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ORIGINAL ARTICLE

Role of genetic investigation in the diagnosis of short stature in a cohort of Italian children

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Abstract

Background Short stature (SS) is defined as height more than 2 standard deviations below the mean for age and sex. Hypothyroidism, celiac disease, growth hormone deficiency, hormonal abnormalities, and genetic conditions are among its causes. A wide range of conditions often due to largely unknown genetic variants can elude conventional diagnostic workup.

Aim We used next-generation sequencing (NGS) to better understand the etiology of SS in a cohort of Italian children.

Patients and methods The study sample was 125 children with SS of unknown origin referred to our Institute between 2015 and 2021. All had undergone complete auxological and hormonal investigations to exclude common causes of SS. Genetic analysis was performed using a NGS panel of 104 genes. Clinical data were reviewed to clarify the pathogenicity of the variants detected.

Results In this cohort, 43 potentially causing variants were identified in 38 children. A syndromic genetic condition was diagnosed in 7: Noonan syndrome in 3, Leri–Weill syndrome in 3, and hypochondroplasia in 1. Moreover, 8 benign variants and other 37 like benign variants were found. In 88 children, 179 variants of uncertain significance (VUS) were identified. No variant was found in 16 children.

Conclusion Genetic analysis is a useful tool in the diagnostic workup of patients with SS, in adapting management and treatment, and in identifying syndromes with mild atypical clinical features. The role of VUS should not be underestimated, particularly when multiple VUS with possible mutual worsening effects are present in the same child.

Keywords Short stature (SS) · Next-generation sequencing (NGS) · Genetic analysis · Variants of uncertain significance (VUS) · Growth

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Introduction

Normal height is determined according to age, sex, ethnic origin, and family context [1]. Growth is a dynamic process; normal height at a given age does not necessarily mean that short stature (SS) may occur some time later during development [1]. In brief, growth can be considered one of the most important indicators of a child's health, and growth failure the first sign of an acute and/or chronic condition [2].

SS is defined as height less than − 2 standard deviations (SD) of the age- and sex-matched population [3, 4]. This definition is arbitrary, however, since it does not take into account parental target height (TH) or variation in bone rate maturation. Since children may be short for age but not for osseous maturation, the most common criteria for SS in clinical practice are: height below – 2 SD for age, sex, and ethnic origin; normal height (between ± 2 SD for the

general population) but > 2 SD below the growth curve of the patient's TH; a projected adult height (prediction of adult height) > 2 SD below the TH; and persistent low growth velocity [1].

Initial evaluation of a child with SS will include patient and family history taking, complete physical examination, and determination of bone age (BA). Some authors suggest that laboratory tests should be guided by clinical features rather than as routine in all patients with SS [5], while others recommend a screening set of laboratory investigations [1, 6]. Laboratory analysis may help to differentiate between primary causes of SS, such as syndromic and/or genetic defects (i.e., Turner syndrome, *SHOX* defects, skeletal dysplasia) [7–9], and secondary growth deficits due to endocrine or other chronic disorders such as celiac disease, Crohn's disease, malnutrition, and renal diseases [10, 11]. If no signs of disease are found after complete evaluation by a pediatric endocrinologist, including stimulated growth hormone (GH) levels, the SS is defined as idiopathic [12–14].

Idiopathic short stature (ISS) comprises a wide range of conditions associated with SS that elude conventional diagnostic workup and often result from still largely unknown genetic variants. In the last decade, with advances in diagnostic techniques, researchers have discovered causal variants in the genes involved in the function of the GH/insulin-like growth factor-I (IGF-1) axis and in growth plate physiology. A genetic cause has recently been identified in 44% of syndromic short patients and in approximately 14% of patients with ISS but without a specific clinical phenotype [15, 16]. Summarizing, identifying the genetic etiology of SS can aid in clinical management and improvement of final stature. For this study, we applied genetic testing to better understand the etiology of SS in a cohort of Italian children referred to our pediatric endocrinology service.

Patients and methods

Patients

For this retrospective study, the sample was 125 children with SS referred to the Pediatric Endocrinology Division, Hospital of Verona, Italy, between 2015 and 2021. Genetic evaluation was performed only in children aged between 2 and 18 years of age with SS of unknown origin.

Patients met at least one of the following inclusion criteria:

- height below – 2.5 SD for age, sex, and ethnic origin
- height > 2 SD below the growth curve of the patient's TH
- a projected adult height > 2 SD below the TH
- family history for SS in first- or second-degree relatives
- idiopathic GH deficiency unresponsive to replacement therapy after at least 1 year of treatment
- non-specific dysmorphisms for a particular disorder but suggestive of genetic etiology

Children with known causes that could explain their SS were excluded. All patients were examined by local pediatric endocrinologists and had undergone auxological, hormonal, and imaging investigations, to exclude common causes of SS. Evaluation included measurement by the same investigator of body weight (kg), height (cm) using a Harpenden stadiometer, Body Mass Index (BMI, weight in kg divided by height in meters squared), sitting height (cm), arm span (cm), and signs of pubertal development. The children were examined for features of body asymmetry and disproportion, microcephaly or relative macrocephaly, heart murmur, cryptorchidism, and muscular hypertrophy. TH was determined by calculating the mid-parental height according to the formula: ([father's height cm – 13 cm] + mother's height cm)/2 for girls; ([mother's height cm + 13 cm] + father's height cm)/2 for boys [17].

Laboratory analysis included blood count, renal and liver function tests, electrolytes, IGF-1, TSH, fT4, anti-transglutaminase, total IgA, and GH stimulation tests when a GH deficiency was clinically suspected. When dysmorphic features or disproportionate growth suggested a genetic cause and an endocrine cause was highly unlikely, radiography of the spine, pelvis, and knee was performed to evaluate clinical signs suggestive of skeletal dysplasia. X-ray films were evaluated by the same clinician with experience in bone genetic disorders. Patients with inconclusive results not clearly attributable to a known form of skeletal dysplasia were included in the study. At first evaluation and every year thereafter, all children underwent radiography to determine BA, evaluated by the same pediatric endocrinologist using the Greulich and Pyle method [18]. Final adult height was estimated based on BA, according to Bayley and Pinneau tables [19].

Gestational age (GA), birth weight (BW), and birth length (BL) were retrospectively evaluated. Children born small for gestational age (SGA) were categorized according to Bertino Neonatal Anthropometric Charts [20]. We defined SGA a newborn with a BW and/or BL less than – 2 SD according to the Consensus Statement of the International Societies of Pediatric Endocrinology and of the Growth Hormone Research Society [21].

The study was conducted in compliance with the terms of the Helsinki II Declaration. The Institutional Ethics Committee of the provinces of Verona and Rovigo, Italy, took note of the retrospective design of the study and approved the results for publication. Written informed consent was obtained from the parents or the guardians of each patient.

Assay

Serum IGF-1 was measured using a one-step sandwich chemiluminescence immunoassay (CLIA, LIAISON Analyzer, DiaSorin, Vercelli, Italy). Analytical sensitivity was 3 ng/mL. Intra-assay and inter-assay coefficients of variation were 4.3% and 7.1%, respectively.

The other assays for the detection of GH deficiency are described elsewhere [22].

Genotype

Genomic DNA was extracted from peripheral blood leucocytes by means of a DNA Blood Midi Kit via the QIASymphony platform (Qiagen, Hilden, Germany). Nucleic acid quantity/quality was checked using a Nanodrop and Qubit® 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Genetic screening was performed using a multigene next-generation sequencing (NGS) panel, comprising a total of 104 genes related to SS. We used a SOPHiA Panel Custom ID: CSSD – 2242 (Sophia Genetics, Saint-Sulpice, CH), a capture-based system that simultaneously identifies single nucleotide variants (SNVs), indels, and copy number variations (CNVs) in genes with very high coverage uniformity. Library runs were performed on a 600-cycle format V3 flowcell, sequenced via an Illumina MiSeq DX platform according to the Illumina and the SOPHiA Genetics protocol.

The sequencing data were simultaneously processed using SOPHiA DDM software (DDM), updated to the last available version at the time of sequencing.

Variants are reported using the international standard Human Genome Variation Society (HGVS) nomenclature and classified into five categories according to the American College of Medical Genetics and Genomics criteria [23]: pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB), and benign (B). For this purpose, we used the NCBI database (ClinVar, dbSNP) and the Human Gene Mutation Database (HGMD). To evaluate the potential pathogenicity of VUS we used "in silico" predictors such as PolyPhen-2, MutationTaster, SIFT and the Human Splice Finder (HSF3.1), when available.

All gene variants interpreted as P or LP were confirmed by Sanger sequencing performed with predesigned primers and the BigDye Direct Cycle Sequencing Kit (Thermo Fischer Scientific). They were sequenced on an Applied Biosystems 3730xL Genetic Analyzer platform (Thermo Fisher Scientific) and the results were analyzed with SeqScape3 software (Thermo Fisher Scientific). When NGS identified big deletions/duplications of the regions (data analyzed with Sophia DDM software, minimum resolution 1 exon), CNVs were confirmed by MLPA (MRC-Holland, Amsterdam, NL) and analyzed with Coffalyser.Net software or Real-Time PCR (LightCycler® 480 Instrument, Roche, Basel, CH).

Patients with variants in genes known to be associated with SS were re-evaluated for associated clinical features in the respective instances by clinicians.

The genes in the NGS panel for SS were: *ACAN* (NM_013227.3); *ALMS1* (ENST000000264448.6); *ANKRD11* (NM_001256182.1); *ARID1A* (NM_006015.4); *ARID1B* (NM_001346813.1); *ARNT2* (NM_014862.3); *ATR* (NM_001184.3); *ATRIP* (NM_130384.2); *BLM* (NM_000057.2); *BRAF* (NM_004333.4); *CBL* (NM_005188.2); *CCDC8* (NM_032040.4); *CENPJ* (NM_018451.4); *CEP152* (NM_001194998.1); *CEP63* (NM_025180.3); *CHD7* (NM_017780.3); *COL2A1* (NM_001844.4); *COL9A1* (NM_001851.4); *COL9A2* ((NM_1852.3); *COL9A3* (NM_1853.3); *COL10A1* (NM_000493.3); *COMP* (NM_000095.2); *CREBBP* (NM_004380.2); *CRIPT* (NM_014171.4); *CUL7* (NM_001168370.1); *DNA2* (NM_1080449.2); *DVL1* (NM_004421.2); *EP300* (NM_001429.3); *ERCC8* (NM_000082.3); *FGD1* (NM_004463.2); *FBN1* (NM_000138.4); *FGF8* (NM_033163.3); *FGFR1* (NM_023110.2); *FGFR3* (NM_000142.4); *GH1* (NM_000515.4); *GHR* (NM_000163.4); *GHRHR* (NM_000823.3); *GLI2* (NM_005270.4); *GLI3* (NM_000168.5); *GNAS* (NM_080425.3); *GPR161* (NM_001267609.1); *HDAC8* (NM_018486.2); *HESX1* (NM_003865.2); *HMGA2* (NM_003483.4); *HRAS* (NM_005343.2); *HSPG2* (NM_001291860.1); *IGF-1* (NM_001111283.2); *IGF1R* (NM_000875.3); *IGF2* (NM_001127598.2); *IGFALS* (NM_001146006.1); *IGSF1* (NM_001170961.1); *IHH* (NM_002181.3); *KDM6A* (NM_001291415.1); *KRAS* (NM_033360.2); *LARP7* (NM_001267939.1); *LHX3* (NM_016564.4); *LHX4* (NM_033343.3); *LMNA* (NM_170707.2); *MATN3* (NM_002381.4); *MLL2/KMT2D* (NM_003482.3); *NIPBL* (NM_133433.3); *NPR2* (NM_003995.3); *NRAS* (NM_002524.3); *NSMCE2* (NM_001349486.1); *OBSL1* (NM_015311.2); *OTX2* (NM_021728.3); *PAPPA2* (NM_020318.2); *PCNT* (NM_006031.5); *PDE4D* (NM_001146031.1); *PITX2* (NM_001204397.1); *POC1A* (NM_015426.4); *POU1F1* (NM_001122757.2); *PRKR1A* (NM_001276289.1); *PROK2* (NM_001126128.1); *PROKR2* (NM_144773.2); *PTPN11* (NM_002834.3); *RAD21* (NM_006265.2); *RAF1* (NM_001354689.1); *RBBP8* (NM_002894.2); *RIT1* (NM_001256821.1); *RNPCR* (NM_017619.3); *ROR2* (NM_004560.3); *SHH* (NM_000193.3); *SHOC2* (NM_007373.3); *SHOX* (NM_006883.2); *SMARCA4* (NM_001128849.1); *SMARCAL1* (NM_014140.3); *SMARCB1* (NM_003073.3); *SMARCE1* (NM_003079.4); *SMC1A* (NM_006306.3); *SMC3* (NM_005445.3); *SOCS1* (NM_003745.1); *SOS1* (NM_005633.3); *SOX2* (NM_003106.3); *SOX3*

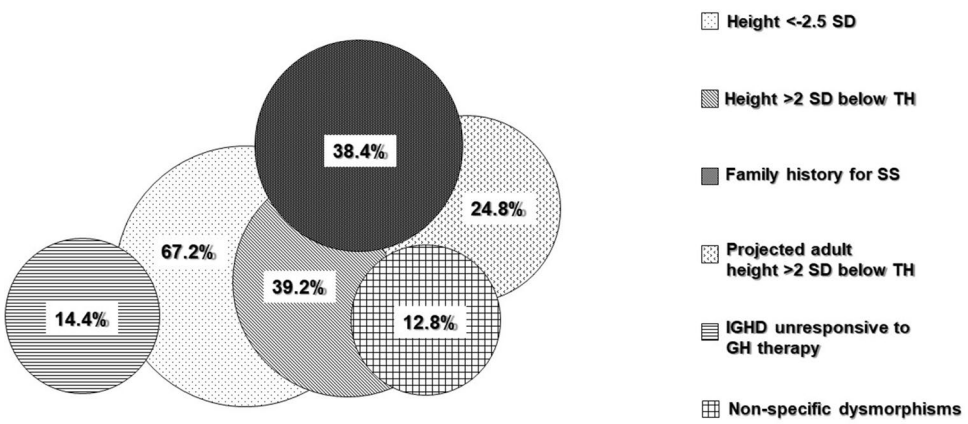

Fig. 1 Cohort characteristics in relation to the inclusion criteria. *TH* denotes target height, *SS* short stature, *IGHD* idiopathic growth hormone deficiency, *GH* growth hormone

Table 1 Neonatal data of the cohort

Characteristic	Total ($n=125$)	Females ($n=58$)	Males ($n=67$)
Gestational age (week)	37.96 ± 2.74	38.17 ± 2.28	37.77 ± 3.09
Birth weight (g)	2818.5 ± 673.41	2819.0 ± 574.60	2818.1 ± 753.99
SDS birth weight	−0.64 ± 1.08	−0.58 ± 0.95	−0.70 ± 1.19
Birth length (cm)	47.72 ± 3.09	47.71 ± 1.76	47.72 ± 3.88
SDS birth length	−0.75 ± 1.08	−0.67 ± 0.84	−0.81 ± 1.25
Head circumference (cm)	33.17 ± 2.76	33.24 ± 2.06	33.10 ± 3.33
SGA (n^o)	20	6	14
SGA for weight (n^o)	15	3	12
SGA for length (n^o)	11	3	8

SGA denotes small for gestational age, *SDS* standard deviation score, n^o number

(NM_005634.2); *SOX9* (NM_000346.3); *SOX11* (NM_003108.3); *SRCAP* (NM_006662.2); *STAT5B* (NM_012448.3); *TRAIP* (NM_005879.2); *TRIM37* (NM_015294.3); *WDR11* (NM_018117.11); *WNT5A* (NM_003392.4); *XRCC4* (NM_022406.2).

Statistical analysis

Statistical analysis was performed using IBM Corp 2017, IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA. Normal distribution was determined with the Kolmogorov–Smirnov test. Groups were compared using either the two-tailed Student's *t* test or the Mann–Whitney *U* test, when appropriate. Data are expressed in numbers with frequency, median plus range, or mean ± standard deviation (SD), as appropriate. Statistical significance was set at $p<0.05$; all tests were two-sided.

Results

Descriptive results

The study cohort was 125 children (53.6% boys and 46.4% girls). Figure 1 presents the sample characteristics in relation to the inclusion criteria. Table 1 presents the neonatal

Table 2 Auxological data at the enrollment

Characteristic	Total ($n=125$)	Females ($n=58$)	Males ($n=67$)
Age (years)	11.93 ± 3.43	11.65 ± 3.19	12.18 ± 3.63
SDS height	−2.77 ± 0.79	−2.67 ± 0.73	−2.86 ± 0.84
Target height (cm)	163.96 ± 8.12	157.37 ± 5.24	169.79 ± 5.03
SDS target height	−1.01 ± 0.85	−0.92 ± 0.89	−1.08 ± 0.81
ΔSDS Target height—height	−1.77 ± 0.04	−1.76 ± 0.50	−1.79 ± 0.50

SDS denotes standard deviation score

data and Table 2 the auxological data at enrollment. GH deficiency was diagnosed in 41 (32.8%) children but only 18 (14.4%) began GH treatment. Since growth rate was regular and constant in the remaining children, no treatment was initiated in accordance with the Italian guidelines for GH treatment [24]. Based on genetic analysis, other 14 (11.2%) children began GH treatment. Radiographic evaluation of the spine, pelvis, and knee was performed in 12 (9.6%) children because their clinical evaluation suggested skeletal dysplasia.

Genetic results

Overall, 267 gene variants were identified, of which 43 (16.1%) might be classified as potentially causing variants; these variants were identified in 38 (30.4%) children. Thirteen were P (4.9%), already described in the literature. Eight variants (3.0%) were categorized as probably causing variants because either not present in the common reference databases or classified as VUS, which previous studies associated with SS or a parent with SS and the same mutation as the proband. Finally, we identified other 22 (8.2%) variants as possibly causing variants because, although not present in the common reference databases or classified as VUS, they are described as pathogenic in all the in silico prediction models analyzed.

In detail, the P variants were identified in 12 (9.6%) children (1 child had 2 P variants); probably causing variants were identified in 7 (5.6%) children (since 1 child with this type of variant also had a P variant), and the possibly causing variants in 19 (15.2%) children (since 1 patient with this type of variant also had a P variant and 2 other patients had 2 variants of this type each). The characteristics of the genes in which these variants were identified are presented in supplementary table 1. Table 3 presents the clinical data of the children in which a variant was identified.

A syndromic condition underlying SS was identified in 7 children: Noonan syndrome in 3; Leri–Weill syndrome in 3; and hypochondroplasia in 1. Although the girl affected by hypochondroplasia presented a typical phenotype of this clinical condition, characterized by SS, stocky build, disproportionately short arms and legs and macrocephaly, a previous specific genetic analysis excluded this clinical condition. As consequence, this child was enrolled in the present study, and a P variant identified. Two of 3 children affected by Noonan syndrome showed a PTPN11 variant; both displayed a relatively mild clinical expression of the syndrome without cardiac defect or specific dysmorphisms. One of them had a GH deficiency, for which she was already being treated with poor results. The other child with a Noonan syndrome presented a variant in NRAS gene and has not the clinical features typical of the syndrome. His twin brother had normal height. Finally, 3 children with Leri–Weill syndrome presented the SS as the only clinical sign of their clinical condition, but all 3 had fathers with SS. One of them had a GH deficiency, for which he had already been treated at traditional dose with poor results.

Potentially causing variants were most frequently found in *ACAN*, *SHOX* and *FBN1* genes (Fig. 2). None of the children with these variants displayed a classic phenotype. Clinical features of familial osteochondritis dissecans were noted only in one of the children with *ACAN* variants: his mother had the same variant and presented a similar phenotype, and his sister was also affected but presented only SS as phenotype. The mean height of children with potentially causing variants was -2.70 ± 0.82 SD; 18.4% presented a variable degree of psychomotor delay and 15.8% were born SGA. GH deficiency unresponsive to the therapy was found in 7 children (18.4%). No significant differences were found in the auxological parameters between these children and those in which no potentially causing variants were identified.

Eight variants were classified as B (3.0%) and 37 as LB variants (13.8%) (Supplementary table 2). B variants were more frequently detected in *ARID1B* [3] and *FGFR3* [2] genes, whereas LB variants were found mainly in *PAPPA2* [6] and *ACAN* [4] genes. The remaining 179 variants (67%) were classified as VUS and identified in 88 (70.4%) children. The effects of VUS remain uncertain. *HSPG2* [13], *ACAN* [8], and *KMT2D* [8] genes presented more frequently VUS. Only VUS were detected in 45 (36%) children and only B or LB variants in 6 (4.8%). P or LP variants were detected in 28 (11.2%) children along with VUS or B or LB variants. Most of the children with VUS presented only one VUS, but 61.4% of them (and 43.2% of the whole cohort) presented two or more VUS; this association might play a more complex role in the etiology of SS (Fig. 3).

No variant in any of the genes was detected in 16 (12.8%) children. Their mean height was -3.14 ± 0.91 SD. Severe SS (< – 4 SDS) was noted in 2 children; moreover, 2 were born SGA and 1 child was born moderately premature at 28 weeks GA. Their SDS height was much lower than their SDS TH compared to the children with potentially causing variants and those with VUS or B and LB variants ($p < 0.05$). Psychomotor delay was noted in 3 children and mild dysmorphic features in 2. GH deficiency with very poor response to treatment was recorded for 3 children.

A possible classification of patients, according to the different types of variant detected, is summarized in Table 4.

Table 3 Clinical data of the patients in which pathogenic or likely pathogenic variants were identified

Pat	Incl. Crit	Age (y)	Sex	TH	H	ΔTH-H	ΔBA-A	BW	BL	SGA	GHD	GH treat	IGF-1	Gene	DNA variant	Protein variant	ZYG	INHER	Other variants	Note
Pathogenic variants																				
1	1, 4	3.08	M	−1.54	−3.15	−1.61	0	−0.17	−1.29	No	No	No	−1.65	ACAN	Del nt2267-10-nt2695 + nucleotidic variant c.2652del	p.(gln885Serfs*60)	Het	AD	–	
2	1, 3, 4	8.75	M	−0.73	−2.99	−2.26	0.25	−0.53	−0.73	No	Yes	No	−0.13	ACAN	c.706C > T	p.(Arg236*)	Het	AD	–	Advanced bone age
3	4, 5	12.38	M	−1.37	−2.48	−1.11	0	−1.92	−1.76	No	Yes	Yes	−1.54	SHOX	Del L25088-L30792		Het	AD	–	
4	4	8.89	F	−2.49	−2.31	0.18	1	−0.32	0.37	No	Yes	No	1.29	SHOX	Dupl L25088-L25091		Het	AD	KMT2D SMARCA 4 ROR2 PCNT	ADHD, facial dysmorphism
5	1, 4	6.01	F	−2.49	−2.77	−0.28	0	0.47	−0.6	No	Yes	No	0.58	SHOX	Dupl L25088-L25091		Het	AD	KMT2D SMARCA 4 ROR2 PCNT	Beta thalassemia, facial dysmorphism
6	4	7.41	M	−1.05	−2.05	−1	−1.25	−0.02	0.26	No	No	No	0.04	COL9A1	c.2260-2A > G	p.?	Het	AD	COL2A1[c]ATRIP SRCAP EP300	
7	1, 2, 3	4.32	F	−0.79	−3.11	−2.32	−0.75	−0.48	−1.31	No	Yes	Yes	−4.55	GH1	c.626G > A	p.(Arg209His)	Het	AR/AD	-	
8	3	10.14	M	−0.81	−1.67	−0.86	0.67	−0.29	−0.29	No	No	No	1.62	NPR2 DNA2	c.2761C > T c.2083G > T	p.(Gly695*) p.(Arg921*)	Het Het	AR/AD AR	PROKR2[a], ALMS[a], COL9A3[a], HSPG2[b]	
9	1	5.4	F	−0.87	−2.59	−1.72	−2.0	0.95	0.71	No	Yes	No	0.62	PTPN11 OBSL1	c.205G > C c.1273dupA	p.(Glu69Gln) p.(Thr425Asnfs*40)	Het Het	AD AR	SMARCE1 GHR HDAC8	
10	1, 3, 5	6.83	M	−0.57	−2.68	−2.11	0	1.13	−0.1	No	Yes	No	−1.38	PTPN11	c.923A > G	p.(Asn308Ser)	Het	AD	ATR LMNA XRCC4	Psychomotor retardation
11	1	7.63	M	−1.37	−2.59	−1.22	−1.25	−0.5	−0.8	No	Yes	No	0.19	CENPJ	c.2117_2118del	p.(Ser706*)	Het	AR	ATRIP, GNAS[b]	
12	4	2.09	F	−2.41	−1.46	0.95	0.5	0.87	0.48	No	No	No	−1.37	FGFR3	c.806G > T	p.(Ser269Ile)	Het	AD	–	–
Probably causing variants																				
13	1	11.93	M	−1.21	−3.18	−1.97	−5.0	−0.74	−1.21	No	Yes	Yes	−0.8	ACAN	c.7342G > A	p.(Gly2448Arg)	Het	AD	GHRHR	
14	1, 3, 4	9.19	M	−1.05	−3.39	−2.34	−3.25	−0.93	−1.31	No	No	No	1.37	NRAS	c.226G > T	p.(Glu76*)	Het	AD	ACAN ANKRD11[a] KMT2D[a] SOX3[a] CUL7[b] GLI3[a]	
15	1, 2	6.22	M	−0.4	−4.43	−4.03	NA	−2.18	−1.29	Yes	No	No	−0.37	CUL7	c.3293 T > G	p.(Leu1098Arg)	Het	AR	COL2A1[a]	Spondylometaephyseal dysplasia
16	1, 4	7.41	M	−1.54	−2.94	−1.40	0	−0.84	−1.26	No	No	No	−1.64	SMC3	c.1825A > G	p.(Met609Val)	Het	AD	–	
17	1, 6	8.32	M	NA	−2.88	NA	−3.0	−2.37	−1.50	Yes	No	No	1.20	GLI2	c.4332_4333delinsAT	p.(Met1444_Leu1445delinsIlePhe)	Het	AD	HSPG2, PDE4D	
18	1, 2, 3	9.7	M	−1.5	−3.95	−2.45	−2.0	0.47	0.46	No	No	No	−2.12	BLM	c.2371C > T	p.(Arg791Cys)	Het	AR	–	

Table 3 (continued)

Pat	Incl. Crit	Age (y)	Sex	TH	H	ΔTH-H	ΔBA-A	BW	BL	SGA	GHD	GH treat	IGF-1	Gene	DNA variant	Protein variant	ZYG	INHER	Other variants	Note
19	4	7.30	M	−1.32	−1.87	−0.55	NA	−1.70	NA	Yes	No	No	0.49	GHRHR	c.29 T > G	p.(Val10Gly)	Het	AR	GPR161 FGFR1	
Possibly causing variants																				
20	1, 2, 3, 4	5.73	F	−0.79	−4.06	−3.27	0.25	−1.13	−1.23	No	No	No	1.06	COMP	c.1860del	p.(Tyr621Ilefs*20)	Het	AD	CHD7 PCNT	–
21	1, 3	10.74	F	−0.44	−2.5	−2.06	−0.25	−0.51	−0.73	No	No	No	−0.45	COMP	c.700C > T	p.(Pro234Ser)	Het	AD	–	–
22	1	8.84	F	0.24	−1.5	−1.74	1.75	0.35	−0.12	No	No	No	2.34	ANKRD11	Del16q24.3 (89,295,307)-16q24.3(89,446,407)		Het	AD	OBSL1	Precocious puberty
23	1, 4	7.28	M	−2.26	−3.92	−1.66	−3.25	−0.10	−1.29	No	Yes	No	−1.53	FBN1	c.2780 T > C	p.(Val927Ala)	Het	AD	SOX3 FBN1	
24	1, 4	3.99	F	−2.66	−2.97	−0.31	−1.00	−0.70	NA	No	No	No	0.84	COL11A1	c.1645A > C	p.(Thr549Pro)	Het	AD	SRCAP TRIM37	
25	1, 2, 4	2.06	F	−1.64	−2.68	−1.04	NA	−2.44	−1.61	Yes	No	No	−0.74	NPR2	c.2644G > A	p.(Val882Ile)	Het	AD/AR	WNT5A[a]	
26	1,2, 4	3.41	M	−1.21	−3.47	−2.26	−1.5	0.16	−0.80	No	No	No	−0.71	IHH	c.904C > T	p.(Gln302*)	Het	AR	GLI3[a]	
27	1	4.38	M	−1.45	−3.33	−1.88	−1.0	−0.62	−1.23	No	No	No	−1.97	GHR	c.899C > T	p.(Pro300Leu)	Het	AR	PAPPA2	
28	2	5.1	M	−0,16	−2.4	−2.24	−1.5	−2.51	NA	Yes	No	No	0.39	PCNT	c.366 + 2del	p.?	Het	AR	GLI3[a], IGF1R, SMARCA4[a]	
29	1	10.26	F	−1.47	−2.91	−1.44	−1.5	0.61	−0.9	No	Yes	No	−1.78	FBN1; PCNT	c.5123G > A; c.8044C > T	p.(Gly1708Glu); p.(Arg2682Trp)	Het	AD; AR	IGFALS NPR2 WDR11 XRCC4[b]	Papillary breast lump
30	1, 2	13.07	F	−0.7	−3.79	−3.09	−0.5	−0.46	−0.9	No	Yes	Yes	−1.78	WDR11	c.377A > G	p.(Asp126Gly)	Het	AD	CHD7 ARID1A PAPPA2	
31	2	10.41	M	0	−1.86	−1.86	−0.75	1.22	0.95	No	Yes	No	0.91	SOS1; FNB1	c.734 T > C; c.2017C > T	p.(Ile245Thr); p.(Pro673Ser)	Het	AD	HSPG2[a] NIPBL RNPC3 ACAN GLI3[a]	Intellectual disability
32	1	8.75	M	−1.21	−2.74	−1.53	−1.13	−1.25	−0.4	No	Yes	No	1.22	FGFR1	c.231C > G	p.(Asn77Lys)	Het	AD	PROKR2 COL9A3 GLI3[b] BLM[a] HSPG2	
33	2	5.38	M	0,4	−2.38	−2.78	−2.40	−0.59	−0.33	No	Yes	No	−0.86	TRAIP	c.863_873del	p.(Val288Glyfs*21)	Het	AR	SRCAP[b], ATR	Obstructive hydrocephalus, hypospadias, strabismus, pes planus, inguinal hernia
34	2	2.26	M	0.49	−2.28	−2.77	−1.0	0.38	−0.51	No	Yes	No	−1.59	ACAN	c.922C > T	p.(Arg308Cys)	Het	AD	SRCAP IGF1R	
35	2	9.7	M	−0.16	−2.04	−1.88	0.5	1.59	NA	No	Yes	No	−0.60	COL2A1	c.2453G > C	p.(Arg818Pro)	Het	AD	COL10A1	Cortisol deficiency

Table 3 (continued)

Pat	Incl. Crit	Age (y)	Sex	TH	H	ΔTH-H	ΔBA-A	BW	BL	SGA	GHD	GH treat	IGF-1	Gene	DNA variant	Protein variant	ZYG	INHER	Other variants	Note
36	1	5.94	M	−1.86	−2.63	−0.77	−0.75	−1.55	−0.29	No	Yes	No	−0.19	GLI3	c.908G > A	p.(Ser303Asn)	Het	AD	ACAN GLI3 WDR11 CHD7[a]	Chondrodysplasia
37	6	12.16	M	−0.16	−0.17	−0.01	0.70	2.71	2.76	No	No	No	NA	ROR2	c.2089 T > C	p.(Ser697Pro)	Het	AD	–	Congenital right brachydactyly
38	2	6.50	M	0.09	−2.34	−2.25	−0.80	−3.54	NA	Yes	No	No	−1.00	CBL	c.305A > T	p.(Tyr102Phe)	Het	AD	–	

Pat patient, *Incl. Crit* inclusions criteria, *M* male, *F* female, *TH* target height, *H* height, Δ*TH-H* difference between target height and height, Δ*BA-A* difference between bone age and age, *BW* birth weight, *BL* birth length, *SGA* short for gestational age, *GHD* growth hormone deficiency, *GH treat* growth hormone treatment, *IGF-1* insulin-like growth factor 1, *ZYG* zygosis, *INHER* inheritance, *HET* heterozygote, *NA* data not available, *AR* autosomic recessive, *AD* autosomic dominant

[1]Height below -2.5 SD for age, sex and ethnic origin

[2]Height > 2 SD below the growth curve corresponding to the patient's target height,

[3]A projected adult height > 2 SDS below the target height,

[4]Familiarity for hyposomy in first- or second-degree relatives

[5]Idiopathic GH deficiency unresponsive to replacement therapy after at least one year of treatment

[6]Non-specific dysmorphisms for a particular disorder but suggestive of a possible genetic etiology

[a]VUS described as likely benign variant

[b]VUS described as benign variant

[c]VUS described but described as pathogenic in all the in silico prediction models

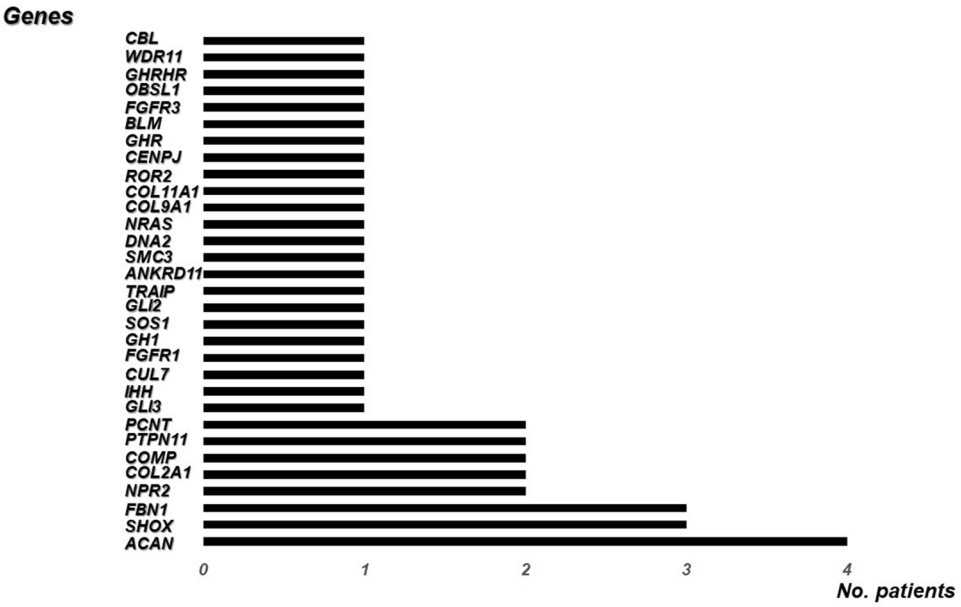

Fig. 2 Genes in which pathogenic and/or likely pathogenic variants were identified

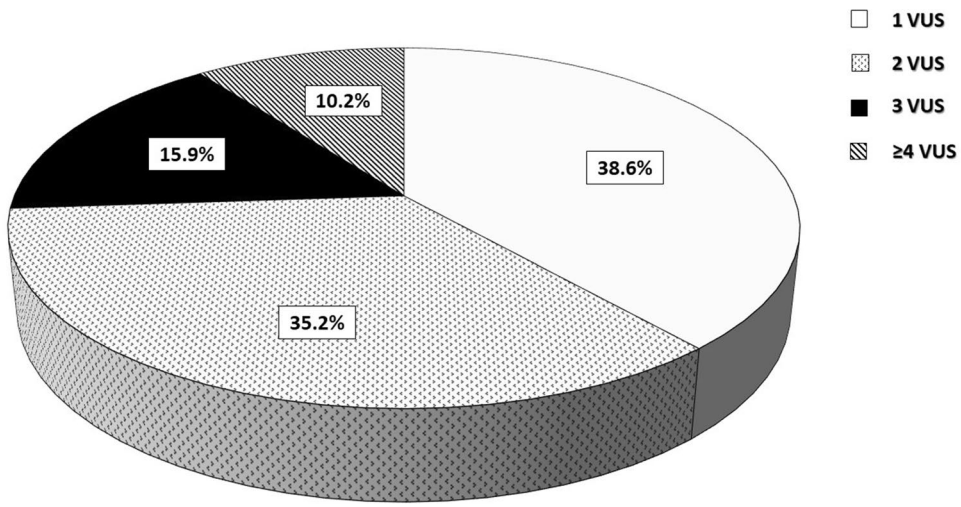

Fig. 3 Number of variants of uncertain significance identified in the cohort

Table 4 Representation of patients, in relation to the different types of variant detected

Group	Patients ($n=125$)	Males ($n=67$)	Females ($n=58$)
Children with P variants	12	7	5
Children with probably causing variants	7	7	0
Children with possibly causing variants	19	12	7
Children with syndromic condition	7	3	4
Children with B variants	8	2	6
Children with LB variants	30	16	14
Children with VUS	88	46	42
Children with only VUS	45	22	23
Children without variants and VUS	16	9	7

Discussion

Our study findings provide evidence that genetic analysis is useful in elucidating the etiology of SS in children without clinical signs or dysmorphic features suggestive of a specific pathology. With use of a specific NGS panel, we were able to identify potentially causing variants in 30.4% of the cohort. Nevertheless, these variants account for only 16.1% of those identified in the cohort. Most were VUS and often multiple VUS with a possible mutual worsening effect in the same child, suggesting that VUS may play a role in the genesis of SS. To our knowledge, ours is the first study to identify common variants associated with SS in a large cohort of Italian children.

Final height is known to be influenced by hormonal, nutritional, environmental, and genetics factors [1, 25]. Growth plate chondrogenesis is regulated by endocrine and paracrine factors, chondrocyte proliferation, and secretion of the cartilage extracellular matrix [26, 27]. Establishing a genetic etiology can aid in gaining a better understanding of the evolution of SS, weighing therapeutic options, predicting response to GH therapy, improving medical management and prognosis and recurrence risk counseling for patients and their family members [5, 28–30]. Finally, a definitive genetic diagnosis reassures families, which can finally deal with a recognized cause for the condition of their offspring; moreover, it allows an esteem of the height the children will achieve and a definition of the risk of the features that may be developed over lifetime [31]. Based on recently published data [5, 11], we can state that genetic investigation combined with clinical and hormonal examination plays a fundamental role in the diagnosis of SS. Obviously, given the complexity of the genomic analyses, we do not suggest to carry out genetic diagnostic research blindly, reducing the role of the clinic and the radiology. On the contrary, the genomics, the microarrays and the gene sequencing are required to complete a diagnostic process that includes the patient's visit, the evaluation of family pathologies, TH, hormonal values and BA. Therefore, genetic analysis takes on the duty of detecting variants that would otherwise would remain unknown.

Although the chance of identifying a genetic diagnosis increases correspondently to the severity of the SS, such as < – 3 SDS, not all children with SS need to undergo genetic testing. Our cohort reflects the recommended criteria for genetic testing: height < – 3 SD from the population or from mid-parental TH, syndromic features, body disproportion, and children born SGA without adequate catch-up growth [5, 31–33]. Moreover, children whose phenotype suggests a genetic cause should be included in the diagnostic workup: that is, children with congenital anomalies, dysmorphic features or intellectual disability [5, 30, 31, 34, 35]. When the preliminary clinical and laboratory analysis suggest a well-definite endocrine disorder, a specific molecular diagnosis is made, even though in the case of our patient affected by hypochondroplasia the first specific genetic analysis resulted negative. Anyway, most of SS children have not a peculiar phenotype which can allow an immediate diagnostic suspect; in some cases, typical dysmorphic features of a specific clinical condition may be absent, making it difficult, if not impossible, to raise a diagnostic suspicion. In these cases, NGS panel or the whole exome might be used. NGS panel include genes causing similar phenotype and other genes that impact on hormonal and basic cellular growth, permitting to elucidate the etiology of a growth disorder in 13.6–52% of cases; on the contrary, the whole-exome approach is more complete but also more challenging [32, 33, 36]. In this study, we used the NGS approach and when variants were described as pathogenic in all the in silico prediction models we analyzed, we considered them as possibly causing variants although they are not present in the common reference databases nor have been classified as VUS; the above reported percentage reflects this decision.

The percentage of short children in this cohort in which there was a potential genetic finding was slightly higher than that reported in previous studies [15, 37–41], though a molecular etiology was detected in up to 40% of cases involving selected populations that included syndromic patients [42–45]. Nevertheless, unlike other studies, our cohort was very heterogeneous because it included children born SGA, children presenting GH deficiency unresponsive to GH treatment, and children with several non-specific dysmorphisms. Furthermore, stature was also variable, within the normal range but at least 2 SD below the TH in some cases. These aspects render our findings more intriguing than previous studies that involved patient series with stature lower than – 2 SD.

Moreover, the most frequent syndromic conditions detected in our cohort were Noonan and Leri–Weill syndrome. Due to its wide phenotypic spectrum, even after complete pediatric endocrinology workup, Noonan syndrome may remain unrecognized and diagnosed as ISS or isolated GH deficiency [41]. Two patients presented variants in *PTPN11* and one presented a variant in the *NRAS* gene, all displaying mild features of the syndrome without heart disease. Finally, one child had a mutation in the *CBL* gene, which might be associated with a Noonan syndrome-like disorder [46]. The child had no evident dysmorphism and was born with low BW, which is unusual for Noonan syndrome typically characterized by postnatal growth retardation.

The three children with Leri–Weill syndrome presented alteration of the *SHOX* gene. The frequency of this genetic disorder is 2.4% in a population with SS [15] and 2–15% in children with ISS [26, 47]. *SHOX* haploinsufficiency usually affects growth plate function and is more often caused by copy number variant than by single nucleotide variants [48]. Good response can be achieved with higher GH dosage [47]. In our cohort, response to standard dose GH therapy was poor in one child initially diagnosed with GH deficiency. In contrast, initial benefit was obtained with higher dose (0.05 mg/kg/day) in two sisters who began GH therapy after genetic diagnosis. Summarizing, genetic analysis in short children can play a key role in adjusting the GH dose to promote growth, especially in children with *SHOX* deficit.

The most frequent P variants detected in our cohort concerned the *ACAN* gene; this observation is shared by recent studies on populations of diverse ethnicities that reported *ACAN* to be the most commonly mutated SS–associated gene [11, 49]. In addition, we identified the highest number of non-pathogenic variants in the *ACAN* gene. Such variants are associated with a range of severe to mild growth

defects in children born SGA or with normal birth size [26]. Typically, children with *ACAN* gene variants show advanced BA and reach an adult height of 150–152 cm without further dysmorphic features; GH treatment may be moderately effective [50, 51]. Some presented with joint problems and/or arthritis or osteochondritis dissecans [49]: only one child with variants in this gene presented advanced bone maturation, as reported elsewhere [49, 52, 53]. The child also displayed osteochondritis dissecans, a clinical condition shared by his mother. Three of the four patients with *ACAN* variants underwent GH therapy: one before, receiving a diagnosis of GH deficiency, and the other two after genetic diagnosis, but with poor outcome in all three cases. These findings suggest that the phenotype of patients with *ACAN* variants may be more variable than previously thought and that it probably depends on the type of variants identified.

Only six children born SGA (4.8% of our cohort) presented a potentially causing variant. This low proportion contrasts with previous data, according to which the frequency of genetic causes appears to be higher in short children born SGA than in children with ISS [53, 54]. A positive diagnostic yield of 15% was reported by a study involving 55 unexplained cases of SS in children born SGA, in which a targeted gene panel or exome was sequenced [32] and another more recent study raised this percentage to 42% on a total sample of 176 SGA children with SS [55]. However, such children may have methylation and array comparative genomic hybridization (CGH) abnormalities, which we did not investigate [56]. In 3 of the 6 SGA children in which we detected a potentially causing variant, the genes were associated with intrauterine growth restriction [57], whereas the *CBL*, *GHRHR* and *GLI2* genes are not usually correlated with this condition. Finally, our data confirm the hypothesis that short children born SGA are generally noted to have primary growth disorders due to growth plate alteration [11, 55, 58].

Only 3 (2.4%) patients carried potentially causing variants in the genes regulating the GH-IGF-1 axis, confirming the notion that variants in this axis are rarely a monogenic cause of SS. Genes regulating growth plate function are more often involved in SS [27]. Linear growth takes place in the growth plate; variants in some of these genes impair not only growth plate development and/or function but also non-skeletal structures in some cases, resulting in congenital anomalies, as seen in some children in this cohort. We speculate that mild forms of skeletal dysplasia, often interpreted as ISS, might be the most frequent cause of SS.

Not all patients in which we found a potentially causing variant manifested all the symptoms usually associated with the variant. Plausible explanations are the lack of clinical evidence for such new variants and their variable expressivity, resulting in a very mild phenotype and SS as the sole clinical manifestation. Finally, because some variants (4 in detail as shown in Table 3) are recessive and heterozygous, they alone cannot produce the clinical phenotype. In this context, we describe a child with a heterozygous probably causing variant in the *BLM* gene. If the child had been homozygous for this variant and, therefore, affected by Bloom syndrome [59], GH treatment would have been contraindicated. In such cases, genetic analysis is essential for identifying conditions in which GH treatment must not be considered.

The numerous VUS detected in our cohort raise curiosity. The main question is whether the presence of multiple VUS together or associated with a potentially causing variant might influence the phenotype of these patients: 73.7% of the children with a potentially causing variant presented at least one VUS; and 36% of the entire cohort presented only VUS, with most presenting two or more VUS simultaneously. The functional and clinical relevance of VUS identified by genetic testing remains to be elucidated. Nevertheless, we believe that multiple VUS with possible mutual worsening effect in the same child may have a role in the genesis of SS. Theoretically, functional studies are necessary to assess VUS pathogenicity. Furthermore, bio-informatic analysis is helpful and phenotype/genotype correlation or familial segregation is critical for interpretation of causation, as evidenced by our data analysis [5, 23].

No variant in any of the genes was found in 12.8% of the present cohort, although these children presented with severe SS and other features (dysmorphisms, SGA, prematurity) suggestive of a genetic etiology of their clinical condition. In addition, their height was much lower than their TH, further confirming our hypothesis for a genetic cause of their SS. We speculate that the SS was of polygenic etiology in many and that they had probably inherited common gene variants from both parents with small, multiple negative effects on stature [30]. While true that human height is heterogeneous and its heritability is reported to be approximately 80% [60], in some of our patients, particularly in those born SGA, methylation analysis or a full-exome study may be useful to identify other disorders such as Silver Russel or Temple syndrome [61]. However, Silver Russel syndrome was excluded in the children in which there was an elevated clinical suspicion of the syndrome. Finally, these children might benefit from a genetic evaluation and a genome-wide study.

Our study has several limitations. This heterogeneous cohort included children with SS in relation to their TH but not less than -2 SD and so did not meet the strict criteria for a diagnosis of ISS. Nevertheless, we believe that such heterogeneity reflects the population of short children with no definitive diagnosis. Another limitation may be the use

of a NGS panel rather than whole-exome sequencing for molecular analysis. The reasons for this choice were: exome sequencing was seldom used and quite expensive when we started the study; the primary aim was to determine the variants in the genes most frequently correlated with growth and not the incidence of all potential genetic defects in this cohort. That being said, the our NGS panel identified copy number variants and deletions and/or duplications of the exons; in some situations, such as when SHOX insufficiency was suspected, MLPA analysis was performed to better investigate deletions and/or duplications of the relevant gene coding regions. Finally, parental DNA samples were analyzed for some children with potentially causing variants but not for all. This choice was dictated solely by financial reasons, although we are aware that the examination of all the relatives could have clarified the role of some of the variants we identified.

In conclusion, the NGS panel enabled us to identify potentially causing variants in 30.4% of the present cohort. Genetic analysis proved useful in the diagnosis of SS in children without clinical signs or dysmorphic features suggestive of a specific pathology, in modifying clinical management and treatment decisions, and in identifying syndromes with mild, atypical clinical features, which had initially been erroneously diagnosed as ISS. Obviously, given the complexity of the genetic analysis, we do not suggest to carry out genetic research blindly, which instead should come as the last part of a complex diagnostic process including clinical, laboratory and radiological tests. Finally, we detected VUS in 70.4% of the cohort, and multiple VUS with possible mutual worsening effect in the same child in some cases. The functional and clinical relevance of this finding remains to be elucidated. We speculate that VUS, especially if numerous, may influence the growth process and be a cofactor in the etiology of the SS seen in this cohort.

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Author contributions All the authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Moreover, all the authors read and approved the final manuscript. In particular: PC conceived of the study, contributed to the preparation and critical review of the manuscript; SM, MA and RG wrote the manuscript; AG, AMB, MM, DC and AG contributed in the genetic analysis; FA, GP and AP conceived the study and participated in its coordination.

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Data availability The data is available in our university hospital, without difficulty. We have tried to report as much data as possible in the tables and supplementary files.

Declarations

Conflict of interest The authors declare no conflict of interest that could compromise the impartiality of the research reported and that no financial support was requested for this study.

Research involving human participants and/or animals The Institutional Ethics Committee of the provinces of Verona and Rovigo, Italy, took note of the retrospective design of the study and approved the results for publication.

Informed consent Written informed consent was obtained from the parents or the guardians of each patient.

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