RESEARCH

Diagnostic yield of a multigene sequencing approach in children classified as idiopathic short stature

Nathalia Liberatoscioli Menezes Andrade¹,², Mariana Ferreira de Assis Funari², Alexsandra Christianne Malaquias³, Paulo Ferrez Collett-Solberg⁴, Nathalia L R A Gomes⁵, Renata Scalco⁶,⁷, Naiara Castelo Branco Dantas¹, Raissa C Rezende², Angelica M F P Tiburcio⁵, Micheline A R Souza⁶, Bruna L Freire¹,², Ana C V Kreisch⁸, Carlos Alberto Longui³, Antonio Marcondes Lerario⁹, Ivo J P Arnhold¹,², Alexander A L Jorge¹,² and Gabriela Andrade Vasques¹,²

¹Unidade de Endocrinologia Genetica (LIM 25), Hospital das Clinicas da Faculdade de Medicina, Universidade de Sao Paulo (USP), Sao Paulo, Brasil
²Unidade de Endocrinologia do Desenvolvimento, Laboratorio de Hormonios e Genetica Molecular (LIM42), Hospital das Clinicas da Faculdade de Medicina, Universidade de Sao Paulo (USP), Sao Paulo, Brasil
³Departamento de Pediatria, Faculdade de Ciencias Medicas da Santa Casa de Sao Paulo, Sao Paulo, Brasil
⁴Disciplina de Endocrinologia, Facultade de Medicina (USP), Sao Paulo, Brasil
⁵Serviço de Endocrinologia, Unidade de Crescimento, Santa Casa de Belo Horizonte, Belo Horizonte, Minas Gerais, Brasil
⁶Departamento de Medicina, Faculdade de Ciencias Medicas da Santa Casa de Sao Paulo, Sao Paulo, Brasil
⁷Servico de Endocrinologia do Instituto de Puericultura e Pediatria Martagao Gesteira/Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
⁸Centro de Pesquisa em Genoma Humano e Células-Tronco, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de Sao Paulo, São Paulo, Brasil
⁹Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Correspondence should be addressed to A A L Jorge: alexj@usp.br

Abstract

Objective: Most children with short stature remain without an etiologic diagnosis after extensive clinical and laboratory evaluation and are classified as idiopathic short stature (ISS). This study aimed to determine the diagnostic yield of a multigene analysis in children classified as ISS.

Design and methods: We selected 102 children with ISS and performed the genetic analysis as part of the initial investigation. We developed customized targeted panel sequencing, including all genes already implicated in the isolated short-stature phenotype. Rare and deleterious single nucleotide or copy number variants were assessed by bioinformatic tools.

Results: We identified 20 heterozygous pathogenic (P) or likely pathogenic (LP) genetic variants in 17 of 102 patients (diagnostic yield = 16.7%). Three patients had more than one P/LP genetic alteration. Most of the findings were in genes associated with the growth plate differentiation: IHH (n = 4), SHOX (n = 3), FGFR3 (n = 2), NPR2 (n = 2), ACAN (n = 2), and COL2A1 (n = 1) or involved in the RAS/MAPK pathway: NF1 (n = 2), PTPN11 (n = 1), CBL (n = 1), and BRAF (n = 1). None of these patients had clinical findings to guide a candidate gene approach. The diagnostic yield was higher among children with severe short stature (35% vs 12.2% for height SDS ≤ −3; P = 0.034). The genetic diagnosis had an impact on clinical management for four children.

Conclusion: A multigene sequencing approach can determine the genetic etiology of short stature in up to one in six children with ISS, removing the term idiopathic from their clinical classification.

Key Words

idiopathic short stature
multigene sequencing analysis
 genetic
 mutation

Endocrine Connections
(2022) 11, e220214

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https://doi.org/10.1530/EC-22-0214

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Introduction

Short stature affects 2.3% of children and is a frequent complaint in pediatric endocrinology visits. However, more than 60% of these children have clinical and laboratory evaluations with no relevant findings justifying their growth impairment and are classified as having idiopathic short stature (ISS) (1). This term is widely used but this strict definition excludes children with evidence of systemic, endocrine, nutritional or chromosomal abnormalities, and those born small for gestational age (SGA). On the other hand, it includes familial short stature (2), which may suggest a major genetic basis.

It is known that human height has a high degree of heritability (>80%) (3). Thousands of common single nucleotide polymorphisms (minor allele frequency (MAF) ≥ 5%) have a low individual impact on height variability (less than 1–2 mm), while some rare variants (MAF < 1%) have been associated with a higher individual impact on stature (about 20 mm) (4, 5). This demonstrates that short stature has a complex inheritance and that adult height is primarily determined by the sum of the effects of common and rare variants (5, 6).

In the last 20 years, many monogenic defects have been established as causes of isolated short stature in patients initially classified as ISS (7). The genetic defects associated with ISS are mainly located in genes that regulate the endochondral ossification process in the growth plate and in genes related to the GH–IGF-1 axis (8, 9, 10). Also, defects in genes associated with the RAS-MAPK pathway (PTPN11 and NF1) were reported in children with short stature without other characteristics (11, 12), with clear relevance for the patient’s follow-up.

The individuals carrying these monogenic conditions exhibit variable degrees of short stature with nonspecific additional findings, limiting the use of a candidate gene approach. Therefore, genetic evaluation through multigene sequence analysis has gained importance, and several studies have already been published using this methodology in children with short stature of unknown cause (8, 10, 13, 14, 15, 16). However, the previous studies assessed pre-selected samples and/or included children born small for gestational age (SGA) or children with syndromic short stature. Thus, the present study aimed to evaluate a large number of homogeneous children classified as ISS, using a targeted gene panel as part of the initial investigation. We evaluated the diagnostic yield of this comprehensive genetic approach in 102 children with ISS.

Patients and methods

Patients

We enrolled 102 children initially classified as ISS (2) for genetic investigation. The ethics committee for the analysis of research projects of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo approved the present study (approval no. 3.555.875). All procedures followed the ethical standards of our ethics committee and the Declaration of Helsinki. The patients and/or their tutors provided written informed consent after receiving a full explanation of the purpose and nature of all procedures.

All children underwent clinical and laboratory assessment defined by the ISS consensus (2). The inclusion criteria were short stature (height standard deviation score (SDS) ≤ –2 (17)) and clinical assessment compatible with a healthy child. We excluded children born preterm (<37 weeks) and/or with low birth weight, as well as patients with significant dysmorphic features, major malformations (18), microcephaly, neurodevelopmental delay, intellectual disabilities, skeletal dysplasia, chronic systemic, nutritional, and endocrine diseases, including growth hormone deficiency (19).

In total, 364 children classified as ISS and who met the inclusion criteria were evaluated between August 1994 and February 2020. Of these, 262 were not included because they lost clinical follow-up (n = 198) and/or had already been genetically evaluated in previous studies using a candidate gene approach (n = 64) (20, 21, 22, 23). A total of 102 children were enrolled for this study. Fifty-nine children without previous genetic studies were selected for the genetic study during the follow-up in our outpatient clinic (retrospective cohort). Forty-three children were sequentially enrolled from March 2018 to February 2020 (prospective cohort). For these 43 children, the present genetic analysis was performed just after the first clinical and laboratory evaluation (Fig. 1).

The clinical assessment included measurements of weight (measured using an electronic scale) and standing and sitting height (mean of three measurements using a calibrated wall-mounted stadiometer to the nearest 0.1 cm). The results were converted to SDS using age- and sex-specific norms for height, BMI, and sitting height:total height ratio (SH:H) (17, 24). The heights of the parents were obtained during the patients’ visit or were obtained from the medical records reported by the family. The target height of the child was calculated as ((father’s height+mother’s height ± 13 cm)/2) and expressed as SDS. Patients were characterized as having familial short...
stature when at least one of the parents had height SDS \leq -2. Bone age was assessed by the method of Greulich and Pyle, using left hand and wrist x-rays (25). All children underwent laboratory tests, including a complete blood count, creatinine, electrolytes, alkaline phosphatase, albumin, screening for celiac disease, TSH, free T4, and IGF-1 levels determination. A karyotype was performed in all girls to exclude Turner syndrome. Growth hormone deficiency (GHD) was excluded in all children based on normal IGF-1 SDS level (IGF-1 SDS \geq -1) (26), normal growth velocity for age (n = 100), and/or a GH peak at stimulation test > 5 µg/L (n = 59).

Genetic analysis

Genomic DNA was isolated from peripheral blood leukocytes from all patients and relatives using standard procedures. Targeted panel sequencing was performed only for the index patients. We developed a customized targeted panel (Agilent SureSelect XT assay; Agilent Technologies). The complete list of included genes is available in Supplementary Table 1 (see section on supplementary materials given at the end of this article). It contains genes expressed in the growth plate, involved in fundamental cellular processes and/or in the GHRH-GH-IGF1 pathway (7). In addition, we included SHOX genomic and enhancer regions to improve the analysis of this locus by next-generation sequencing (NGS). Our customized targeted panel had similar accuracy to the standard evaluation of SHOX defects (27). In-house bioinformatic analysis was performed as previously reported (12). The sequences were aligned to the human reference genome sequence (GRCh37/hg19). Copy number variation (CNVs) analysis was also performed for all patients using the program Copy Number Targeted Resequencing Analysis (CONTRA) to identify the presence of deletions or duplications (28).

Variant assessment

For data analysis, we searched for rare variants located in exonic regions and consensus splice site sequences, with MAF < 0.1% in public international (gnomAD (29)) and national databases (AbraOM (30); SELAdb (31)). We prioritized variants according to their potential to be pathogenic, including loss-of-function variants, variants predicted to be pathogenic by multiple in silico tools (Supplementary Table 2), or previously reported in Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/). The sequencing reads carrying candidate variants were visually inspected using the Integrative Genomics Viewer to reduce false-positive findings. The assessment of gene function was performed using the OMIM (https://www.omim.org/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/) databases. Sanger sequencing was performed to validate the candidate variants identified and for segregation analysis. The CNVs related to the SHOX gene identified by the CONTRA analysis were confirmed and segregated by multiplex ligation-dependent probe amplification (MLPA) (27). MLPA analysis was carried out using the SALSA MLPA P018 SHOX (MRC Holland, Amsterdam, Netherlands). The other CNVs of interest were confirmed and segregated in the family using the CGH-array technique. All variants were classified following the American College of Medical Genetics and Genomics/Association for Molecular Pathology variant pathogenicity guidelines (32).

Statistical analysis

Qualitative (nominal) variables were reported as absolute values and percentages. Continuous variables were

![Flowchart of the approach used in this study to investigate a group of patients with idiopathic short stature.](https://doi.org/10.1530/EC-22-0214)
expressed as mean ± s.d. or median and interquartile range (IQR) (p25–p75). To compare children who had a genetic diagnosis with those without a genetic diagnosis and with those children who harbor a variant of uncertain significance (VUS), we used the ANOVA followed by Tukey test or Kruskal–Wallis, one-way ANOVA on ranks for numerical variables, as appropriate. Nominal variables were compared by chi-square or Fisher's exact test, as appropriate. A P-value <0.05 was used for statistical significance. All analyses were done with SigmaStat software (RRID:SCR_010285, version 3.5; Systat Software, San Jose, CA).

Results

Cohort description

We analyzed data from 102 unrelated children initially classified as ISS. The clinical characteristics of these children are summarized in Table 1. Children who were included in the genetic study during their follow-up (retrospective cohort) or at the initial evaluation (prospective cohort) had similar characteristics (Supplementary Table 3). Our cohort was characterized by the predominance of boys (66%) and patients with familial short stature (59%). The mean age at the first evaluation was 8.9 ± 3.7 years, and 78% of the selected children were prepubertal. The average height was −2.6 ± 0.8 SDS, with 70% of the children having a height SDS < −2.5 and 20% a height SDS ≤ −3.0. Most of the children were eutrophic (80%; BMI SDS ≥ −2 and ≤1), 11% had low BMI (BMI SDS < −2), and 9% were overweight (BMI SDS > 1 and ≤2). The mean difference between bone and chronological age was −1.9 ± 1.4 years.

Genetic variants detected in the targeted panel

Eight pathogenic (P) and 12 likely pathogenic (LP) heterozygous variants, including 4 CNVs (16 single nucleotide variants and 4 CNVs; total = 20), were identified in 17 of 102 patients. Three patients (patients 1, 4, and 7) had more than one P/LP variant (Fig. 2). The genetic results are described in Fig. 1 and Table 2. Ten single nucleotide variants were located in genes related to the growth plate (four in IHH, two in NPR2, two in FGFR3, one in ACAN, and one in COL2A1), one variant was in a gene associated with the GH–IGF1 axis (GHSR), and five variants were in genes related to the RAS-MAPK pathway (two in NFI, one in CBL gene, one in PTEN, one in BRAF). In addition, we observed an allelic imbalance suggesting mosaicism in one NFI1 de novo mutation (Tables 2 and 3). Three CNVs were heterozygous deletions related to the SHOX gene, and one is a complex rearrangement involving the ACAN gene (Supplementary Table 4). Considering the 20 variants identified in this study, 13 were found in patients from the prospective cohort and 7 in patients from the retrospective cohort.

Additionally to P/LP variants, 13 variants were classified as VUS in 12 patients (Table 4). One patient had more than one VUS (patient 26), and another carried one P and one LP variant besides the VUS (patient 4). Eight VUSs were in

Table 1 Clinical characteristics of 102 patients with isolated short stature.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>P/LP</th>
<th>VUS</th>
<th>Negative</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospective: retrospective cohort</td>
<td>43:59</td>
<td>10:7</td>
<td>66</td>
<td>27:47</td>
<td>0.230</td>
</tr>
<tr>
<td>Familial short stature</td>
<td>58 (56.9%)</td>
<td>13 (76%)</td>
<td>5 (45%)</td>
<td>40 (54%)</td>
<td>0.175</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>67:35</td>
<td>12:5</td>
<td>65</td>
<td>49:25</td>
<td>0.672</td>
</tr>
<tr>
<td>Chronological age</td>
<td>8.9 ± 3.6</td>
<td>7.3 ± 3.8</td>
<td>8.9 ± 2.5</td>
<td>9.2 ± 3.7</td>
<td>0.164</td>
</tr>
<tr>
<td>Bone age – chronological age (years)</td>
<td>−1.9 ± 1.4</td>
<td>−1.4 ± 1.8</td>
<td>−2.0 ± 0.9</td>
<td>−2.0 ± 1.4</td>
<td>0.340</td>
</tr>
<tr>
<td>Height SDS</td>
<td>−2.65 ± 0.9</td>
<td>−2.98 ± 0.6</td>
<td>−2.68 ± 0.4</td>
<td>−2.57 ± 0.9</td>
<td>0.198</td>
</tr>
<tr>
<td>Severe short stature</td>
<td>20 (19.6%)</td>
<td>7 (41.2%)</td>
<td>1 (9.1%)</td>
<td>12 (16.2%)</td>
<td>0.042</td>
</tr>
<tr>
<td>Height SDS corrected by TH SDSD</td>
<td>−1.41 ± 1.2</td>
<td>−1.39 ± 0.9</td>
<td>−1.91 ± 0.8</td>
<td>−1.33 ± 1.2</td>
<td>0.308</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>−0.44 ± 1.1</td>
<td>0.18 ± 1.0</td>
<td>−0.27 ± 1.0</td>
<td>−0.61 ± 1.1</td>
<td>0.021</td>
</tr>
<tr>
<td>Sitting height/height (SH:H) SDS</td>
<td>0.78 ± 1.3</td>
<td>0.94 ± 1.6</td>
<td>0.48 ± 0.8</td>
<td>0.78 ± 1.3</td>
<td>0.665</td>
</tr>
<tr>
<td>GH peak at stimulation test</td>
<td>8.8 (n = 65) (6.5–14.4)</td>
<td>13.4 (n = 10) (9.4–18.6)</td>
<td>6.5 (n = 8) (4.4–10.0)</td>
<td>8.8 (n = 47) (6.8–14.8)</td>
<td>0.017</td>
</tr>
<tr>
<td>IGF-1 SDS</td>
<td>−0.9 ± 1.1</td>
<td>−0.91 ± 1.1</td>
<td>−0.83 ± 1.1</td>
<td>−0.9 ± 1.1</td>
<td>0.865</td>
</tr>
</tbody>
</table>

*ANOVA test performed to compare P/LP, VUS, and negative groups; 4When affected children had at least one of the parents with height SDS ≤ −2; 5Height SDS ≤ −3; 6Height SDS – target height (TH) SDS; Tukey test post hoc: P = 0.018 for P/LP vs negative; Tukey test post hoc: P = 0.039 for P/LP vs VUS.

GH, growth hormone; IGF-1, insulin growth factor 1; LP, likely pathogenic; P, pathogenic; SDS, standard deviation score; TH, target height; VUS, variant of uncertain significance.

Bold indicates statistical significance, P < 0.05.
genes related to the growth plate (three in NPR2, three in IHH, one in FGFR3, and one in COL2A1), four were in genes associated with the GH-IGF1 axis (three in IGF1R and one in GHR), and one in NF1 gene. Among these 13 variants, 6 were found in patients from the prospective cohort and 7 were found in the retrospective cohort.

Phenotype of patients harboring variants associated with idiopathic short stature

The clinical characteristics of the children who had P/LP variants (n = 17) are summarized in Tables 1 and 3. Twelve children (71%) inherited the variant from a parent with short stature (height SDS from −4.2 to −2.0). Seven patients had mild disproportionate short stature (SH:H SDS > 2) and a normal skeletal survey; all of them had defects in genes related to the growth plate (FGFR3, IHH, SHOX, and ACAN). Eight had nonspecific skeletal abnormalities, and seven of them with a variant associated with the growth plate. Two of four patients carrying a variant in IHH gene (patients 2 and 11) had a shortening of the middle phalanx of the fifth finger with cone-shaped epiphyses identified in the x-ray for bone age. None of the patients who had CNVs (n = 3) related to the SHOX gene had Madelung deformity, and only one girl had disproportionate short stature. Most of the children had a bone age (BA) compatible with chronological age (58%) or slightly delayed BA (37%). Considering the two girls that harbored pathogenic variants in ACAN, only one had advanced bone age (CA 5.4 years; BA: 7.6 years), the other one had a bone age compatible with her chronological age (CA 9.3 years; BA: 8.6 years). The boys with pathogenic variants in CBL, PTPN11, and BRAF had no clinical criteria that could suggest Noonan syndrome or other RASopathies. Similarly, the two patients who had the NF1 variants did not have any clinical findings that suggested neurofibromatosis type 1 at the age of 2.7 and 9.6 years. One of them had mosaicism for the NF1 variant, which could explain the absence of additional signs.

Although children harboring LP/P variants had clinical characteristics that overlapped with children with negative results, as a group, these patients had a higher BMI and GH peak. There were no differences in the clinical characteristics of patients with VUS compared to patients with negative result (Table 1). Eight inherited the VUS from a parent with short stature (height SDS from −4.2 to −2.0), and only one had mild body disproportion.

Diagnostic yield of the targeted gene panel

We identified 20 P/LP variants and/or CNVs in 17 of the 102 children with idiopathic short stature, corresponding...
to a diagnostic yield of 16.7% (95% CI: 9.2–24.4%). The diagnostic yield was higher among children with severe short stature (height SDS < –3; 35%) in comparison with children with less severe short stature (12.2%; P = 0.034). There was no significant difference in the rate of a positive genetic diagnosis in the group of patients with familial short stature (with 22.4%, without 9.1%; P = 0.129) or with mild body disproportion (present 30.4%, absent 14.1%, P = 0.116). The positivity in the prospectively evaluated group was higher: in this group, 10/43 patients (23.3%) had the cause of short stature elucidated vs 7/59 patients (11.9%) in the retrospective group. Despite this, there was no statistical significance when comparing the results of the two groups (P = 0.333).

**Discussion**

The assessment of children with ISS is puzzling. Despite the wide amount of reported new causes of growth disorders in children, those labeled as ISS remain without a specific diagnosis. The genetic investigation has emerged as a potential tool to establish the etiology of short stature. Therefore, we performed a comprehensive genetic study as part of the initial investigation of a large cohort of 102 children with ISS. We identified 20 P/LP variants and/or CNVs in 17 children with ISS, which corresponds to a diagnostic yield of 16.7%, including 3 patients with more than 1 P/LP finding (Figs 1 and Table 2). Additionally, 13 variants were classified as of uncertain significance (VUS) in 12 patients. However, the definitive classification of VUS is a challenge, demanding the genotyping of several family members, functional assays and/or population studies. Thus, in our study, we preferred to be cautious and to consider patients with VUS belonging to the group of patients with negative results when we calculated the diagnostic yield. Consequently, this diagnostic yield may increase in the future if, some VUS are reclassified with additional data.

The diagnostic yield observed in our study was above the median reported in previous studies that used a multigene sequencing approach to assess children with short stature of unknown cause (average diagnostic rate of 12.5%, ranging from 8.7 to 19.5%; reviewed by Sentchordi-Montané et al. (10)). Nevertheless, several of these studies were not restricted to patients with ISS and included patients who were born SGA, with syndromic conditions, neurodevelopmental disorders, and/or skeletal dysplasia (8, 11, 12, 14, 33, 34).
Table 3  Clinical characteristics at first evaluation of patients with pathogenic or likely pathogenic genetic alterations.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>Sex</th>
<th>Age (years)</th>
<th>BA-CA (years)</th>
<th>H SDS</th>
<th>SH:H SDS</th>
<th>BMI SDS</th>
<th>Father (H SDS)</th>
<th>Mother (H SDS)</th>
<th>Additional findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACAN/SHOX</td>
<td>F</td>
<td>5.4</td>
<td>2.3</td>
<td>-2.1</td>
<td>-0.9</td>
<td>0.7</td>
<td>-2.2</td>
<td>-1.4</td>
<td>Advanced bone age</td>
</tr>
<tr>
<td>2</td>
<td>IHH</td>
<td>M</td>
<td>4.0</td>
<td>-0.9</td>
<td>-3.1</td>
<td>2.0</td>
<td>0.6</td>
<td>1.5</td>
<td>-0.9</td>
<td>Shortening of the middle phalanx of the fifth finger at x-ray for bone age</td>
</tr>
<tr>
<td>3</td>
<td>NPR2</td>
<td>M</td>
<td>4.8</td>
<td>-1.6</td>
<td>-2.5</td>
<td>0.7</td>
<td>-0.1</td>
<td>-0.3</td>
<td>-1.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CBL/SHOX</td>
<td>M</td>
<td>12.9</td>
<td>-2.9</td>
<td>-3.2</td>
<td>-0.5</td>
<td>-2.8</td>
<td>-0.4</td>
<td>-4.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GHSR</td>
<td>M</td>
<td>4.1</td>
<td>-0.3</td>
<td>-2.3</td>
<td>-0.5</td>
<td>0.3</td>
<td>-2.0</td>
<td>0.7</td>
<td>Prominent forehead</td>
</tr>
<tr>
<td>6</td>
<td>PTPN11</td>
<td>M</td>
<td>4.0</td>
<td>-0.3</td>
<td>-2.6</td>
<td>-0.5</td>
<td>-0.5</td>
<td>1.5</td>
<td>0.0</td>
<td>Epicanthus</td>
</tr>
<tr>
<td>7</td>
<td>COL2A1/NF1</td>
<td>F</td>
<td>2.7</td>
<td>-0.1</td>
<td>-3.4</td>
<td>-0.1</td>
<td>1.4</td>
<td>-2.5</td>
<td>-0.4</td>
<td>Clinodactyly</td>
</tr>
<tr>
<td>8</td>
<td>ACAN</td>
<td>F</td>
<td>9.3</td>
<td>-0.7</td>
<td>-3.3</td>
<td>3.6</td>
<td>0.2</td>
<td>-3.7</td>
<td>-3.9</td>
<td>High palate, epicanthus</td>
</tr>
<tr>
<td>9</td>
<td>SHOX</td>
<td>F</td>
<td>9.3</td>
<td>0.5</td>
<td>-2.3</td>
<td>2.2</td>
<td>-0.1</td>
<td>-2.7</td>
<td>-0.3</td>
<td>Slight triangulation of the distal epiphysis of the radius at x-ray for bone age</td>
</tr>
<tr>
<td>10</td>
<td>FGFR3</td>
<td>F</td>
<td>17.2</td>
<td>NA</td>
<td>-3.9</td>
<td>3.6</td>
<td>1.4</td>
<td>-0.7</td>
<td>-3.9</td>
<td>Slight accentuation of lumbar lordosis at x-ray</td>
</tr>
<tr>
<td>11</td>
<td>IHH</td>
<td>M</td>
<td>7.5</td>
<td>-2.5</td>
<td>-2.6</td>
<td>0.0</td>
<td>0.3</td>
<td>-2.0</td>
<td>-0.6</td>
<td>Shortening of the middle phalanx of the fifth finger at x-ray for bone age</td>
</tr>
<tr>
<td>12</td>
<td>NPR2</td>
<td>M</td>
<td>9.7</td>
<td>-3.7</td>
<td>-2.8</td>
<td>-1.3</td>
<td>0.3</td>
<td>-2.2</td>
<td>-1.5</td>
<td>Mild epicanthus</td>
</tr>
<tr>
<td>13</td>
<td>BRAF</td>
<td>M</td>
<td>8.7</td>
<td>-2.2</td>
<td>-3.0</td>
<td>-0.7</td>
<td>-0.9</td>
<td>-2.0</td>
<td>-3.1</td>
<td>Slight accentuation of thoracic kyphosis</td>
</tr>
<tr>
<td>14</td>
<td>IHH</td>
<td>M</td>
<td>3.4</td>
<td>-0.7</td>
<td>-2.8</td>
<td>2.3</td>
<td>1.6</td>
<td>-1.2</td>
<td>-2.2</td>
<td>High palate, mild epicanthus, short fifth metacarpal</td>
</tr>
<tr>
<td>15</td>
<td>IHH</td>
<td>M</td>
<td>7.1</td>
<td>-1.3</td>
<td>-2.8</td>
<td>2.2</td>
<td>0.8</td>
<td>-0.2</td>
<td>-1.2</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>NF1</td>
<td>M</td>
<td>9.6</td>
<td>-5.9</td>
<td>-4.1</td>
<td>1.5</td>
<td>0.0</td>
<td>-3.0</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>FGFR3</td>
<td>M</td>
<td>5.1</td>
<td>-1.0</td>
<td>-2.8</td>
<td>2.2</td>
<td>-0.1</td>
<td>-1.5</td>
<td>-3.5</td>
<td>Mild epicanthus</td>
</tr>
</tbody>
</table>

* Affecting parent from who the patient inherited the variant; † This patient also has a VUS in the NPR2 gene; ‡ This patient carried only the SHOX variant; § This patient carried only the COL2A1 variant.

BA, bone age; CA, chronological age; H, height; SDS, standard deviation score; SH, sitting height.

In one remarkable study, Hauer et al. (8) evaluated 200 children with short stature by whole-exome sequencing (WES). A genetic diagnosis was obtained in 19 (14.2%) of 134 children initially classified as isolated short stature but which encompassed children born SGA or with syndromic features. In a recent study, Fan et al. (13) analyzed 561 patients with short stature by WES. In a subgroup of 257 patients classified as having isolated short stature due to the absence of associated organ malformation(s) or other clinical evidence for multisystem involvement, a genetic diagnosis was obtained in 11.3%. However, among the positive results, mutations in genes associated with hypopituitarism, skeletal dysplasia, and chronic and metabolic disorders were reported, making it unlikely that all investigated patients met the ISS criteria (13). In another recent study, Sentchordi-Montané et al. (10) evaluated 108 children with short stature and minor skeletal anomalies with a customized skeletal dysplasia NGS panel. Although

Table 4  Variants of uncertain significance identified by targeted panel sequencing and CNV analysis in 12/102 patients with ISS.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>Variant</th>
<th>MAF*</th>
<th>Inheritance pattern</th>
<th>ACMG/AMP (32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>NPR2</td>
<td>NM_003995.4:c.1922C&gt;T (p.Ser641Leu)</td>
<td>9.0 × 10⁻⁵</td>
<td>Mother&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VUS PM1, PM2, BP4</td>
</tr>
<tr>
<td>18</td>
<td>IGF1R</td>
<td>NM_000875.5:c.1139T&gt;G (p.Leu390Arg)</td>
<td>7.4 × 10⁻³</td>
<td>Unavailable</td>
<td>VUS PM3, BP5</td>
</tr>
<tr>
<td>19</td>
<td>NF1</td>
<td>NM_000267.3:c.169G&gt;A (p.Gly57Ser)</td>
<td>1.0 × 10⁻⁴</td>
<td>De novo</td>
<td>VUS PM6, BP2, BP4</td>
</tr>
<tr>
<td>20</td>
<td>IGF1R</td>
<td>NM_000875.5:c.3932C&gt;T (p.Ser1311Leu)</td>
<td>4.0 × 10⁻⁴</td>
<td>Father&lt;sup&gt;b&lt;/sup&gt;</td>
<td>VUS PM2, PP2, BP3</td>
</tr>
<tr>
<td>22</td>
<td>NPR2</td>
<td>NM_000399.5:c.2644G&gt;A (p.Val882Ile)</td>
<td>2.0 × 10⁻³</td>
<td>Unavailable</td>
<td>VUS PM3, BP4</td>
</tr>
<tr>
<td>23</td>
<td>COL2A1</td>
<td>NM_000184.5:c.550G&gt;A (p.Ala184Thr)</td>
<td>4.3 × 10⁻³</td>
<td>Mother&lt;sup&gt;e&lt;/sup&gt;</td>
<td>VUS PM1, PM2, BP4</td>
</tr>
<tr>
<td>24</td>
<td>IGF1R</td>
<td>NM_000875.5:c.2719G&gt;T (p.Ala976Ser)</td>
<td>9.0 × 10⁻⁴</td>
<td>Mother&lt;sup&gt;e&lt;/sup&gt;</td>
<td>VUS PM2, PP2, BP3</td>
</tr>
<tr>
<td>25</td>
<td>GHR</td>
<td>NM_000163.5:c.1156C&gt;T (p.Arg386Cys)</td>
<td>4.9 × 10⁻³</td>
<td>Mother&lt;sup&gt;e&lt;/sup&gt;</td>
<td>VUS PM2, PP2, BP, BS4</td>
</tr>
<tr>
<td>26</td>
<td>IGF1R</td>
<td>NM_000184.5:c.229A&gt;C (p.Arg757Ser)</td>
<td>1.0 × 10⁻⁴</td>
<td>Mother&lt;sup&gt;e&lt;/sup&gt;</td>
<td>VUS PM2, BP2, PP, BS4</td>
</tr>
<tr>
<td>27</td>
<td>NPR2</td>
<td>NM_000399.5:c.838C&gt;T (p.Arg280Trp)</td>
<td>5.9 × 10⁻⁵</td>
<td>Mother&lt;sup&gt;e&lt;/sup&gt;</td>
<td>VUS PM2, PP2, BP, BS4</td>
</tr>
<tr>
<td>28</td>
<td>IHH</td>
<td>NM_000218.4:c.685G&gt;A (p.Val229Met)</td>
<td>0</td>
<td>Father&lt;sup&gt;b&lt;/sup&gt;</td>
<td>VUS PM2, PP1, PP2, BP3</td>
</tr>
</tbody>
</table>

Patients 11, 14, 15 (22), and 28 (44) have been reported in recent studies as clinically characterized children with IHH variants.

*MAF, minor allele frequency based on the higher frequency observed in gnomAD (29) and ABraOM (30) database; † Affecting parent with short stature (height SDS ≤ 2).
most of these children met the criteria for ISS, about a third were born SGA. Additionally, defects in the SHOX were ruled out before patients had been included in the study. The diagnostic yield was 11.1%, considering only P/LP variants (10).

We can attribute the higher diagnostic yield of our study in part to the systematic analysis of CNVs. This analysis included the evaluation of the SHOX gene and its regulatory regions, whose haploinsufficiency is a well-known cause of short stature in patients with ISS (20). Three patients had P/LP SHOX-related CNVs, corresponding to 2.9% of our cohort. Additionally, our cohort had an enrichment of familial cases, which can be associated with an increased possibility to identify an autosomal-dominant genetic cause for short stature (9, 35). Since our protocol was not pre-selected based on the patients’ parental height, we hypothesized that there was a referral bias of familial cases to our outpatient clinic in a tertiary center. Nevertheless, the severity of short stature was the only variable significantly associated with a higher diagnostic rate in our cohort.

Most of the genetic defects found in our cohort of ISS (70%) were identified in genes related to the growth plate (ACAN, IHH, SHOX, NPYR2, COL2A1, FGFR3), similarly to previous studies (8, 12, 36). This finding emphasizes the role of defects in the endochondral ossification process leading to ISS phenotype. Children with defects in these genes usually inherited the genetic defect from a parent with short stature (9 of 13 cases); had mild body disproportion (7 of 13) and/or nonspecific skeletal abnormalities (7 of 13). However, these findings did not allow a differentiation between patients with alterations in the IHH, NPYR2, ACAN, or SHOX genes. Additionally, three children with a causative defect in a growth plate gene had normal body proportion and no skeletal findings; and two children with de novo germline mutations had parents with normal height. Thus, clinical and radiological data may be insufficient to guide a candidate gene approach due to their nonspecificity (Table 3).

It is noteworthy that five children had genetic variants related to the RAS-MAPK pathway. Three of them had a de novo mutation, including one detected in a mosaic state and two inherited the variant from a parent with ISS (Table 2). These children may have a mild presentation in a spectrum of phenotypes caused by defects in these genes. Other studies using the genomic approach in patients with short stature also identified NFI1 and PTPN11 mutations in patients without the typical clinical features (8, 11, 13, 14, 15). This data highlight the importance of the genetic diagnosis as it has an impact on the clinical follow-up and in the decision to start growth hormone therapy for these children.

Three patients have more than one P/LP genetic alteration that explains the short stature phenotype (Fig. 2). Multiple genetic diagnoses can occur in 1.4–7.2% of patients with Mendelian disorders (37). Additionally, two non-overlapping genetic diagnoses of growth disorders were previously reported in patients with syndromic short stature (8, 13, 38). The exact role of each variant in our patients’ phenotype was not well established.

In summary, a multigene sequencing approach is able to determine a genetic etiology of short stature in up to one in six children with ISS, removing the term idiopathic from their clinical classification (35). Knowing the genetic basis of these patients short stature can allow precise genetic counseling and clinical follow-up. It is particularly relevant to children harboring variants that can support the indication or be a contraindication of rhGH therapy. Based on the significant and progressive reduction of the costs of the genetic studies and their increasing diagnostic yield, we expect to reach the time when this diagnostic tool will be routinely offered in the assessment of children with ISS.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0214.

Declaration of interest
A A L J has received an independent research grant from BioMarin and consulting fees from Novo Nordisk. The other authors declare that they have no competing financial interest to declare.

Funding
This work was supported by Grants 2013/03236-5 (to A A L J) from the São Paulo Research Foundation (FAPESP); Grant 303294/2020-5 (to A A L J) from the National Council for Scientific and Technological Development (CNPq) and by Coordination of Superior Level Staff Improvement (CAPES; Finance Code 001 to N C B D, N L M A, and R C R).

Author contribution statement
N L M A and M F and A A L J and G A V contributed equally to this study.

Acknowledgements
The authors thank the Laboratorio de Sequenciamento em Larga Escala for their assistance with targeted panel sequencing and all collaborators, patients, and family members who made this study possible.

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