High prevalence of variants in skeletal dysplasia associated genes in individuals with short stature and minor skeletal anomalies

Lucía Sentchordi-Montané[1,2,3,4], Sara Benito-Sanz[1,5,6], Miriam Aza-Carmona[1,4,5], Francisca Díaz-González[1,4], Silvia Modamio-Høybjør[1,4], Carolina de la Torre[1,4], Julián Nevado[1,5,6], Pablo Ruiz-Ocaña[7], Carolina Bezanilla-López[8], Pablo Prieto[9], Pilar Bahíllo-Curieses[10], Atilano Carcavilla[4,11], Inés Mulero-Collantes[12], Ana C Barreda-Bonis[4,11], Jaime Cruz-Rojo[13], Joaquín Ramírez-Fernández[14], José Antonio Bermúdez de la Vega[15], André M Travessa[16], Jesús González de Buitrago Amigo[17], Angela del Pozo[1,5,6], Elena Vallespín[1,5], Mario Solís[1], Carlos Goetz[18], Ángel Campos-Barros[1,5], Fernando Santos-Simarro[1,4,5,6], Isabel González-Casado[4,11], Purificación Ros-Pérez[19], Manuel Parrón-Pajares[4,20] and **Karen E Heath[1,4,5]**

[1]Institute of Medical and Molecular Genetics (INGEMM), IdiPAZ, Hospital Universitario La Paz, UAM, Madrid, Spain, [2]Department of Pediatrics, Hospital Universitario Infanta Leonor, Madrid, Spain, [3]Department of Pediatrics, School of Medicine, Complutense University of Madrid, Madrid, Spain, [4]Skeletal Dysplasia Multidisciplinary Unit (UMDE) and ERN-BOND, Hospital Universitario La Paz, Madrid, Spain, [5]CIBERER, ISCIII, Madrid, Spain, [6]ERN-ITHACA, Hospital Universitario, Hospital La Paz, Madrid, Spain, [7]Department of Pediatrics, Hospital Universitario Puerta del Mar, Cádiz, Spain, [8]Department of Pediatrics, Hospital Universitario Fundación Alcorcón, Madrid, Spain, [9]Department of Pediatrics, Hospital Universitario Clínico Salamanca and Biomedical Research Institute of Salamanca (IBSAL), Salamanca, Spain, [10]Department of Pediatrics, Hospital Clínico Universitario de Valladolid, Valladolid, Spain, [11]Department of Pediatric Endocrinology, Hospital Universitario La Paz, Madrid, Spain, [12]Department of Pediatrics, Hospital Universitario Río Hortega, Valladolid, Spain, [13]Department of Pediatric Endocrinology, Hospital Universitario 12 de Octubre, Madrid, Spain, [14]Department of Pediatrics, Hospital Universitario Príncipe de Asturias, Alcalá de Henares, Madrid, Spain, [15]Department of Pediatrics, Hospital Universitario Virgen de la Macarena, Sevilla, Spain, [16]Medical Genetics Service, Department of Pediatrics, Hospital de Santa Maria and Faculty of Medicine, University of Lisbon, Lisbon, Portugal, [17]Department of Pediatrics, Hospital Universitario San Pedro de Alcántara, Cáceres, Spain, [18]PeRTICA SAS Consultant, Madrid, Spain, [19]Department of Pediatrics, Hospital Universitario Puerta de Hierro, Majadahonda, Madrid, Spain, and [20]Department of Radiology, Hospital Universitario La Paz, Madrid, Spain

Correspondence should be addressed to K E Heath
Email karen.heath@salud.madrid.org

European Journal of Endocrinology

Abstract

Objective: Next generation sequencing (NGS) has expanded the diagnostic paradigm turning the focus to the growth plate. The aim of the study was to determine the prevalence of variants in genes implicated in skeletal dysplasias in probands with short stature and mild skeletal anomalies.

Design: Clinical and radiological data were collected from 108 probands with short stature and mild skeletal anomalies.

Methods: A customized skeletal dysplasia NGS panel was performed. Variants were classified using ACMG recommendations and Sherloc. Anthropometric measurements and skeletal anomalies were subsequently compared in those with or without an identified genetic defect.

Results: Heterozygous variants were identified in 21/108 probands (19.4%). Variants were most frequently identified in *ACAN* (n = 10) and *IHH* (n = 7) whilst one variant was detected in *COL2A1*, *CREBBP*, *EXT1*, and *PTPN11*. Statistically significant differences ($P < 0.05$) were observed for sitting height/height (SH/H) ratio, SH/H ratio standard deviation score (SDS), and the SH/H ratio SDS >1 in those with an identified variant compared to those without.

Conclusions: A molecular defect was elucidated in a fifth of patients. Thus, the prevalence of mild forms of skeletal dysplasias is relatively high in individuals with short stature and mild skeletal anomalies, with variants in *ACAN* and *IHH* accounting for 81% of the cases. An elevated SH/H ratio appears to be associated with a greater probability in detecting a variant, but no other clinical or radiological feature has been found determinant to finding a genetic cause. Currently, we cannot perform extensive molecular studies in all short stature individuals so detailed clinical and radiological phenotyping may orientate which are the candidate patients to obtain worthwhile results. In addition, detailed phenotyping of probands and family members will often aid variant classification.

European Journal of Endocrinology (2021) **185**, 691–705

https://eje.bioscientifica.com
https://doi.org/10.1530/EJE-21-0557

Published by Bioscientifica Ltd.

Introduction

Short stature, defined as height below −2 standard deviation score (SDS) for sex, age, and ethnicity, is a paramount problem for clinicians but also to the parents and the patient themselves. It is one of the most common reasons for referral to a pediatric endocrinologist. The high prevalence of short stature of unknown etiology has also driven scientists and clinicians to investigate its origin and pathogenic mechanisms. Initially, the research concentrated on the somatotrophic axis with growth hormone (GH) playing a critical role in human growth, primarily through its regulation of insulin growth factor I (IGF-I) production. Genetic disorders have been identified throughout the GH/IGF-I axis, ranging from growth hormone deficiency, either in isolation or as part of combined pituitary hormone deficiency, to primary IGF-I deficiency, IGF-I resistance, or dysregulation of IGF-I availability. However, genetic defects are only found in a small proportion (<10%) of cases (1, 2).

Since the first description, in 1997, of the implication of *SHOX* in idiopathic short stature (ISS) (3), extensive research has been performed. *SHOX* is located in the pseudoautosomal region 1 (PAR1) of the sexual chromosomes, thus, all normal stature males and females have two active copies. *SHOX* encodes a transcription factor which regulates growth in the growth plate (4). Heterozygous defects in *SHOX* and/or its enhancers have been identified in a small proportion, approximately 2.5%, of individuals with ISS (5), and in a significantly higher proportion, approximately 70%, in Léri-Weill dyschondrosteosis (LWD) (6). LWD is a skeletal dysplasia characterized by short stature, mesomelic shortening of the limbs, and the classic Madelung deformity of the wrist. Clinical heterogeneity is high and the skeletal defects often become more evident during puberty and, thus, can be missed in young children (7). *SHOX* genetic testing in our laboratory has resulted in the identification of a *SHOX* or enhancer defect in approximately 16% of all *SHOX* testing referrals (n= 7300 since 2008, unpublished data). Thus, *SHOX* alterations are the most common genetic defect in short stature.

During the last decade, the implementation of next generation sequencing (NGS) has expanded the diagnostic paradigm, simultaneously permitting the analysis of a large number of genes or the entire exome or genome. The detection of genetic variants in genes expressed in the growth plate in ISS patients has encouraged us to search for skeletal traits (8, 9, 10). Mild or minor skeletal traits are often overlooked during the initial evaluation of short stature patients and skeletal surveys do not form part of routine clinical workups. Yet, the increasing rate of heterozygous variants identification in various skeletal dysplasia genes, such as *FGFR3*, *NPR2* and more recently, *ACAN* and *IHH* (9, 10, 11, 12, 13, 14, 15, 16), in children with disproportionate or proportionate short stature with or without mild skeletal abnormalities, has significantly contributed to widen the phenotypic spectrum of many skeletal dysplasias.

Although many monogenic causes of growth disorders have been identified, the diagnostic yield remains low and we continue to investigate the genetic contribution in short stature. In this study, we set out firstly, to determine the prevalence of variants in skeletal dysplasia genes in a cohort of pediatric probands with short stature and mild skeletal abnormalities, and to subsequently evaluate which clinical and radiological variables increase the probability of identifying the underlying genetic defect.

Subjects and methods

Study subjects

All participants or parents provided informed consent for the performed studies and ethical approval was obtained from the Hospital Universitario La Paz ethical committee.

The study included children less than 18 years old, who all met two principal criteria: short stature of unknown etiology (defined as height ≤ −2 SDS for sex, age, and ethnic group) and the presence of a skeletal anomaly defined as the occurrence of at least one of the following: body disproportion, mild skeletal anomaly, or a short stature parent with body disproportion or mild skeletal anomaly. Patients with several dysmorphic features or major malformations, indicative of a syndrome were excluded from this study. 'Minor malformations' refers to unusual morphologic features found in the general population causing no serious medical or cosmetic significance to the affected individual (17). Following this concept, we define mild skeletal defects as unusual skeletal features both unpainful and non-disabling for the affected individual (see list in Supplementary Table 1, see section on supplementary materials given at the end of this article).

The children were recruited using an extensive questionnaire (Supplementary data) which pediatric endocrinologists and clinical geneticists from various Spanish and Portuguese hospitals were asked to complete. All cases were subsequently reviewed by the same pediatric endocrinologist, L.S.-M., and pediatric radiologist, M.P.-P.,

both experienced in skeletal dysplasia evaluations. A total of 108 unrelated probands were selected for the study. Clinical (medical records, physical examination, and anthropometric values), radiological (bone age and skeletal survey), and family data (height and skeletal traits of both parents) were then collected from all selected probands. Anthropometric measurements were made by the referring clinician. Height SDS and parental height SDS were calculated according to Spanish reference data 2010, as well as small for gestational age (SGA), defined as birth weight and/or height below −2 SDS (18). Sitting height/height (SH/H) ratio SDS were estimated according to Fredriks charts (19). Arm span/height (A/H) ratio SDS were calculated according to Maastricht standards (20). Bone age was determined by Greulich and Pyle method (21). Body disproportion was considered as having at least an A/H ratio ≤ 0.96 and/or SH/H ≥ 0.55.

Systemic and endocrine disorders including somatotropic axis related conditions were excluded by laboratory tests including hemogram, biochemistry including bone markers, IGF-I, IGFBP-3, FT4, TSH, and celiac disease screening. Karyotyping was also performed in girls. All participants had also been previously excluded for *SHOX* alterations using multiplex ligation probe amplification (MLPA P018G2, MRC Holland) and high-resolution melting (HRM) and/or Sanger sequencing.

Genetic analysis

Blood samples were obtained from the probands and analyzed using a custom designed skeletal dysplasia NGS panel, SKELETALSEQ.V4-8 (n= 327–416 genes, gene lists are available upon request). All coding exons, intron: exon junctions including ±10 bp) were extrapolated to the variant calling file (VCF) whilst the binary alignment map (BAM) files were manually assessed for variants within approximately 100 bp of intronic sequence from the intron:exon boundaries for the following genes: *ACAN, IHH, NPR2, FGFR3, COL2A1,* and *PTPN11*. The identified variants were assessed for amino acid conservation, *in silico* pathogenicity prediction analysis: CADD V1.4 (http://cadd.gs.washington.edu/), SIFT, Polyphen, MutationTaster, various splicing programmes available in Alamut V2.14 (Interactive Biosoftware, France); and allelic frequencies in gnomAD (https://gnomad.broadinstitute.org/). Copy number variant (CNV) analysis was performed using in house software, LACONv tool release 0.0., developed for calculating dosage for each exon present in targeted gene panels, after GC sequence content had been corrected. The dosage for each captured region of the test sample is compared against all other samples in the same panel run and P-values were calculated using Mann–Whitney U tests. CNVs were considered significant with $P > 0.05$.

All detected variants were subsequently validated by Sanger sequencing. The identified *IHH* deletion was confirmed by a single nucleotide polymorphism (SNP) array (Infinium CytoSNP-850K v1.2 BeadChip, Illumina) but due to low probe density in this region, we further characterized the deletion using a custom-designed *IHH* MLPA (10). Family testing was performed, where possible. Kinship was confirmed using microsatellite marker analysis (Devyser Complete QF-PCR, Stockholm, Sweden). Variants were classified according to American College of Medical Genetics and Genomics (ACMG) guidelines (22) and Sherloc variant classification (23).

Statistical analysis

Clinical, radiological, and familiar features were analyzed for the total cohort and subdivided into two groups depending on the presence or absence of a genetic defect.

Statistical analyzes were performed using SAS software V9.4 (SAS Institute Inc., Cary NC). Quantitative values are expressed as means, medians (non-Gaussian distribution), standard deviations (SD), and ranges (min–max). Categorical variables were used to describe absolute frequencies and percentages. Comparisons between groups were performed by Student's t-tests and Fisher's exact tests for quantitative variables whilst chi-square tests were used for qualitative variables. Results were considered statistically significant with a two-sided significance level of $P < 0.05$.

For identifying which clinical or anthropometric indicators could predict the presence and the identification of a genetic variant, logistic multi-regression analyzes were performed with varied sets of explanatory variables, applying full models and stepwise selection models.

Results

A total of 108 probands (61 female, 47 male) were included in the study. Mean proband age was 8.59 years (range: 1.5–18), mean height SDS was −2.97 (range: −4.6 to −2.0), and the mean parental height was −1.69 SDS (range: −3.6 to +1.41). An overview of clinical, radiological and familial features, and frequencies of the total cohort and the sub-groups (variant vs no identified variant) are shown in Table 1.

A total of 20 heterozygous variants, classified as pathogenic, likely pathogenic or variants of unknown

Table 1 Summary of the main clinical and radiological features of the 108 probands. Parents height and skeletal anomalies are presented below.

Clinical characteristics	**Total cohort** (*n* = 108)		**Variant** (*n* = 21)		**No variant** (*n* = 87)		***P*-value**
	n (%) or mean ± S.D.	Median (range)	*n* (%) or mean ± S.D.	Median (range)	*n* (%) or mean ± S.D.	Median (range)	
Sex							0.294
Female	61 (56.5)		14 (66.7)		47 (54)		
Male	47 (43.5)		7 (33.3)		40 (46)		
Age (y)	8.59 ± 3.76	8.00 (1.5 to 18)	8.08 ± 4.15	7.00 (1.5 to 16)	8.71 ± 3.67	8.00 (2 to 18)	0.414
Height SDS	−2.97 ± 0.59	−2.99 (−4.6 to −2)	−3.02 ± 0.61	−3.10 (−4 to −2.1)	−2.96 ± 0.59	−2.98 (−4.6 to −2)	0.557
Parental height SDS	−1.69 ± 1	−1.82 (−3.6 to 1.41)	−1.80 ± 1.15	−1.92 (−3.6 to 1.41)	−1.67 ± 0.97	−1.79 (−3.54 to 0.53)	0.484
SH/H	0.54 ± 0.03	0.54 (0.45 - 0.60)	0.56 ± 0.02	0.55 (0.52 - 0.60)	0.54 ± 0.03	0.540 (0.580 - 0.450)	**0.021**
SH/H SDS	0.32 ± 0.99	0.35 (−3 to 2.3)	0.81 ± 0.56	0.87 (−0.23 to 1.74)	0.21 ± 1.03	0.27 (−3 to 2.3)	**0.016**
SH/H SDS ≥ 1	19 (26.2)		6 (60)		13 (20)		**0.015**
A/H	0.97 ± 0.04	0.97 (0.90 to 1.06)	0.97 ± 0.03	0.98 (0.90 to 0.99)	0.98 ± 0.04	0.970 (0.90 to 1.06)	0.348
A/H SDS	−0.74 ± 1.77	−0.7 (−4.37 to 4.60)	−0.95 ± 1.97	−0.21 (−4.37 to 1.41)	−0.7 ± 1.75	−0.88 (−4.28 to −4.60)	0.644
A/H SDS ≤ −1	36 (54.3)		5 (33.3)		31 (47.6)		0.318
Body disproportion†	46 (52.2)		10 (62.5)		36 (50)		0.365
Bone age (y)*	−0.79 ± 1.57	0 (−4 to 3)	−0.36±1.38	0 (−3 to 2)	−0.89 ± 1.61	−1.5 (−4 to 3)	0.139
SGA	29 (28.4)		8 (38)		21 (25.9)		0.271
Skeletal defects							
Abnormal skeletal survey	83 (78.3)		18 (85.7)		65 (65.85)		0.357
Brachydactyly	70 (67.3)		17 (80.9)		53 (63.8)		0.194
Spine	10 (9.6)		3 (13.6)		7 (8.5)		0.418
Hip	23 (22.3)		4 (19)		19 (23.1)		0.775
Knee	12 (11.6)		4 (19)		8 (9.7)		0.259
Other	21 (19.8)		4 (19)		17 (20)		1.000
Dysmorphic features	31 (29)		6 (28.5)		25 (29)		0.960
Microcephaly	12 (12.6)		4 (19)		8 (9.3)		0.246
Minor malformations	9 (9.1)		1 (4.7)		8 (9.3)		0.685
Father's height SDS	−1.70 ±0.31	−1.68 (−4.88 to 1.34)	−2.17 ± 1.44	−2.26 (−4.50 to 1.06)	−1.58 ± 1.26	−1.36 (−4.88 to 1.34)	0.063
Mother's height SDS	−1.75 ± 1.28	−1.96 (−4.8 to 1.44)	−1.45 ± 1.43	−1.18 (−4.2 to 1.44)	−1.82 ± 1.24	−1.86 (−4.80 to 1.16)	0.241
Father's skeletal anomalies	20 (21.5)		10 (50)		10 (13.6)		**0.0005**
Mother's skeletal anomalies	33 (33)		5 (25)		28 (35)		0.440

P-values < 0.05 are in bold. *Bone age (years) related to chronological age; †SH/H ≥0.55 and/or A/H ≤0.96.

significance (VUS), were identified in 21/108 probands (19.4%) (Fig. 1A and Tables 2, 3). Variants were most frequently identified not only in *ACAN* (n= 10), followed by *IHH* (n= 7) (Fig. 1B) but also in four other genes, *CREBBP*, *COL2A1*, *EXT1*, and *PTPN11*. A total of 11/20 (55%) variants were classified as pathogenic or likely pathogenic (35% and 20%, respectively) whilst 9/20 (45%) were classified as VUS. Heterozygous variants were inherited in 17/20 probands (80.9%) whilst *de novo* events were likely to have occurred in 3/20 probands (14.2%), although germline mosaicism cannot be entirely excluded. Inheritance pattern could not be determined in proband 21 as the paternal sample was unavailable.

Clinical and molecular features of all probands with a pathogenic/likely pathogenic/VUS are shown respectively in Tables 2 and 3. The most characteristic radiographic traits of probands with *ACAN* and *IHH* variants are shown in Fig. 2. Pedigrees and radiographs from all probands with identified variants are shown in Supplementary Figs 1, 2, 3, 4, 5, 6, 7 and 8, respectively.

Statistically significant values were observed between those with a genetic variant and SH/H ratios, SH/H ratio SDS, SH/H ratio SDS >1, and father's skeletal traits (Table 1). Logistic regression analysis revealed a high concordance index (c) for SH/H ratio (c = 0.697) and SH/H ratio SDS (c = 0.706) and a moderate concordance index for father's skeletal traits (c = 0.682) and father's height (c = 0.631).

Discussion

A pathogenic, likely pathogenic, or VUS was identified in a skeletal dysplasia associated gene in 19.4% of the cohort of 108 probands with short stature and mild skeletal anomalies. The majority of the variants were identified in two genes, *ACAN* and *IHH.* Although initial observations suggested that *ACAN* variants were associated with short stature and advanced bone age, more recent work showed that they also occur in children with appropriate or delayed bone age with respect to chronological age (8, 9). In the 10 probands with *ACAN* variants in this cohort, 2 had advanced bone age, 6 had a bone age equal to their chronological age and two had delayed bone age. Both mild skeletal anomalies and/or dysmorphic facial features may occur, with brachydactyly being the most frequent finding in previous studies (9) and this was indeed true for all 10 probands with *ACAN* variants in this cohort. In individuals with *IHH* variants, brachydactyly was the only skeletal anomaly observed in addition to the short stature, as observed previously (10).

A total of 11/20 variants (55%) were classified as pathogenic or likely pathogenic, however, 9/20 (45%) were classified as VUS, 7 of these in *ACAN* and 2 in *IHH*. As with many genetic disorders, upgrading the classification of these variants is difficult for various reasons: 1) Performing functional studies is not often possible and indeed, to date, very few functional studies have been performed for variants in these genes (24); 2) Genotype: phenotype cosegregation studies are often hampered as assortative mating occurs in short stature; 3) gnomAD data are not stratified for height and these mild skeletal phenotypes remain undisclosed. As a consequence, many of these gene variants including clearly pathogenic variants are included in both the general and control data.

Single variants were also found in *COL2A1*, *CREBBP*, *EXT1*, and *PTPN11*. Proband 11 had a pathogenic *de novo* *COL2A1* variant, NM_001844.4:c.2059G>A; p.(Gly687Ser), located in a glycine rich repeat of the triple helix of the alpha-1(II) chains of COL2A1. The girl presented with a slightly shortened trunk and mild kyphosis correlating with a type 2A collagenopathy. *COL2A1* variants have been observed previously in children with short stature without the expected skeletal features (25, 26, 27). Proband 12 was found to have a pathogenic *de novo* variant in *CREBBP,* NM_004380.2:c.6324C>G; p.(Tyr2108*). The read depth of the variant was 107, 60 wild type: 47 variant, thus, somatic mosaicism was not considered but no other tissue was tested. Mild skeletal and dysmorphic features, many of which do occur in Rubinstein-Taybi syndrome (MIM 180849) were present in the young girl, but her intellectual development was normal. Atypical Rubinstein-Taybi syndrome and variable expression have also been previously reported (28, 29, 30) and somatic mosaicism has been reported, although rare (31). Thus, both cases have indeed subtle and non-specific radiological signs which maybe attributable to their early age or mild forms of the dysplasia.

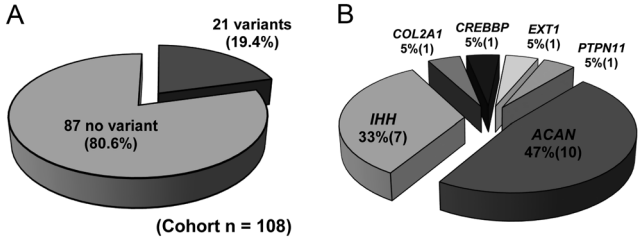

Figure 1

Summary of genetic findings in the cohort of 108 probands (n = 108).

Table 2 Clinical, radiological, and genetic features of the 21 probands with an identified variant.

Proband	Gene	Variant	ACMG clasification and Sherloc	Inheritance (PAT/MAT)	Sex (M/F)	Age (years)	Height SDS	BA: CA	SGA	Mid-parental height SDS (father/mother's height SDS)	SH/H (SH/H SDS) A/H (A/H SDS)	Skeletal survey observations	Dysmorphic features
1	*ACAN*	c.371G>A p.(Arg124His)	VUS	AD (PAT)	F	8	−3.7	−2.0	No	−2.5 (−2.7/−2.2)	0.547 (0.57) 0.99 (0.23)	Brachydactyly, hiperlordosis, and coxa valga	Broad nose and filtrum, thin lips, and high arched palate
2	*ACAN*	c.903G>C p.(Trp301Cys)	VUS	AD (PAT)	F	7	−3.5	0	Yes	−3.6 (−4.4/−2.8)	NA	Brachydactyly, slender femora, coxa valga, and mild osteochondral knee defects	Frontal bossing, midface hypoplasia, and depressed nasal bridge
3	*ACAN*	c.1608C>A p.(Tyr536*)	P	AD (PAT)	F	4.5	−3.5	0	No	−3 (−4.5/−1.1)	0.603 (1) 0.98 (0.71)	Brachydactyly and mild osteochondral knee defects	No
4	*ACAN*	c.1608C>A p.(Tyr536*)	P	AD (PAT)	F	2.5	−3.0	1.5	No	−2.2 (−3.7/−0.7)	0.553 (0.88) 0.98 (1.4)	Brachydactyly	No
5	*ACAN*	c.1930G>A p.(Gly644Ser)	VUS	AD (PAT)	F	16	−2.1	0	No	−2 (−3.7/0.1)	0.57 (1.74) 0.93 (−3.5)	Brachydactyly, Madelung deformity, short femoral necks, and mild epiphyseal knee defects	No
6	*ACAN*	c.1948G >A p.(Val650Met)	VUS	AD (PAT)	M	12	−2.6	−3.0	No	−1.4 (−2.3/−0.7)	0.544 (1.24) 0.96 (−2.1)	Brachydactyly	Depressed nasal bridge, thin lips, and epicantus
7	*ACAN*	c.2218A>T p.(Thr740Ser)	VUS	AD (MAT)	M	4	−3.2	0	Yes	−2.2 (−0.5/−3.7)	0.543 (−0.23†) 0.98 (−0.6†)	Brachydactyly	Frontal bossing, mid-face hypoplasia, high-arched palate, and triangular face
8	*ACAN*	c.2369C>G p.(Ser790*)	P	DE NOVO	M	14.5	−2.2	2.0	No	−0.6 (−1.7/0.4)	0.520 (0.42†) NA	Brachydactyly	Broad nose and filtrum, and hypertelorism
9	*ACAN*	c.6142C>G p.(Pro2048Ala)	VUS	AD (PAT)	F	12.5	−2.2	0	Yes	−2.1 (−2.1/−2.1)	NA	Syndactyly, polydactyly, and brachydactyly	No

10	*ACAN*	c.7276G>A p.(Glu2426Lys)	VUS	AD (PAT)	F	8.5	−2.5	0	Yes	−1.8 (−1.1/−2)	NA 0.966 (−1.5)	Brachydactyly, hyperlordosis, short femoral neck, and cone-shaped epiphysis	Frontal bossing and depressed nasal bridge
11	*COL2A1*	c.2059G>A p.(Gly687Ser)	P	DE NOVO	F	7	−3.8	0	No	−0.3 (0/−0.9)	0.549 (0.44) NA	Mild kyphosis and short trunk	Arched palate
12	*CREBBP*	c.6324C>G p.(Tyr2108*)	P	DE NOVO	F	7	−2.5	−2.0	Yes	−0.8 (−0.8/−0.8)	NA	Brachydactyly, broad thumbs, coxa valga, and genu valgo	Frontal bossing, synophrys, hyperlaxity, mild dysmorphia, hypertrichosis, and dental anomalies
13	*EXT1*	c.608A>G p.(Tyr203Cys)	LP	AD (PAT)	F	10.5	−2.5	ND	No	NA (−2.2/NA)	NA	None **	No
14	*IHH*	c.482_510del p.(Asn161Serfs*6)	LP	AD (PAT)	M	1.5	−3.8	0	Yes	−1.2 (−2.1/0)	NA	5th finger clinodactyly	No
15	*IHH*	c.797dupC p.(Arg267Thrfs*15)	P	AD (PAT)	F	8	−3.1	0	No	−2.6 (−3.3/−1.7)	0.555 (0.85) NA	Brachydactyly	No
16	*IHH*	c.823C>A p.(His275Asn)	VUS	AD (MAT)	F	7	−2.7	1.5	No	−1.6 (−2/−1.2)	0.56 (1) 0.979 (−0.06)	Brachydactyly	No
17	*IHH*	c.887_890del p.(Ser296Thrfs*68)	LP	AD (MAT)	M	5.5	−3.3	0	Yes	−2.4 (−1.6/−3.4)	NA 0.897 (−4.37)	Brachydactyly	No
18	*IHH*	c.892G>A p.(Val298Met)	LP	AD (MAT)	M	16	−2.3	0	No	−1.4 (−0.8/−2.1)	0.557 (1.66) 0.93 (−3.99)	Brachydactyly	No
19	*IHH*	c.1202T>C p.(Phe401Ser)	VUS	AD (PAT***)	M	8	−4.0	−3.0	No	−3.5 (−3/−4.2)	0.545 (0.5) 0.99 (−0.21)	Brachydactyly	No
20	*IHH*	Complete deletion	P	AD (PAT)	F	5.7	−3.2	0	No	−1.8 (−3.1/−0.5)	0.58 (1.21) 0.99 (0.8)	Short 5th metacarpal, short 4th and 5th metatarsals Brachytelephalangia	No
21	*PTPN11*	c.794G>A p.(Arg265Gln)	P	ND	F	4	−3.5	0	Yes	1.4 (1.06/1.44)	0.556 (0.08) 0.98 (0.87)	None	No

*Transcripts: *ACAN* NM_013227.3; *COL2A1* NM_001844.4; *CREBBP* NM_004380.2; *IHH* NM_002181.3; *PTPN11* NM_002834.3. **Patient 13's father showed osteochondromas in both upper and lower limbs which had resulted in pain-free deformities. Variant classification was according to ACMG recommendations and Sherloc variant classification (22, 23). Inheritance: AD, autosomal dominant; PAT, paternally inherited; MAT, maternally inherited; AD PAT*** (predicted inheritance, two siblings with the genetic variant and the mother tested normal); †values in parentheses is S.D.; DE NOVO, de novo inheritance; ND, not determined due to lack of parental DNA samples. Sex: M, male; F, female; BA:CA: bone age related to chronological age according to Greulich and Pyle method (21). SGA, small for gestational age. SH/H, sitting Height/Height ratio. SH/H SDS, sitting height/Height ratio SDS according to Fredriks (19). Arm span/Height SDS (Arm span divided by height of the Maastricht reference population (20); Not available data is represented with NA.

Table 3 Genetic evaluation of the 20 heterozygous variants identified in the 21 probands in the cohort.

Proband	Gene	Heterozygous variant	Variant type	**Inheritance** (number of tested affected members)	**gnomAD ALL MAF**	**Insilico analysis CADD V1.4/SIFT/Polyphen/ MutationTaster**	**ACMG variant classification and Sherloc variant criteria**
1	*ACAN*	NM_013227.3:c.371G>A p.(Arg124His)	Missense	AD PAT (1)	0.0000201	24.1/Del/PbD/Dis	VUS (PP3)
2	*ACAN*	NM_013227.3:c.903G>C p.(Trp301Cys)	Missense	AD PAT (2)	Absent	33/Del/PbD/Dis	VUS (PM2 and PP3)
3, 4[†]	*ACAN*	NM_013227.3:c.1608C>A p.(Tyr536*)	Nonsense	AD PAT (1) AD PAT (1)	Absent	42/–/–/–	Pathogenic (PVS1, PM2, and PP3)
5	*ACAN*	NM_013227.3:c.1930G>A p.(Gly644Ser)	Missense	AD PAT (1)	0.0000686	26.1/Del/PbD/Dis	VUS (PP3)
6	*ACAN*	NM_013227.3:c.1948G>A p.(Val650Met)	Missense	AD PAT (1)	0.0001617	28.2/Del/PbD/Dis	VUS (PP3)
7	*ACAN*	NM_013227.3:c.2218A>T p.(Thr740Ser)	Missense	AD MAT (1)	0.0000397	23.9/Del/PbD/Dis	VUS (PP3)
8	*ACAN*	NM_013227.3:c.2369C>G p.(Ser790*)	Nonsense	DE NOVO	Absent	35/–/–/–	Pathogenic (PVS1, PS2, PM2, and PP3)
9	*ACAN*	NM_013227.3:c.6142C>G p.(Pro2048Ala)	Missense	AD PAT (1)	0.0001284	17.4/Del/PbD/Poly	VUS (PP3)
10	*ACAN*	NM_013227.3:c.7276G>A p.(Glu2426Lys)	Missense	AD PAT (1)	0.0001426	33/Del/PbD/Dis	VUS (PP3)
11	*COL2A1*	NM_001844.4:c.2059G>A p.(Gly687Ser)	Missense	DE NOVO	Absent	32/Del/PbD/Dis	Pathogenic (PS2, PM1, PM2, PM5, PP3, and PP4)
12	*CREBBP*	NM_004380.2:c.6324C>G p.(Tyr2108*)	Nonsense	DE NOVO	Absent	38/–/–/–	Pathogenic (PVS1, PS2, PM2, and PP3)
13	*EXT1*	NM_002181.3:c.608A>G p.(Tyr203Cys)	Missense	AD PAT (1)	Absent	32/Del/PbD/Dis	Likely pathogenic (PM1, PM2, PP3, and PP5)
14	*IHH*	NM_002181.3:c.482_510del p.(Asn161Serfs*6)	Frameshift	AD PAT (2)	Absent	–/–/–/–	Likely pathogenic (PVS1 and PM2)
15	*IHH*	NM_002181.3:c.797dup p.(Arg267Thrfs*15)	Frameshift	AD PAT (1)	Absent	–/–/–/–	Pathogenic (PVS1 and PM2)
16	*IHH*	NM_002181.3:c.823C>A p.(His275Asn)	Missense	AD MAT (1)	Absent	25.8/Del/PbD/Dis	VUS (PM2, PP1, PP2, and PP3)
17	*IHH*	NM_002181.3:c.887_890del p.(Ser296Thrfs*68)	Frameshift	AD MAT (1)	Absent	–/–/–/–	Likely pathogenic (PVS1and PM2)
18	*IHH*	NM_002181.3:c.892G>A p.(Val298Met)	Missense	AD MAT (4)	0.0000243	33/Del/PbD/Dis	Likely pathogenic (PM2, PP1, PP2, PP3, and PP4)
19	*IHH*	NM_002181.3:c.1202T>C p.(Phe401Ser)	Missense	AD PAT* (1)	Absent	29.2/Tol/PbD/Dis	VUS (PM2 and PP3)
20	*IHH*	Complete deletion	Deletion	AD PAT (1)	Absent	–/–/–/–	Pathogenic (PVS1, PS2, and PM2)
21	*PTPN11*	NM_002834.3.c.794G>A p.(Arg265Gln)	Missense	NA	0.0000324	32/Del/PbD/Dis	Pathogenic (PS3, PS4, PM1, PP2, PP3, and PP5)

[†]Although probands 3 and 4 share the same variant, they belong to different families – the variant was inherited from the father in both cases (AD PAT). Variants were classified according to ACMG classification criteria and Sherloc variant classification (22, 23).

AD, autosomal dominant; MAT, maternal; PAT, paternal; NA, not available; AD PAT* (predicted inheritance, two siblings with the genetic variant and the mother tested normal); SIFT: Del, deleterious; Polyphen: PbD, probably damaging; MutationTaster: Dis, disease-causing, Poly, polymorphism.

Proband 13 carries a likely pathogenic variant in *EXT1*. No osteochondromas or abnormal skeletal findings were detected in the female proband; nonetheless, her father was reported to have supposedly Madelung deformity. We subsequently requested to review his radiographs, where we indeed observed osteochondromas in both upper and lower limbs, which had resulted in pain-free deformities. Proband 21 had mildly disproportionate short stature and microcephaly. A pathogenic *PTPN11* variant, p.(Arg265Gln) was identified, which has been shown to increase the protein tyrosine phosphatase catalytic activity (31). This variant has been found in children with a relatively mild form of Noonan syndrome, characterized by a low prevalence of cardiac defects, and cognitive and behavioral issues, as well as less evident typical facial features (32). The unaffected mother tested negative for the variant. Unfortunately, the father was not available for testing to determine if it was *de novo* or inherited but, he had normal height (+1 SDS) and no Noonan-related clinical features. After the diagnosis, a cardiac ultrasound and an audiological examination were performed, both of which were normal. These cases illustrate how phenotypes of well-known conditions can be atypical or mild and how NGS resolved the molecular cause.

Surprisingly no pathogenic or likely pathogenic or VUS were identified in *NPR2* or *FGFR3* in this cohort. A *NPR2* variant, NM_003995.3:c.2644G>A p.(Val882Ile), initially classified as a VUS was identified in one proband, but after performing a three generation cosegregation analysis, the variant was reclassified as likely benign. Thus, it is important to perform either extensive cosegregation studies or functional studies. Children with hypochondroplasia may either have a more defined phenotype, not compliance with the inclusion criteria defined for this study, and/or have been previously screened for the *FGFR3* hotspot regions by direct sequencing, available in many laboratories. The true prevalence of heterozygous *NPR2* and *FGFR3* variants is unknown. Other studies similar to this one but

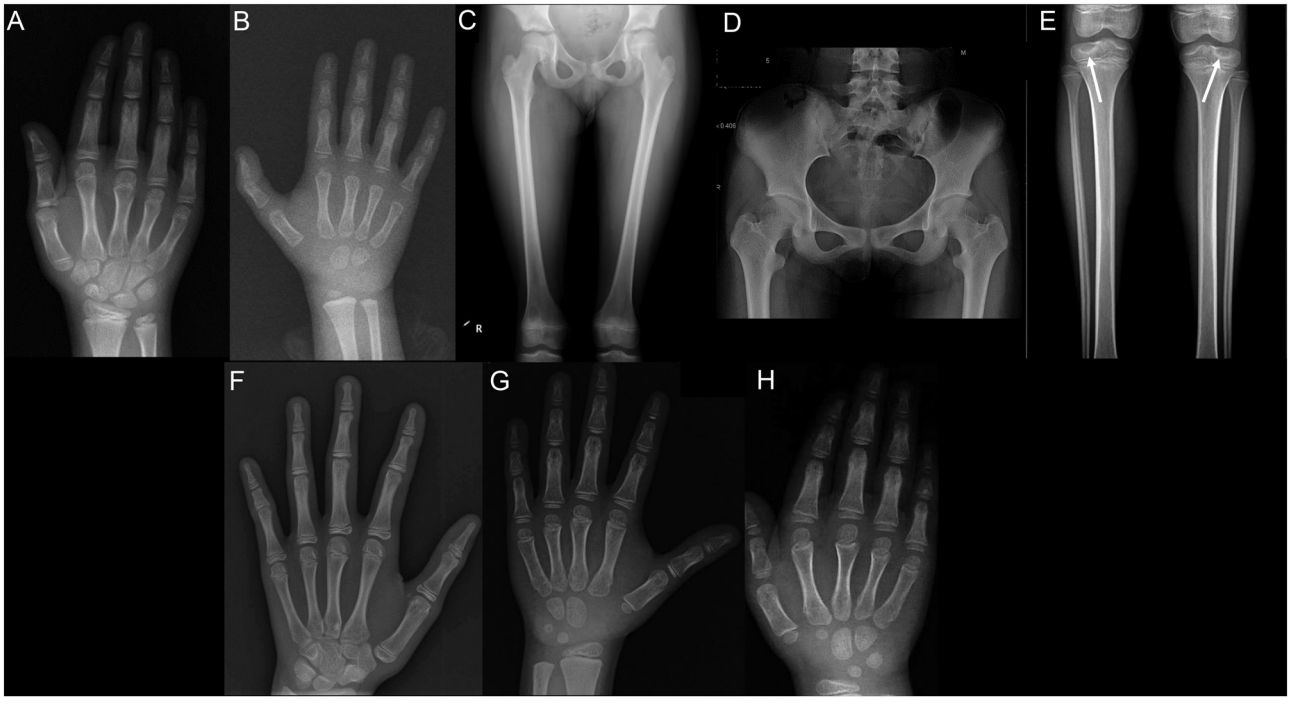

Figure 2

Summary of the main skeletal findings in probands with *ACAN* and *IHH* variants. Radiographs from patients with *ACAN* variants: (A) Hand radiograph proband 2 (female 7 years): mild brachydactyly; (B) Hand radiograph proband 7 (male 4 years): mild brachydactyly; (C) Hip radiograph proband 2 (female 7 years): Coxa valga, slender femora; (D) Hip radiograph proband 5 (female 14 years): short femoral necks; (E) Knee radiograph proband 2 (female 7 years): osteochondral defects in both proximal tibiae (white arrows). Hand radiographs from patients with *IHH* variants: (F) Proband 16 (female 7 years): middle phalanx shortening of 5th finger; (G) Proband 19 (male 8 years): Mild middle phalanx shortening of 5th finger; (H) Proband 20 (female 5.7 years): middle phalanx shortening of 2nd and 5th fingers.

Table 4 Summary of previous published NGS studies (2021–2013) performed in short stature children.

Reference	Cohort number and inclusion criteria	Clinical assessment	Screening methodology	Additional genetic studies	Variants identified in following genes	ACMG variant classification and Sherloc	% Diagnostic yield (nº positive/total)
Current study	108 children with short stature and mild skeletal defects present in proband or parent (*SHOX* defects previously excluded)	SD specialized pediatric endocrinology and radiology assessments	Targeted skeletal dysplasia panel (327–416 genes)	Family testing by Sanger, SNP arrays, or MLPA	*ACAN* (10), *COL2A1*, *CREBBP*, *EXT1*, *IHH* (7), and *PTPN11*	Pathogenic 7 Likely pathogenic 4 VUS 9	19.4% (21/108)
Fan *et al.* (33)	561 short stature children (257 isolated short stature and 304 syndromic short stature)	NR	WES	-	Isolated short stature (ISS): ***ACAN*** (3), *AQP2, BLM,* ***COL2A1***, *FGFR3, FBN1, GH1, GLI2, IGFR1, NF1, NPR2, PHEX, POU1F1,* ***PTPN11*** (3), *ROR2,* and *STAT5B*	Pathogenic (NR) Likely pathogenic (NR)	ISS: 11.3% (29/257) Syndromic short stature: 34.9% (106/304)
Perchard *et al.* (34)	263 prepubertal ISS children (IGF-1 deficiency, dysmorphic signs, and disproportionate short stature)	Pediatric endocrinology assessment	Targeted growth panel (232 genes)	-	***ACAN*** (2), *FANCB, GDF5, GH1, GNAS* (2), *HRAS, IGF1R* (5), *IKBKG, LHX4, MMP13* (2), *NOG, NPR2* (3), *OBSL1,* ***PTPN11***, *RUNX2,* and *TP63* (18 variants belonging to growth plate genes)	Pathogenic 2 Likely pathogenic 25	10% (27/263)
Homma *et al.* (27)	44 children born SGA with short stature and additional features, such as dysmorphic face, major malformation, developmental delay, and/or intellectual disability.	Clinical dysmorphology assessment	WES	Candidate gene testing, array-CGH and/or target panel sequencing	*ACTG1, AFF4, ANKRD11, BCL11B, BRCA1, CDKN1C,* ***COL2A1*** (2), *GINS1, INPP5K, KIF11, KMT2A, POC1A,* and *SRCAP* (2)	Pathogenic 11 Likely pathogenic 4	34% (15/34)
Freire *et al.* (26)	55 isolated short stature children (SGA cohort: minor malformations and disproportion not excluded)	Pediatric endocrinology assessment	WES or targeted panel (388 genes)	-	***ACAN***, ***IHH*** (2), *NF1, NPR2* (2), *SHOX,* and ***PTPN11***	Pathogenic 3 Likely pathogenic 5	14.5% (8/55)

Huang *et al.* (35)	114 severe short stature children (heterogenous cohort; 15.8% isolated short stature)	Pediatric endocrinology assessment	WES or targeted panel	Array-CGH	*ALPL, BLM, BRAF, COL1A1, COL1A2, COMP,* ***CREBBP****, DCHS1, ERCC8, FBN1, FGFR3* (6)*, GALNS, GH1, GLB1, HDAC8, HRAS, KIF22, KMT2A, KRAS, MATN3, NAA10, OBSL1, PYCR1, RAF1* (2)*, RUNX2, SLC12A3* (2)*, SPINK5, SRCAP,* and *VPS13B*	Pathogenic 19 Likely pathogenic 13 VUS 4	33.3% (38/114)
Yang *et al.* (36)	91 short stature children (SGA excluded)	Clinical assessment (not specified)	Targeted growth panel (166 genes)	NR	***ACAN***, ***COL2A1****, COMP, HOXD13,* ***PTPN11*** (3), and *SOS*	Pathogenic 6 Likely pathogenic 2	8.7% (8/91)
Hauer *et al.* (25)	200 short stature children (isolated or syndromic*; (after extensive genetic evaluation)	Clinical geneticist	WES	Target genetic testing (when suspected)	***ACAN*** (5)*, ANKRD11, CASK, CLCN5,* ***COL2A1*** (2)*, CUL7, FGD1, FGFR3, FLNB, GHSR, HDAC6, IFT140, IGFA1R,* ***IHH****, KAT6B, KDM6A* (2)*, MAP2K1, MATN3, NF1, NPR2* (3)*, PDE3A, PDE4D,* ***PTPN11****, SLC26A2,* and *TRIM37*	Pathogenic 19 Likely pathogenic 14	16.5% (33/200)
Kim *et al.* (37)	11 syndromic short stature children*	Clinical assessment (not specified)	TruSight One Panel (4813 genes)	Karyotyping, array-CGH, Sanger (based on suspicion)	*CDT1, DYRK1A, NBAS, RPS6KA3,* and *TRPS,*	Pathogenic 5	45% (5/11)
Hattori *et al.* (38)	86 ISS probands Exclusion criteria: SGA, skeletal malformations and dysmorphic features (*SHOX* defects previously excluded)	Clinical assessment (not specified)	Targeted panel (10 genes: *ACAN, FGFR3, NPR2, GHRHR, GH1, GHR, STAT5B, IGF1, IGFALS,* and *IGF1R*)		***ACAN*** (4)*, FGFR3* (2)*, GHRHR (2), GHR,* and *IGFALS*	Likely pathogenic 4 VUS 6	11.6 % (10/86)
Guo *et al.* (39)	14 syndromic severe short stature children	Pediatric endocrinology ± genetic assessment	WES	Array-CGH or SNP array	*B4GALT7, CUL7, FAM111A, SRCAP,* and *OBSL1*	NR	35.7% (5/14)
Wang *et al.* (40)	192 syndromic short stature children	NR	Targeted: panel (1077 genes-short stature/ growth plate biology)	NR	*IGF1R,* ***PTPN11*** (3), and *TRPV4*	Pathogenic 5	2.6% (5/192)

*Syndromic short stature refers to a child with additional features such as dysmorphic features, medical comorbidities, body disproportion, developmental delay or other accompanying anomalies.
Variant classification according to ACMG recommendations and Sherloc variant classification (22, 23). Genes marked in bold represent those in which variants were also detected in our study.
NR, not reported; SD, skeletal dysplasia; VUS, variant of unknown significance.

including larger cohort sizes (see Table 4) have identified a few *NPR2* and *FGFR3* variants in ISS and SGA probands. However, data from our diagnostic referrals and those of others (25, 34) suggest that the incidence of *NPR2* variants is lower than originally reported (15, 41). The increasing use of whole genome sequencing may reveal the presence of deep-intronic variants which affect splicing or variants in non-coding regulatory regions such as those identified recently in *POU1F1* (42).

Statistical analysis was performed on the obtained data for the entire cohort and for two subgroups, those with and without an identified genetic defect. Lower limb shortening (determined by the SH/H ratio) and a father with a skeletal anomaly were found to be statistically significant. A total of 12/16 (75%) variants were inherited from the father but we consider this incidental rather than a true factor. Other features that were important for inclusion into the cohort did not reach statistical significance, for example, A/H ratio, brachydactyly, which was the most frequent observed trait, or an abnormal skeletal survey. We hypothesize that the small number of patients with a genetic defect ($n = 21$), the wide age range and ranges of some of the variables, for example, A/H SDS ranged from -4.37 to +4.60 and the lack of data in some categories make the results difficult to interpret.

With the aim of forming a homogeneous study cohort, the clinical and radiological characterization of the 108 probands has been extensive. Unlike other studies with more heterogeneous cohorts or less restrictive selection criteria (summarized in Table 4) (25, 26, 27, 33, 34, 35, 36, 37, 38, 39, 40), we pursued an accurate estimation of the prevalence of genetic variants in skeletal dysplasia genes in such individuals. However, study limitations do still exist: (1) The probands have been assessed by different clinicians, although the completed questionnaires and x-rays were subsequently examined by the same pediatric endocrinologist and radiologist, both with experience in skeletal dysplasia evaluation; (2) Mild skeletal defects are difficult to evaluate post growth plate closure so parental x-ray evaluation is hampered; (3) Statistical analysis is always impaired due to missing data; and (4) Lack of a validated criteria to define brachydactyly which is considered either as a clinical/anthropometric feature or as a radiological trait (43). To minimize these biases, the skeletal surveys were reviewed by the same pediatric radiologist.

A criticism of the molecular analysis performed in this study is that a large customized skeletal dysplasia NGS panel was utilized rather than whole exome sequencing (WES). However, we wanted to determine the incidence of variants in skeletal dysplasia genes not the incidence of all genetic defects in this cohort so clearly WES would be the ideal technique but this was not undertaken at the onset of the project.

Previously published studies using NGS to investigate the genetic cause of short stature are summarized in Table 4, although the design, technology, and even the aim of the studies are different between them (25, 26, 27, 33, 34, 35, 36, 37, 38, 39, 40). Some include syndromic patients and others are more similar to ours, examining ISS, isolated short stature or short stature with mild dysmorphias/skeletal defects children with different clinical assessments. As expected, the diagnostic yield is higher when syndromic short stature probands are tested, with detection rates ranging from 33–45% (25, 27, 33, 35, 37, 39, 40) compared to non-syndromic cohorts, 8.7–19.5% (26, 33, 34, 36, 38, this study). This is particularly shown by a recent WES study which included both groups, whereby the detection rate was 11.3% for ISS cases ($n = 257$) but 34.9% for syndromic short stature cases ($n = 304$; (33)). In the studies of non-syndromic cases, three used targeted panels (including ours) resulting in diagnostic yields of 8.7–19.5%; one of the studies used a combination of targeted panels and WES and obtained a yield of 14.5%, and the remaining study performed WES and obtained a diagnostic yield of 11.3%, thus, no differences were observed between different NGS methodologies in non-syndromic cases. On the other hand, in syndromic cohorts, the majority of the studies included WES or WES/targeted panels with yields of 33–45% whilst in one study from 2013, which included the screening of 192 severe syndromic short stature children using a large panel of 1077 genes implicated in short stature and growth plate biology, the yield was only 2.6% (40). This data suggests that panels or WES virtual panels may be adequate for non-syndromic cases whilst WES improves the detection rate in syndromic cases.

The search for the underlying pathogenic cause in the remaining 80.6% of probands remains a challenge. WES or whole genome sequencing (WGS) may reveal variants in other genes but probably in a small proportion. As short stature is present in 3% of the population, WES or WGS is still not realistically feasible to perform in all these children especially as the likelihood of identifying a single causative genetic defect remains low plus short stature is often a polygenic trait (44, 45). Further studies should be focused on discovering new genes as well as other etiopathogenic mechanisms. WGS in combination with transcriptome sequencing and sequencing-based DNA methylation

analysis of the whole genome, will hopefully provide additional information.

Classical guidelines have been created focusing on the medical evaluation of children with ISS, SGA, or GHD (46, 47, 48) but they rely upon standard physical examination and laboratory parameters that assess organic causes of growth failure, such as renal dysfunction, hypothyroidism, celiac disease, inflammatory disorders, and assessment of the GH axis, either via measurement of GH-dependent factors, such as IGF-I or IGF binding protein-3, or through direct measurement of GH levels after stimulation. Recent guidelines propose an approach based on medical history, detailed physical examination, and analysis of individual growth curves with the aim of collecting diagnostic clues before conducting laboratory exams and left hand–wrist x-ray (49). After this first evaluation the clinician should have enough data to suspect primary or secondary growth disorders or by contrast an idiopathic short stature before planning the next diagnostic steps.

The high detection of variants in skeletal dysplasia genes in our study outlines the importance of a detailed clinical and radiological examination, looking for clues for primary growth disorders. Dominant inheritance of short stature and family history of early osteoarthritis or discopathy should also be investigated in family members. Complete anthropometric measurements and screening of external habitus (scoliosis, hyperlordosis, genu varum/valgum, size, and shape of hands, etc) should be performed in not only the child but also their parents and siblings. A careful radiological hand examination in the patient (not only bone maturation) is frequently very informative. Indications for performing a skeletal survey have not been established in short stature patients with mild skeletal defects. According to this study, one should be performed when a child has disproportionate short stature and/or the presence of a mild skeletal anomaly or if a parent has these features. In order to minimise radiation exposure, expert recommendations suggest a series of x-rays in patients with suspected bone disease: skull (anterior–posterior (AP) and lateral), thoracolumbar spine (AP and lateral), thorax, pelvis, one upper limb, one lower limb, and both hands (50). Other projections should be performed when other skeletal features are present.

The authors, therefore, encourage pediatric endocrinologists and other specialists to closely examine for skeletal anomalies, both in the child and in their parents, which may orientate them as to whether they should perform NGS genetic testing of skeletal dysplasia genes and if performed, to aid variant classification.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-21-0557.

Declaration of interest

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

Funding

This work was supported from a grant (SAF2017-84646-R and PID2020-116263RB-I00) from the Ministerio de Economia, Industria y Competividad (to K E H), and cofunded by the 'Fondo Europeo de Desarrollo Regional' (FEDER). L S-M and K E H have received awards from Fundación SEEP. F D-G was supported by an FPU studentship from the Spanish Ministry of Education (MEC).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

The authors would like to thank the families for their participation in this study.

References

1 Grosse G, Hilger A, Ludwig M, Reutter H, Lorenzen F, Even G, Holterhus PM, Woelfle J & German GHI Study Group. Targeted resequencing of putative growth-related genes using whole exome sequencing in patients with severe primary IGF-I deficiency. *Hormone Research in Paediatrics* 2017 **88** 408–417. (https://doi.org/10.1159/000480505)

2 Blum WF, Klammt J, Amselem S, Pfäffle HM, Legendre M, Sobrier ML, Luton MP, Child CJ, Jones C, Zimmermann AG *et al.* Screening a large pediatric cohort with GH deficiency for mutations in genes regulating pituitary development and GH secretion: frequencies, phenotypes and growth outcomes. *EBiomedicine* 2018 **36** 390–400. (https://doi.org/10.1016/j.ebiom.2018.09.026)

3 Rao E, Weiss B, Fukami M, Rump A, Niesler B, Mertz A, Muroya K, Binder G, Kirsch S, Winkelmann M *et al.* Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. *Nature Genetics* 1997 **16** 54–63. (https://doi.org/10.1038/ng0597-54)

4 Marchini A, Marttila T, Winter A, Caldeira S, Malanchi I, Blaschke RJ, Häcker B, Rao E, Karperien M, Wit JM *et al.* The short stature homeodomain protein SHOX induces cellular growth arrest and apoptosis and is expressed in human growth plate chondrocytes. *Journal of Biological Chemistry* 2004 **279** 37103–37114. (https://doi.org/10.1074/jbc.M307006200)

5 Marchini A, Ogata T & Rappold GA. A track record on SHOX: From basic research to complex models and therapy. *Endocrine Reviews* 2016 **37** 417–448. (https://doi.org/10.1210/er.2016-1036)

6 Benito-Sanz S, Aza-Carmona M, Rodríguez-Estevez A, Rica-Etxebarria I, Gracia R, Campos-Barros A & Heath KE. Identification of the first PAR1 deletion encompassing upstream SHOX enhancers in a family with idiopathic short stature. *European Journal of Human Genetics* 2012 **20** 125–127. (https://doi.org/10.1038/ejhg.2011.210)

7 Binder G. Short stature due to SHOX deficiency: genotype, phenotype, and therapy. *Hormone Research in Paediatrics* 2011 **75** 81–89. (https://doi.org/10.1159/000324105)

8 Gkourogianni A, Andrew M, Tyzinski L, Crocker M, Douglas J, Dunbar N, Fairchild J, Funari MF, Heath KE, Jorge AA *et al.* Clinical characterization of patients with autosomal dominant short stature due to aggrecan mutations. *Journal of Clinical Endocrinology and Metabolism* 2017 **102** 460–469. (https://doi.org/10.1210/jc.2016-3313)

9 Sentchordi-Montané L, Aza-Carmona M, Benito-Sanz S, Barreda-Bonis AC, Sánchez-Garre C, Prieto-Matos P, Ruiz-Ocaña P, Lechuga-Sancho A, Carcavilla-Urquí A, Mulero-Collantes I *et al.* Heterozygous aggrecan variants are associated with short stature and brachydactyly: description of 16 probands and a review of the literature. *Clinical Endocrinology* 2018 **88** 820–829. (https://doi.org/10.1111/cen.13581)

10 Sentchordi-Montané L, Benito-Sanz S, Aza-Carmona M, Pereda A, Parrón-Pajares M, de la Torre C, Vasques GA, Funari MFA, Travessa AM, Dias P *et al.* Clinical and molecular description of 16 families with heterozygous IHH variants. *Journal of Clinical Endocrinology and Metabolism* 2020 **105**. (https://doi.org/10.1210/clinem/dgaa218)

11 Bober MB, Bellus GA, Nikkel SM & Tiller GE. Hypochondroplasia. In *GeneReviews®*. Eds MP Adam, HH Ardinger, RA Pagon, SE Wallace, LJ Bean, G Mirzaa & A Amemiya. Seattle (WA): University of Washington, 1993.

12 Kant SG, Cervenkova I, Balek L, Trantirek L, Santen GW, de Vries MC, van Duyvenvoorde HA, van der Wielen MJ, Verkerk AJ, Uitterlinden AG *et al.* A novel variant of FGFR3 causes proportionate short stature. *European Journal of Endocrinology* 2015 **172** 763–770. (https://doi.org/10.1530/EJE-14-0945)

13 Rappold G, Blum WF, Shavrikova EP, Crowe BJ, Roeth R, Quigley CA, Ross JL & Niesler B. Genotypes and phenotypes in children with short stature: clinical indicators of SHOX haploinsufficiency. *Journal of Medical Genetics* 2007 **44** 306–313. (https://doi.org/10.1136/jmg.2006.046581)

14 Benito-Sanz S, Royo JL, Barroso E, Paumard-Hernández B, Barreda-Bonis AC, Liu P, Gracía R, Lupski JR, Campos-Barros Á, Gómez-Skarmeta JL *et al.* Identification of the first recurrent PAR1 deletion in Léri-Weill dyschondrosteosis and idiopathic short stature reveals the presence of a novel SHOX enhancer. *Journal of Medical Genetics* 2012 **49** 442–450. (https://doi.org/10.1136/jmedgenet-2011-100678)

15 Hisado-Oliva A, Garre-Vázquez AI, Santaolalla-Caballero F, Belinchón A, Barreda-Bonis AC, Vasques GA, Ramirez J, Luzuriaga C, Carlone G, González-Casado I *et al.* Heterozygous NPR2 mutations cause disproportionate short stature, similar to Léri-Weill dyschondrosteosis. *Journal of Clinical Endocrinology and Metabolism* 2015 **100** E1133–E1142. (https://doi.org/10.1210/jc.2015-1612)

16 Vasques GA, Funari MFA, Ferreira FM, Aza-Carmona M, Sentchordi-Montané L, Barraza-García J, Lerario AM, Yamamoto GL, Naslavsky MS, Duarte YAO *et al.* IHH gene mutations causing short stature with nonspecific skeletal abnormalities and response to growth hormone therapy. *Journal of Clinical Endocrinology and Metabolism* 2018 **103** 604–614. (https://doi.org/10.1210/jc.2017-02026)

17 Hoyme HE. Minor malformations. Significant or insignificant? *American Journal of Diseases of Children* 1987 **141** 947. (https://doi.org/10.1001/archpedi.1987.04460090024014)

18 Sánchez González E, Carrascosa Lezcano A, Fernández García JM, Ferrández Longás A, López de Lara D & López-Siguero JP. Estudios españoles de crecimiento: situación actual, utilidad y recomendaciones de uso. *Anales de Pediatría* 2011 **74** 193.e1–193.e16. (https://doi.org/10.1016/j.anpedi.2010.10.005)

19 Fredriks AM, van Buuren S, van Heel WJ, Dijkman-Neerincx RH, Verloove-Vanhorick SP & Wit JM. Nationwide age references for sitting height, leg length, and sitting height/height ratio, and their diagnostic value for disproportionate growth disorders. *Archives of Disease in Childhood* 2005 **90** 807–812. (https://doi.org/10.1136/adc.2004.050799)

20 Gerver WJ & de Bruin R. *Paediatric Morphometrics: A Reference Manual*, 2nd ed. Maastricht: University Press Maastricht, 2001.

21 Pyle SI & Greulich WW. *Radiographic Atlas of Skeletal Development of the Hand and Wrist*, 2nd ed. Stanford: Stanford University Press, 1950.

22 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine* 2015 **17** 405–424. (https://doi.org/10.1038/gim.2015.30)

23 Nykamp K, Anderson M, Powers M, Garcia J, Herrera B, Ho YY, Kobayashi Y, Patil N, Thusberg J, Westbrook M *et al.* Sherloc: a comprehensive refinement of the ACMG-AMP variant classification criteria. *Genetics in Medicine* 2017 **19** 1105–1117. (https://doi.org/10.1038/gim.2017.37)

24 Ma G, Yu J, Xiao Y, Chan D, Gao B, Hu J, He Y, Guo S, Zhou J, Zhang L *et al.* Indian hedgehog mutations causing brachydactyly type A1 impair Hedgehog signal transduction at multiple levels. *Cell Research* 2011 **21** 1343–1357. (https://doi.org/10.1038/cr.2011.76)

25 Hauer NN, Popp B, Schoeller E, Schuhmann S, Heath KE, Hisado-Oliva A, Klinger P, Kraus C, Trautmann U, Zenker M *et al.* Clinical relevance of systematic phenotyping and exome sequencing in patients with short stature. *Genetics in Medicine* 2018 **20** 630–638. (https://doi.org/10.1038/gim.2017.159)

26 Freire BL, Homma TK, Funari MFA, Lerario AM, Vasques GA, Malaquias AC, Arnhold IJP & Jorge AAL. Multigene sequencing analysis of children born small for gestational age with isolated short stature. *Journal of Clinical Endocrinology and Metabolism* 2019 **104** 2023–2030. (https://doi.org/10.1210/jc.2018-01971)

27 Homma TK, Krepischi ACV, Furuya TK, Honjo RS, Malaquias AC, Bertola DR, Costa SS, Canton AP, Roela RA, Freire BL *et al.* Recurrent copy number variants associated with syndromic short stature of unknown cause. *Hormone Research in Paediatrics* 2018 **89** 13–21. (https://doi.org/10.1159/000481777)

28 Bartsch O, Locher K, Meinecke P, Kress W, Seemanová E, Wagner A, Ostermann K & Rödel G. Molecular studies in 10 cases of Rubinstein-Taybi syndrome, including a mild variant showing a missense mutation in codon 1175 of crebbp. *Journal of Medical Genetics* 2002 **39** 496–501. (https://doi.org/10.1136/jmg.39.7.496)

29 Bartsch O, Kress W, Kempf O, Lechno S, Haaf T & Zechner U. Inheritance and variable expression in Rubinstein-Taybi syndrome. *American Journal of Medical Genetics: Part A* 2010 **152A** 2254–2261. (https://doi.org/10.1002/ajmg.a.33598)

30 Menke LA, van Belzen MJ, Alders M, Cristofoli F, DDD Study, Ehmke N, Fergelot P, Foster A, Gerkes EH, Hoffer MJ *et al. Crebbp* mutations in individuals without Rubinstein-Taybi syndrome phenotype. *American Journal of Medical Genetics: Part A* 2016 **170** 2681–2693. (https://doi.org/10.1002/ajmg.a.37800)

31 de Vries TI, Monroe GR, van Belzen MJ, van der Lans CA, Savelberg SM, Newman WG, van Haaften G, Nievelstein RA & van Haelst MM. Mosaic crebbp mutation causes overlapping clinical features of Rubinstein-Taybi and Filippi syndromes. *European Journal of Human Genetics* 2016 **24** 1363–1366. (https://doi.org/10.1038/ejhg.2016.14)

32 Pannone L, Bocchinfuso G, Flex E, Rossi C, Baldassarre G, Lissewski C, Pantaleoni F, Consoli F, Lepri F, Magliozzi M *et al.* Structural, functional, and clinical characterization of a novel *PTPN11* mutation cluster underlying Noonan syndrome. *Human Mutation* 2017 **38** 451–459. (https://doi.org/10.1002/humu.23175)

33 Fan X, Zhao S, Yu C, Wu D, Yan Z, Fan L, Song Y, Wang Y, Li C, Ming Y *et al.* Exome sequencing reveals genetic architecture in patients with isolated or syndromic short stature. *Journal of Genetics and Genomics* 2021 **48** 396–402. (https://doi.org/10.1016/j.jgg.2021.02.008)

34 Perchard R, Murray PG, Payton A, Highton GL, Whatmore A & Clayton PE. Novel mutations and genes that impact on growth in short stature of undefined aetiology: the EPIGROW study. *Journal of*

the Endocrine Society 2020 **4** bvaa105. (https://doi.org/10.1210/jendso/bvaa105)
35 Huang Z, Sun Y, Fan Y, Wang L, Liu H, Gong Z, Wang J, Yan H, Wang Y, Hu G *et al.* Genetic evaluation of 114 Chinese short stature children in the next generation era: a single center study. *Cellular Physiology and Biochemistry* 2018 **49** 295–305. (https://doi.org/10.1159/000492879)
36 Yang L, Zhang C, Wang W, Wang J, Xiao Y, Lu W, Ma X, Chen L, Ni J, Wang D *et al.* Pathogenic gene screening in 91 Chinese patients with short stature of unknown etiology with a targeted next-generation sequencing panel. *BMC Medical Genetics* 2018 **19** 212. (https://doi.org/10.1186/s12881-018-0730-6)
37 Kim YM, Lee YJ, Park JH, Lee HD, Cheon CK, Kim SY, Hwang JY, Jang JH & Yoo HW. High diagnostic yield of clinically unidentifiable syndromic growth disorders by targeted exome sequencing. *Clinical Genetics* 2017 **92** 594–605. (https://doi.org/10.1111/cge.13038)
38 Hattori A, Katoh-Fukui Y, Nakamura A, Matsubara K, Kamimaki T, Tanaka H, Dateki S, Adachi M, Muroya K, Yoshida S *et al.* Next generation sequencing-based mutation screening of 86 patients with idiopathic short stature. *Endocrine Journal* 2017 **64** 947–954. (https://doi.org/10.1507/endocrj.EJ17-0150)
39 Guo MH, Shen Y, Walvoord EC, Miller TC, Moon JE, Hirschhorn JN & Dauber A. Whole exome sequencing to identify genetic causes of short stature. *Hormone Research in Paediatrics* 2014 **82** 44–52. (https://doi.org/10.1159/000360857)
40 Wang SR, Carmichael H, Andrew SF, Miller TC, Moon JE, Derr MA, Hwa V, Hirschhorn JN & Dauber A. Large-scale pooled next-generation sequencing of 1077 genes to identify genetic causes of short stature. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** E1428–E1437. (https://doi.org/10.1210/jc.2013-1534)
41 Vasques GA, Arnhold IJP & Jorge AAL. Role of the natriuretic peptide system in normal growth and growth disorders. *Hormone Research in Paediatrics* 2014 **82** 222–229. (https://doi.org/10.1159/000365049)
42 Gergics P, Smith C, Bando H, Jorge AAL, Rockstroh-Lippold D, Vishnopolska SA, Castinetti F, Maksutova M, Carvalho LRS, Hoppmann J *et al.* High-throughput splicing assays identify missense and silent splice-disruptive POU1F1 variants underlying pituitary hormone deficiency. *American Journal of Human Genetics* 2021 **108** 1526–1539. (https://doi.org/10.1016/j.ajhg.2021.06.013)
43 Temtamy SA & Aglan MS. Brachydactyly. *Orphanet Journal of Rare Diseases* 2008 **3** 1–16.
44 Marouli E, Graff M, Medina-Gomez C, Lo KS, Wood AR, Kjaer TR, Fine RS, Lu Y, Schurmann C, Highland HM *et al.* Rare and low-frequency coding variants alter human adult height. *Nature* 2017 **542** 186–190. (https://doi.org/10.1038/nature21039)
45 Hauer NN, Popp B, Taher L, Vogl C, Dhandapany PS, Büttner C, Uebe S, Sticht H, Ferrazzi F, Ekici AB *et al.* Evolutionary conserved networks of human height identify multiple Mendelian causes of short stature. *European Journal of Human Genetics* 2019 **27** 1061–1071. (https://doi.org/10.1038/s41431-019-0362-0)
46 Collett-Solberg PF, Ambler G, Backeljauw PF, Bidlingmaier M, Biller BMK, Boguszewski MCS, Cheung PT, Choong CSY, Cohen LE, Cohen P *et al.* Diagnosis, genetics, and therapy of short stature in children: a Growth Hormone Research Society International Perspective. *Hormone Research in Paediatrics* 2019 **92** 1–14. (https://doi.org/10.1159/000502231)
47 Argente J, Tatton-Brown K, Lehwalder D & Pfäffle R. Genetics of growth disorders – which patients require genetic testing? *Frontiers in Endocrinology* 2019 **10** 602. (https://doi.org/10.3389/fendo.2019.00602)
48 Cohen P, Rogol AD, Deal CL, Saenger P, Reiter EO, Ross JL, Chernausek SD, Savage MO, Wit JM & 2007 ISS Consensus Workshop Participants. Consensus statement on the diagnosis and treatment of children with idiopathic short stature: a summary of the Growth Hormone Research Society, the Lawson Wilkins Pediatric Endocrine Society, and the European Society for Paediatric Endocrinology Workshop. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 4210–4217. (https://doi.org/10.1210/jc.2008-0509)
49 Wit JM, Kamp GA, Oostdijk W & on behalf of the Dutch Working Group on Triage and Diagnosis of Growth Disorders in Children. Towards a rational and efficient diagnostic approach in children referred for growth failure to the general paediatrician. *Hormone Research in Paediatrics* 2019 **91** 223–240. (https://doi.org/10.1159/000499915)
50 Offiah AC & Hall CM. Radiological diagnosis of the constitutional disorders of bone. As easy as A, B, C? *Pediatric Radiology* 2003 **33** 153–161. (https://doi.org/10.1007/s00247-002-0855-8)

Received 26 May 2021
Revised version received 29 July 2021
Accepted 10 September 2021