). PIK3R1 heterozygous mutations have been identified in several patients affected with SHORT syndrome (200-203). Persistently low levels of IGF-I with insufficient response to rhGH has been shown in some patients, indicating some degree of GHI.

Targeted disruption of the Pik3r1 gene in mice leads to increased insulin sensitivity and hypoglycemia due to increased glucose transport both in muscle and adipocytes (204). In addition, liver-specific deletion of Pik3r1 in mice also results in increased hepatic and peripheral insulin sensitivity (205). Zebrafish embryos injected with morpholinos to reduce pik3r1 levels displayed angiogenesis defects with variable shortening of intersegmental vessel (ISV) length and were otherwise overtly normal (206).

IGF1 gene (OMIM 147440), growth retardation with deafness and mental retardation due to IGF-I deficiency (OMIM 608747)

The first molecular defect in the IGF1 gene was described in 1996 in a 15-year-old boy presenting severe intrauterine growth retardation, postnatal growth failure, sensorineural deafness, mental retardation, microcephaly, and delayed puberty (207). The patient was homozygous for a deletion of exon 4 and 5 in the IGF1 gene. Marked insulin-resistance was also present, likely related to the abnormally high GH levels and a functional GH receptor. The few other reported patients with IGF1 gene mutations present pre- and postnatal growth impairment, mental retardation, and hearing loss (208-211). A homozygous missense mutation (p.Val44Met) detected in a 55-year-old patient presenting severe intrauterine and postnatal growth retardation, microcephaly, and sensorineural deafness was functionally inactive with a 90-fold reduced affinity for the IGF-I receptor (209). The classical phenotype with prenatal growth retardation was observed in those cases with both affected alleles. A less severe phenotype without intrauterine growth retardation, microcephaly, or deafness, has been described in several members of a family, carrier of a frameshift mutation that, if expressed, resulted in a truncated and presumably inactive protein (210). A patient with non-dysmorphic phenotype and less severe pre- and postnatal growth retardation was homozygous for a missense mutation that reduces two- to three-fold the affinity of the mutant IGF-I for the IGF-1 receptor (211). Molecular defects of the IGF1 gene are rare, and only about 9 patients have been described (212).

In the mouse, targeted disruption of Igf1 gene resulted in birth weight of about 60% compared to normal mice. Depending on the genetic background, Igf1(-/-) dwarf mice die shortly after birth or survive and reach adulthood (213). To further explore the role of liver-produced IGF-I, the major contributor of circulating IGF-I, Yakar *et al* (28) used the Cre/loxP recombination system to delete the igf1 gene exclusively in the liver (LID mouse). Although the LID mouse showed a severe reduction in circulating IGF-I levels, body weight, body length, and femoral length did not differ from wildtype littermates. However, due to the reduction of negative feed-back, this animal presents high circulating GH levels that could partially compensate for the reduction of circulating IGF-I levels. This study suggests an important role for locally produced IGF-I (autocrine/paracrine IGF-I) in longitudinal growth. The only study that used morpholinos to knockdown igf1 in zebrafish led to embryonic abnormalities that were not possible to discern from nonspecific toxic effects from the morpholino itself (214).

IGFALS gene (OMIM 601489), acid-labile subunit deficiency (OMIM 615961)

The acid-labile subunit (ALS), a member of the leucine-rich repeats proteins, is a circulating protein that plays an important role in maintaining high circulating levels of IGF-I. Although ALS has no discernible affinity for IGF-I, it is capable of binding binary complexes formed by IGF-I or IGF-II with IGFBP-3 or IGFBP-5, forming ternary complexes (215).

Thus, ALS could be considered a binding protein of binary complexes. The main role of ALS is to maintain up to 80-90% of the circulating IGFs in this ternary complex, extending the half-life of free IGF-I from 10 min to more than 12 hours (216).

The first description of complete ALS deficiency was reported in a 17-year-old boy with delayed onset of puberty, slow pubertal progress, and markedly reduced IGF-I and IGFBP-3 levels that remained unchanged after GH stimulation (217). The patient was homozygous for a frameshift mutation in the IGFALS gene (p.Glu35Lysfs*87). Once the clinical characteristics and biochemical phenotype of ALS deficiency became recognized, severe IGF-I and IGFBP-3 deficiencies associated with moderate growth retardation (a "mismatch" between the severity of IGF-I and IGFBP-3 deficiencies and the mild effect on growth), several reports communicated at least 62 patients with this defect (218-228). In these patients, whereas circulating levels of IGF-I are dramatically decreased, local production appears to be preserved. Local expression of IGF-I, under the control of normal and/or increased GH levels, could be responsible for the preservation of linear growth near normal limits (229-231). Functional *in vitro* characterization of several IGFALS variants has shown that pathogenic variants result in the absence of ALS synthesis or intracellular retention of the mutant protein (232,233). In children with apparent GHI, systematic genetic characterization by candidate gene approach or WES has shown that mutations in the IGFALS gene, resulting in complete ALS deficiency, is the second most frequent gene defect, second only to GHR gene defects (234).

It is noteworthy that heterozygous IGFALS gene mutations are present in a subgroup of children with idiopathic short stature presenting partial ALS deficiency (235,236). Characterization of children with partial ALS deficiency may prove clinically relevant, because these patients have shown responsiveness to rhGH treatment, increasing IGF-I levels and accelerating growth velocity (236,237). Whether this initial response results in an increase in adult height remains to be determined.

Homozygous null mice for Igfals are 13% smaller than their wildtype littermates at 10 weeks of age (238). This modest phenotype, despite marked reductions of IGF-I and IGFBP-3 levels in plasma, support the importance of locally produced IGF-I in growth.

The zebrafish igfals morphants have not yet been reported.

IGF-I Insensitivity

There are only a few molecular defects resulting in impairment of IGF-I action (**table 4**).

IGF1R gene (OMIM 147370), insulin-like growth factor I, resistance to (OMIM 270450)

A specific IGF-I receptor was first characterized in 1977 (239), but it was not until 1986 that the complete cDNA sequence for this receptor was published (240). Although several patients with intrauterine growth retardation presenting elevated levels of GH and IGF-I, suggestive of some degree of IGF-I resistance, were reported in the 1980s and 1990s, (241-243), it was not until 2003 that the first patients with IGF1R gene mutations were reported (244). The first mutations in this gene were detected in patients with intrauterine growth retardation or short stature and elevated IGF-I levels (244). This original report was essentially the result of two separate

Table 4. IGF-I resistance

Gene OMIM	Phenotype OMIM	Inher.	Human phenotype	Mice phenotype	Zebrafish Phenotype
IGF1R 147370	Resistance to IGF-I 270450	AR AD	Intrauterine growth retardation, postnatal growth retardation with normal/ elevated IGF-I levels	Mice are 45% smaller than widtype at birth with general organ hypoplasia	Reduced embryonic growth, arrested development, and increased lethality. Defects in retina, innear ear, heart, and motor neurons
PAPPA2 -	PAPPA2 deficiency -	AR	Mild postnatal growth retardation with high levels of IGF-I, IGFBP-3, and ALS	Postnatal growth retardation	Ventral curvature of embryos, notochord defects, reduced jaw and angiogenesis defects
IGF2 147470	Severe growth restriction with distinctive facies 616489	Epigenetic	Severe intrauterine and postnatal growth restriction and a Silver-Russell syndrome-like phenotype	Heterozygous males have growth defects while females are normal. Homozygous males and females have growth defects	Knockdown of either ortholog or both leads to ventralized embryos with reduced growth, reduced eyes, disrupted brain structures and a defective cardiovascular system

studies published together. The first group consisted of 42 patients with intrauterine growth retardation and subsequent short stature. One girl was a compound heterozygote for a point mutation in exon 2 of the IGF1R gene (p.Arg108Gln/p.Lys115Asn). Cultured fibroblasts from the patient had decreased IGF-I-receptor function. In the second cohort of 50 children with short stature and elevated circulating IGF-I levels, the authors identified one boy with a nonsense mutation (p.Arg59stop) that resulted in a reduced number of IGF-I receptors in fibroblasts. Both children had intrauterine growth retardation and poor postnatal growth. It is likely that, as is observed in the mouse, complete absence of IGF1R in humans may be lethal. This could explain why, except for two compound heterozygous cases (244,245), and two homozygous patients (246,247), only heterozygous cases have been reported. The few patients presenting mutations in both IGF1R alleles appear to retain some degree of IGF1R activity. Functional *in vitro* studies of naturally occurring IGF1R mutations suggest that different mechanisms could explain the impairment of IGF action: receptor haploinsufficiency, decreased biosynthesis, reduced binding affinity, interference of transmembrane signaling, and disruption of the tyrosine kinase activity (248). The impact of IGF1R mutations on intrauterine growth is variable, but is frequently more severe when maternally inherited, indicating that maternal IGF-I resistance during pregnancy is one factor contributing to the severity of growth retardation, possibly by decreasing placental growth (249). As many as 20 patients have been described with IGF1R mutations (250-256). These patients have shown a poor to moderate clinical response to rhGH treatment (248).

Targeted disruption of the Igf1r in mice led to death shortly after birth due to respiratory failure and 45% smaller birth size than wildtype mice. They also have global organ hypoplasia (213). Zebrafish have 2 igf1r genes (igf1ra and igf1rb). Using either morpholinos or a dominant negative igf1r fusion protein to target these 2 genes in zebrafish resulted in reduced embryonic growth, arrested development, and increased lethality. In addition, these embryos had defects in the retina, inner ear, heart, and motor neurons (257).

Pregnancy-associated plasma protein A2 deficiency (PAPP-A2)

A completely new syndrome has been recently described, involving the first genetic defect in a protease. Pregnancy-associated plasma protein-A2 (PAPP-A2) is a serum and tissue protease responsible for proteolysis of IGFBP-3 and IGFBP-5, regulating the bioavailability of IGF-I and IGF-II to their target tissues (258). Five affected subjects from two families presenting moderate growth retardation and elevated circulating levels of IGF-I, IGF-II, IGFBP-3, IGFBP-5, and ALS, were found to be homozygous for two different mutations in the PAPPA2 gene (p.Asp643fs25* and p.Ala1033Val) (259). *In vitro* analysis of IGFBP cleavage demonstrated that both mutations cause a complete absence of PAPP-A2 proteolytic activity. Size exclusion chromatography showed a significant increase in IGF-I bound in its ternary complex, and decrease in free and bioactive IGF-I concentrations. Other clinical findings included characteristic thin long bones most notable in the fibulae, tibiae, and femurs. While bone age was according to chronological age, bone mineral density (BMD) was decreased at the lumbar spine, and fasting glucose concentrations were normal with mild hyperinsulinemia. Interestingly, a one-year treatment with rhIGF-I resulted in a clear increase in growth velocity and height in two siblings. Bioactive IGF-I was increased, and spontaneous GH secretion was diminished after acute administration of rhIGF-1, whereas serum total IGF-I and IGFBP-3 levels remained elevated (260). Besides the moderate increase in growth velocity, one-year treatment with rhIGF-I resulted in a reduction of insulin resistance and an increase in total body BMD (261).

The finding of PAPPA2 mutations as an etiological cause of short stature has both clinical and physiological consequences. The molecular diagnosis resulted useful for the selection of the proper therapeutic agent to increase adult height and, on the other hand, illustrate the important physiological role of the IGFBPs and their specific proteases in the regulation of IGF-I bioavailability (262).

The Pappa2a knockout mouse was of normal size at birth but had postnatal growth retardation (263). The knockout mice also display disproportionally reduced dimensions of specific bones, including skull and mandible (264). Knockdown of zebrafish papp-a2 results in ventral curvature of embryos as well as notochord defects implicating this protein in notochord development. In addition, the jaw is significantly reduced, indicating a role for papp-a2 in cranial cartilage development. Finally, embryos also have defects in angiogenesis (265).

IGF2 gene (OMIM 147470), severe growth restriction with distinctive facies (OMIM 616489)

In 2015 Begemann *et al*. reported an IGF2 nonsense variant (p.Ser64*) in a multigenerational family with four members presenting growth restriction (266). Only transmission of the paternally affected allele resulted in growth impairment in those tissues involved in growth, confirming the monoallelic expression of the maternally imprinted IGF2 gene. The affected patients have severe intrauterine and postnatal growth restriction and a Silver-Russell syndrome (SRS)-like phenotype. More recently, two independent reports described two patients with a frameshift (p.Leu37Glnfs*31) and a missense (p.Gly34Asp) de novo mutation in the IGF2 gene, presenting a characteristic SRS-phenotype, (267,268), indicating that this alteration could arise as a de novo condition in non-familial patients affected with SRS. Remarkably, in these last cases the mutations resided in the

paternal allele, an expected result considering the paternal monoallelic expression of the IGF2 gene in non-hepatic tissues. Two out of three patients showed a satisfactory growth increase after chronic rhGH treatment (267).

Targeted disruption of Igf2 in mice led to heterozygous male mice with growth defects yet phenotypically normal heterozygous females. Homozygous female mutants resemble their heterozygous growth defective male siblings (269). In contrast, the zebrafish genome contains 2 co-orthologs of mammalian IGF2 gene (igf2a and igf2b). Knockdown of either gene using morpholinos led to ventralized embryos characterized by reduced growth, reduced eyes, disrupted brain structures and a defective cardiovascular system. Knockdown of both genes simultaneously increased the severity of the phenotype. This implicates both genes in dorso-ventral patterning during development in zebrafish (270,271).

Conclusions

From the molecular characterization of the first genetic defect in the GH/IGF axis, a complete GH1 gene deletion in patients with severe isolated GHD and profound growth retardation by Phillips III and their colleagues in 1981, mutations in more than 48 different genes have been described all along the GH/IGF axis. These defects result in alteration of GH synthesis/secretion (isolated or associated to other pituitary hormones), defects in GH action (alteration at the level of the GH receptor, the intracellular signaling pathway, or the transport of IGFs), or IGF-I action (alteration of IGF-I synthesis or transport). Most of these molecular defects were discovered by the candidate gene approach, by using clinical data and biochemical profiles to select the more likely candidate gene(s) to be studied. Since 2012, with the development of next generation sequencing (NGS) techniques, capable of determining the WES or even the whole genome sequence (WGS) within weeks, new genetic clinical conditions have been elucidated in patients where clinical and biochemical data did not suggest an obvious candidate gene or where several likely candidate genes had to be explored and the conventional sequencing of each one would be more expensive and time consuming than the NGS approach. In addition, this last approach has revealed novel genetic defects, previously unknown or unsuspected given the clinical and biochemical characteristics of the subjects under study. It has also been shown that in a small percentage of cases, more than one gene could be affected, resulting in a more complex clinical presentation, usually presenting overlapping phenotypic features (272). It has been proposed that genetic evaluation of short stature is indicated in those cases that present severe GHD, multiple pituitary hormone deficiency, unequivocal GH insensitivity, small for gestational age without catch-up growth, additional congenital anomalies or dysmorphic features, evidence of skeletal dysplasia, associated intellectual disability, microcephaly, and severe growth retardation (273). Even with careful selection of patients, a genetic diagnosis is obtained in only 30-40% of patients with IGHD, CPHD, apparent GH or IGF-I insensitivity (13,234). Due to increased accessibility to NGS, a significant number of likely pathogenic variants have been described in novel genes in patients with short stature and defects in the GH/IGF axis. These variants appear both in genes previously associated with these conditions as well as in completely novel genes. Characterization of these variants by functional *in vitro* assays and *in vivo* animal models is required to determine the real contribution of these findings.

Disclosure

The authors do not have any commercial or financial relationships that could be considered as a potential conflict of interest.

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