

RESEARCH

Optimising diagnosis in children with short stature: an integrated clinical and NGS approach

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Abstract

Short stature (SS) is one of the most frequent reasons for referral to paediatric endocrinologists. Linear growth is a multifactorial process, with genetic variation representing the principal determinant of height differences. Between 2018 and 2022, 102 children referred to our clinic for growth failure were identified as having SS of unknown aetiology. The cohort comprised 57 children with idiopathic GH deficiency (GHD-SS) and 45 with idiopathic short stature (ISS). Children born small for gestational age and those with known genetic conditions were excluded. All patients underwent a single next-generation sequencing (NGS) analysis using a custom-designed targeted gene panel for SS. When variants were detected, segregation analysis was performed through parental testing. The overall diagnostic yield of NGS was 14.9%, with variants considered causative of the SS phenotype detected in 14.3% of GHD-SS patients and 15.6% of ISS patients. Detection rates were comparable between isolated GHD and combined pituitary hormone deficiency. Among ISS patients, a genetic diagnosis was achieved in 23.8% of familial cases and in 8.7% of sporadic cases. Variants of uncertain significance were identified in approximately half of the cohort. In conclusion, a first-line targeted NGS approach, applied in routine clinical practice to a carefully selected cohort of children with SS of unknown aetiology, demonstrated a competitive diagnostic yield. Accurate phenotypic assessment remains critical to improving the diagnostic performance of molecular testing and refining the aetiological evaluation of SS. Moreover, identification of the underlying genetic cause provides valuable insights for predicting clinical evolution and guiding therapeutic strategies.

Keywords: short stature; genetic testing; GH deficiency; ISS

Introduction

Short stature is one of the most common reasons for referral to paediatric endocrinologists and may significantly impact the quality of life of affected children, who are more vulnerable to developmental, social, and educational challenges (1, 2). Genome-wide association studies have established that human height is a polygenic trait influenced by more than 600 common variants clustered at hundreds of loci (3).

However, monogenic disorders also contribute to growth impairment in a subset of patients (4, 5).

For decades, linear growth impairment was attributed almost exclusively to dysfunctions in the growth hormone (GH)-insulin-like growth factor-1 (IGF-1) axis. It is now well recognised, however, that the aetiology of SS is highly heterogeneous and includes disruptions in a

wide range of biological pathways involved in the proliferation and differentiation of growth plate chondrocytes (6, 7). Pathogenic mechanisms may be extrinsic or intrinsic to the growth plate. Extrinsic factors include nutritional deficiencies, hormonal imbalances, and dysregulation of inflammatory cytokines, whereas intrinsic factors involve abnormalities of the extracellular matrix as well as alterations in intracellular and paracrine signalling pathways (8, 9, 10).

Advances in molecular biology, nanotechnology, and bioinformatics have significantly accelerated our understanding of the molecular mechanisms underlying growth and facilitated the identification of genetic causes of SS. The application of molecular genetic techniques has not only improved diagnostic capabilities but has also contributed to more accurate phenotypic characterisation of affected individuals (11), whose clinical presentation may vary widely (8), ranging from severe syndromic phenotypes to apparently idiopathic SS (ISS) (8, 12).

Genetic alterations in the GH-IGF1 axis – the most important extrinsic regulator of growth plate activity – can lead to a broad spectrum of growth abnormalities from severe to milder phenotypes, depending on the degree of axis involvement (13). The phenotype may be further complicated by the presence of combined pituitary hormone deficiencies (CPHD), often associated with midline brain defects and additional congenital anomalies (14, 15). Moreover, a single gene mutation can result in overlapping phenotypic presentations (14, 15).

Similarly, abnormal phenotypes with short stature arising from disruptions in local signalling pathways acting directly on growth plate chondrocytes (intrinsic mechanisms) are also characterised by wide phenotypic variability (8).

Growth deficiency associated with skeletal dysplasias has long been considered distinct from other forms of short stature. However, in the past decade, increasing evidence has demonstrated that these conditions represent a continuum within the short stature phenotype, ranging from syndromic to idiopathic SS with mild or minimal radiographic signs of skeletal dysplasia (8). The severity of clinical manifestations largely depends on the critical role of the affected gene in the growth plate and the impact of the genetic anomaly on protein function, which depends on both the type and zygosity of the specific pathogenic variants.

Given this complexity, molecular characterisation in patients suspected of having a genetic form of SS has become increasingly important, and genetic testing is now recommended to identify the specific diagnosis (16). Although various clinical features have been proposed to help identify individuals most likely to have a monogenic cause of SS (17, 18, 19), such indicators have

not yet been rigorously validated (19). Auxological evaluation, laboratory testing, and detailed personal and family medical history remain essential components of the diagnostic work-up. Nevertheless, establishing a specific diagnosis is often challenging, as most affected individuals are non-syndromic and exhibit mild, non-specific features. On the other hand, identifying the genetic aetiology of SS provides valuable information for predicting phenotype evolution, guiding treatment strategies, optimising personalised clinical management, and enabling appropriate genetic counselling.

The aim of this study was to improve the diagnostic assessment of children with short stature of unknown aetiology by applying targeted next-generation sequencing (NGS) of a custom-designed gene panel as a first-line test, following rigorous clinical selection criteria within routine diagnostic practice. In addition, genotype–phenotype correlations were also explored.

Materials and methods

Study design and participants

This retrospective study involved children with pathological SS referred to the Paediatric Endocrinology Unit of the University Hospital of Padova (Italy) between 2018 and 2022, who were studied by targeted sequencing of a single extensive gene panel associated with SS. Inclusion criteria for patients were: short stature, defined as height <2 SDS below the mean height for age, sex, and ethnic group, or height <2 SDS below the parental target height (TH). Exclusion criteria were: secondary GH deficiency, being born small for gestational age (SGA), and previously established diagnoses of genetic conditions associated with SS (including *SHOX* gene copy number variants detected by MLPA).

A comprehensive auxological characterisation was performed in all selected patients at the time of SS diagnosis, including the evaluation of: standing height (H), sitting height (SH), SH/H ratio, height velocity (HV), bone age (BA), parental height, and TH as predicted by parental stature. Standing and SH (trunk/lower limbs ratio) were measured using a Harpenden stadiometer (accuracy of 0.1 cm) to identify body disproportion; non-physiological SH/H ratio was defined according to age and sex (20). Height and HV SDS were calculated according to reference growth charts specific for the population of the study (21, 22); CDC charts were used for non-Italian children (23, 24, 25, 26). Severe SS was defined as height ≤ -3 SDS for age and sex. HV was calculated as the annual increase in height (cm/year). BA was assessed using the Greulich and Pyle method (27). TH was calculated according to the following formulae: ((father's height in cm – 13 cm) + mother's height in cm)/2 for girls; ((mother's height in cm + 13 cm) + father's height in cm)/2 for boys (28).

All patients underwent comprehensive clinical evaluation to exclude common causes of growth impairment, such as coeliac disease, hypothyroidism, chronic diseases, severe nutritional deficiency, and prolonged corticosteroid therapy; the assessment also aimed to identify and exclude children whose physical features were suggestive of a syndromic condition. Following clinical work-up, patients were stratified into two main groups based on GH secretion status: the first group included children with idiopathic GH deficiency (GHD-SS), and the second one included those with non-GH-deficient ISS (NGHD-ISS). GHD diagnosis was confirmed according to the guidelines issued by the National Health Authority, based on clinical-auxological and laboratory parameters. GH secretion was assessed by two different stimulation tests: the first by administering arginine and the second using either insulin or glucagon. A GH peak <8 ng/mL was required for diagnosis, in the presence of adequate blood glucose and cortisol responses. Testosterone priming was applied in boys with delayed puberty.

For children included in the GHD-SS group, the following additional data were collected: severity of GH secretion deficiency after stimulation tests (GH peak $<$ or >5 ng/mL), IGF1 plasma levels, presence of additional pituitary hormone deficiencies, and brain MRI findings. To account for the age- and sex-dependency as well as the skewed distribution of IGF1 concentrations, IGF1 SDS values were derived using normative data from a large multicentre cohort of healthy Caucasian subjects with a modified LMS method, as proposed by Bidlingmaier *et al.* (29) MRI scans were evaluated by experienced neuroradiologists, with particular attention to midline brain structures. Pituitary gland and stalk measurements were interpreted based on age- and sex-specific reference data by Sari *et al.* (30).

Molecular genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes of all patients included in the study. A custom-designed targeted gene panel comprising 82 genes associated with SS (Supplementary Table 1 (see section on [Supplementary materials](#) given at the end of the article)) was analysed using NGS technology. Genes were selected based on evidence from the published literature, specifically those implicated in the regulation of growth plate function (31). Both extrinsic and intrinsic regulatory genes were considered. Extrinsic regulators included genes encoding early transcription factors involved in the morphogenesis of brain midline structures as well as genes associated with the GH/IGF-1 axis. Intrinsic regulators encompassed genes involved in paracrine signalling within the growth plate, extracellular matrix organisation, and key intracellular signalling pathways. The coding regions of the selected genes were isolated and captured using the SureSelect Target Enrichment systems (Agilent Technologies, USA);

indexed DNA fragment libraries were prepared following the manufacturer's protocol and sequenced on the Illumina NextSeq 550 platform (Illumina, USA). Variant calling and annotation were conducted using SureCall software (Agilent Technologies). All variants with a satisfactory sequencing depth and quality were filtered according to their frequency in the general population (as reported in gnomAD; <http://gnomad.broadinstitute.org/>). Common polymorphisms with an allele frequency $>1\%$ were excluded from the study, while rare variants were retained for further analysis. Variant interpretation was performed in accordance with the American College of Medical Genetics and Genomics (ACMG) guidelines (32). Public databases including ClinVar and LOVD, as well as bioinformatic platforms such as VarSome (<https://varsome.com/>) and Franklin (<https://franklin.genoox.com/>), were used to annotate and assess the clinical relevance of variants. All potentially pathogenic variants were investigated in the parental DNA, when available, by Sanger sequencing to assess their inheritance pattern and further support variant classification. Pathogenic (P) and likely pathogenic (LP) variants were considered causative of the patients' phenotype. Variants of uncertain significance (VUS) were designated as 'hot VUS' when a high level of supporting evidence was available but no further affirmation of pathogenicity could be obtained to upgrade the classification (33). These variants were considered to have a high probability of contributing to the pathological phenotype.

The study was conducted in compliance with the terms of the Helsinki II Declaration and with the approval of the local Ethics Committee. Written informed consent was obtained from the parents or legal guardians of all participants before genetic testing, in accordance with institutional and ethical standards.

Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation (SD), while categorical variables are reported as absolute frequencies and percentages. Comparisons between groups were performed using the Mann-Whitney U test or the Kruskal-Wallis one-way ANOVA for continuous variables, and Fisher's exact test for categorical variables. A P -value <0.05 was considered statistically significant. Sex was not included as a variable in the statistical analysis.

Results

Characteristics of the cohort

A total of 102 patients with short stature were selected for the study: 57 children (55.9%) with idiopathic GHD (35 males and 22 females) and 45 (44.1%) with ISS (27 males and 18 females). Among GHD subjects, 42 (73.7%)

Table 1 Clinical characteristics of study participants ($n = 102$)*.

Clinical feature	n (%) or mean $\pm$ SD
GH deficiency*	57
Isolated GHD	42 (73.7%)
CPHD	15 (26.3%)
NGHD-ISS	45
Familial short stature	21 (46.7%)
Sporadic short stature	23 (51.1%)
Adopted child	1 (2.2%)
Height	
> -3 SD	74 (72.5%)
≤ -3 SD	28 (27.5%)
Sitting height/height ratio	
Harmonic	84 (82.4%)
Disharmonic	18 (17.6%)
Bone-chronological age gap (months)	-17.1 ± 14.5

*Included one couple of siblings with isolated GHD deficiency.

presented with isolated GHD, while 15 patients (26.3%) displayed a combined pituitary hormone deficiency (CPHD). Among ISS subjects, 21 had a familial SS (46.7%), 23 a sporadic SS (51.1%), and one child was adopted with unknown family history (2.2%) (Table 1). With respect to ethnic background, 84 patients (82.3%) included in the study were of Italian origin, while the remaining individuals were of European Caucasian descent. Familial forms of SS were observed predominantly among patients of Italian origin, with only a single case identified in a patient of Albanian descent. No parental consanguinity was reported in any of the included cases.

Clinical features of GHD and ISS patients tested by NGS analyses

Overall, there was no statistically significant difference in mean height SDS between the GHD group (-2.67 SDS) and the ISS group (-2.52 SDS) ($P = 0.25$). Although a higher proportion of children in the GHD group presented with severe SS (i.e. ≤ -3 SD) compared to the ISS group (35.1 vs 17.8%), this difference was not statistically significant ($P = 0.07$). The disproportionate trunk/lower limb ratio was instead significantly more frequent among ISS patients (26.7% of cases) compared to GHD patients (10.5%) ($P = 0.04$). The BA/chronological

age gap was comparable between the two groups (Table 2).

The mean HV was -1.84 SDS in the GHD group and -1.42 SDS in the ISS group ($P = 0.15$); the mean IGF1 level was -1.86 SDS in GHD patients and -0.16 SDS in ISS patients ($P < 0.05$).

Approximately 30% of both isolated GHD and CPHD subgroups of children presented a severe form of SS (Table 3); most of them had a severe GH secretion deficiency (i.e. <5 ng/mL), specifically 60% of the isolated GHD patients and 83.3% of the CPHD patients ($P = 0.179$).

All children with CPHD had a normal SH/height ratio, whereas in very few children with isolated GHD, auxological evaluation resulted in a ratio slightly above normal limits; one of these carried a pathogenic variant in the *GNAS* gene.

Regarding brain imaging, a structural anomaly was detected in the large majority (80%) of the patients with CPHD compared to those with isolated GHD (16.7%, $P = 0.0002$), as shown in Table 3. Among patients with isolated GHD, in six out of seven cases the observed anomalies consisted of mild to marked hypoplasia of the anterior pituitary gland, occasionally associated with a thin stalk and ectopic posterior pituitary gland. Two cases presented with more complex malformations (Chiari malformation type I in one patient and optic chiasm malformation with cerebellar hypoplasia in another).

In the CPHD subgroup, all pathological MRI findings involved complex abnormalities of the pituitary region, including severe hypoplasia or agenesis of the adenohypophysis associated with thin or absent pituitary stalk and ectopic posterior pituitary gland. Only one patient with CPHD exhibited a less complex presentation with isolated anterior pituitary hypoplasia.

No pathological brain anomalies were observed in children with ISS who underwent MRI; in particular, no morphological abnormalities of the hypothalamic-pituitary structures were detected (Supplementary Table 2).

Table 2 Clinical features of patients according to GH secretion impairment.

Clinical feature	GH deficiency ($n = 57$)*	NGHD-ISS patients ($n = 45$)	<i>P</i> -value
Height†			
> -3 SD	37 (64.9%)	37 (82.2%)	0.07
≤ -3 SD	20 (35.1%)	8 (17.8%)	
Sitting height/height ratio†			
Harmonic	51 (89.5%)	33 (73.3%)	0.04
Disharmonic	6 (10.5%)	12 (26.7%)	
Bone-chronological age gap (months)‡	-17.6 ± 13.5	-16.6 ± 15.8	0.60

Bold indicates statistical significance.

*Included one couple of siblings. † n (%). ‡Mean $\pm$ SD.

Table 3 Clinical features of patients with isolated and combined GH deficiency tested by NGS analyses.

Clinical feature	Isolated GH deficiency (n = 42)*	Combined GH deficiency (n = 15)	P-value
GH peak value [†]	n = 40	n = 12	
5–7.9 ng/mL	16 (40%)	2 (16.7%)	0.179
≤5 ng/mL	24 (60%)	10 (83.3%)	
Height [†]			
> –3 SD	27 (64.3%)	10 (66.7%)	1.000
≤ –3 SD	15 (35.7%)	5 (33.3%)	
Sitting height/height ratio [†]			
Harmonic	36 (85.7%)	15 (100%)	0.325
Disharmonic	6 (14.3%)	0 (0%)	
Bone-chronological age gap (months) [‡]	–18.4 ± 13.2	–15.4 ± 14.4	0.435
Brain MRI [†]			
Normal	14 (33.3%)	2 (13.3%)	0.0002
Mildly abnormal	14 (33.3%)	1 (6.7%)	
Pathological	7 (16.7%)	12 (80%)	
Not performed or not available	7 (16.7%)	0 (0%)	

Bold indicates statistical significance.

*Included one couple of siblings. [†]n (%). [‡]Mean ± SD.

Diagnostic yield of NGS analyses

The results of NGS analyses are shown in Table 4. Overall, P/LP variants and ‘hot’ VUS considered associated with the patients’ phenotype were identified in 15 cases (14.9%): eight patients with GH deficiency (14.3%) and seven with NGHD-ISS (15.6%) ($P = 1.000$). These variants and the associated phenotypes are listed in Tables 5 and 6.

Other VUS were more frequently identified among ISS children (62.2%) than GHD patients (53.6%), but this difference did not reach statistical significance ($P = 0.423$).

Regarding the group of patients with GHD, a genetic diagnosis was established in 6/41 (14.6%) cases with isolated GHD and in 2/15 (13.3%) cases with CPHD. Among patients with isolated GHD, variants considered associated with the phenotype were identified in three cases within genes encoding factors involved in GH synthesis and secretion (*GH1* and *GHRHR*); the remaining three cases carried variants in genes implicated in intracellular signalling and paracrine activity (*CREBBP* and *GNAS*), underscoring the diversity of biological mechanisms contributing to SS.

Several auxological parameters and clinical features (including mean height SDS, mean HV SDS, mean BA/chronological age gap, mean GH peak level, and presence of brain anomalies) were comparable

between patients with and without a confirmed genetic diagnosis in both the isolated GHD and CPHD groups (Table 7).

The rate of patients carrying only VUS was much higher in cases with isolated GHD (63.4 vs 26.7%) ($P = 0.017$) (Supplementary Table 3). However, excluding cases with variants considered associated with the patients’ phenotype, the identification of VUS did not correlate with the severity of SS, regardless of their number (Supplementary Table 4).

In the ISS group, a genetic diagnosis was reached in 23.8% of familial cases and in 8.7% of sporadic cases; in approximately 60% of cases, variants were inherited from an affected parent. Clinically relevant variants were detected in a number of genes usually associated with specific genetic syndromes or conditions, including *NPR2* (skeletal dysplasias with dominant or recessive inheritance), *COL2A1* (Stickler syndrome and other skeletal dysplasias), *DVL1* (Robinow syndrome), *PTPN11* (Noonan syndrome), *COMP* (skeletal disorders including multiple epiphyseal dysplasia and pseudoachondroplasia), *IGF1R* (severe growth deficiency due to resistance to insulin-like growth factor I), and *PROKR2* (hypogonadotropic hypogonadism with or without anosmia) (Tables 5 and 6). As observed in isolated GHD and CPHD groups, several auxological parameters (mean height SDS, mean HV SDS, mean BA/chronological age gap) were comparable between patients with and without a confirmed genetic diagnosis (Table 7). The rate of patients carrying only VUS was high in both familial and sporadic cases, though it was higher in patients with sporadic ISS (47.6 vs 73.9%). However, this difference did not reach statistical significance ($P = 0.219$) (Supplementary Table 3). Similarly to the GHD group, the identification of VUS and their number did not correlate with the severity of SS in the ISS group (Supplementary Table 4).

Table 4 Results of NGS analyses (n = 101).

Genetic test	GH deficiency (n = 56)	NGHD-ISS (n = 45)	Total (n = 101)
Short stature gene panel sequencing			
P/LP variants and ‘hot’ VUS	8 (14.3%)	7 (15.6%)	15 (14.9%)
Other VUS	30 (53.6%)	28 (62.2%)	58 (57.4%)
Non-relevant variants	18 (32.1%)	10 (22.2%)	28 (27.7%)

Table 5 Variants considered associated with the patients' phenotype (P/LP variants and 'hot' VUS), detected by SS gene panel sequencing.

Case ID	Phenotype	Gene	Ref. seq	Variant	Zygosity	ACMG criteria	ACMG class	Genes with VUS
1	GHD	GH1	NM_000515.5	Whole gene deletion	Hom	PVS1, PM2, PM3, PP5	P	ENPP1
2	GHD	GHRHR	NM_000823.4	c.880G>A p.(Gly294Arg); c.1146G>A p.(?)	Compound het	PM1, PM2, PP3 PM2, PP3	Hot VUS Hot VUS	
3	GHD	GHRHR	NM_000823.4	c.57 + 1G>A p.(?); c.713C>A p.(Ser238*)	Compound het	PVS1, PM2, PM3, PP5 PVS1, PM2, PM3, PP5	P P	
4	GHD	GNAS	NM_000516.7	c.1174G>A p.(Glu392Lys)	Het, mat	PM1, PM2, PP3, PP4, PP5	LP	GNAS, CUL7, KMT2D CHD7
5	GHD	CREBBP	NM_004380.3	c.778C>T p.(Gln260*)	Het, <i>de novo</i>	PVS1, PS2, PM2	P	
6	GHD	GNAS	NM_000516.7	c.478C>T p.(Arg160Cys)	Het, <i>de novo</i>	PS2, PM1, PM2, PP3, PP5	P	LHX4, ACAN
7	CPHD	LHX3	NM_014564.5	c.-43A>T p.(?); c.641G>C p.(Arg214Pro)	Compound het	PM2, PP4 PM1, PM2, PP3, PP4	Hot VUS LP	
8	CPHD	LHX3	NM_014564.5	c.134A>T p.(Asp45Val); c.194A>G p.(Asp65Gly)	Compound het	PM1, PM2, PP3, PP4 PM1, PM2, PP3, PP4	LP LP	
9	NGHD-ISS	NPR2	NM_003995.4	c.2175C>G (Tyr725*)	Het, mat	PVS1, PM2	P	SHOX, IGF1R
10	NGHD-ISS	COL2A1	NM_001844.5	c.3644G>C p.(Gly1215Ala)	Het, pat	PM1, PM2, PP3	Hot VUS	
11	NGHD-ISS	DVL1	NM_001330311.2	c.1255_1262del p.(Ala419Argfs*113)	Het, <i>de novo</i>	PS2, PM2	LP	
12	NGHD-ISS	COMP	NM_000095.3	c.2161C>T p.(Arg721Cys)	Het, mat	PM1, PM2, PP3	Hot VUS	OBSL1 FGF8
13	NGHD-ISS	IGF1R	NM_000875.5	c.3601G>A p.(Val1201Ile)	Het, <i>de novo</i>	PS2, PM1, PM2	LP	
14	NGHD-ISS	PROKR2	NM_144773.4	c.868C>T p.(Pro290Ser)	Het, pat	PS3, PM1, PM2, PP3	LP	
15	NGHD-ISS	PTPN11	NM_002834.5	c.188A>G p.(Tyr63Cys)	Het, <i>de novo</i>	PS2, PM1, PM2, PP3, PP5	P	

Zygosity: heterozygous (Het), homozygous (Hom), maternal (mat), paternal (pat).

ACMG (American College of Medical Genetics) class: pathogenic variant (P), likely pathogenic variant (LP), variant of uncertain significance (VUS).

Table 6 Phenotype of patients with clinically relevant genetic variants.

Case ID	Mutated gene	Phenotype
1	<i>GH1</i>	Congenital isolated GH deficiency. – 5 SDS. Normal adeno- and neurohypophysis. Radiological evidence of Chiari malformation type 1. Normal SH/H ratio.
2	<i>GHRHR</i>	Congenital isolated GH deficiency. – 3.3 SDS. Normal adeno- and neurohypophysis. Normal SH/H ratio.
3	<i>GHRHR</i>	Congenital isolated GH deficiency. – 4 SDS. Mild hypoplasia of the adenohypophysis. Normal SH/H ratio.
4	<i>GNAS</i>	Isolated GH deficiency diagnosed at the age of 9. – 3.5 SDS. Familial short stature (both mother and father). Normal brain MRI. Regular psychomotor and cognitive development. Slightly increased SH/H ratio.
5	<i>CREBBP</i>	Isolated GH deficiency diagnosed at the age of 8. – 2.2 SDS. Mild hypoplasia of adenohypophysis. Learning difficulties. No congenital malformations. Normal SH/H ratio.
6	<i>GNAS</i>	Isolated GH deficiency diagnosed at the age of 9. – 2.2 SDS. Adenohypophysis size at the lower normal limits. Normal SH/H ratio. Learning difficulties. Normal pubertal development. The patient is also affected by tooth agenesis due to compound heterozygous mutations in <i>WNT10A</i> gene.
7	<i>LHX3</i>	Congenital central hypothyroidism. GH deficiency diagnosed at the age of 2. –2.6 SDS. ACTH deficiency since the age of 8 and hypogonadotropic hypogonadism. Adenohypophysis hypoplasia, thin stalk and neurohypophysis lacking physiological hyperintensity.
8	<i>LHX3</i>	GH deficiency diagnosed at the age of 6. –2.9 SDS. TSH deficiency since the age of 10 and hypogonadotropic hypogonadism. Adenohypophysis hypoplasia, normal stalk and neurohypophysis.
9	<i>NPR2</i>	Familial short stature. Proband's height at –2.6 SDS. Mother carrier of the same genetic variant, with height at –2 SDS. Slightly increased SH/H ratio in both.
10	<i>COL2A1</i>	Familial short stature. Proband's height at –2.2 SDS. Father carrier of the same genetic variant, with height at –2.5 SDS. Normal SH/H ratio in both. No dysmorphic features or skeletal abnormalities.
11	<i>DVL1</i>	Sporadic short stature at –2.7 SDS. Normal SH/H ratio. No congenital malformations. No dysmorphic features or skeletal abnormalities.
12	<i>COMP</i>	Familial short stature. Proband's height at –2.4 SDS. Mother carrier of the same genetic variant, with height at –2.1 SDS. Normal SH/H ratio in both. No skeletal abnormalities.
13	<i>IGF1R</i>	Sporadic short stature at – 3 SDS. Normal SH/H ratio. Occasionally elevated IGF1 values (+3.2 SDS), normal GH secretion. Hypogonadotropic hypogonadism. Absent olfactory bulbs, normal hypothalamus-pituitary region. Mild behavioural difficulties.
14	<i>PROKR2</i>	Sporadic short stature at –3.6 SDS. Normal SH/H ratio. Pubertal delay. Father carrier of the same genetic variant, with normal height (–1.4 SDS) and delayed but spontaneous pubertal development.
15	<i>PTPN11</i>	Sporadic short stature at –2.9 SDS. Normal SH/H ratio. Minor dysmorphic facial features. No cardiovascular anomalies.

Discussion

Phenotypes associated with SS are highly heterogeneous and show wide variability in expressivity, which often leads to misdiagnosis when based solely on clinical criteria. The introduction of NGS into clinical practice has significantly improved the diagnostic accuracy and clinical management of patients with SS in recent decades. NGS-based approaches enabled the simultaneous analysis of multiple genes, facilitating the identification of both novel SS-associated genes and previously unrecognised pathogenic variants. This has significantly contributed to the expansion of the current understanding of the genetic architecture underlying growth disorders.

Several studies published over the past decade have investigated the genetic basis of pathological SS in children using NGS approaches (34, 35, 36, 37, 38). The diagnostic yield of these investigations varies considerably, depending on study design, patient selection, and the specific NGS methodology – ranging from targeted gene panels to whole exome sequencing (WES) or whole genome sequencing. Detection rates of variants considered causative of the patients' short

stature range from 10 to 50%, largely depending on the patient selection criteria and the genetic approach. Higher diagnostic yields are generally observed in patients presenting with additional syndromic features (e.g. dysmorphic traits, developmental delay) or a positive family history of SS or related conditions.

In our cohort of patients with GHD-SS and NGHD-SS of unknown aetiology, we adopted a targeted gene panel approach. The analysis focused on the identification of P/LP variants and 'hot' VUS in genes involved in key biological processes related to growth, acknowledging the complex and heterogeneous genetic architecture of SS (31). A recent study on an Italian cohort of patients with SS similarly employed a gene panel NGS approach and reported a higher diagnostic yield than observed in our study. This difference is likely attributable to their broader inclusion criteria, which encompassed children born SGA and patients with non-specific clinical or skeletal features suggestive of a genetic aetiology (39).

Interestingly, in our patients with GHD-SS, we identified pathogenic variants not only in genes involved in GH synthesis and secretion but also in genes associated with intracellular signalling and paracrine regulation

Table 7 Comparison between patients with and without a confirmed genetic diagnosis.

Clinical feature	Genetic aetiology	Unknown aetiology	P-value
Height SDS (mean)			
CPHD	−2.73	−2.55	0.59
Isolated GHD	−3.30	−2.62	0.22
NGHD-ISS	−2.76	−2.48	0.21
Height velocity SDS (mean)			
CPHD	−1.58	−2.50	0.66
Isolated GHD	−2.38	−1.56	0.10
NGHD-ISS	−1.68	−1.36	0.59
Bone-chronological age gap (months) (mean)			
CPHD	−26.5	−13.7	0.66
Isolated GHD	−17.5	−19.1	0.72
NGHD-ISS	−24.7	−15.0	0.34
Brain MRI anomalies (%)			
CPHD	100%	85%	1.00
Isolated GHD	67%	55.9%	1.00
GH peak value (ng/mL) (mean)			
CPHD	3.08	2.57	0.87
Isolated GHD	3.39	4.37	0.45

(*CREBBP* and *GNAS*), underscoring the diversity of biological mechanisms contributing to SS. These genes may not only directly affect the proliferation and differentiation of growth plate chondrocytes but also interfere with the GH–IGF1 axis. Thus, multiple mechanisms may act simultaneously in the same individual, contributing in a synergistic manner to the development of the pathological phenotype of SS.

These results are consistent with those reported in a recent study by Plachy *et al.* (40), who performed NGS analysis in a cohort of 52 children with isolated GHD. Causative variants were identified in 15 patients (29%), yet only three of these involved genes directly associated with the GH–IGF1 pathway; the majority of the remaining variants were found in genes related to primary growth plate disorders. The authors suggested that the response to GH stimulation tests in patients carrying mutations in growth plate-related genes may have been influenced by factors such as pubertal stage, obesity, or other individual clinical characteristics. Consequently, the SS phenotype observed in the subgroup of GHD patients with variants in genes implicated in primary growth plate disorders may, in certain cases, be GH-independent. However, in individuals rigorously selected to minimise known confounding factors, this phenotype may result from a dual mechanism involving both mild GH deficiency and an intrinsic growth plate dysfunction related to the underlying genetic anomaly.

The percentage of patients with CPHD who tested positive for causative variants in our study was similar to or only slightly higher compared to other recent works (15, 41). Due to the small size of this subgroup of patients, we identified pathogenic variants only in the *LHX3* gene,

which plays important roles as a transcriptional regulator of the embryonic development of the pituitary gland.

Regarding the neuroradiological findings, the higher prevalence of complex brain malformations observed in patients with CPHD compared to those with isolated GHD is consistent with previous reports in the literature (15, 42, 43). This difference reflects the underlying aetiology of the two conditions: CPHD may be associated with congenital brain malformations and/or with pathogenic variants in genes encoding transcription factors that regulate the expression of numerous downstream genes involved in various physiological processes. In contrast, isolated GHD is typically linked to mutations in genes encoding factors specifically involved in the GH–IGF1 axis function.

It is noteworthy that isolated GHD may represent either the initial or the sole clinical manifestation in children carrying variants in genes involved in pituitary development, including those encoding early (e.g. *OTX2*, *HESX1*, *SOX2*, *SOX3*) and late (e.g. *PROP1*, *POU1F1*) transcription factors (44), whose mutations are usually associated with CPHD. In such cases, the presence of brain malformations serves as a predictive marker for the potential evolution towards multiple pituitary hormone deficiencies. In particular, the size and location of the ectopic posterior pituitary gland have been proposed as early radiological indicators of future progression to CPHD (45, 46).

Considering ISS patients, the diagnostic yield across studies published in the literature varied from approximately 10% to 20%. The identified genetic variants predominantly involved genes related to cartilage development through various growth-related processes. The most frequently implicated genes include *SHOX*, *ACAN*, *IGF1R*, and *NPR2*.

The diagnostic yield of NGS analysis in NGHD-ISS patients included in the present study was comparable to that reported in the literature. The relatively low diagnostic yield, especially in sporadic cases, is likely attributable to the multifactorial and polygenic nature of human growth, as well as the numerous epigenetic factors modulating gene expression (36). Moreover, we cannot exclude the possibility that pathogenic variants in genes not included in the selected sequencing panel may also contribute to the phenotype.

Nevertheless, while molecular investigations are now routinely included in the diagnostic work-up of patients with specific clinical conditions – such as SS associated with isolated GHD or CPHD – individuals with non-specific phenotypes such as ISS are those who may benefit most from the availability of novel genetic testing approaches.

In our cohort, the majority of clinically relevant variants were identified in genes involved in intrinsic mechanisms of the growth plate (Table 5) (8). Among these, we identified a novel heterozygous truncating variation in

the *NPR2* gene in both a proband and her mother, whose phenotype was characterised by mild disharmonic ISS (Table 6). In the literature, *NPR2* mutations have been reported in patients with both autosomal recessive and dominant skeletal dysplasias (OMIM # 602875, 615923), as well as in patients with ISS (OMIM # 616255) (47). Regarding the *de novo* *DVL1* frameshift variant we detected, it is noteworthy that nearly all known pathogenic variants associated with Robinow syndrome are located in exon 14; however, in our case, the variant was found in exon 12, suggesting a potential new genotype–phenotype correlation. A heterozygous variant in the *COMP* gene, which we classified as a ‘hot’ VUS, was also found in a patient and her mother, both presenting with ISS (Table 6). Although we were unable to definitively demonstrate its pathogenicity, several factors – including familial segregation, rarity of the variant, its location within a critical functional domain, and supportive computational predictions – suggest a high likelihood of its involvement in the pathogenesis of short stature in this family.

Interestingly, we did not find any P/LP variants in *ACAN*, which is currently considered one of the most frequently mutated genes associated with autosomal dominant ISS, with a variable frequency ranging between 1.4 and 6% (36, 39, 48, 49). The absence of pathogenic variants in this gene is likely due to the relatively small number of patients with ISS tested in our study. Notably, three *ACAN* mutations were detected in our cohort; however, based on their characteristics – such as type of variant, benign *in silico* prediction, and inheritance from parents with normal stature – they could not be considered causative of the patients’ phenotypes. These findings highlight the critical role of segregation analysis in the accurate classification and interpretation of genetic variants.

The identification of gene variants associated with autosomal dominant syndromes both in cases with GHD and ISS raises the question of whether the classification of the patients’ phenotype before genetic testing was sufficiently accurate or could have been further refined. It is important to emphasise that the use of broad gene panels can facilitate diagnosis, especially in affected patients who do not display clinical features suggestive of such genetic conditions. A re-evaluation of the phenotype confirmed the absence of clinical features indicative of syndromic disorders in all patients, with the exception of the child with the *de novo* *PTPN11* mutation, who displayed only minor facial dysmorphisms associated with RASopathies (Table 6). These findings underscore the wide phenotypic spectrum associated with gene variants, which can be misleading even when detailed pre-test phenotyping is performed by experienced endocrinologists and clinical geneticists.

The role of the numerous VUS identified through NGS analysis remains controversial, as also observed in our cohort. While it is tempting to hypothesise that VUS may contribute to modulating the phenotype of patients with

P/LP variants or even cause SS in patients without clearly pathogenic variants, such hypotheses require validation through studies with sufficient statistical power in large cohorts and/or functional *in vitro* or *in vivo* experiments. The analysis of a large number of genes inevitably leads to the identification of an increasing number of VUS, often by chance alone. In our cohort, we observed a high rate of patients carrying at least one VUS, particularly among those with isolated GHD and ISS; however, the presence of VUS did not correlate with the severity of SS, regardless of the number of variants identified. Familial segregation analysis remains crucial for improving the clinical interpretation of VUS, although it is often not sufficient. In the near future, advanced bioinformatic tools may contribute to clarifying the functional relevance of these variants and to identifying specific genotype–phenotype correlations.

A key strength of our study lies first in the careful phenotypic selection and detailed clinical evaluation of a patient cohort comprising both idiopathic GHD and NGHD individuals with SS of unknown aetiology, two groups that are not frequently studied in parallel in the literature. Second, all selected subjects were analysed using the same NGS approach, based on an integrated analytic pipeline designed to detect both small sequence variants and CNVs affecting the coding regions of targeted genes. In addition, the potentially relevant variants were examined in parental DNA to enable proper classification according to the ACMG criteria (32).

Some limitations of our study should also be acknowledged. Small differences among the sub-phenotypes within the cohort may have gone undetected due to the limited sample size, which does not allow for sufficient statistical power. This limitation may partially affect the generalisability of our findings. In addition, the use of a custom-designed gene panel may have reduced the diagnostic yield, as variants in genes not included – yet potentially associated with short stature – would have been missed. This limitation could be addressed by adopting a WES approach. However, WES may result in the identification of a higher number of VUS, particularly in genes whose functions and associated phenotypes are not yet well defined. In addition, WES may also uncover incidental findings.

Comparison with other studies is challenging due to the highly variable inclusion criteria across published cohorts. Furthermore, the classification of variant pathogenicity and causality remains complex, and according to current guidelines, some previously published findings may need to be reinterpreted.

In conclusion, our results achieved the objective of implementing a first-line, custom-designed gene panel sequencing approach for short stature, which demonstrated a good and currently competitive detection rate in a cohort of carefully selected GHD and NGHD patients with SS evaluated in routine clinical practice for growth deficiency without a clear aetiology. Nevertheless, we were unable to identify

specific clinical features that reliably predict a positive genetic test result due to the wide clinical variability, lack of specificity, and genetic heterogeneity of SS. This emphasises the utility of large, yet carefully selected, gene panel analysis in uncovering cases with subtle phenotypic expressions of specific genetic disorders. In addition, our findings highlight the importance of analysing the same extensive gene panel in both GHD-related and idiopathic SS cases, as multiple mechanisms may concurrently contribute to the pathogenesis of the phenotype.

The current literature provides evidence of positive responses to GH treatment in SS patients without GH deficiency but with genetic defects affecting pathways involved in the regulation of the bone growth plate (50, 51, 52, 53). A close collaboration between endocrinologists and geneticists enhances the diagnostic assessment of pathological SS and enables the identification of underlying genetic causes in a larger proportion of patients, who may benefit from GH therapy, leading to improvement in their final height and quality of life.

Supplementary materials

This is linked to the online version of the paper at
<https://doi.org/10.1530/EC-25-0229>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work reported.

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Ethics statement

The study complies with the Helsinki Declaration and has been approved by the local ethics committee of the Central Eastern Veneto region at the University Hospital of Padua with the code 6159/AO/24. Written informed consent was obtained from the parents or legal guardians of all participants before genetic testing, in accordance with institutional and ethical standards.

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