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Short stature: Beyond growth hormone

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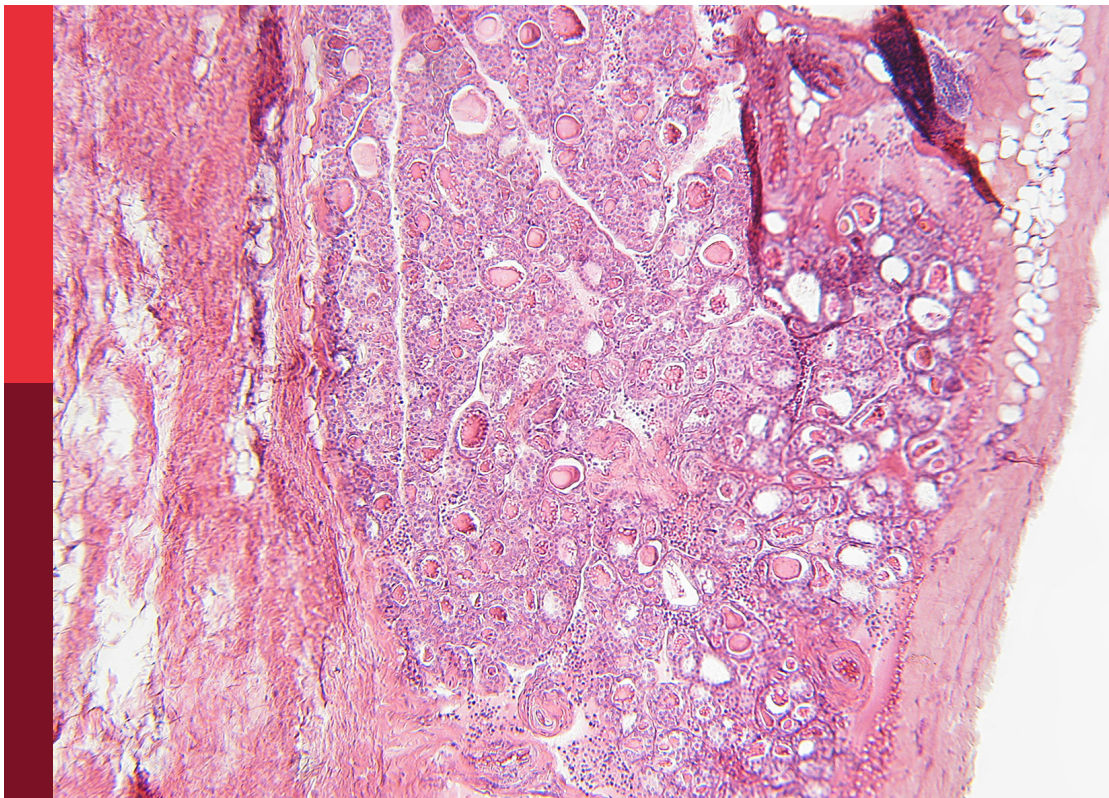

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Short stature: Beyond growth hormone

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Editorial: Short stature: beyond growth hormone

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Editorial on the Research Topic
Short stature: beyond growth hormone

Short stature is the most common cause of referral to pediatric endocrinology units (1) and can be defined as a multifactorial condition regulated by genetic, epigenetic, and environmental factors (2). Traditionally, growth hormone (GH) has been considered the main regulator of growth. However, as understanding of short stature pathogenesis advances, a new concept has been proposed: the role of GH in the regulation of growth is only one of many factors influencing growth plate physiology (3). Moreover, the diagnostic methods currently used to diagnose GH deficiency (GHD) are known to have low specificity, leading to frequent false positive results (4). Children with diagnosed GHD are therefore believed to have variable etiology of their growth disorder, frequently independent of GH secretion (5).

One of the major topics in current pediatric endocrinology is whether it is possible to improve the poor accuracy of GH stimulation tests. One possibility might be the optimization of sex-steroid priming (6). Partepone et al. presented a comprehensive review regarding this topic. The authors highlighted a close link between sex steroids and GH secretion leading to a higher probability of false positive results in children with delayed onset of puberty and consequent GH overtreatment. The same mechanism, on the other hand, may lead to a non-physiological GH peak, resulting in missing the diagnosis in children with real GH deficiency (GHD) in case sex-steroid priming is performed. So far, there is no agreement Regarding the indication and management of sex-steroid priming. Another issue that might lead to an inaccurate diagnosis of GHD is bone age (BA) evaluation. Delayed BA is mandatory before making the GHD diagnosis in some countries (7), however, the subjective nature of the evaluation is considered its main disadvantage. Maratova et al. evaluated an automated software for BA evaluation and proved its good accuracy.

A lasting controversy in the current way to diagnose GHD was supported by Plachy et al. Using next-generation sequencing methods, the authors genetically examined children with isolated growth hormone deficiency (GHD) and familial short stature. Interestingly, the genetic results frequently did not correspond with the previous diagnosis of GHD – 67% of children with a clinical diagnosis of GHD and a genetic etiology of short stature had proven primary growth plate disorder. Another point of view

on the same topic was presented by Lanzetta et al. In their retrospective analysis of children with a clinical and laboratory diagnosis of GHD, they compared children with or without an identifiable genetic, functional, or anatomical cause of GHD, namely definite GHD or short stature unresponsive to stimulation tests (SUS). These two groups differed significantly in pretreatment IGF-1 concentration and their increase after GH treatment initiation, in prevalence of pathological retesting, and of being overweight/obese at the end of treatment. However, the response to GH treatment in terms of near-adult height did not differ between the groups. Despite lasting doubts regarding the accuracy of GHD diagnostics, children diagnosed with "GHD" might profit from GH therapy even when another etiology of short stature is suspected.

The etiology of short stature other than GHD was covered by two other articles in our Research Topic. Mastromauro et al. wrote a review presenting growth hormone insensitivity (GHI) as a broad spectrum of disorders with a variable clinical picture. Since Laron described homozygous mutations in the gene for the GH receptor as the first mechanism causing GHI, many novel causes of GHI have been described, demonstrating the complexity of GHI and its role in the growth regulation. Another numerous and etiologically highly variable group of children are those born small for gestational age (SGA) with persistent short stature (8). In a retrospective study, Becker et al. compared clinical features and responses to GH treatment of SGA children with and without syndromic signs. They discovered that syndromic SGA children were shorter at the initiation of GH treatment, started GH therapy earlier, and reached a shorter adult height despite receiving higher doses of GH.

The etiology of growth disorders is, therefore, more complex than originally expected and is not just a matter of hormones. To understand it better, we must think far beyond GH.

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ORIGINAL RESEARCH
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Children With Short Stature Display Reduced *ACE2* Expression in Peripheral Blood Mononuclear Cells

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Background: The cause of short stature remains often unknown. The renin-angiotensin system contributes to growth regulation. Several groups reported that angiotensin-converting enzyme 2 (*ACE2*)-knockout mice weighed less than controls. Our case-control study aimed to investigate if children with short stature had reduced *ACE2* expression as compared to controls, and its significance.

Materials and Methods: children aged between 2 and 14 years were consecutively recruited in a University Hospital pediatric tertiary care center. Cases were children with short stature defined as height SD ≤ –2 diagnosed with growth hormone deficiency (GHD) or idiopathic short stature (ISS), before any treatment. Exclusion criteria were: acute diseases, kidney disease, endocrine or autoimmune disorders, precocious puberty, genetic syndromes, SGA history. *ACE* and *ACE2* expression were measured in peripheral blood mononuclear cells, angiotensins were measured by ELISA.

Results: Children with short stature displayed significantly lower *ACE2* expression, being 0.40 fold induction (0.01-2.27) as compared to controls, and higher *ACE/ACE2*, with no differences between GHD and ISS. *ACE2* expression was significantly and inversely associated with the risk of short stature, OR 0.26 (0.07-0.82), and it had a moderate accuracy to predict it, with an AUC of 0.73 (0.61-0.84). The cutoff of 0.45 fold induction of *ACE2* expression was the value best predicting short stature, identifying correctly 70% of the children.

Conclusions: Our study confirms the association between the reduction of *ACE2* expression and growth retardation. Further studies are needed to determine its diagnostic implications.

Keywords: ACE2, growth hormone deficiency, short stature, growth retardation, Renin-Angiotensin System, angiotensin, ANP

INTRODUCTION

Short stature is one of the most common reasons parents seek consultation with a growth specialist (1). Despite standard clinical and laboratory evaluation, a pathological cause is usually not found in up to 50-90% of cases, and children are eventually diagnosed as having constitutional delay of growth, familial short stature, or idiopathic short stature (2). Growth regulation is important not only per se but also because it seems associated with adult disease. In particular, fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women (3). It is well known that embryogenesis, fetal development, and postnatal growth are controlled by the coordinated action of different hormonal regulators. In addition to traditional growth hormones (GHRH/GH/IGF-1 axis), other peptide hormones, such as angiotensins, have been implicated in growth regulation (4).

The renin angiotensin system (RAS) is a pivotal regulator of vascular homeostasis. It is composed of different enzymes and peptides whose main function is the dynamic control of vascular function, blood pressure and fluid balance (5). Many of these components have opposing functions, such as angiotensin converting enzyme (ACE) that forms the vasoconstrictor Angiotensin II (AngII) and ACE2 that cleaves AngII, producing the vasodilator Angiotensin 1-7 (Ang1-7). In addition to the regulation of vascular function, AngII promotes inflammation, fibrosis and apoptosis, while Ang1-7 is associated with the opposite beneficial effects (5). Overall, RAS final effects depend on the activity of both ACE and ACE2, which determines the amount of circulating and tissue AngII and Ang1-7 (6). Interestingly, it has been argued that ACE2 may be even more important than ACE in some settings, such as the regulation of local levels of AngII and Ang1-7. For instance, in *ACE*-knockout mice, tissue AngII is not significantly modified because it is generated by non-ACE pathways (7), while in *ACE2*-knockout mice tissue AngII increases significantly, due to the lack of alternative pathways to ACE2 (8).

ACE2 was discovered in 2000 (9), and this was followed by the generation of *ACE2*-knockout mice to characterize its physiological functions. The first studies reported that these mice appeared healthy and fertile, apart from a marked defect in cardiac contractility that was observed by some Authors (8) and not by others (10), possibly due to a difference in *ACE2*-knockout genetic backgrounds. Further works were carried out in different laboratories to establish other functions of ACE2 (11–13). When we were studying the effects of ACE2 deficiency on glucose metabolism, we found that *ACE2*-knockout mice receiving a standard diet were smaller than wild-type mice, and this was not associated with differences in food intake, locomotor activity or heat production (13). Over time, several other groups have reported that *ACE2*-knockout mice weighed less than wild-type mice (14–19).

It remains to be clarified if there is an association between ACE2 deficiency and a defect of human growth. Based on this background, here we investigated if children with short stature displayed reduced expression of *ACE2* in peripheral blood mononuclear cells as compared to controls and its implications.

MATERIALS AND METHODS

Study Design and Population

This is an observational case-control study, aiming to compare children with short stature (either idiopathic or due to growth hormone deficiency) to respective controls. Subjects were consecutively recruited between October 2019 and June 2021 among the children aged between 2 and 14 years referred to the Clinica Pediatrica of the Institute of Maternal and Child Health 'Burlo Garofolo'. Cases were children with persistent short stature [height ≤ −2 standard deviations (SD)] after the second year of life, diagnosed with growth hormone deficiency or idiopathic short stature, before starting any treatment. GHD was diagnosed on the basis of failure to respond to 2 provocative tests of GH secretion (20). Controls were children with normal growth (height SD > −2 after the second year of life), mostly recruited among healthy children undergoing allergy testing. Exclusion criteria were history of any acute disease in the 3 weeks prior to enrollment, history of kidney disease, other endocrine or autoimmune disorders, precocious puberty, small for gestational age, as well as genetic syndromes. In particular, we excluded patients with dysmorphic features, major malformations, microcephaly, neurodevelopmental delay, intellectual disabilities, or skeletal dysplasia. Although the protocol was written before COVID-19 outbreak, after February 2020 we excluded also children with history of COVID-19 (including history of positive PCR test for SARS-CoV2 from nasal swab).

After providing the informed consent, children underwent a medical visit. History and anthropometric parameters were recorded. These included: birth weight, weight, height, body mass index (BMI), sitting height/height ratio (SH/H ratio), arm span and the ratio between upper and lower segment (U/L ratio), as well as systolic and diastolic blood pressure (SBP and DBP). Standard deviations (SD) of weight, height, and BMI were calculated with the Growth4 software and following the Italian growth charts reported by Cacciari et al. (21). The following laboratory parameters were also recorded: full blood count, erythrocyte sedimentation rate (ESR), glucose, creatinine and electrolytes, bicarbonate, alkaline phosphatase (ALP), albumin, TSH, free T4 (FT4), IGF-1, anti transglutaminase Ab and total IgA levels. Standard deviation of IGF-1 was calculated with the following formula: IGF-1 SD (Z-score)=[(log IGF-1 ng/L)-(log mean for age and sex)]/log mean SD (22). Then, all the children underwent a fasting blood sampling, after a day of rest, to collect 5 ml of whole venous blood and 5 ml of serum.

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board and Ethics Committee (CEUR-2019-Sper-115).

PBMC Isolation, Gene Expression Analysis, and ELISA

The gene expression of *ACE* and *ACE2* was measured in isolated peripheral blood mononuclear cells (PBMC). To isolate PBMC, blood samples were collected in EDTA-tubes and added to the same volume of Ficoll-PaqueTM Plus (Cytiva Sweden AB) and then centrifuged at 2400 rpm for 30 minutes at room

temperature. The mononuclear cell layer that was obtained was used to extract RNA.

PBMC were homogenized with 500 µl of Trizol (Invitrogen) per $5*10^5$ cells. In order to isolate mRNA, 100 µl of chloroform/isoamyl alcohol were added to each tube and the samples were vortexed for 15 seconds and left at room temperature for 5 minutes. Then samples were centrifuged at 13000 rpm for 20 minutes at 4°C and the upper aqueous phase was carefully collected to new tubes. In order to precipitate RNA, 250 µl of isopropanol were added to each tube and the tubes were briefly vortexed and left at −20°C overnight. The day after, samples were centrifuged at 13000 rpm for 15 minutes at 4°C to pellet the RNA precipitate. The supernatant was then carefully discarded and RNA was washed with 1ml of 75% ethanol and then centrifuged for 15 minutes at 13000 rpm. The supernatant was entirely removed, the RNA was resuspended in 20 µl of RNAse-free water and incubated at 55°C for 5 minutes, before quantifying RNA. RNA was treated with DNAse to eliminate DNA contamination (#AM-1906, Ambion DNA-free product), and 1.2 µg of treated RNA were subsequently used to synthesize cDNA with Superscript First-Strand synthesis system for RT-PCR (Gibco BRL). The expression of *ACE*, *ACE2* and *AT1R* (AngII type1 receptor) was evaluated with the TaqMan Gene Expression Assay (Life Technologies). Fluorescence for each cycle was quantitatively analyzed by StepOnePlus real-time PCR system (Applied Biosystems). Gene expression was normalized to 18s (TaqMan), and reported as a ratio compared with the level of expression in controls, which were given an arbitrary value of 1.

Serum AngII (Elabscience, E-EL-H0326) and Ang1-7 (Elabscience, E-EL-H5518), were measured by ELISA, according to manufacturer's instructions. Briefly, 50 µl of each standard or samples were added to the respective (AngII or Ang1-7) pre-coated plate and, immediately after, 50 µl of specific biotinylated detection antibody were added to each well. The plate was incubated at 37°C for 45 minutes. Fluid was aspirated and the plate washed for 3 times with the wash buffer before adding 100 µl of Avidin-Horseradish Peroxidases solution for 30 minutes at 37°C. Fluid was aspirated and plate washed for 3 times with wash buffer before 90 µl of substrate reagent for 15 minutes at 37°C, and then 50 µl of stop solution to end the reaction. Absorbance was taken at 450 nm.

Statistical Analyses

Sample size was calculated with openepi.com. To detect a mean difference in *ACE2* expression of 2 cycles (SD = 2) with a two-sided significance level of 5% and power of 80% with equal allocation to two groups would require 20 patients in each group. Based on this estimate, we decided to double this number taking into account the presence of GHD and ISS patients.

All statistical analyses were carried out in R system for statistical computing (Version 4.0.2; R development Core Team, 2020). Statistical significance was set at $p<0.05$. Shapiro-Wilk test was applied to continuous variables to check for distribution normality. Quantitative variables were reported as median with range (min-max) or mean ± standard deviation, depending on distribution. Categorical variables were reported as absolute frequencies and/or percentages. Continuous variables were compared by Mann-Whitney test (and Kruskall-Wallis test) or student t-test (and ANOVA), depending on data distribution and number of groups. Two multivariate regression models were performed: the first linear regression was performed to evaluate factors influencing *ACE2* expression and the second logistic regression to investigate if *ACE2* expression could predict being a child with short stature. Receiver operating characteristic (ROC) curves were evaluated to investigate the level of discrimination of *ACE2* expression in predicting short stature. The area under the curve (AUC) was calculated, with higher values indicating better discriminatory ability. The optimal thresholds for *ACE2* to differentiate between cases and controls, were calculated using Youden's index method. Sensitivity, specificity, and positive and negative predictive values with 95% confidence interval (CI) were then calculated (R packages: pROC and OpimalCutpoints).

RESULTS

General Characteristics

A total of 39 cases and 35 controls were recruited, whose characteristics are reported in **Table 1**. Median age of cases was 11 (3-14) years and median age of controls was 7.5 (3-14) years, as most of them were recruited among healthy children undergoing allergy testing. There were no differences in the proportion of boys and girls. Groups differed in terms of standard deviations (SD) of weight, height, and BMI, which were significantly lower in the group of children with short stature. This is consistent with the report that idiopathic prepubertal short stature might be associated with low BMI (23, 24). Hemoglobin, ESR, glucose, creatinine, electrolytes, bicarbonate, alkaline phosphatase, albumin, TSH values were within reference ranges in both groups. Screening for coeliac disease was negative in both groups. Children with short stature displayed significantly lower IGF-1 (SD) levels, being -1.81 [-4.21, 0.5] as compared to 0.17 (-2.42, 2.24) in the control group.

Renin-Angiotensin System Expression

Also *ACE2* gene expression was significantly lower in the group of children with short stature, being 0.40 (0.01-2.27) fold increase in cases and 1.00 (0.25-5.49) in controls (p-value <0.001), **Figure 1A**. Consistent with this, *ACE*/*ACE2* ratio was significantly higher in the group of children with short stature, being 3.85 (0.43-172.07) in cases vs 1.2 (0.13-10.11) in controls (p-value <0.001), **Figure 1B**. There were no differences in terms of *ACE* and *AT1R* expression, as well as AngII and Ang1-7 circulating levels and their ratio between the two groups.

Subgroup Analyses

To exclude that the difference in *ACE2* expression was confounded by the age of cases and controls, we identified a subgroup of 29 cases and a subgroup of 17 controls, who were matched by age (**Table 2**). Median age of cases was 11 (5-14) years and median age of controls was 9 (5-14) years. There were no differences in the proportion of boys and girls. Cases exhibited

TABLE 1 | General characteristics of whole cohort.

	Controls (n = 35)	Cases (n = 39)	p-value
Age (year)	7.5 [3, 14]	11 [3, 14]	<0.001
M/F (%)	40/60	51/49	0.33
Height (SD)	0.06 [-1.92, 2.40]	-2.36 [-3.16, -2.00]	<0.001
Weight (SD)	0.12 [-2.65, 1.54]	-2.29 [-3.91, -0.9]	<0.001
BMI (SD)	0.2 [-2.48, 1.46]	-1,2 [-2.27, 0.16]	<0.001
SH/H ratio (SD)	0.24 [-2.25, 2.33]	0.42 [-1.55, 2.86]	0.21
U/L ratio	1.15 [0.93, 1.44]	1.11 [0.97, 1.34]	0.06
Arm span/H	0.97 [-0.06, 1.06]	0.98 [0.92, 1.04]	0.34
SBP (mmHg)	103 [84, 135]	95 [70, 129]	0.23
DBP (mmHg)	62 [51, 75]	60.5 [45, 84]	0.29
Hb (g/dL)	13 [11, 15]	13 [10, 15]	0.59
ESR (mm/h)	9 [2, 52]	12 [2, 83]	0.29
Glucose (mg/dL)	90 [73, 99]	85 [54, 104]	0.32
Creatinine (mg/dL)	0.40 [0.27, 0.56]	0.46 [0.26, 0.76]	0.30
Na^+ (mEq/L)	139 [135, 142]	138 [130, 141]	0.61
K^+ (mEq/L)	4.21 [3.91, 4.87]	4.33 [3.90, 5.01]	0.17
HCO_3^- (mEq/L)	24 [22, 28]	24 [20, 28]	0.88
Ca^{2+} (mg/dL)	9.89 [9.34, 11.03]	10.00 [9.53, 10.58]	0.21
Phosphate (mg/dL)	4.97 [3.38, 6.11]	4.57 [3.37, 5.48]	0.28
ALP (U/L)	235 [41, 458]	227 [35, 462]	0.31
Albumin (g/dL)	4.32 [3.87, 4.81]	4.41 [3.89, 4.87]	0.38
TSH (μU/mL)	1.81 [0.90, 4.46]	2.05 [0.46, 4.37]	0.75
FT4 (pg/mL)	9 [7, 13]	9 [6, 11]	0.32
IGF1 (μg/L)	154 [49, 436]	122 [48, 242]	0.02
IGF1 (SD)	0.17 [-2.42, 2.24]	-1.81 [-4.21, 0.5]	<0.001

Data are expressed as median (min-max). SD is for standard deviation.

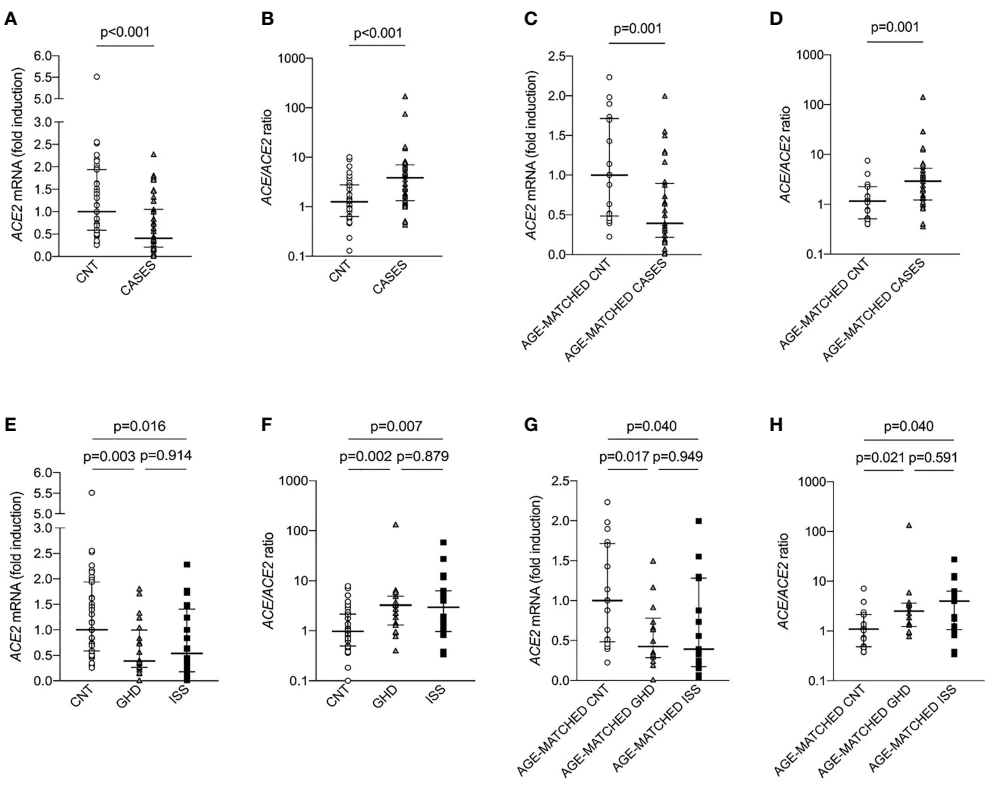

FIGURE 1 | *ACE2* expression and *ACE/ACE2* ratio in children with short stature (CASES) and controls (CNT). Gene expression was measured as mRNA fold induction as compared to controls (CNT). *ACE* is for angiotensin converting enzyme, *ACE2* is for angiotensin converting enzyme 2, GHD is for growth hormone deficiency and ISS is for idiopathic short stature. **(A–D)** Mann-Whitney test, **(E–H)** Kruskall-Wallis test.

TABLE 2 | General characteristics of age-matched subgroups.

	Controls (n = 17)	Cases (n = 29)	p-value
Age (year)	9 [5, 14]	11 [5, 14]	0.55
M/F (%)	53/47	48/52	0.76
Height (SD)	0.04 [-1.92, 2.40]	-2.46 [-3.16, -2.00]	<0.001
Weight (SD)	-0.21 [-2.65, 1.54]	-2.24 [-3.77, -0.91]	<0.001
BMI (SD)	-0.20 [-2.48, 1.37]	-1.21 [-2.27, 0.12]	0.006
IGF1 (µg/L)	193 [90, 436]	127 [52, 242]	0.003
IGF1 (SD)	-0.11 [-2.42, 2.24]	-1.57 [-2.81, 0.50]	<0.001

Data are expressed as median (min-max). SD is for standard deviation.

significantly lower weight (SD), height (SD), BMI (SD), IGF-1 levels (SD). In line with our previous results, children with short stature displayed significantly lower *ACE2* expression, being 0.39 (0.01-1.99) fold increase in cases as compared to 1 (0.22-2.23) in controls (p-value <0.01), **Figure 1C**. Consistent with this, *ACE*/*ACE2* ratio was significantly higher in the group of children with short stature, being 2.91 (0.36-142.21) in cases and 1.16 (0.40-7.57) in controls (p-value <0.01), **Figure 1D**. There were no differences in terms of *ACE* expression, AngII and Ang1-7 circulating levels as well as their ratio between the two groups.

Idiopathic Short Stature and Growth Hormone Deficiency

Children with short stature were further divided into two subgroups: children with growth hormone deficiency (GHD, n=19), and children with idiopathic short stature (ISS, n=20). These two subgroups did not differ in terms of height, weight, and BMI. It has to be noted that not only GHD but also ISS is regarded as a disorder of the GH-IGF-1 axis, falling between GH deficiency and GH insensitivity in the so-called GH-IGF-1 axis continuum model (25), but differing from GHD for the response to GH stimulation test (20, 25). As compared to controls, *ACE2* expression was significantly reduced in both groups and there were no differences between children with GHD and children with ISS (p=0.914), **Figure 1E**. Also, *ACE/ACE2* ratio was significantly increased in both groups and there were no differences between the two groups (p=0.879), **Figure 1F**. These results were maintained also after matching the children for age (**Figures 1G, H**).

Regression Analyses

To investigate the relationship between being a children with short stature and *ACE2*, as the response variable, we performed linear regression analyses taking into account the whole cohort (35 controls and 39 cases) as well as the subgroups matched by age (17 controls and 29 cases). Our data showed that *ACE2* expression was significantly and independently correlated to belonging to the short stature group as well as sex, while it was not correlated to age or BMI (**Table 3**). *ACE*/*ACE2* expression did not show any correlation with age, sex, and short stature. Then, to understand if *ACE2* expression was associated with the risk of short stature we performed a multivariate logistic regression using *ACE2* as a predictor variable and child status (control=0, case=1) as the response variable. The odds ratio (OR) for *ACE2* as predictor of belonging to the short stature group was 0.26, [95% confidence interval (CI) (0.07-0.82)], meaning that an increase of one unit in *ACE2* expression would be associated with a 74% decrease in the odds of being a child with short stature, regardless of age, sex, and BMI. This was maintained after matching for age. *ACE*/*ACE2* was associated with the risk of short stature only after matching for age.

ROC Analysis

On ROC curve analysis (**Figure 2**), we found that *ACE2* expression had an AUC of 0.73 (0.61-0.84), indicating a moderate accuracy to predict short stature. We found the same AUC when we considered only the subgroups of 17 controls and 29 cases, as *ACE2* expression had an AUC of 0.73 (0.58-0.88). The cutoff value of *ACE2* expression with highest specificity and sensitivity was 0.73, allowing to correctly classify 63.5% of children with short stature. However, the cutoff value best predicting being a child with short stature was 0.45 fold induction, allowing to classify correctly 70.3% of the children, with sensitivity of 0.54 and specificity of 0.88.

DISCUSSION

This study shows for the first time that children with short stature, being idiopathic or linked to growth hormone deficiency, had lower *ACE2* expression in PBMC with subsequent increase of the ratio *ACE/ACE2*. *ACE2* expression was associated with the risk of being a children with short stature, regardless of age, sex, and BMI, which suggests that ACE2 should not being related to adiposity, being BMI the most common anthropometric index to estimate adiposity (26). In addition, *ACE2* expression had a moderate accuracy to predict short stature, and the cutoff of 0.45 fold induction was the value of *ACE2* expression best predicting being a child with short stature with specificity of 88% and sensitivity of 54%, allowing to classify correctly 70% of the children.

This finding is consistent with the reports that *ACE2*-knockout mice are smaller than wild-type controls (13–19), and the concept that RAS contributes to growth regulation. Accumulating scientific evidence indicates that RAS is involved in pre-natal growth. Early studies have demonstrated that this system is expressed in both maternal and fetal tissues. AngII levels are higher in uterine venous than arterial blood, or peripheral venous blood in pregnant women (27). The AT1R (AngII type1 receptor) is expressed across all trimesters of pregnancy in the placental syncytiotrophoblast, cytotrophoblast, and the fetal vascular endothelium (28). In

TABLE 3 | Regression models.

A) LINEAR REGRESSION				
Dependent variable: *ACE2* expression				
WHOLE COHORT (35 cnt vs 39 cases)				
Predictive variables	β-estimate	95%CI	Standard error	p-value
Age	0.02	[-0.04, 0.08]	0.03	0.56
Sex [M]	-0.80	[-1.15, -0.46]	0.13	<0.001
Group [CNT]	0.41	[-0.04, 0.87]	0.14	0.07
BMI_SD	0.11	[-0.11, 0.34]	0.11	0.31
AGE-matched SUBGROUPS (17 cnt vs 29 cases)				
Predictive variables	β-estimate	95%CI	Standard error	p-value
Age	-0.01	[-0.08, 0.05]	0.03	0.71
Sex [M]	-0.54	[-0.86, -0.22]	0.16	0.01
Group [CNT]	0.51	[0.14, 0.87]	0.18	0.01
BMI_SD	-0.03	[-0.22, 0.17]	0.09	0.77
Dependent variable: *ACE/ACE2* expression				
WHOLE COHORT (35 cnt vs 39 cases)				
Predictive variables	β-estimate	95%CI	Standard error	p-value
Age	-0.39	[-1.76, 0.96]	0.68	0.56
Sex [M]	3.07	[-4.93, 11.07]	4.01	0.45
Group [CNT]	-9.66	[-20.05, 0.73]	5.21	0.68
BMI_SD	1.58	[-3.57, 6.73]	2.58	0.54
AGE-matched SUBGROUPS (17 cnt vs 29 cases)				
Predictive variables	β-estimate	95%CI	Standard error	p-value
Age	0.22	[-2.48, 2.92]	1.34	0.87
Sex [M]	6.10	[-7.28, 19.50]	6.63	0.36
Group [CNT]	-9.48	[-25, 6.04]	7.68	0.22
BMI_SD	1.79	[-6.43, 10.01]	4.07	0.66
B) LOGISTIC REGRESSION				
Dependent variable: CNT vs CASE				
WHOLE COHORT (35 cnt vs 39 cases)				
Predictive variables	OR	95%CI		p-value
Age	1.25	[1.01, 1.59]		0.04
Sex [M]	0.83	[0.16, 3.97]		0.82
***ACE2* mRNA**	0.26	[0.07, 0.82]		0.03
BMI_SD	0.18	[0.07, 0.41]		<0.001
AGE-matched SUBGROUPS (17 cnt vs 29 cases)				
Predictive variables	OR	95%CI		p-value
Age	0.98	[0.68, 1.38]		0.92
Sex [M]	0.36	[0.03, 2.74]		0.36
***ACE2* mRNA**	0.10	[0.01, 0.55]		0.02
BMI_SD	0.21	[0.06, 0.54]		<0.01
WHOLE COHORT (35 cnt vs 39 cases)				
Predictive variables	OR	95%CI		p-value
Age	1.28	[1.02, 1.64]		0.04
Sex [M]	1.42	[0.29, 6.70]		0.65
***ACE/ACE2* mRNA**	1.27	[1.03, 1.92]		0.18
BMI_SD	0.19	[0.07, 0.42]		<0.001
AGE-matched SUBGROUPS (17 cnt vs 29 cases)				
Predictive variables	OR	95%CI		p-value
Age	1.01	[0.72, 1.40]		0.95
Sex [M]	0.35	[0.04, 2.54]		0.32
***ACE/ACE2* mRNA**	1.97	[1.15, 4.39]		0.04
BMI_SD	0.19	[0.04, 0.52]		<0.01

addition, on the fetal side, autoradiographic analysis of ^{125}I-labeled AngII, showed intense binding in the skin, mesenchymal and connective tissues, and skeletal muscle in the later period of gestation, overlapping with the sites reported for IGF-2 in the rat fetus (4). Also *ACE2* and Ang1-7 are expressed in the rat uterus (29). Ghadhanafar et al. found that both *ACE2* and Ang1-7 expression were reduced in the placenta of dexamethasone-exposed rats and this was associated with low birth weight of the offspring. Consistent with this, Bharadwaj et al. showed that ACE2 deficiency resulted in 3-fold higher AngII content in the

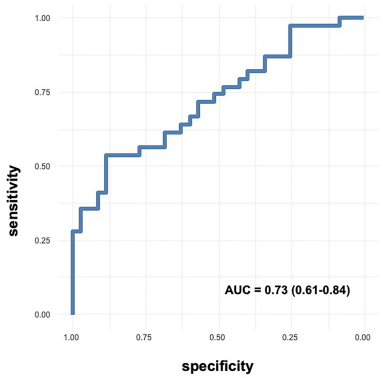

FIGURE 2 | ROC curves of *ACE2* expression in predicting short stature. Accuracy of *ACE2* expression as predictor of short stature.

placenta, and this was associated with reduced gestational weight gain and significant inhibition of fetal growth, as *ACE2*-knockout pups had significantly lower body weight and length than controls (30). It has been argued that ACE2 deficiency might result in inhibition of fetal growth due to the increase of AngII in the placenta, leading to placental ischemia, consistent with the finding that the chorionic villi of the placenta of pre-eclamptic women displayed increased AngII (31). Recently, a negative correlation was found between birth weight centiles and circulating ACE2 levels (32), depending on ACE2 shedding and tissue loss (33).

Nevertheless, in our study, the children with short stature had no history of being small for gestational age babies, suggesting that they suffered from a post-natal growth defect, which is also influenced by the RAS activity. Animal studies have shown that an infusion of AngII markedly reduced plasma IGF-1 levels (by 56% after 1 week of treatment and by 41% after 2 weeks of treatment) with a parallel reduction of hepatic *IGF-1* mRNA, and body weight, which decreased by 18% after 1 week of treatment (34). These effects were mediated by the AT1R, as they were blocked by its antagonist losartan (34). Consistent with these findings, also the treatment with ACE inhibitors for 3 years was associated with significantly higher levels of IGF-1 in a cohort of 1154 subjects aged > 65 years (35). Nevertheless, in our study, blood pressure as well as circulating AngII and Ang1-7 levels did not significantly differ between the groups. This suggest that the mechanisms underlying the association between ACE2 deficiency and short stature might involve an unbalance on tissue - rather than circulating - AngII and Ang1-7 levels, in organs that are critically involved in growth regulation, such as the pituitary or the liver. For instance, it has been shown that the regulation of somatotrope cell function depends on paracrine processes within the pituitary, which involve peptide hormones such as AngII (36) acting as a signaling molecule (37). In addition to AngII and Ang1-7 tissue levels, ACE2 deficiency affects other peptide hormones, such as atrial natriuretic peptide (ANP). We have shown that both acquired and genetic ACE2 deficiency significantly reduced renal ANP (12), and that tissue ANP production was induced by Ang1-7 (12). ANP reduction could be another mechanism underlying the association between ACE2 reduction and growth retardation, given that natriuretic peptides stimulate endochondral bone growth in animal studies, and natriuretic peptides have gained increasing attention as potential stimulants to skeletal growth (38).

Apart from short stature, *ACE2* expression was influenced by the sex of participants, being higher in the female group, as we have recently found in a cohort of young adults (39). This is due to the fact that *ACE2* gene is located on the X chromosome, and the X chromosome inactivation, which should silence the transcription from one of the two X chromosomes in female mammalian cells, is often incomplete (40). It has been shown that incomplete X chromosome inactivation affects at least 23% of X-chromosomal genes, resulting in sex-biases in gene expression underlying sex-related phenotypic diversity (40).

The limitations of this study include the fact that *ACE* and *ACE2* expression was measured in PBMC, as they represent the most easily accessible tissue in children to perform these analyses, while *ACE* and *ACE2* gene expression in other tissues and activity levels were not assessed. Another issue is the fact that we measured circulating and not tissue peptides, as this would have required the use invasive procedures. Nevertheless, this is the first study evaluating *ACE2* expression in children with short stature, recruited and managed according to current guidelines in a University Hospital pediatric tertiary care center (1).

In conclusion, our study shows for the first time that *ACE2* expression is significantly lower in children with short stature, with potential diagnostic implications, as *ACE2* expression had a moderate accuracy in predicting short stature. In addition, our findings shed light onto potential mechanisms underlying growth retardation, including changes of angiotensins and natriuretic peptides in the organs regulating skeletal growth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Institute for Maternal and Child Health IRCCS Burlo Garofolo and Regional Ethics Committee (CEUR-2019-Sper-115). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GT, BF, and SB, study conception and design, data analysis. FT and BT, data collection, data analysis, database organization, figures and tables. GT, FN, and EB, patient recruitment. FG, statistical analysis. EB and BF, important intellectual content.

SB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Sex steroid priming in short stature children unresponsive to GH stimulation tests: Why, who, when and how

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Despite decades of experience, the diagnosis of growth hormone deficiency (GHD) remains challenging, especially in peripubertal children. Failure to respond to GH stimulation tests (GHSTs) is needed to confirm GHD, but long-standing controversies regarding the number of tests needed and the interpretation of GH peaks are still a matter of debate worldwide. Diagnostic workup is even more problematic in short children with slow growth and delayed sexual development: they often exhibit low GH peaks under GHST, which often normalize as puberty progresses. Consequently, this transient suboptimal response to GHST may result in GH overtreatment, carrying both health and economic concerns. Considering the complex and bound link between GH axis and sex steroids, the use of sex steroid priming prior to GHST might be helpful in peripubertal setting. However, its use is still controversial. There is no consensus regarding patient selection, timing, dose, and preparation of sex steroids. In this review, we aim to overview the use of sex steroid priming in clinical practice, highlighting the need to develop appropriate guidelines in order to overcome diagnostic pitfalls in peripubertal age.

KEYWORDS

pubertal delay, sex steroid priming, GH deficiency (GHD), short stature, peripubertal age, growth hormone stimulation test (GHST)

Introduction

Despite decades of experience, the diagnosis of growth hormone deficiency (GHD) remains a challenge for the paediatric endocrinologist. It should result from "auxologic, anatomical and laboratory data", as stated in the recent document from Growth Hormone Research Society Workshop (1), and therefore appropriate selection of

patients eligible for growth hormone (GH) investigation is crucial. Family and previous medical history should be taken into account, as well as accurate physical examination to rule out body disproportions and syndromic features and to evaluate pubertal status. Radiological findings such as brain MRI for hypothalamus-pituitary study and hand-wrist X-ray for bone age assessment do also contribute to the diagnostic evaluation. Finally, serum IGF-I and IGFBP 3 values are supportive biochemical findings. Since measurement of random serum GH concentrations are useless, except for neonates (2), failure to respond to GH stimulation tests (GHSTs) is needed to confirm GHD, when an alternative aetiology for short stature is not evident.

Long-standing controversies continue to generate debate, regarding how to perform and interpret GHSTs (3–7). Arbitrary and not universally adopted cut-off levels, reliability and reproducibility of these tests are the main issues. In a study by Marin et al. investigating GH response to provocative tests in prepubertal children with normal stature, 61% of them failed three different tests with a cut-off fixed at 7 mcg/l (8). Difficulties in distinguishing partial GHD from idiopathic short stature (ISS) or constitutional delay of growth and puberty (CDGP) have already been extensively highlighted (9–12), showing normalization of GH peaks at early retesting (10). Both GH and sex steroids are required for the pubertal growth spurt and there is strong evidence, at least in boys, that sex steroids are a potent stimulus facilitating GH release (13). Diagnostic workup is challenging in short children with slow growth velocity and delayed sexual development: they often exhibit low GH peaks under GHST, which reverts to normal levels as puberty progresses. As a consequence, this transient suboptimal response to GHST may result in GH overtreatment, with both health and economic concerns (4).

According to the 2019 GH Research Society guidelines, the use of sex steroid priming prior to GHST might be helpful in the peripubertal setting. It was first introduced in 1968 to reduce the percentage of false positive results to GHST, since availability of GH treatment was limited at that time (14). However, its use is still controversial. There is no consensus regarding patient selection, timing, dosage, preparation, and administration of sex steroids.

In this review, we aim to overview the use of sex steroid priming in clinical practice, highlighting the need to develop appropriate guidelines in order to overcome diagnostic pitfalls in peripubertal age.

Rationale for use of priming

After minipuberty occurs, during childhood the hypothalamus-pituitary-gonadal system becomes quiescent. A significant change and maturation in the hypothalamic "gonadostat" occur in girls at approximately 10.5 years and in boys at 11.5 years. GnRH neurons amplify their signal to increase amplitude and frequency of FSH and LH pulsatile release by the pituitary gonadotropic cells with a prominent nocturnal rhythm (15). This in turn triggers sex steroid production by gonads with feedback regulation of gonadotropin secretion by both testosterone and oestrogen (16). In girls growth acceleration starts with the onset of breast development (Tanner Stage B2), whereas in boys the rate of growth increases significantly only after achieving Tanner stage III-IV (with approximately 10 ml of testicular volume) (17, 18). The different timing of puberty onset between sexes may be related to an increased sensitivity of the gonadotrophs to GnRH in girls or to a greater bioactivity of oestrogen in prepubertal females compared to prepubertal males (19).

Historical data have demonstrated a complex and close link between GH axis and sex steroids both in animals models and humans (20–24). The hypothalamic regulation of GH secretion results primarily from a stimulating control by GH-releasing hormone (GHRH) and by an inhibiting control by somatostatin. On one hand, sex steroids are known to potentiate GH responsiveness to GHRH in somatotroph cells in the anterior pituitary gland; on the other hand, GH modulates pubertal development by stimulating local production of insulin-like growth factors in gonads and by enhancing gonadal response to gonadotrophin secretion and these axes constitute a regulated network whose feedback relationships manifest important changes at the time of puberty.

The use of sex steroid priming in the diagnosis of GHD is based on three considerations:

A) GH levels increase physiologically during puberty.

Rose et al. (25) analysed circadian GH secretion of 132 normal children and adolescents (every 20 minutes for 24 hours) and found that spontaneous GH secretion increases during puberty, with a peak during early-mid puberty in girls (sometimes before the earliest clinical signs of puberty) and during mid-late puberty in boys, corresponding to their peak of growth velocity. If correlated with bone maturation, mean GH levels and pulse amplitude increased in girls beyond a bone age of 8 years, whereas a decrease in growth velocity was observed in boys till bone age of 11 years. This means that the interpretation of GH levels according to chronological age may be misleading and generate a high amount of false GHD diagnosis.

Similarly, Mauras et al. (26) confirmed that prepubertal boys showed lower GH concentrations compared to sexually mature boys of same age and these findings were secondary to variations in amplitude rather than in the pulse frequency of GH secretions. A study from a cross-sectional group of healthy North American males showed that mean 24-hour GH concentration of young adult is similar to that in the prepubertal state, suggesting that the relative impact of sex steroids on GH concentrations is limited to the last stages of puberty (27).

The role of IGF-I as a modulator of pubertal timing is increasingly recognized (28, 29). High GH secretion is most

certainly responsible for the increased IGF-I levels during puberty; nevertheless, previous studies have found a suboptimal growth response to GH stimulation test in girls with central precocious puberty (30, 31). Negative feedback of IGF-I levels on pituitary may be reduced in puberty, emphasizing their synergic anabolic role during growth spurt. IGF-I levels peak 2 years after growth spurt and might play a role in gonadal and secondary sexual characteristics maturation (32).

B) Sex steroids regulate GH secretion and actions, both directly or *via* modulators, through paracrine or endocrine signalling (33).

The evidence of high levels of oestrogen receptors in hypothalamus and pituitary demonstrates that oestrogens act as regulator of GH secretion by reducing somatostatin receptor expression, increasing the number of GHRH-binding sites and increasing ghrelin-induced GH production (34). Moreover, 80% of the somatotropes in human pituitary co-express aromatase, and in patients with aromatase deficiency the GH response to stimulation is substantially blunted (35). Similarly, late pubertal boys receiving oestrogen receptor blocker (Tamoxifen) to evaluate the role of endogenous oestrogens in the control of GH secretion showed a significant decrease in GH production rates, in mean GH pulse amplitudes, and in serum IGF-I levels (36). These data support the paracrine effect of oestrogens derived from aromatization of androgens in men. Peripherally, oestrogen exerts tissue-specific effect: for example, in bones it potentiates GH signalling *via* SOCS-2 pathway promoting osteoblast proliferation and bone growth (37). Testosterone also acts peripherally, amplifying GH-mediated secretion of IGF-I, sodium retention, substrate metabolism and protein anabolism, while exhibiting similar but independent actions of its own and interacts directly with GH in the liver to regulate protein metabolism by enhancing GH receptor expression (38). Contrary to androgens, oestrogens do not influence whole body protein anabolism, and this may explain sex differences in muscle bulk. Sex steroids modulate GH secretion during lifespan. Evidence of a regulatory role of sex steroids on GH comes from association studies in children and adults. Physiologically, in children, a positive correlation between sex steroid and GH status has been proved from the evidence of a threefold increase in GH secretion along with an increase in gonadal steroid concentrations during puberty (39).

C) Exogenous sex steroids stimulate GH synthesis, release, and action.

Sex steroids administration exerts a stimulatory effect on GH secretory episodes. In the above mentioned study by Marin et al. 40 mcg/m2 oral ethynil oestradiol given two days before GHST increased GH peak in normal prepubertal children from 1.9-20 mcg/L to 7.2-40.5 mcg/L, reaching similar levels of pubertal children (8-63.2 mcg/L) (8). Low doses of ethinyloestradiol (0.1 mcg/kg/day) could rise GH concentrations after 1 to 5 weeks and improve height gain in patients with Turner Syndrome, without significantly advancing bone maturation (40). The effect of oestrogen on GH secretion is dependent on the route of administration. When administered orally, oestrogen reduces hepatic IGF-I production as a result of first-pass effect. The fall in IGF-I after oral oestrogen therapy reduces negative feedback on GH secretion, as seen in postmenopausal women (41). In men with hypogonadism, testosterone replacement stimulates GH/IGF-I system peripherally and enhances tissue responsiveness to GH. Importantly, non-aromatizable androgens do not stimulate GH secretion (42).

Concerns and benefits

A recent audit among nine American and European expert paediatric endocrinologists showed that priming is recommended in 5 out of 9 countries (the UK, the Netherlands, Denmark, Spain, and Germany), but protocols differ significantly (43). The prevalence was higher (up to 85%) among tertiary endocrine centres in UK (44). In contrast, different data result from a French population-based registry (45): in 2,165 patients with idiopathic GHD sex steroid priming was used in only 2% of patients before GHST. Pubertal development has been reported not to increase GH reserve when evaluated by GHST (3, 46). It should be noted, however, that these studies were performed decades ago with different GH assays and sometimes with obsolete and unreliable diagnostic test (i.e. treadmill exercise). Soliman et al. found that the mean GH response to provocative testing did not differ between primed and not primed-children, although testosterone intramuscular injections were administered at a lower dose compared to other reports; this study however included younger children (starting from 9 years old) compared to other papers and this could had influenced the results (47). Another concern against routine use of priming is that primed GH peak may be unphysiological and transient, therefore many peripubertal GH deficient patients may be not identified, preventing them from receiving appropriate and potentially beneficial treatment (13, 48, 49). The existence of "transient GHD" in adolescents with delayed puberty is still debated, as the underlying pathology is more often consistent with sex hormones deficiency rather than GHD. The majority of the patients with idiopathic GHD show normal GH secretion when retested after achieving of final height, whereas the likelihood of permanent GHD is higher in adults with congenital panhypopituitarism and acquired pituitary lesions (50, 51). For this reason some authors suggested the need to retest patients with idiopathic GHD after one year of therapy: the Belgian Study Group for Paediatric Endocrinology reported normal GH peak in 44% of cases (52). 28 out of 33 GHD patients of an Italian cohort with normal pituitary morphology at brain MRI normalized GH secretion even before commencement of GH treatment (10). Recently published data suggested patients with isolated GHD without a hypothalamic-pituitary abnormality on MR scanning (including small anterior pituitary) can also be considered for early retesting of the GH axis once they are established in puberty

(Tanner stages B2/3 in girls & 6-12 ml testes in boys) (53). In addition, GH treatment seems to have little effect on final height in adolescents with transient GHD (54).

On the contrary, many other studies reported that sex steroid priming could improve diagnostic efficacy of GHSTs in peripubertal patients. Molina et al. demonstrated that 53.8% of short children who underwent clonidine stimulation test normalized GH secretion after priming (55); these data were confirmed also among children affected by ISS compared to GHD when micronized oestradiol was administered before GHST (56). A prospective study including 50 boys with poor growth who failed to respond to unprimed GHST showed that some of them normalized GH response to GHST after testosterone priming with different protocols (57): 31/50 boys after single low dose testosterone (62.5 mg/m2), 11/50 after single conventional dose (125 mg/m2) and 8/50 boys after multiple-dose testosterone (62.5 mg/m2 weekly for 4 weeks). Mean peak GH increased from 4.9 ± 3.0 to 19.3 ± 5.9 mcg/L in the low dose group, from 5.4 ± 2.1 to 17.0 ± 5.9 mcg/L in the conventional dose group, and from 5.1 ± 2.1 to 15.4 ± 5.1 mcg/L in the multiple-dose group. There was no statistical difference among mean peak GH level of the three groups before and after priming. Most relevantly, those subjects were able to reach a final height well within their genetic target (mean final height -1.27 +/-0.72 SDS versus mean mid-parental height -1.38 +/-0.72 SDS) without any rhGH treatment. More recently, a retrospective study among ENDO-ERN centres confirmed that sex steroid priming enhanced the specificity of GHST in differential diagnosis between GHD and CDGP in a cohort of 184 peripubertal children (74 females), selecting children who may benefit the most from priming. In fact, those children diagnosed as GHD upon a primed GHST reached a greater final height compared to untreated CDGP (primed CDGP vs GHD FH: -1.5 vs -0.81; p = 0.023) and closer to their midparental target (primed CDGP vs GHD ΔSDS FH-TH: -0.74 vs 0.12, p = 0.025), whereas those diagnosed upon an unprimed GHST, final height was similar between GHD children treated with rhGH and untreated CDGP children (unprimed CDGP vs GHD FH: -0.9 vs -0.93, p =n.s.)

(58). Lastly, two recent retrospective Italian studies on short pre/peripubertal boys primed with a prolonged low-dose testosterone protocols (either with intramuscular or transdermal preparation) showed an increase in height and growth velocity SDS and a normalization of GHST peaks compared to untreated boys (59, 60).

A summary of the clinical research studies included in this review is reported in Table 1.

Use of priming in clinical practice

The actual limitation for use of priming is the current absence of standardized protocol for sex steroids administration with reference to patients' age, type, dose, and timing. The 2019 international audit (43) revealed that priming may be used in boys between the ages of 10 to 13 years and in girls between the ages of 8 to 12 years. According to 2016 Pediatric Endocrine Society Guidelines priming should be considered in prepubertal boys older than 11 and in prepubertal girls older than 10 years with final height prognosis > −2 SD of the reference population (68). Another adopted strategy is to prime all prepubertal children (boys >9 years, girls >8 years, either on chronological age or bone age) (4). Lazar and Phillip (49) advocated the use of priming only in selected cases, i.e. girls aged > 11.5–12 years and boys aged > 13–13.5 years with any or only initial signs of puberty. Similarly, the recent update from GH Research Society advised to limit its use to adolescents with delayed puberty only, but did not provide any age cut-off due to lack of consensus (1).

Published data revealed a large heterogeneity in current practice about dose and type of sex steroid preparations across centres and countries, as listed in Tables 2, 3.

A reasonable, easy to use and commonly used approach in both boys and girls would be 2 mg (1 mg for body weight <20 kg) of β-oestradiol orally on each of the 3 evenings preceding the test, as indicated in the BSPED UK Consensus National Guidelines for Sex Hormone Priming (71). Alternatively, for instance in case of lack of supply of β-oestradiol, children of both sexes can be primed with oral Stilboestrol (1 mg twice a day for 2 days before the test). Promising results have been proven for boys primed with transdermal testosterone 2% (59, 60).

As previously mentioned, clinical and experimental data strongly suggest that oestrogens control the feedback amplification of GH levels during puberty even in males and that the modulation of GH production by androgens is mainly secondary to their aromatization. Moreover, the use of oral or transdermal preparations is suitable for needle-phobic patients and could possibly increase patient compliance to treatment. Several oestrogen and testosterone products are available and their effects and pharmacokinetics may vary according to different route and strength, so that a comparison is not always possible (72, 73). For example, oral ethynyloestradiol elicits a sharp response on IGF-I and achieves its peak plasma concentration within 0.5-1.5 hours with a half-life of approximately 12-14 hours, whilst transdermal formulations result in lower oestrogen metabolite concentrations. In a pilot study by Borghi et al. oestrogen patches are considered safe and viable since they deliver a continuous release of oestradiol, guaranteeing stable plasma levels for 72 hours (70). In addition, this route of administration avoids the first passage hepatic effect and does not directly affect IGF-I synthesis. Testosterone transdermal gel are commonly accepted for puberty induction in boys with CDGP and hypopituitarism (74) and their use can be theorized for priming prior to GHST as suggested in the study by Mastromattei et al. 2022 (60).

For research purposes, Radetti et al. reported that priming GHST with Pegvisomant, a GH receptor antagonist, would

TABLE 1 Summary of the main clinical studies included in the review.

STUDY	STUDY TYPE	COHORT SIZE	SEX STEROID	FINDINGS
Marin et al., 1994 (8)	Randomized control trial	84	Both boys and girls: EE 40 mcg/m^2 divided into 3 doses For 2 days prior to GHST	Priming increased GH response to GHST in pubertal and prepubertal children with normal height
Drop et al., 1982 (13)	Control study	8	Boys: TU 120 mg twice a day For 5 days prior to GHST Girls: EE 50 mcg twice a day For 5 days prior to GHST	Priming (unlike spontaneous puberty) did not increase GH response to GHST in GHD children
Soliman et al., 2014 (47)	Randomized control trial	92	Boys: TD 25 mg 7-10 days before GHST Girls: Conjugated oestrogens 1.25 mg daily For 3 days prior to GHST	Priming did not increase GH response to GHST in prepubertal children (age 9-13 years)
Chalew et al., 1988 (48)	Control study	8, all boys	TD 200 mg once a month for 4-5 months before assessment of spontaneous GH secretion	Testosterone transiently increases spontaneous GH secretion in boys with CDGP
Molina et al., 2008 (55)	Control study	39	Boys: TD 100 mg 5-8 days prior to GHST Girls: EV 1-2 mg daily For 3 days prior to GHST	Priming normalized GH response to GHST in both GHD and CDGP
Martinez et al., 2000 (56)	Randomized control trial	59	Both boys and girls: EV 1-2 mg daily For 3 days prior to GHST	Priming increases GH response to GHST in both GHD and ISS children
Gonc et al., 2008 (57)	Retrospective cohort study	50, all boys	Testosterone Esters (Sustanon®) 62.5 or 125 mg/m^2 7 days before GHST In non-responders (stimulated GH peak <10 ng/ml): multiple-dose priming (62.5 mg/m^2 monthly injections for 3-4 months)	Priming normalized GH response to GHST in 42/50 peripubertal boys. 8/50 elicit normal GH peaks after multiple dose priming. All 50 patients were able to achieve a final height within their mid-parental target, regardless of priming regimen.
Galazzi et al., 2020 (58)	Retrospective cohort study	184	Mixed regimes: Boys: TD - Low dose: 50mg - High dose: 100mg 4-7 days before GHST Girls: EE 100 mcg daily For 3 days prior to GHST or Stilboestrol 1 mg twice a day For 2 days prior to GHST	Priming played a key role in identifying children who may benefit most from recombinant GH treatment in terms of final height. Priming enhances the diagnostic accuracy of GHST in the differential diagnosis between peripubertal GHD and CDGP.
Chioma et al., 2018 (59)	Retrospective study	73, all boys	- TD 50 mg every 4 weeks for 3 months - Transdermal testosterone 2% 10 mg daily for 3 months	Both testosterone preparations (intramuscular and transdermal) were able to increase SDS height and SDS growth velocity compared to placebo, helping in the differential diagnosis between peripubertal GHD and CDGP.
Mastromattei et al., 2022 (60)	Retrospective study	246, all boys	- TD 50 mg every 4 weeks for 3 months - Transdermal testosterone 2% 10 mg daily for 3 months	3-month low dose priming with testosterone (either intramuscular or transdermal) increased height and growth velocity and normalized almost all GHST and IGF-1 levels in short pre/peripubertal boys. Testicular enlargement and LH increase was more evident with transdermal testosterone preparations.
Moll et al., 1986 (61)	Control study	23	Both boys and girls: EE 20 mcg/m^2 daily For 1-2 days prior to GHST	Higher priming EE regimen increased GH response to GHST in prepubertal children (age 3-15 years)
Borghi et al., 2006 (62)	Control study	22	Both boys and girls: Transdermal EE 50 mcg For 3 days prior to GHST	Priming increased GH response to GHST in non-GHD short children

(Continued)

TABLE 1 Continued

STUDY	STUDY TYPE	COHORT SIZE	SEX STEROID	FINDINGS
Bacon et al., 1969 (63)	Control study	26	Both boys and girls: Stilboestrol 5 mg twice a day For 3 days prior to GHST	Oestrogen increased GH secretion similarly to arginine test
Ross et al., 1987 (64)	Control study	14	Both boys and girls Stilboestrol 1 mg twice a day For 2 days prior to GHST	Priming increased GH response to GHST through the hypothalamus presumably by increasing endogenous GHRH release
Lanes et al., 1986 (65)	Control study	144	Both boys and girls: Conjugated oestrogens 1.25 mg daily For 3 days prior to GHST	Priming did not seem to alter the GH response to exercise in prepubertal children
Wilson et al., 1993 (66)	Control study	73	Both boys and girls: Conjugated oestrogens 2.5 mg 1 dose the evening before and 1 dose the morning of the GHST	Priming did not increase GH response to GHST (clonidine)
Gonc et al., 2001 (67)	Control study	84, all boys	Testosterone Esters (Sustanon®) 62.5 or 125 mg/m^2 7 days before GHST Or 62.5 mg/m^2 monthly (three doses in total) last dose 7 days before GHST)	Priming either with low or high dose increased GH response to GHST in peripubertal boys. Multiple-dose priming is useful in those patients who failed to respond to a GHST after a single-dose priming

EE, Ethinyloestradiol; TU, Testosterone undecanoate; TD, Testosterone depot; TP, Testosterone Propionate; EV, oestradiol valerate.

TABLE 2 Sex steroid priming regimens for girls.

	Dose	Route of administration	Timing prior to GHST
β-oestradiol (oestradiol valerate)	1 mg < 20 kg daily (bedtime) 2 mg >20 kg (55, 56, 68)	Oral	For 2 (58) – 3 (55, 56) days
Ethinyloestradiol	10 or 20 or 30 or 50 mcg (69) 50 mcg twice a day (13) 100 mcg (58, 70) 20 mcg/m2 daily (bedtime) (61) 40 mcg/m2 daily (8) 50 mcg patch (62)	Oral Oral Oral Oral Oral Oral Transdermal	For 2 or 3 or 5 or 7 days For 5 days For 3 days For 1-2 days For 2 days For 2 days 1 patch to be kept on for 3 days
Stilboestrol	5 mg twice a day (63) 1 mg twice a day (58, 64)	Oral Oral	For 3 days For 2 days
Premarin® (Conjugated oestrogen)	1.25 daily (47, 65) 2.5 mg (66)	Oral Oral	For 3 days 1 dose the evening before and 1 dose the morning of the GHST

enhance the accuracy of the test, although this approach has not been extensively confirmed in clinical practice (75).

Side effects

Data are lacking on potential side effects of sex steroid priming. Albrecht et al. analysed the consequences of priming with testosterone enanthate i.m. (50 mg, 125 mg, 250 mg) given 7 days before GHST on 188 prepubertal boys. Overall, only 5 subjects displayed side effects (2.7%), irrespective of testosterone plasma levels: 2/188 developed severe priapism requiring cavernosal aspiration (after testosterone 125 mg single dose), 1/188 mild self-limiting priapism, 2/188 complained testicular pain (after testosterone 50 mg single dose (76). Other common adverse effects related to intramuscular administration are local inflammation and pain at injection site. It is worth mentioning that, as cottonseed or sesame/peanut oil are the formulation vehicle, testosterone vials are contraindicated in case of known hypersensitivity/allergy to nuts or soy (74). Side effects such as severe nausea and vomiting are frequently observed following priming with Stilboestrol. Transient breast tenderness has been reported as well (49).

The need for a structured approach

Since GH production and release are significantly and physiologically influenced by androgen and oestrogen milieu during puberty, sex steroid priming has been proposed and

TABLE 3 Sex steroid priming regimens for boys.

	Dose	Route of administration	Timing prior to GHST
Testosterone depot (Testosterone Enanthate)	25 mg (47) 50 – 100 mg (58, 68) 100 mg (4, 55)	Intramuscular	7-10 days 7 days 5-8 days (55), 7-10 days (4)
Sustanon® (mixture of Testosterone esters: propionate, phenylpropionate, isocaproate, decanoate)	50-100 mg (69) 62.5 mg/m2 (57, 67) 125 mg/m2 (57, 67) MDT (multiple dose testosterone) 62.5 mg/m2 monthly for 3 doses (67)	Intramuscular	3-5 days 7 days 7 days Last injection 7 days before test
Testosterone undecanoate	120 mg daily (13)	Oral	5 days
Testosterone gel 2%	10 mg daily for 6 months (59, 60)	Transdermal	3-6 months before test
β-oestradiol (oestradiol valerate)	1 mg < 20 kg 2 mg >20 kg daily (bedtime) (52, 56)	Oral	For 2 (68) – 3 (52) days
Ethinyloestradiol	10 or 20 or 50 or 100 mcg (69) 20 mcg/m2 daily (bedtime) (61) 40 mcg/m2 daily (8) 50 mcg patch (62)	Oral Oral Oral Transdermal	For 3 days For 1-2 days For 2 days 1 patch to be applied and kept on for 3 days
Stilboestrol	5 mg twice a day (63) 1 mg twice a day (64)	Oral Oral	For 3 days For 2 days
Premarin® (Conjugated oestrogen)	1.25 daily (61, 65) 2.5 mg (66)	Oral Oral	For 3 days 1 dose the evening before and 1 dose the morning of the GHST

proved to improve diagnostic performance of GH provocation tests. We therefore recommend including priming to all protocols for diagnostic workup of short patients. However, there is no consensus on who, when and how to use it. Nowadays, the availability of biosynthetic GH has eased limitations for GH prescription and has led to the conclusion that sex hormone priming is not necessary in the routine evaluation of every prepubertal child. It should be considered only in a subgroup of adolescents with delayed puberty (e.g. Tanner stages 1 and 2 in girls older than 12 years and boys older than 13 years) in order to prevent unnecessary GH treatment of children with CDGP. Assessment of bone age is warranted to evaluate pubertal delay and to select candidates for sex steroid priming. Several protocols have been suggested for priming and no one demonstrated evident superiority over others. Nevertheless, among different preparations and dosages, oral oestrogen seems preferable in both girls and boys as oestrogen plays a pivotal role in the regulation of GH secretion also in males.

Conclusion

Although sex hormone priming prior to GHST is not mandatory to diagnose GHD, several evidence recommend its use in peripubertal children in order to select those who may benefit the most from rhGH treatment, avoiding redundant treatment in CDGP, who can either achieve normalization of their auxological parameters with a low dose short course of sex steroids. Large prospective studies following patients until final height are still needed to clarify the optimal priming regimen and the correct timing of these preparations during their growth, especially in girls.

Author contributions

CP drafted the manuscript. AA and EG critically revised the manuscript. LP supervised the whole process. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association between uric acid and height during growth hormone therapy in children with idiopathic short stature

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Background: Serum uric acid (UA) within appropriate levels is reported to be beneficial in patients with idiopathic short stature (ISS). This study aimed to evaluate the association between serum UA levels and height standard deviation scores (SDS) in patients with ISS during growth hormone (GH) therapy.

Methods: A longitudinal study (LG Growth Study) of 182 children (mean age: 7.29±2.60 years) with ISS was performed. All participants were in the prepubertal stage and treated with GH, and the data within a treatment period of 30 months were analyzed.

Results: In the adjusted Pearson's correlation, UA was significantly correlated with height SDS after controlling for sex, age, and body mass index (BMI) SDS (r=0.22, p=0.007). In the adjusted multiple regression analyses, the height SDS was significantly associated with UA after controlling for sex, age, and BMI SDS (β=0.168, p=0.007). Within the 30-month treatment period, the UA levels significantly increased as the height SDS increased, and the mean UA levels at baseline and 30 months after treatment were 3.90±0.64 mg/dL and 4.71 ±0.77 mg/dL, respectively (p=0.007).

Discussion: In conclusion, UA is related to height SDS, and GH treatment leads to a significant increase in UA without hyperuricemia. Elevated UA is considered a favorable outcome of GH therapy, and further studies are needed to determine its role as a monitoring tool.

KEYWORDS

uric acid, children, short stature, growth hormone treatment, height

Introduction

The number of studies on the association of uric acid (UA) on growth is extremely limited compared with that on the association between UA and metabolic problems. The general UA level in children gradually increases from birth to adolescence and that the fastest increase in UA occurs during puberty (1, 2). Sexual differences in serum UA levels begin at puberty and partially result from the direct influence of the muscle mass (3). A previous study investigated the association between UA concentrations and the standard deviation scores (SDS) of insulin-like growth factor 1 (IGF-1) in children and adolescents with idiopathic short stature (ISS); IGF-1 SDS was positively associated with appropriate serum UA concentrations, whereas serum UA levels that were too high or too low were associated with lower IGF-1 SDS values (4). IGF-1 is also well known as an important regulator of muscle mass (5, 6). Obesity is associated with hyperuricemia, and a negative relationship has been reported between hyperuricemia and peak growth hormone (GH) levels in obese children and adolescents (7, 8). Studies have suggested a link between UA and GH status, suggesting that serum UA levels may be related to height during growth. We hypothesized that GH treatment would increase IGF-1 levels and possibly lead to an increase in muscle mass, along with an increase in height, leading to elevated serum UA levels. However, previous studies on the association between serum UA levels and height during the growth of children and adolescents have not yet been reported. Therefore, this study was aimed at analyzing the association between serum UA levels and height in Korean children with ISS and investigating the role of UA on growth by measuring the changes in serum UA levels relative to the height increases following GH treatment.

Methods

Study design and participants

We used data from a registry study (LG Growth Study, LGS) designed to investigate the efficacy and long-term safety of GH treatment in short Korean children and adolescents with ISS, growth hormone deficiency (GHD), Turner syndrome, small for gestational age (SGA) without catch-up growth, or chronic renal failure. LGS was registered at ClinicalTrials.gov (identifier: NCT01604395). LGS is a multicenter, non-interventional study, and detailed descriptions of its background have been provided in previous publications introducing cohort characteristics and study protocols (9, 10). Written informed consent was obtained from patients and their parents. This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Hallym University Kangdong Sacred Heart Hospital (IRB No. 2021-18-019).

A total of 367 patients with ISS were registered in the LGS between November 2011 and March 2017. The inclusion criteria were:1) height <3rd percentile for sex and age according to the data from the 2017 Korean National Growth Charts for children and adolescents (11); 2) normal GH secretion confirmed by GH levels of ≥10 ng/mL in at least one GH stimulation test; 3) prepubertal children before and during GH treatment (without breast development in girls and testicular volume lower than 4 mL in boys); and 4) treatment period longer than 1 year. ISS patients were treated with GH at a dose of 0.37 mg/kg/wk. The exclusion criteria were: 1) children born SGA; 2) chronic diseases, including chronic kidney disease and malnutrition; 3) brain diseases, such as epilepsy, cerebral palsy, and brain tumor; 4) endocrine diseases, such as GHD, hypothyroidism, precocious puberty, and diabetes mellitus; and 5) chromosomal abnormalities, such as Turner syndrome. A total of 182 patients with ISS met these criteria and were included.

Measurements

Owing to the nature of the enrolled multicenter and non-interventional studies, all laboratory analyses were performed in accordance with the local standard procedures at each site, without the use of a central laboratory. Other laboratory tests, including glucose, blood urea nitrogen (BUN), creatinine, UA, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, total cholesterol (T-C), triglyceride, IGF-1, and IGF-binding protein 3(IGF-BP3) were performed prior to test drug administration in the GH stimulation test. The pediatric endocrinologists at each center performed the physical examinations to determine the Tanner stage and GH stimulation tests by selecting two of the commonly used methods in clinical practice, such as insulin-induced hypoglycemia, L-dopa, clonidine, and glucagon tests. In all research institutions, height was measured to the nearest 0.1 cm using a Harpenden stadiometer (Holtain Ltd., Crymych, Wales, UK), while body weight was measured to the nearest 0.1 kg using a digital scale. BMI was calculated by dividing the weight by height in meters squared (kg/m^2). The SDSs for height, weight, and BMI were calculated based on the 2007 Korean National Growth Charts using the LMS method (SDS = [measured value/M]$^{1/L}$/LS; L, lambda for the Box-Cox power for skewness; M, mu for the median; S, sigma for the generalized coefficient of variation) (11). The IGF-1 SDS and IGFBP-3 SDS were calculated based on the reference values for Korean children and adolescents (12). Anthropometric and laboratory measures were obtained at 6-month intervals during the period of GH treatment, and these data were used to analyze the variables related to the changes in UA levels.

Statistical analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) and the R statistical software (https://www.r-project.org). To compare the statistical significance between groups, Fisher's exact test or chi-square test was used for categorical variables, while the t-test or Mann-Whitney U test was used for continuous variables. Pearson's correlation tests were performed at baseline to examine the correlation between UA and other anthropometric and biochemical variables, while partial correlation was used after adjusting for sex, age, and BMI SDS. Multiple linear regression analysis was conducted to investigate the independent association between UA and height SDS, after adjusting for sex, age, and BMI SDS at baseline. The clinical variables were expressed as mean ± standard deviation. Statistical significance was set at $p<0.05$.

Results

Baseline clinical characteristics of the participants

The baseline clinical characteristics of the patients are shown in Table 1. A total of 182 patients (53.3% were boys) with ISS were included in this study. The mean age of the participants was 7.29 ± 2.60 years. The mean UA levels were 3.95 ± 0.78 mg/dL in boys and 3.79 ± 0.84 mg/dL in girls at baseline, and no significant difference was observed in the UA levels between the two groups.

Correlation between UA and anthropometric and biochemical variables at baseline

Pearson's correlation analysis was performed to analyze the relationship between UA levels and anthropometric and biochemical variables. As shown in Table 2, UA levels had a significantly positive correlation with height SDS in the unadjusted (r=0.18, p=0.023) and adjusted (r=0.22, p=0.007) correlation analyses. Other variables, including weight, SDS, BUN, total protein, albumin, and T-C, were significantly positively correlated with UA levels in the adjusted correlation analysis ($p<0.05$).

Association of height SDS with anthropometric and biochemical variables by multiple linear regression analyses at baseline

Results of the unadjusted and adjusted multiple linear regression analyses of the association between UA and the anthropometric and biochemical variables are shown in

TABLE 1 Baseline clinical characteristics of the study participants.

Variable	Total (n=182)	Male (n= 97)	Female (n= 85)	*p*
Age, years	7.29 ± 2.60	7.26 ± 2.74	7.32 ± 2.45	0.877
Height, cm	113.68 ± 12.96	112.49 ± 13.34	115.13 ± 12.44	0.214
Height SDS	−2.45 ± 0.66	−2.45 ± 0.45	−2.46 ± 0.85	0.911
Weight, kg	20.87 ± 6.01	21.06 ± 6.65	20.64 ± 5.15	0.671
Weight SDS	−1.93 ± 0.88	−1.86 ± 0.89	−2.01 ± 0.86	0.307
BMI	15.88 ± 1.84	16.16 ± 1.80	15.53 ± 1.83	0.039
BMI SDS	−0.64 ± 1.04	−0.53 ± 1.05	−0.77 ± 1.02	0.182
Glucose, mg/dL	91.41 ± 12.76	92.22 ± 12.14	90.48 ± 13.45	0.361
BUN, mg/dL	12.61 ± 3.02	12.91 ± 2.88	12.28 ± 3.15	0.169
Creatinine, mg/dL	0.44 ± 0.13	0.44 ± 0.13	0.45 ± 0.12	0.853
AST, U/L	30.19 ± 7.45	29.79 ± 6.69	30.65 ± 8.25	0.448
ALT, U/L	14.69 ± 5.94	15.07 ± 6.42	14.26 ± 5.34	0.358
UA, mg/dL	3.88 ± 0.81	3.95 ± 0.78	3.79 ± 0.84	0.188
T-C, mg/dL	168.78 ± 24.40	166.62 ± 26.03	171.19 ± 22.36	0.239
TG, mg/dL	82.72 ± 39.21	78.53 ± 40.83	86.70 ± 38.23	0.522
IGF-1	159.81 ± 85.10	155.83 ± 95.70	164.43 ± 71.27	0.534
IGF-1 SDS	−0.58 ± 0.99	−0.40 ± 1.12	−0.79 ± 0.77	0.013
IGF-BP3	2,924.50 ± 1,372.55	2,758.57 ± 1,206.45	3,129.15 ± 1,549.90	0.275
IGF-BP3 SDS	0.44 ± 2.44	0.41 ± 2.41	0.49 ± 2.52	0.892
Peak GH, ng/mL	18.14 ± 10.08	17.82 ± 8.08	18.56 ± 12.23	0.681

BMI, body mass index; SDS, standard deviation score; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-C, total cholesterol; TG, triglyceride; IGF-1, insulin-like growth factor-1; IGF-BP3, insulin-like growth factor-binding protein 3; GH, growth hormone.

TABLE 2 Unadjusted and adjusted correlation analyses between uric acid and anthropometrical and biochemical variables at baseline.

	Unadjusted model		Adjusted model	
	r	*p*	r	*p*
Sex	-0.10	0.188	–	–
Age	0.00	0.977	–	–
BMI SDS	0.07	0.394	–	–
Height	0.03	0.687	0.15	0.071
Height SDS	0.18	0.023	0.22	0.007
Weight	0.1	0.213	0.24	0.003
Weight SDS	0.17	0.040	0.22	0.009
Glucose	-0.15	0.037	-0.13	0.113
BUN	0.26	<0.001	0.17	0.043
Cr	0.11	0.136	0.16	0.054
Total protein	0.17	0.021	0.31	<0.001
Albumin	0.10	0.163	0.18	0.028
AST	0.10	0.182	0.15	0.072
ALT	0.08	0.270	0.04	0.667
T-C	0.13	0.104	0.20	0.030
TG	0.07	0.667	0.03	0.875
IGF-1 SDS	0.11	0.194	0.16	0.090
IGF-BP3 SDS	-0.02	0.853	0.05	0.739
Peak GH	0.23	0.007	0.11	0.245

BMI, body mass index; SDS, standard deviation score; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-C, total cholesterol; TG, triglyceride; IGF-1, insulin-like growth factor-1; IGF-BP3, insulin-like growth factor-binding protein 3; GH, growth hormone.
The adjusted model was used after controlling for sex, age, and BMI SDS score.

Table 3. In the unadjusted multiple linear regression analysis, the height SDS was significantly and positively associated with UA levels (β=0.156, p=0.023). Height SDS also had a significantly positive association with UA levels in the adjusted multiple linear regression analysis after controlling for sex, age, and BMI SDS (β=0.168, p=0.007). The other variable associated with height SDS in the unadjusted and adjusted multiple linear regression analyses was the weight SDS.

Change in anthropometric and biochemical variables during GH treatment

The different variables monitored within the 30-month GH treatment period are listed in Table 4 and Figure 1. The treatment period was subdivided as follows: before treatment and at 6, 12, 24, and 30 months after treatment. As expected, height, height SDS, IGF-1, and IGF-1 SDS continued to increase from baseline to 6, 12, 24, and 30 months after treatment (p<0.001). The UA levels increased significantly during the treatment period, in line with the increasing trend of these factors (p<0.001). The BMI also steadily increased significantly during the period of GH treatment (p<0.05), but the BMI SDS did not show a statistically significant change.

Discussion

Our findings identified an independent correlation between height SDS and serum UA levels in children with ISS, as confirmed by multiple regression analysis after adjusting for age, sex, and BMI SDS. More importantly, the serum UA levels significantly increased with elevation in height SDS during the 30-month GH treatment. No hyperuricemia was observed in the GH-treated patients.

The reference range for UA levels in healthy children and adolescents has been reported to have a specific distribution. According to a study of UA reference values for Brazilian children and adolescents, UA slowly increases with age throughout childhood and remains the same in both sexes, and sexual differences in distribution begin at puberty (3). The results of this study also revealed no difference in UA levels between boys and girls before puberty. The relationship between UA and body composition components showed that body fat percentage seemed to have a partial effect in girls, and that the only factor contributing to UA elevation in both sexes was muscle mass. Muscle mass is considered the largest source of purines in the body, and as muscle mass increases, the supply of nucleic acids and purines to the liver increases, resulting in increased UA production (13). Alvim et al. recently reported an association between muscle mass and

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TABLE 3 Association of height SDS with anthropometric and biochemical variables using unadjusted and adjusted multiple linear regression analyses at baseline.

	Unadjusted model			Adjusted model		
	β	SE	*p*	β	SE	*p*
Age	−0.022	0.014	0.103	–	–	–
Sex	−0.065	0.074	0.382	–	–	–
BMI SDS	0.033	0.034	0.331	–	–	–
Weight	0.007	0.005	0.154	0.181	0.014	<0.001
Weight SDS	0.300	0.035	<0.001	1.332	0.026	<0.001
Glucose	0.007	0.003	0.331	0.006	0.003	0.333
BUN	0.007	0.013	0.604	0.015	0.013	0.250
Cr	0.336	0.333	0.314	0.519	0.353	0.143
Uric acid	0.156	0.068	0.023	0.168	0.061	0.007
Total protein	0.010	0.091	0.277	0.065	0.088	0.457
Albumin	0.154	0.152	0.312	0.026	0.144	0.859
AST	−0.001	0.006	0.831	−0.001	0.006	0.992
ALT	0.001	0.007	0.827	0.006	0.007	0.437
T-C	−0.001	0.002	0.971	0.003	0.002	0.835
TG	0.115	0.002	0.053	0.002	0.002	0.311
IGF-1 SDS	0.088	0.045	0.052	0.039	0.043	0.365
IGF-BP3 SDS	0.089	0.028	0.002	0.049	0.026	0.061
Peak GH	−0.005	0.005	0.270	−0.008	0.005	0.105

BMI, body mass index; SDS, standard deviation score; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-C, total cholesterol; TG, triglyceride; IGF-1, insulin-like growth factor-1; IGF-BP3, insulin-like growth factor-binding protein 3; GH, growth hormone.
Multiple analysis was adjusted for age, sex, and BMI SDS.

UA level in children and adolescents. After adjusting for age and fat mass, both sexes with higher muscle mass showed higher UA (3). Previous reports have shown that the body composition of short-stature children has different characteristics than that of normal children. In a case-control study comparing muscle mass between children with short stature and normal controls, fat-free mass, such as protein and bone minerals, was reported to be lower in children with short stature (14).

GH treatment results in improvements in body composition and long-term beneficial effects on muscle and adipose tissues (15, 16). Matusik et al. reported that severe GH deficiency is associated with an increase in adipose tissue and a decrease in muscle mass (17). An increase in muscle volume and strength

TABLE 4 Changes in anthropometric and biochemical variables during recombinant human growth hormone treatment.

	Before treatment	After treatment				*p*
		6 months	12 months	24 months	30 months	
Height	114.34 ± 13.74	119.10 ± 13.62[a]	123.44 ± 13.81[b]	129.62 ± 14.79[c]	134.00 ± 15.84[d]	<0.001
Height SDS	−2.52 ± 0.60	−2.11 ± 0.65[a]	−1.78 ± 0.60[b]	−1.44 ± 0.69	−1.20 ± 0.53[d]	<0.001
Weight	21.28 ± 6.71	23.20 ± 7.05[a]	25.65 ± 8.24[b]	30.02 ± 11.00	31.96 ± 11.06[d]	<0.001
Weight SDS	−2.02 ± 0.92	−1.75 ± 0.80[a]	−1.47 ± 0.75[b]	−1.11 ± 0.79	−0.99 ± 0.83[d]	<0.001
BMI	15.88 ± 2.00	15.95 ± 1.83	16.32 ± 2.02	17.16 ± 2.84	17.30 ± 2.72[d]	0.007
BMI SDS	−0.71 ± 1.06	−0.76 ± 0.91	−0.69 ± 0.84	−0.46 ± 0.91	−0.51 ± 1.00	0.628
Uric acid	3.90 ± 0.64	4.04 ± 0.76[a]	4.13 ± 0.87[b]	4.37 ± 0.77[c]	4.71 ± 0.77[d]	<0.001
IGF-1	154.27 ± 74.35	295.87 ± 133.35[a]	341.94 ± 158.81[b]	401.19 ± 185.38[c]	434.52 ± 193.68[d]	<0.001
IGF-1 SDS	−0.70 ± 0.78	0.79 ± 1.29[a]	1.01 ± 1.35[b]	1.02 ± 1.30[c]	1.41 ± 1.64[d]	<0.001
IGF-BP3	2,818.26 ± 1,232.73	3,342.67 ± 1,307.78[a]	3,655.20 ± 1,434.28[b]	3,657.40 ± 1,648.21	3,221.25 ± 1,553.00	0.204
IGF-BP3 SDS	0.28 ± 2.23	1.19 ± 2.34[a]	1.56 ± 2.46[b]	1.58 ± 3.24	0.99 ± 3.47	0.452

Before treatment–12 months after treatment: [a]p<0.001; 6 months after treatment–12 months after treatment: [b]p<0.001; 12 months after treatment–24 months after treatment: [c]p<0.001; 24 months after treatment–30 months after treatment: [d]p < 0.001.

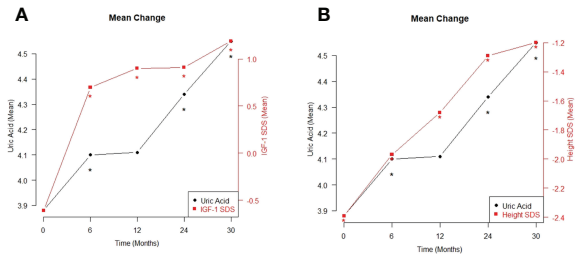

FIGURE 1
The mean changes in between uric acid and IGF-1 SDS **(A)**, and between uric acid and height SDS **(B)** during the period of growth hormone treatment *$p < 0.05$.

was observed in adults with GHD treated with GH (16). In children with isolated GHD, GH treatment plays an important role not only in the promotion of linear growth but also in the development of muscles and bones (18). GH treatment in children with SGA, Prader–Willi syndrome, and ISS had a positive effect on muscle mass and strength (19–21). Taken together, this evidence suggests that GH treatment in short-stature children will improve body composition, with long-term beneficial effects on muscle and adipose tissue, and that the increase in muscle mass will eventually lead to an increase in UA levels.

However, little is known regarding the association of GH therapy with UA metabolism. Dixit et al. reported an increase in UA levels after GH treatment in children with short stature aged 4–17 years (22). The serum UA level was 3.4 ± 0.4 mg/dL before GH treatment. The mean elevation of UA was 1.4 ± 1.4 mg/dL after GH treatment and correlated with the mean duration of treatment (2.7 ± 2.1 years). Studies on UA in children with short stature are also rare. Recently, Wang et al. reported an association between UA and IGF-1 SDS in Chinese children and adolescents with ISS (4). They showed a positive correlation between IGF-1 and UA between 2.82 mg/dL and 5.06 mg/dL. However, an inverse correlation was observed between IGF-1 SDS values and UA concentration that were either above or below threshold values; the IGF-1 SDS value decreased in response to changes in UA concentrations that were either less than 2.82 mg/dL or more than 5.06 mg/dL. The present study attempted to reproduce the nonlinear distribution results presented in a previous study using stepwise statistical analysis, but the distribution of serum UA was not characteristic. Moreover, although the increasing trends of IGF-1 SDS and UA levels were similar, no statistical association was observed. Instead, our study showed that UA was significantly associated with height SDS, and that UA increased significantly from 3.90 mg/dL to 4.71 mg/dL after GH treatment. This change in UA level corresponds to the appropriate UA range, as suggested in previous studies. Wilcox et al. reported a mean UA value of 4.1 ± 1.0 mg/dL at age 5–10 years, and Kubota et al. reported that it was 4.2 ± 0.9 mg/dL at age 7– 9 years (23, 24). The 50th percentile of UA for Korean children and adolescents is 5.1 mg/dL, but it cannot be directly compared because it is a value for the distribution at ages 10–18 years (25).

This study, as well as the study by Wang et al. (4), suggests that an increase in UA within an appropriate reference range can have a positive effect on the growth of patients with ISS. This study confirmed that changes caused by GH treatment, including increases in IGF-1 SDS and height SDS, were not different from previously reported results. Based on the results presented above, we predicted that GH treatment in ISS patients would result in an increase in muscle mass along with an increase in height SDS, which would eventually lead to an increase in UA. The results of this study suggest that UA may serve as a monitoring tool for GH treatment.

A major limitation of this study was that data such as dual-energy X-ray absorptiometry related to body composition measurements were not available. Therefore, we could not confirm an association between UA elevation and changes in body composition. Another limitation is that UA can be affected by food intake; however, it was not possible to analyze this because of the nature of this study. Nevertheless, this study had several strengths. In this study, to minimize the factors affecting UA, we included children with no underlying medical conditions such as chronic disease, endocrine disease, brain disease, or chromosomal abnormalities. Furthermore, considering that the characteristics of UA are affected by puberty, only prepubertal patients were included; thus, it is considered a meaningful result compared with other studies that included pubertal patients. The characteristics of multicenter long-term prospective cohort studies may also lend greater significance to the statistical results of this study.

In conclusion, serum UA levels are associated with height SDS. The result of a significant elevation in serum UA with an increase in height SDS after GH treatment was an interesting feature found during GH treatment. Further studies are needed to determine whether changes in serum UA levels caused by GH treatment are associated with changes in height and body

composition, and to determine the role of UA in monitoring GH treatment.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Hallym University Kangdong Sacred Heart Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

JSY contributed to the data research and analyses and drafted the initial manuscript; YJS, EBK, and HJL designed the study and interpreted the findings; MJK critically reviewed the manuscript and contributed to the conception of the study; and ITH designed and conceptualized the study and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Isolated growth hormone deficiency in children with vertically transmitted short stature: What do the genes tell us?

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Introduction: The growth hormone deficiency (GHD) diagnosis is controversial especially due to low specificity of growth hormone (GH) stimulation tests. It is therefore believed that children diagnosed with GHD form a heterogeneous group with growth disorder frequently independent on GH function. No study evaluating the complex etiology of growth failure in children with diagnosed GHD has been performed thus far.

Aims: To discover genetic etiology of short stature in children with diagnosed GHD from families with short stature.

Methods: Fifty-two children diagnosed with primary GHD and vertically transmitted short stature (height SDS in the child and his/her shorter parent <-2 SD) were included to our study. The GHD diagnosis was based on growth data suggestive of GHD, absence of substantial disproportionality (sitting height to total height ratio <-2 SD or >+2 SD), IGF-1 levels <0 for age and sex specific SD and peak GH concentration <10 ug/L in two stimulation tests. All children were examined using next-generation sequencing methods, and the genetic variants were subsequently evaluated by American College of Medical Genetics standards and guidelines.

Results: The age of children at enrollment into the study was 11 years (median, IQR 9-14 years), their height prior to GH treatment was -3.0 SD (-3.6 to -2.8 SD), IGF-1 concentration -1.4 SD (-2.0 to -1.1 SD), and maximal stimulated GH 6.3 ug/L (4.8-7.6 ug/L). No child had multiple pituitary hormone deficiency or a

midbrain region pathology. Causative variant in a gene that affects growth was discovered in 15/52 (29%) children. Of them, only 2 (13%) had a genetic variant affecting GH secretion or function (*GHSR* and *OTX2*). Interestingly, in 10 (67%) children we discovered a primary growth plate disorder (*ACAN*, *COL1A2*, *COL11A1*, *COL2A1*, *EXT2*, *FGFR3*, *NF1*, *NPR2*, *PTPN11* [2x]), in one (7%) a genetic variant impairing IGF-1 action (*IGFALS*) and in two (12%) a variant in miscellaneous genes (*SALL4*, *MBTPS2*).

Conclusions: In children with vertically transmitted short stature, genetic results frequently did not correspond with the clinical diagnosis of GH deficiency. These results underline the doubtful reliability of methods standardly used to diagnose GH deficiency.

KEYWORDS

short stature, growth hormone, growth hormone deficiency, genetics, next-generation sequencing

Introduction

The correct production, secretion, and function of growth hormone (GH) is important for the physiological growth and optimal functioning of the human organism (1, 2). For people with growth hormone deficiency (GHD), treatment with recombinant GH is essential to achieve normal adult height and, in cases of severe GHD, prevent repeated episodes of hypoglycaemia or other possible consequences of impaired metabolic GH function (1, 3). Precise diagnosis of individuals with GHD allowing early GH treatment is therefore crucial (2).

The diagnosis of GHD is complex combining auxological, laboratory and radiological examination. Growth hormone stimulation tests are performed for the confirmation of the diagnosis (3, 4). However, these tests are known to have low specificity, potentially causing false positive results (5–8). Consequently, children diagnosed with GHD likely form a rather heterogeneous group with different etiology of growth disorder frequently independent of GH production or function (9). However, no studies evaluating the complex genetic etiology of growth failure in children that have been clinically classified as having GHD have been performed so far.

Importantly, modern genetic methods including next-generation sequencing (NGS) have shown their potential to discover the causes of growth disorders on a molecular basis (9, 10). The boom in genetic diagnostics in the last two decades has led to a substantial progress in understanding the etiology of short stature (9, 11). In our study, we aimed to search for genetic background of short stature in children diagnosed as GHD from families with short stature.

Materials and methods

Patients

Inclusion criteria

According to the records database, 747 children are currently treated with GH in our center. After excluding children with Prader-Willi syndrome, Turner syndrome, and those with known secondary cause of their growth disorder (e.g., chronic kidney disease, secondary GHD caused by intracranial tumor, surgery and/or irradiation), 528 patients remained for further evaluation. Within this group, 419 individuals were diagnosed with primary GHD. Out of these, 70 had vertically transmitted short stature defined as a height SDS ≤-2 SD in both the child and his/her shorter parent and therefore, were chosen for the study. A total of 52 study participants/their legal guardians signed written informed consent before genetic examination and were included in the study. The study was approved by the institutional Ethics Committees of the 2nd Faculty of Medicine (approval number EK-753.3.5/21) of Charles University in Prague, Czech Republic.

Clinical evaluation

The heights and body proportionality (sitting height to total height [SHH] ratio) of all participants were obtained during anthropometric measurements. Data regarding birth parameters were obtained from the medical records. The parents' heights were measured to the nearest 1 mm and the heights of other relatives were obtained from the parents. All the data was standardized according to recent normative values (12–14).

Bone age was evaluated using the Tanner-Whitehouse method (15).

Diagnostics of growth hormone deficiency

Growth hormone deficiency was diagnosed using current guidelines (3, 4). In all children with auxological data suggestive of GHD (i.e., current height <-3 SD below the mean, current height <-1.5 SD below the midparental height and/or current height <-2 SD below the mean combined with a decrease in height >0.5 SD over one year in a child older than 2 years), IGF-1 levels <0 SD (reference ranges standardized for sex and age) and no substantial disproportionality (SHH ratio <-2 SD or >+2 SD) GH stimulation tests were performed. Children with a maximum GH concentration <10 ug/L in both the clonidine and insulin hypoglycemia tests were classified as having GHD. Sex-steroid priming was performed in children aged 9 years and older.

Genetic testing

Genomic DNA was extracted from peripheral blood (QIAamp Blood Mini Kit, Quiagen, Hilden, Germany) in all children included in the study. Firstly, some children underwent basic genetic testing. Turner syndrome and SHOX haploinsufficiency were examined in all girls by fluorescence *in situ* hybridization (FISH). In children with a clinical suspicion of a specific genetic disorder, targeted genetic testing was performed. Children with no genetic cause of short stature elucidated at this point were subsequently examined using the NGS methods: whole-exome sequencing (WES) or custom-targeted NGS panel containing 398 genes associated with growth (Supplementary Table 1). All variants from the NGS were confirmed by Sanger sequencing as we described previously (16). The method of genetic examination we described in detail in previous studies (11, 17, 18).

All variants with potential clinical importance were evaluated by American College of Medical Genetics and Genomics (ACMG) standards and guidelines (19). For variant evaluation, we also used the ACMG criteria implemented into the VarSome software (20) and Franklin software (https://franklin.genoox.com version date 2nd November 2022) that score each ACMG rule as very strong, strong, moderate, or supporting based on ACMG recommendations and its more up-to-date modifications. In some cases, the strength of the rules was modified according to extended investigation of various databases and clinical evaluation of the patient. To evaluate the segregation of genetic variants with short stature in the families, DNA and height data of other relatives was obtained. The guidelines formulated by Jarvik et al. were followed (21) and applied to co-segregation in the pathogenicity classification. At the end, all genetic variants were classified as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB) or as variants of uncertain significance (VUS).

Results

In total, 52 children with a primary GHD diagnosis and vertically transmitted short stature were enrolled to the study. Their age at study enrolment was 11 years (median; IQR 9-14 years), their pretreatment height was -3.0 SD (-3.6 to -2.8 SD), their shorter parent's height was -2.6 SD (-2.9 to -2.2 SD), their IGF-1 concentration prior to the GH treatment was -1.4 SD (-2.0 to -1.1 SD),their stimulated GH concentration maximum was 6.3 ug/L (4.8-7.6 µg/L), and their bone age was delayed by 1.1 years (0.3-1.7 years). Seventeen children had mild GHD with stimulated GH concentrations of 7.0-9.9 µg/L, 34 children had stimulated GH concentrations 3.0-6.9 µg/L and only one child had severe GHD with stimulated GH concentration <3.0 µg/L. No child had multiple pituitary hormone deficiency or a midbrain region pathology on magnetic resonance imaging. The birth length and birth weight of the children in the study cohort was -1.8 SD (median; IQR -2.4 to -1.2 SD) and -1.3 SD (-2.0 to -0.7 SD), respectively. Twenty-two children were born small for gestational age (SGA) (9 for both birth length and weight, 11 only for birth length, and 2 only for birth weight). The children have been treated with GH for a median 5.0 years (3.5-6.0 years), with a median dose of 32 µg/kg/day (30-34 µg/kg/day) during the first year of treatment.

A monogenic cause of growth failure was elucidated in 15 (29%) of 52 children with diagnosed primary GHD and vertically transmitted short stature who were enrolled to the study. Of them, only 2 (13%) had a genetic variant affecting GH secretion or function (*GHSR* and *OTX2*). Interestingly, 10/15 (67%) children had a genetically proven primary growth plate disorder (4 had extracellular matrix protein defect [genes *ACAN*, *COL1A2*, *COL11A2*, and *COL2A1*], 2 had impaired paracrine regulation of the growth plate [genes *NPR2*, and *FGFR3*], and 4 had a disorder affecting fundamental intracellular processes of the growth plate [*PTPN11* gene in 2 patients, and *EXT2* and *NF1* genes each in a single patient]. Among the remaining children, 1/15 (7%) had altered IGF function (gene *IGFALS*), and 2/15 (13%) had mutations in miscellaneous genes (*MBTPS2* and *SALL4*). Specific genetic variants and phenotypes of the children are summarized in Table 1, data evaluating children with genetically confirmed GHD and those with genetically diagnosed primary growth plate disorder are summarized in Table 2.

Discussion

In our study, we evaluated the complex genetic etiology of growth disorders in children with diagnosed primary GH deficiency from families with short stature. Interestingly, GHD was genetically confirmed as a cause of growth failure only in a minority of children (13% of children with genetic etiology discovered, 4% of the whole study cohort). On the other hand, modern genetic examination

TABLE 1 A table evaluating clinical characteristics and genetic examination results in children with proven monogenic aetiology of their short stature.

Patient	Sex	Age at last follow-up (years)	GH treatment initiation age (years)	GH dose in the first year of GH treatment (ug/kg/day)	Shorter parent's height (SD)	Birth weight (SD)	Birth length (SD)	Height SDS prior to GH treatment	Height SDS after 1 year of GH treatment	Height SDS after 3 years of GH treatment	Growth velocity prior to GH treatment (cm/year)	Growth velocity in the first year of GH treatment (cm/year)	BA prior to GH treatment (difference to CA, years)	IGF1 prior to GH treatment (SD)	Stimulated GH maximum (ug/l)	SHH ratio (SD)	Additional phenotypic features	Genetic examination method	Gene	Variant type	Transcript variant	Protein variant	Classification	ACMG criteria
Impaired growth hormone production																								
1	F	10	7	31	-2.8	-2.6	-3.1	-3.2	-2.1	-1.4	4.2	10.6	-0.6	-2.1	7.2	0.1	Mother HELLP syndrome in pregnancy	WES	GHSR	M/n	c.526G>A	p.Gly176Arg	LP	PM2(m), PP1(m), PP3(m)
2	M	10	6	28	-2.8	-1.2	-1.1	-6.3	-5.0	-3.4	4.5	12.1	0.0	<-3	1.7	0.4	Psychomotor retardation	NGS panel	OTX2	M/n	c.106delC	p.Arg36fs	P	PVS1(vs), PM2(m), PP5(sp)
Alteration of IGF function																								
3	M	11	7	34	-2.8	-1.6	-1.7	-3.5	-2.6	-1.6	5.3	9.8	-0.2	-1.8	6.1	0.6	–	WES	IGFALS	M/n	c.589C>T	p.Arg197Cys	LP	PP1(st), PM2(m)
Primary growth plate disorder																								
Extracellular matrix																								
4	M	14	7	30	-3.6	-1.6	-3.3	-3.7	-2.9	-2.2	3.7	8.6	1.7	-1.6	3.2	0.9	–	Sanger	ACAN	M/n	c.1425del	p.Val478fs*14	P	PVS1(vs), PM2(m), PP1(st)
5	M	12	5	35	-2.0	-2.5	-1.5	-3.2	-2.3	-1.9	3.8	9.9	-2.0	-0.9	6.4	-0.2	Frequent long bone fractures, vertebrae compressive fractures	NGS panel	COL1A2	M/n	c.577G>A	p.Gly193Ser	P	PM2(m), PM6(m), PP2(sp), PP3(st), PP4(sp), PP5(s)
6	M	16	10	35	-2.0	-1.0	-0.7	-3.4	-2.7	-2.1	3.8	7.8	-1.3	-1.2	8.0	0.7	–	NGS panel	COL11A2	M/n	c.3706C>T	p.Arg1236Cys	LP	PM2(m), PP1(st), PP3(sp)
7	M	16	12	33	-2.9	-2.4	-3.0	-3.4	-2.9	-2.2	4.1	8.7	-1.5	-1.6	8.4	1.2	–	WES	COL2A1	M/n	c.3016C>G	p.Arg1036Gly	LP	PM1(sp), PP1(m), PP2(sp), PP3(sp)
Paracrine growth plate regulation																								
8	F	5	4	35	-2.4	-1.6	-2.4	-3.0	-2.9	NA	3.7	6.7	NA	-2.1	4.0	0.3	–	NGS panel	FGFR3	M/n	c.251C>T	p.Ser84Leu	LP	PM2(m), PP1(sp), PP2(sp), PP5(st)
9	M	9	7	32	-2.4	0.9	-1.7	-2.9	-2.2	NA	5.6	10.3	-1.7	-1.4	4.7	0.3	–	NGS panel	NPR2	M/n	c.613C>T	p.Arg205*	P	PVS1(vs), PM2(m), PP3(sp)
Fundamental Intracellular processes of the growth plate																								
10	M	12	8	33	-2.9	-2.4	-3.4	-2.7	-2.0	-1.3	4.6	9.0	0.5	-1.0	5.9	0.5	–	WES	EXT2	M/n	c.2034C>G	p.Asn678Lys	LP	PM2(m), PP1(st), PP3(sp)
11	M	16	11	33	-2.9	1.0	-1.2	-3.9	-2.9	-2.6	2.5	9.2	0.5	-4.3	6.2	0.7	Vitiligo	Sanger	PTPN11	M/n	c.211T>A	p.Phe71Ile	LP	PM1(m), PM2(m), PM5(m), PP2(sp), PP3(sp)
12	M	12	7	33	-2.6	NA	NA	-2.7	-1.9	-1.2	4.0	9.3	-1.6	-1.4	4.8	1.5	–	Sanger	PTPN11	M/n	c.1403C>T	p.Thr468Met	P	PS3(st), PM1(sp), PM2(m), PM5(m), PP2(sp), PP3(sp), PP5(m)
13	F	12	6	33	-3.9	1.0	0.1	-2.5	-1.7	-1.0	4.8	8.7	-0.7	-1.1	1.2	NA	Café-au-lait spots, hamartomas in the brain	Sanger	NF1	M/n	c.4267A>G	p.Lys1423Glu	P	PS3(st), PM1(m), PM2(m), PM5(m), PP2(sp), PP3(sp), PP5(vs)
Genes with miscellaneous function																								
14	M	10	5	34	-2.7	-1.1	-1.8	-4.4	-4.0	-3.6	5.9	7.2	NA	-1.1	6.4	0.9	Immunodeficiency, Hyperkeratosis, cornea scarring	WES	MBTPS2	M/-	c.1538T>C	p.Leu513Pro	LP	PM1(sp), PM2(m), PP3(m), PP5(sp)
15	M	12	7	34	-2.4	-1.8	-1.4	-2.8	-2.1	-1.0	6.3	8.7	NA	-1.0	4.6	-0.3	Radial ray defect. right kidney dystopia	WES	SALL4	M/n	c.1717C>T	p.Arg573*	P	PVS1(vs), PM2(m), PP1(sp), PP4(sp), PP5(sp)

ACMG, American College of Medical Genetics guidelines; BA, bone age; CA, calendar age; F, female; GH, growth hormone; LP, likely pathogenic; M, male; (m), moderate strength of the criterion used; M/- - hemizygote, M/M, homozygote, M/n, heterozygote, NA, not available; NGS, next-generation sequencing; P, pathogenic; SD, standard deviation; (sp), supporting strength of the criterion used; (st), strong strength of the criterion used; SHH, sitting height to height; (vs), very strong strength of the criterion used; WES, whole exome sequencing.

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TABLE 2 Table evaluating clinical data in children with genetically confirmed GH deficiency and those with genetically diagnosed primary growth plate disorder.

	Patient 1 with genetically confirmed GHD	Patient 2 with genetically confirmed GHD	Patients with genetically diagnosed primary growth plate disorder
Gene found	*GHSR*	*OTX2*	NA
Shorter parent's height (SD)	-2.8	-2.8	-2.8 (-2.9 to -2.4)
IGF-1 prior to GH treatment (SD)	-2.1	<-3.0	-1.4 (-1.6 to -1.1)
Maximal stimulated GH concentration (ug/L)	7.2	1.7	5.4 (4.0 to 6.4)
Height prior GH treatment (SD)	-3.2	-6.3	-3.1 (-3.4 to -2.7)
Height after one year of GH treatment (SD)	-2.1	-5.0	-2.5 (-2.9 to -2.0)
Height after 3 years of GH treatment (SD)	-1.4	-3.4	-2.0 (-2.2 to -1.3)
Growth velocity prior to GH treatment (cm/year)	4,2	4,5	3.9 (3.7 to 4.6)
Growth velocity in the first year of GH treatment (cm/year)	10,6	12,1	8.9 (8.6 to 9.3)

The values in children with genetically diagnosed primary growth plate disorder are expressed as medians and interquartile ranges. GH, growth hormone; NA, not applicable; SD, standard deviation.

methods frequently discovered other mechanisms causing growth disorders independent of GH production, secretion or function, further broadening the doubts about the GHD diagnostics.

Studies evaluating the genetic etiology in children classified as GHD are scarce. Depending on the cohort, causative genetic variants were found in approximately 11% of children with primary isolated GHD (22). In the remaining majority of children with diagnosed IGHD, specific causes of their short stature are unknown. These children are traditionally labelled with a descriptive diagnosis of an "idiopathic growth hormone deficiency" (9) and the etiology of their growth disorder remains to be elucidated. In our study, we found genetic causes of GHD in even smaller proportion of children (2/52; 4%). However, unlike in the previous studies, we focused not only on genes causing GHD, but on other possible genetic causes of short stature as well. Using this strategy, we discovered the genetic etiology of growth failure in an additional 25% (13/52) of children. Surprisingly, 67% (10/15) of them had a primary growth plate disorder.

The discrepancy between the original clinical diagnosis of GHD and the genetic finding in most cases raises a question of which method is more accurate. Importantly, current diagnostics of GHD faces many difficulties and is considered as one of the most controversial issues in pediatric endocrinology (5). Growth hormone stimulation tests currently used as golden standard of the GHD diagnosis (4) are not physiological and have a very low specificity and reproducibility. Furthermore, they are affected by pubertal development, obesity, or other characteristics of the examined individual (3, 5). Unfortunately, there is no method reliably proving GHD to validate GH stimulation tests (5). A recent study by Bright et al. calculated an extremely low probability (2.8%) of a true-positive GH stimulation test in a child with short stature (23). Due to all these reasons, it is believed that most children with diagnosed GHD have a non-pituitary etiology of their short stature and are erroneously labelled as GHD (9). The results of our study support this presumption and offers the first insight into the possible non-pituitary causes of growth disorders in such children.

On the other hand, in the absence of a validation method for GHD diagnostics (5), there is no way to prove the diagnosis of GHD is incorrect. We must therefore admit the possibility that GHD might contribute to the patients' short stature in additional to the cause discovered by genetic testing. To correctly interpret the results of our study, we need to consider the possibility of false positive genetic results especially if the likely pathogenic variants are considered causative. However, the probability of a likely pathogenic variant to be truly pathogenic is >90% (19) and are therefore more trustworthy than the methods traditionally used to diagnose GHD.

Our study had several strengths. Firstly, we examined a homogenous population of patients from a single center of an economically stable country with high quality health care, low consanguinity rate, and negligible social causes of growth failure such as malnutrition. Secondly, all GH stimulation tests were

performed by experienced investigators using a defined protocol and the results were analyzed in a single laboratory using the same methodology. However, our study had several limitations as well. Firstly, functional studies were not performed in our study. However, according to ACMG standards and guidelines, the causality of genetic variants can be proven also by other methods (19). In our study evaluating children with vertically transmitted short stature, the segregation of genetic variants in short people within the families was an important factor. Secondly, protein non-coding variants (in the exception of disruption in the exon-intron boundaries) were not captured by the NGS. Thirdly, copy number variants were not evaluated in the current study. Fourthly, although our NGS panel included a large number of genes associated to growth disorders (398), causative variants in the genes not present in the panel could have been missed. Moreover, children with known genetic cause of their short stature prior to the study were not examined using NGS. Finally, only children with vertically transmitted short stature were included in our study. To generalize these results to all children with diagnosed GHD, further studies are warranted.

Conclusion

In children with vertically transmitted short stature, genetic results frequently did not correspond with the clinical diagnosis of GH deficiency. These results underline the doubtful reliability of methods standardly used to diagnose GH deficiency.

Data availability statement

The datasets presented in this article are not readily available for ethical and legal reasons relating to the participants' privacy rights. The raw sequencing data are available upon reasonable request to the corresponding author (lukas.plachy@fnmotol.cz).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committees of the 2nd Faculty of Medicine (approval number EK-753.3.5/21) of Charles University in Prague, Czech Republic. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LuP organized the study, contributed to the study design of the study, helped to obtain clinical data, contributed to the interpretation of the genetic results, contributed to the results interpretation, wrote the manuscript. SA contributed to the study design of the study, contributed to the results interpretation, contributed to the interpretation of the genetic results, contributed to the final version of the manuscript. PD contributed to the study design of the study, performed the genetic examination, supervised the interpretation of genetic results, contributed to the results interpretation, contributed to the final version of the manuscript. KM, DZ contributed to the study design of the study, performed antropometric measurements, contributed to the results interpretation, contributed to the final version of the manuscript. VN, LeP, BO, MS, SK, ZS, JL contributed to the study design of the study, helped to obtain clinical data, contributed to the results interpretation, contributed to the final version of the manuscript SP. Supervised the whole study, contributed to the study design of the study, helped to obtain clinical data, contributed to the genetic results interpretation, contributed to the results interpretation, contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Short stature related to Growth Hormone Insensitivity (GHI) in childhood

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Linear growth during childhood is the result of the synergic contribution of different factors. The best growth determinant system during each period of life is represented by the growth hormone–insulin-like growth factor axis (GH–IGF), even if several other factors are involved in normal growth. Within the broad spectrum of growth disorders, an increased importance has been placed on growth hormone insensitivity (GHI). GHI was reported for the first time by Laron as a syndrome characterized by short stature due to GH receptor (GHR) mutation. To date, it is recognized that GHI represents a wide diagnostic category, including a broad spectrum of defects. The peculiar characteristic of GHI is the low IGF-1 levels associated with normal or elevated GH levels and the lack of IGF-1 response after GH administration. Recombinant IGF-1 preparations may be used in the treatment of these patients.

KEYWORDS

short stature, GH receptor, GH insensitivity, growth hormone, GH-IGF-1 axis, childhood

1 Introduction

Linear growth during childhood is a complex process regulated by both prenatal factors and nutritional, hormonal, environmental, or genetic components, the latter subject to increasing importance. In fact, it is known that adult height is an inheritable trait and results from the synergic contribution of each polymorphism among the genes associated with linear growth. It has been postulated that every single abnormality of these genes could significantly impair linear growth during childhood, and this is related to short stature (1). However, only a small proportion of these genes is recognized to be related to growth disorders during childhood. The newest genetic techniques will probably allow the detection of a rising number of gene mutations, which will explain the underlying cause of short stature.

Within the broad spectrum of growth disorders, an increased importance has been placed on growth hormone insensitivity (GHI). GHI was first described by Laron in 1966. For many years, GHI and Laron syndrome were considered the same entity, and the phenotype characterizing Laron syndrome was the only one recognized among the GHI

syndrome. Particularly, it was known that GHI is first caused by a defect in the GH receptor (GHR). In fact, the mutations of GHR lead to impaired binding of GH to GHR and consequently to the lack of IGF-1 production that is secreted after GHR activation. With the development of molecular techniques that allow the cloning and characterization of the human GHR, the pathophysiology of GHI has been better understood (2, 3). The discovery of novel genes related to growth has allowed a more complete study of genetic abnormalities in the GH–IGF-1 axis, thus providing a better understanding of the complexity of GHI and the physiology of human linear growth. To date, it is recognized that GHI represents a wide diagnostic category, including a broad spectrum of defects affecting the function of the IGF-1 system. These abnormalities may involve gene coding for proteins, both those controlling GH binding or signal transduction and IGF-1 synthesis, transport, or action, and are associated with a variety of phenotypes and biochemical abnormalities (3). The common characteristics of all these defects are represented by short stature, which may be associated with peculiar characteristics specific to each defect, although in the majority of cases the genotype–phenotype correlation is not yet clarified.

The purpose of this review is to describe the common genetic abnormalities related to the spectrum of GHI, focusing on those determining IGF-1 deficiency in order to better clarify each single entity of this complex group and to promote a tailored approach based on genetic features.

2 Physiology of normal growth

Short stature in children is defined as a height standard deviation score (SDS) lower than - 2 SD, more than 2 SD below a population's mean for age and sex (4). Although this definition is well recognized, it is important to state that intrauterine period, infancy, childhood, and adolescence are characterized by different growth patterns that might affect growth differently. In particular, intrauterine growth is mainly influenced by maternal nutrition, placental factors and the intrauterine milieu. During this period, the genetic influence seems to have less importance, as demonstrated by the poor correlation between birth length and adult height (5). The first two years of life are initially characterized by a rapid growth, followed by a successive deceleration. During this period, the child's genetic potential and endocrine factors start to be expressed. Childhood age is characterized by a constant linear growth that rapidly increases soon after the pubertal period (6). However, despite the different factors influencing each period of life, the best growth determinant system at all ages remains the GH–IGF-1 axis, even if several other factors are involved in normal growth (7).

2.1 GH-IGF-1 axis

Regular GH-IGF-1 secretion and functions are fundamental for pre and postnatal growth (3). Human prenatal growth is mainly regulated by nutritional supplies, which influence fetal IGF-1 and, perhaps, IGF-2 (8).

Pituitary GH is encoded by the GH1 gene, and it is secreted in pulsatile manner in the circulation (9). GH production is modulated by neurological, metabolic, and endocrine factors. Various hormonal stimuli, both stimulatory—such as hypothalamic GH-releasing hormone (GHRH), ghrelin, and sex steroids—and inhibitory—such as somatostatin, IGF-1, and glucocorticoids—regulate this balance. GH exists free of or alternatively bound to the GH-binding protein (GHBP), which is a portion of the receptor, thus constituting half of the total GH amount. After secretion, GH binds to a specific receptor, namely, growth hormone receptor (GHR), which is mainly expressed in the liver, bone, muscle, and other target tissues (Figure 1). In turn, after receptor occupation and dimerization, the intracellular effects start. The final result of this complex chain of events is the synthesis of IGF-1 and IGF-2, which through endocrine, paracrine, and autocrine mechanisms stimulates linear growth (10). In particular, IGF-2 and IGF-1 are the major effectors of growth during fetal life, while IGF-1 is produced throughout life. Studies performed on mice have demonstrated that tailored disruption of either IGF-1 or IGF-2 led to a 40% decrease in fetal growth (8). On the other hand, GH and the GH-IGF-1 axis are the major protagonists of human postnatal growth, which may be impaired by mutations affecting every level of this complex axis.

In addition, GH can act in a direct manner despite the indirect actions through the IGF-1 release (7). In fact, the original concept, namely, the "somatomedin hypothesis", postulates that after GH binds to its receptor, IGF-1 is produced and independently influences growth (3, 11). This concept has evolved over the last years, and Green and colleagues proposed the "dual effector hypothesis", postulating that GH regulates the expression of locally produced IGF-1, which then acts in an autocrine/paracrine manner (12). In addition, different studies have suggested local effects of GH that are independent of those mediated by circulating IGF-1. This hypothesis has been confirmed by Isaksson and others who demonstrated that GH stimulates the differentiation of preadipocytes and chondrocytes in the growth plate, while IGF-1 stimulates their clonal expansion (13, 14).

Thereafter, IGF-1 is primarily secreted into the blood and then links to one of six high-affinity IGF-1 binding proteins (IGFBPs). For 75–90%, IGF-1 is linked to IGFBP-3, which is the most abundant among the IGFBPs family, and for 1%, circulates unbound. The linkage between IGF-1 and IGFBP-3 constitutes a complex called the "binary complex". Soon after, this binary complex is stabilized by the linking to an acid-labile subunit (ALS), codified by the gene IGFALS into the liver. The ternary complex reduces IGF-1 and IGF-2 circulating levels and increases their half-life (15). In turn, IGF-1 binds to a membrane-spanning homodimeric receptor (IGF-1R), which determines the autophosphorylation of the intracellular β-subunit of IGF-1R and the stimulation of intracellular signaling pathways (16).

2.2 Growth hormone receptor (GHR)

The GHR mediates the effects of GH on linear growth and metabolism. It is ubiquitously expressed with major concentrations in the liver. The GHR gene is sited on chromosome 5p13-p12 (17). It is a protein composed of 620 amino acids, including an extracellular,

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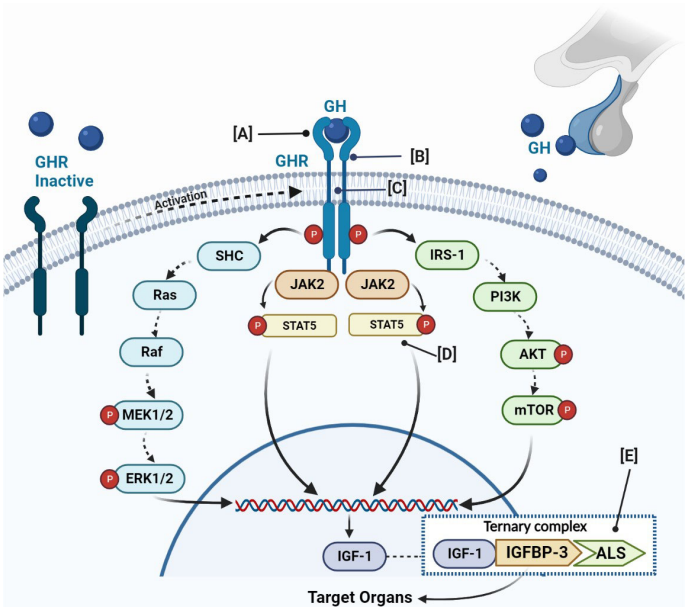

FIGURE 1
Schematic figure of GH-IGF-1 axis, its physiology, and main genetic defects related to GHI. The GH binds to a GHR, which activates JAK2 and promotes the phosphorylation of different members of the signal transducer. The final result is the IGF-1 production. IGF-1 binds to IGFBP-3 or IGFBP-5 and the acid-labile subunit (ALS) and constitutes the ternary complex. **(A)** Defects of the extracellular domain of the GHR; **(B)** defects in GHR dimerization; **(C)** defects of the transmembrane domain of the GHR; **(D)** defects of STAT5b; **(E)** defects of IGFBP. GH, growth hormone; GHR, growth hormone receptor; JAK2, Janus family tyrosine kinase 2; PI3K, phosphatidylinositol-3 kinase; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; IGF, insulin-like growth factor; IGFBP, IGF-binding proteins.; ALS, acid-labile subunit.

transmembrane, and intracellular domain. The binding extracellular domain of 246 amino acids is involved in GH binding through the subdomain 1 and in GHR dimerization across the subdomain 2. The single transmembrane domain is composed of 24 amino acids that anchor the receptor to the cell surface. Finally, the intracellular domain of 350 amino acids is fundamental for GH signaling (18). The receptor is encoded by nine exons, namely from 2 to 10 (19). GH binding to its receptor determines receptor activation through rotation, the changes of the conformation and dimerization thus constituting a ternary complex between two GHR and one GH molecule (20). Thereafter, a cleavage of the GHR determines the release of its extracellular domain, which circulates in blood as a soluble GH-binding protein (GHBP). The boxes 1 and 2, located in the intracellular domain of the GHR, are important for the GHR-GH–IGF-1 axis signaling transduction, since they contain the JAK2 binding sites, which are linked and activated by Janus Kinase 2 (JAK2) (Figure 1). The linkage of JAK2 to GHR is fundamental for controlling the position of the GHR transmembrane helices, its movements, and the crystal structures of the JAK2 kinase. On the other hand, recent studies have revealed that the GH receptor may exist as constitutive dimers rather than being dimerized as a consequence of ligand binding. Binding of the bivalent ligand reorientates and rotates the receptor subunits, determining the conversion from a form with parallel transmembrane domain to one where the transmembrane domain is divided at the point of entry into the cytoplasm (21). This movement slides the pseudokinase inhibitory domain of one JAK kinase away from the kinase domain of the other JAK, allowing the two kinase domains to interact and transactivate (21). This determines the tyrosine phosphorylation within the receptor cytoplasmic domain, which generates docking sites for the SH2 domain that contains proteins such as STAT3 or STAT5, which are phosphorylated and activated (22). Receptor phosphorylation is accompanied by the direct JAK2 phosphorylation of other target proteins (21).

Thus, JAK2 represents a potential modifier of signaling, either by inhibiting activation or activating the receptor in the absence of the ligand. In addition, it has the advantage of activating a receptor that is insensitive to the ligand due to mutations in ligand-binding residues (21).

However, to promote GH action, in addition to this pathway namely Janus kinase-signal transducers and activators of transcription (JAK-STAT), GH signal transduction is mediated by other two pathways: phosphatidylinositol-3 kinase (PI3K) and Mitogen-activated protein kinase (MAPK). The final result of the activation of these signals is represented by IGF-1 production. The normal functions of GHR are essential to ensure the physiological effects of GH (23) not only in terms of linear growth but also in terms of bone mineral density and adiposity, with a greater risk of health consequences like osteoporosis, lipid disorders, and cardiovascular diseases (22).

3 Growth hormone insensitivity (GHI)

Resistance or insensitivity to hormone action is defined when normal concentrations of a specific hormone are unable to induce the usual response; thus, the secretion of the proximal hormone is increased. In detail, GH insensitivity (GHI) is characterized by low IGF-1 levels associated with normal or elevated GH levels and lack

of IGF-1 response after GH administration (24). The pathognomonic biochemical feature of the different entity of GHI is represented by IGF-1 deficiency (25).

Several genetic defects are responsible for the impairment of GH and IGF-1 actions, resulting in short stature that could be manifest during both the prenatal and postnatal period. GHI was reported for the first time by Laron and colleagues in two siblings with the classical clinical appearance of GH deficiency, but presenting elevated levels of GH (26). To date, the spectrum of GH insensitivity has been considerably expanded thanks to advances in terms of genetic diagnosis, leading to the discovery of different mutations affecting every level of the GH-IGF-1 axis (3).

GH insensitivity can be classified as related to primary GH insensitivity, associated with IGF-1 deficiency and IGF-1 insensitivity, and secondary GH insensitivity. The GH insensitivity resulting from IGF-1 deficiency can be categorized into different groups, namely, defects of the GH receptor (GHR), defects of the intracellular GH signaling pathway (STAT5B), and primary defects of the synthesis or activity of IGF-1 and IGF-2 (27), as shown in Table 1.

3.1 Primary GH insensitivity

3.1.1 Growth disorders related to GHR defects

Mutations of GHR represent the most frequent cause of primary GHI syndrome, clinically characterized by severe short stature, with a height up to 10 standard deviations (SDs) below normal, and severe IGF-1 deficiency (28). To date, over 90 different mutations of the GHR gene have been characterized (29). There are various types of mutations, including deletion, RNA-processing defects, translation, and missense mutations, which may affect every step essential for the correct functioning of the pathway, from ligand binding to signal transduction, and which finally determine the failure to stimulate normal growth (22). In each coding exon of GHR, at least one molecular abnormality has been reported, but a poor genotype–phenotype relationship has been demonstrated.

The primary and best-known hormone insensitivity syndrome is known as Laron syndrome. It was described for the first time in 1966 in two siblings among consanguineous Jewish families from Yemen (26). Only after twenty years in 1989 was the cause of the condition identified in the partial deletion of the GHR gene (30). Laron syndrome is a fully penetrant autosomal recessive disease caused by exon deletion (31) or mutations of the GHR (32), leading to the disruption or alteration of the GH-binding site or failure to express the GHR on the cell surface (Figure 1A). Only homozygous and double heterozygous patients for these defects express the typical phenotype. It was characterized as a clinical entity indistinguishable from congenital GH deficiency (GHD). The typical features include severe short stature, dwarfism, obesity, small genitalia, and delayed puberty (33–35). The short stature is severe, with final height ranging from -5 to -10 SDS below a population's mean for age and sex. One of the characteristic features is facial hypoplasia, which has different degrees of severity and is due to underdeveloped facial bones. The obesity starts in childhood and is characterized by high body fat localized in

TABLE 1 The main defects associated with GH insensitivity in children and their clinical and laboratory features.

	DEFECTS	CLINICAL FEATURES	LABORATORY FEATURES
PRIMARY GH INSENSITIVITY	GHR - Extracellular domain - Transmembrane - Intracellular domain - Exon	- Severe/mild short stature - Facial hypoplasia - Obesity - Insulin resistance/glucose intolerance/Type 2 diabetes	GH ↑, IGF-1 ↓, IGFBP-3 N/↓, variable GHBP
	Intracellular GH signaling pathway (STAT5B, STAT3)	- Severe short stature - Immune deficiency	GH↑, IGF-1 ↓↓, IGFBP-3 N/↓↓, GHBP N
	IGF1/IGF2 synthesis	- Short stature - Deafness - Intellectual disability - Microcephaly - Carbohydrate intolerance	GH↑, IGF-1 ↓↓ or absent, IGFBP-3 N, GHBP N
	IGF-1 receptor deficiency/Bioinactive IGF-1	- Severe intrauterine growth restriction - CNS abnormalities	GH↑, IGF-1 ↓↓ or absent, IGFBP-3 N, GHBP N
	IGFBP-3/ALS	- Mild short stature - Delayed puberty - Insulin insensitivity - Decreased bone mineral density	GH↑, IGF-1 ↓, IGFBP3 ↓↓, GHBP N
SECONDARY GH INSENSITIVITY	- Antibodies against GH - Antibodies against GHR - Malnutrition - Inflammatory bowel diseases - Severe disease - Catabolic state - Liver diseases - Poorly controlled diabetes	- Variable short stature - Typical features characterizing the underlying disease	GH ↑, variable IGF-1, GHBP ↓, IGFBP-3 ↓/N

the arms; it is enhanced by insulin resistance that may lead to the development of glucose intolerance and type 2 diabetes (36, 37). In addition, obesity seems to be correlated to leptin levels, which are elevated in patients with homozygous GHI, probably resulting from abnormalities of the body composition and metabolism (15, 38, 39). The laboratoristic features include increased serum GH and low serum IGF-1 that does not increase after rhGH (recombinant human GH) administration.

The vast majority of the recognized molecular defects included in this category are associated with severe GHI and are usually localized in the extracellular domain of the GHR. In particular, among the 93 identified mutations of human GHR, the majority of cases—68 to be exact—are related to the extracellular domain; 13 occur in GHR introns, 10 occur in the intracellular domain, and only 2 occur in the transmembrane domain. The mutation that decrease GH binding are generally associated with the reduction of GHBP, which represents the circulating extracellular domain of the GHR, and it is decreased or undetectable in 80% of cases. The dosage of GHBP may be used as a laboratoristic parameter to differentiate the different mutations of GHR. In fact, mutations affecting the transmembrane or intracellular domains of the GHR are commonly characterized by normal, or even increased, serum concentrations of GHBP.

In addition, among the mutations affecting the extracellular domain, some might preserve GH binding even if they alter GH actions through different mechanisms (40).

The mutations occurring in the subdomain that implicate in the dimerization of GHR should be considered (Figure 1B). Although they preserve GH binding, different mutations are responsible for inability to form a stable GHR dimer, causing a defect of the entire system. To date, the discovered mutations affecting this function are three. The first is E180X (GAA > TAA), which results in a receptor protein with an 8-amino acid deletion in the extracellular dimerization domain. This mutant protein preserves the capacity to homodimerize, but the transferring to the cell surface is abnormal (41). The second is E180 splice, which regards both GH binding and GHR trafficking, producing a non-functional GHR (42). Finally, a deletion of 166 bases of exon 7 in the GHR mRNA was found in a patient affected by neurofibromatosis and concomitant short stature. This mutation resulted in the premature termination of the sequence and thus in a reduced GH-binding affinity to the GHR, hence determining growth failure (43).

However, there are few cases where heterozygous GHR mutations also exert a dominant negative effect (28, 44). These subjects usually present a lesser growth restriction and a milder clinical phenotype (45).

In addition, mutations of the splicing sites result in the improper translation of transcripts into biologically active proteins. Among the defects causing abnormalities of GHR splicing, an intronic base change leading to the activation of a pseudoexon sequence and an insertion of 36 new amino acids within the receptor extracellular domain were first reported in a case of GHI from a consanguineous Pakistani family (46). These defects have been shown to lead to an impaired function of the abnormal protein (47). The phenotypes are variable, from severe to mild short stature (48), since the splice site mutations form heterodimers with the normal GHR and exert a dominant-negative effect on the normal protein (3). A recent study has identified a novel GHR 6Ω pseudoexon inclusion resulting in the loss of GHR function associated with the severe GHI phenotype. This represents a novel mechanism of Laron syndrome and is the first deep intronic variant identified that is related to severe postnatal growth failure (49).

3.1.2 GH signaling defects

The linking of GH to the GHR stimulates signaling cascades, involving different pathways that finally promote GH action, after the phosphorylation of different transduction factors. Among the different pathways, JAK-STAT seems to have a key role in the growth mechanisms, acting after phosphorylation of the GHR. In turn, the receptor phosphorylation promotes STATs phosphorylation and dissociation from the GHR as well as the dimerization and translocation to the nucleus, where it activates specific transcription elements on DNA and regulates the transcription of different genes, including IGF-1 (Figure 1). Although abnormalities in the MAPK pathway and NF-KB pathway may also cause GHI (50), the STATs mutations are the most studied correlation (Figure 1D). Several STATs have been identified, namely, from STATs-1 to STATs-6. All STATs have the same structure composed of five domains, including: an amino-terminus domain, fundamental for nuclear translocation and DNA binding; a coiled-coil domain; a DNA-binding domain; a SH2 domain, needed for receptor-specific recruitment and STAT dimerization; and a COOH-terminal transcriptional activation domain (19). To date, pathophysiological genetic defects have been identified in all STATs except STAT5A (51–53), which is most closely related to STAT5B, sharing >90% amino acid identity (54).

Although the GH signal transduction is mediated in part through STAT1 and STAT3, it seems that the germline STAT5B deficiency is more strongly associated with growth failure due to IGF-1 deficiency. In fact, in a study using mice knockout models (STAT5a–/–, STAT5b–/–, and STAT5a–/–b–/–), it was shown that the mice required STAT5b for the production of IGF-1 after GH treatment and to ensure normal postnatal growth (55). These results were confirmed by studies demonstrating that human STAT5B mutations also cause severe growth failure due to GHI and also demonstrating the critical role exerted by STAT5b signaling in GH-induced IGF-1 production and in normal linear growth (56). The first identification of STAT5B mutations was documented in 2003 in an Argentine 16-years old female born in a consanguineous family and affected by the homozygous missense mutation of the STAT5b gene, which determined the replacement of proline with alanine in the SH2 domain (57). The resultant protein is unable to phosphorylate normal effectors after GH stimulus (58). This patient showed severe short stature with a height lower than 10 SD for age and sex. The biochemical profile is characterized by normal or elevated GH secretion, severe IGF-1, IGFBP-3, and ALS deficiency, which does not increase after GH administration (3). On the other hand, serum GHBP values are normal in the majority of cases, reflecting the normality of the GHR

gene and the produced protein (55). These defects do not affect prenatal growth, as confirmed by the normal birth weight presented by these patients. In contrast, they are characterized by severe postnatal growth failure that does not respond to exogen GH administration (58, 59).

Since the first report, about six other cases of STAT5B mutations have been documented and have been mainly demonstrated in siblings (60). All reported mutations were homozygous and autosomal recessive. With the exception of the first case, a common feature recognized in the majority of cases is immune dysfunction. As a consequence of the immunological abnormalities, the patients were affected by recurrent pulmonary infections occurring since infancy, including episodes of lymphoid interstitial pneumonia and consequently autoimmune disease (58). Other life-threatening infections include chronic diarrhea, severe eczema, herpes keratitis, herpes zoster, severe varicella, and juvenile arthritis (15, 56). However, these patients have normal brain development and cognitive functions.

As mentioned above, it is known that GH activates STAT1 and STAT3, which may regulate genes associated with growth and mediate their metabolic effects (61, 62). An essential role of STAT3 acting through IGF-1 in embryonic and perinatal growth was established (63). Mice lacking one allele of STAT3 showed more perinatal mortality, lower serum IGF-1 levels, and lower birth weight in 10–15% of cases (64). Germline heterozygous STAT3 gain of function mutations result in a heterogeneous phenotype that includes early-onset multiorgan autoimmunity, immunodeficiency, and short stature (64). In addition, the defect is associated with intrauterine growth restriction, delayed puberty, tooth eruption, and sometimes with delayed bone age. Although short stature represents the major clinical feature in these patients, in the majority of cases of STAT3 mutation, only poor data about growth are available. The common laboratoristic characteristic of all cases consists of IGF-1 deficiency associated with normal GH secretion. The molecular mechanism underlying the growth impairment is not completely understood. However, different studies have postulated the influence of chronic disease and immunosuppressive medications on growth failure (65). In addition, some STAT3 mutations have been shown to decrease STAT5B transcriptional activity, suggesting a negative impact in the GH signaling pathway (64).

3.1.3 Primary defects of the synthesis or activity of IGF-1 and IGF-2

This category of defects includes defects of the synthesis of IGF-1 and IGF-2 due to gene mutation, abnormalities of the IGF-1 receptor, bio inactive IGF-1, and defects of IGFBP-3 or ALS.

The first case of growth failure due to the IGF-1 gene defect was described by Woods and colleagues in 1996 (66). It is caused by the homozygous deletion of exons 4 and 5 of the IGF-1 gene. The patients showed both intrauterine and postnatal growth failure, suggesting a key role of IGF-1 in intrauterine growth that seems to be GH-independent (19). The common features of IGF-1 deficiency include severe short stature with a height lower than -3 SDS for age and sex, high GH levels, and extremely low levels of IGF-1, normal IGFBP-3 levels, and slightly delayed bone age (15). At the clinical examination, all the cases with a homozygous IGF-1 deficiency are short and characterized by microcephaly, intellectual development delay, and sensory-neural deafness (67, 68). The microcephaly characterizing these patients allows to distinguish these defects from the others previously described. At the laboratory analysis, the cases may present variable serum IGF-1 levels and particularly those subjects with a mild phenotype present IGF-1 levels that are not extremely decreased (50). However, all the patients have normal or elevated serum IGFBP-3 and ALS levels.

The defects of the IGF-1 receptor determine GHI and severe intrauterine and sensorineural deafness. However, poor studies have been performed in humans. Similarly, the production of bioinactive IGF–1 may cause the same features of the IGF-1 receptor defects and is related to both intrauterine and postnatal growth failure (69).

On the other hand, the bioavailability of IGF-1 is dependent on its release from associated IGFBPs, which have a higher affinity for IGF-1 than IGF1R. IGFBPs thus act both as carriers of IGF, prolonging its half-life, and as modulators of IGF availability and activity (70). No human mutations in IGFBPs have been identified to date (54), but IGFALS may affect IGF-1 bioavailability. In fact, the acid–labile subunit (ALS) deficiency that is needed to form the ternary complex with IGF-1, IGFBP-3, or IGFBP-5 is responsible for IGF-1 deficiency (Figure 1E). However, growth is not extremely compromised in these cases. In fact, serum IGFBP-3 levels are more significantly reduced compared to those of IGF-1. In addition to common features characterizing the IGF-1 deficiency defects, the peculiar characteristic of this defect is represented by the delayed onset of puberty associated with insulin insensitivity and decreased bone mineral density (71). The insulin sensitivity is probably related to the increased GH levels and the low IGF-1 levels (72–74). The poor pubertal growth suggests that the circulating IGF-1 pool, in addition to IGF-1 produced at the growth cartilage, is essential to achieve a normal pubertal spurt (75). This reflects the clinical observations of a lack of growth acceleration during puberty in patients with IGF-1 deletion or resistance where no significant increase in circulating IGF-1 levels occurred. However, normal pubertal growth has been reported for several patients despite persistent low circulating IGF-1 levels (76). Therefore, another possible explanation for the discrepancy in the pubertal growth registered in different studies might be the low estrogen levels detected in these patients, since estrogen plays a direct role in the pubertal spurt. Since IGF-1 is involved in sensitizing the gonads to the action of the gonadotropins, the low estrogen levels might be, in turn, related to the low IGF-1 concentrations (75, 77).

3.2 Secondary GH resistance

In patients treated with rhGH, an immunological reaction may lead to the production of the rhGH antibody that neutralizes GH molecules. However, after ending rhGH treatment or changing the composition of preparation, the GH antibodies rapidly decrease until they disappear in most patients (78, 79). Among the secondary conditions associated with GHR resistance, the antibody that acts

directly against GHR is one of the best known (15). The antibodies are directed in most cases against the extracellular domain and binding protein. The different antibodies may have several effects on the receptor, as an antagonist or agonist (80, 81). Other conditions, such as malnutrition, inflammatory bowel diseases, and chronic diseases, are associated with GH resistance (82). The resulting growth failure may vary from mild to moderate. The laboratory findings that characterize secondary GH resistance are increased GH, variable IGF-1 concentration, low GHBP, and low or normal IGFBP-3 levels (83). Another condition is hepatic resistance in children affected by poor-controlled type 1 diabetes (T1D), which is treated with insulin, resulting in the decrease of IGF-1 levels. In fact, the lack of the negative feedback exerted by IGF-1 causes GH hyper secretion. IGFBP-3 levels increase and GHBP decreases. The results are growth failure varying from mild to severe form. The severe form of growth failure in children with T1D is also called Mauriac syndrome, which is characterized by hepatomegaly and cushingoid features in addition to short stature (84). In these cases, adequate insulin therapy may reverse growth failure and hepatomegaly when present (84).

4 Therapy

The categorization of the defects is important to establish the correct therapeutical strategy. Different studies have demonstrated that high doses of recombinant human GH (rhGH) allow to obtain a mild increase of IGF-1 concentration for a short period. However, after the failure of the compensatory mechanism, IGF-1 production decreases despite treatment and becomes insufficient to assure normal growth, prevent delayed bone age, and affect final height (22).

Alternatively, a combined therapy comprising rhGH plus recombinant human IGF-I (rhIGF-1) appears to be an effective treatment option in some cases. In particular, it may be effective in cases characterized by the presence of heterozygous mutations in the GHR intracellular domain that have mild phenotypes compared with those of the classical GHI syndrome (28). Thus, this therapeutical approach may be useful in cases of less severe GH insensitivity, while in conditions of complete GH insensitivity the rhIGF-1 represents the only therapeutical option to improve linear growth (85).

The treatment with rhIGF-1 is indicated in cases of Laron syndrome and likewise in the mutations of the GH–GHR activation pathway and in the presence of the GH-inhibiting antibody (86). This therapy improves stature by increasing the annual height rate and has a positive effect on dysmorphic facial features typical of patients affected by Laron syndrome. The response to treatment may be more evident in patients with a more severe form of disease. Despite the acceleration of the growth rate, the final height still remains below the third percentile in the majority of cases. However, it has been demonstrated that if the therapy is started early during childhood, a near-normal adult height can be achieved. The adverse effects of the rhIGF-1 therapy include intracranial hypertension occurring in the 5% of cases, headache and transient papilledema, and lipohypertrophy and pain at the injection site (15). One of the most fearsome adverse effects is hypoglycemia, which has been described in 8% of the treated patients (87).

Therefore, it is clear that there are different therapeutical approaches based on the severity of the defect that need to be tailored according to the specific genetic mutation.

5 Conclusions

The GH–IGF-1 axis in humans is fundamental for normal pre and postnatal growth. The mutations at every level of this complex mechanism may result in growth impairment and consequently short stature. GHI was first described by Laron, but following the development of novel and more sophisticated molecular techniques, the pathophysiology of GHI has been better understood. To date, GHI is known to include a wide range of defects, each one with its own clinical and biochemical features that are distinct from one another. However, the exact mechanisms underlying short stature remain unknown in many patients, and the thorough assessment of patients with growth failure should be promoted in order to improve diagnosis and particularly to personalize the correct therapeutical approach.

Author contributions

CG and CM wrote the draft. CG and FC revised the text. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A comprehensive validation study of the latest version of BoneXpert on a large cohort of Caucasian children and adolescents

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Introduction: Automated bone age assessment has recently become increasingly popular. The aim of this study was to assess the agreement between automated and manual evaluation of bone age using the method according to Tanner-Whitehouse (TW3) and Greulich-Pyle (GP).

Methods: We evaluated 1285 bone age scans from 1202 children (657 scans from 612 boys) by using both manual and automated (TW3 as well as GP) bone age assessment. BoneXpert software versions 2.4.5.1. (BX2) and 3.2.1. (BX3) (Visiana, Holte, Denmark) were compared with manual evaluation using root mean squared error (RMSE) analysis.

Results: RMSE for BX2 was 0.57 and 0.55 years in boys and 0.72 and 0.59 years in girls, respectively for TW3 and GP. For BX3, RMSE was 0.51 and 0.68 years in boys and 0.49 and 0.52 years in girls, respectively for TW3 and GP. Sex- and age-specific analysis for BX2 identified the largest differences between manual and automated TW3 evaluation in girls between 6-7, 12-13, 13-14 and 14-15 years, with RMSE 0.88, 0.81, 0.92 and 0.84 years, respectively. The BX3 version showed better agreement with manual TW3 evaluation (RMSE 0.64, 0.45, 0.46 and 0.57).

Conclusion: The latest version of the BoneXpert software provides improved and clinically sufficient agreement with manual bone age evaluation in children of both sexes compared to the previous version and may be used for routine bone age evaluation in non-selected cases in pediatric endocrinology care.

KEYWORDS

bone age, Tanner-Whitehouse, Greulich-Pyle, BoneXpert, validation study

1 Introduction

The status of skeletal maturation is the most reliable indicator of biological age in children and adolescents. Bone age (BA) evaluation is a standard procedure widely used in children with growth failure and puberty disturbances. In addition, it is used in chronically ill patients as a complement to overall clinical health assessment (1, 2). BA is used successfully for the timing of orthopedic surgeries in children with uneven length of extremities or specific bone deformities as well.

An x-ray must comprise of the entire hand and wrist to be able to evaluate the bone age. The rationale for this lies in the fact that this skeletal site includes a large number of short bones of which the order and progression of ossification is very well known. Currently, the most common methods of evaluation are the Greulich and Pyle's method (GP) published in 1959 (3) and the Tanner and Whitehouse 3 method (TW3), where the first edition was published in 1962 (4). While the GP method evaluates the hand as a whole, the TW3 method assigns specific stages of skeletal maturation (1 through 9) to 13 individual pre-determined bones of the hand and wrist (the so-called Radius-Ulna-Short bones, RUS).

Although manual assessment of bone age using both the GP and TW3 methods is reliable if performed by a highly experienced specialist, its main disadvantage is the subjective nature of the procedure. The bone age result of two distinct expert raters may differ by up to a year (5, 6). Thus, automated methods of bone age assessment using software-based morphometric analysis of digitally acquired hand and wrist x-rays have been introduced to clinical practice in the last few years, aiming to eliminate the inherent subjective aspect of the manual work-up and save time. The most sophisticated and currently widely used method of automated bone age analysis works on the platform of the BoneXpert software developed by Visiana (Holte, Denmark). In brief, the software delineates the distal epiphyses of the radius, ulna, metacarpals and phalanges. At least eight bones need to be scored to compute bone age (7). Detailed functioning of the software has been described previously (7, 8).

While the first two commercially released versions of the software already underwent validation with real clinical cases (9, 10), the latest release (issued in 2020) that aimed to improve the limitations of former versions, has not yet been independently tested.

The aims of this study were: 1) to compare manual and automated bone age assessment using BoneXpert software versions BX2 and BX3 using both the GP and TW3 methods in a large cohort of children with various disorders, ages, and sexes, 2) to explore whether the TW3 bone age outcome is affected by differences in the evaluation of individual bones between manual and automated methods.

2 Participants and methods

2.1 Participants

This cross-sectional retrospective study included 1285 radiographs from 1202 non-selected children and adolescents aged 5 to 16 years (657 scans in 612 boys and 628 scans in 590 girls). All radiographs done for the purpose of bone age assessment at Motol University Hospital between January 2018 and January 2019 were collected. Patients with an abnormal bone structure (e.g. skeletal dysplasia) and patients of non-Caucasian ethnicity[1] were excluded from the analysis. The software rejected 8 images for poor quality or having an incorrect hand position. Sex-specific one-year age categories were created for girls between 5 and 15 years and boys between 5 and 16 years. Each one-year category included a minimum of 50 radiographs.

This study was approved by the Ethics Committee of the Motol University Hospital (Reference No.: EK-264/18) and complied with the Declaration of Helsinki.

2.2 Bone age assessment

After the bone age scan of the left hand and wrist was taken, each image was evaluated manually by one of two experienced raters (M.K. or Z.D.) using both the TW3 (4) and GP (3) methods (only patients sex was disclosed, chronological age was calculated after bone age assessment, diagnosis was not provided to the rater). All images were sent in a standard DICOM format (*Digital Imaging and Communications in Medicine*) for evaluation using automated bone age assessment software BoneXpert (Visiana, Holte, Denmark). No post-processing was applied to the x-rays. The software input consists of patient's sex, birth date and date of x-ray scan. The BX2 version was used for the purpose of clinical practice, the same images were then reevaluated by the BX3 version as well. This was used only for the purpose of validating the program (the BX3 version was kindly provided by Visiana in form of a StandAlone program for independent evaluation).

If the absolute difference between the manual and automated bone age assessment was more than 1.5 year (an arbitrarily set cut-off in either the GP or TW3 method) the images were reevaluated by an experienced independent rater (S.P.), a medical anthropologist with no affiliation to the Motol University Hospital. An average of the two manual assessments was used for statistical analysis in these cases (N = 70).

2.3 Statistical analysis

Throughout the analysis, repeated measurements on the same child were treated as independent observations as they were gained at different visits.

The Bland-Altman analysis was used to determine the character of differences between the automated and manual approach. For each patient, Bland-Altman plots the difference between the automated and manual assessment against the mean of the two methods, or alternatively, against the values of one of the two methods. In this analysis, differences were plotted against the results of the manual method. The graphs indicate where the automated

1 According to the 2021 census, the Czech population is homogeneous, the largest minority is of Vietnamese descent and makes up only 0.4% of the population (11).

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method produced higher or lower values in comparison to the manual method, possible bias (mean of differences) and lower and upper limit of accuracy (LOA), computed as bias ± 2×standard deviation (SD). Bias of each method was tested using a one-sample t-test, the bias between BX2 and BX3 were compared using paired t-tests.

To explore the size of differences between manual and automated bone age assessment in general and in various categories (defined by sex and/or age and diagnosis), Root Mean Squared Errors (RMSE) were calculated using the standard formula (12):

$$RMSE = [\sum_{i=1}^{N}(z_{fi} - z_{oi})^2/N]^{1/2}$$

where:

- Σ = summation
- $(z_{fi}\text{-}z_{oi})^2$ = differences, squared
- N = sample size

Confidence intervals for RMSE were computed under the assumption of symmetry of deviations of BoneXpert estimates compared to manual assessment. Accuracy of BX2 with respect to manual assessment was compared to the accuracy of BX3 with respect to manual assessment using the Diebold-Mariano test (13).

In the detailed analysis of the TW3 method, the difference between stages assigned by manual and automated method were compared using ANOVA F-test and *post-hoc* pairwise comparisons with Benjamini-Hochberg correction for multiple comparisons. The differences in assigned bone stages were tested in all available scans divided into 3 groups according to the difference in the final bone age (BX higher than manual by >1.0 year; BX lower than manual by > 1.0 year; BX not different from manual, i.e.<1.0 and > -1.0 year). In bones showing the greatest differences in assigned bone stages, the effect on resulting bone age was tested.

All analyses were performed in statistical language and environment R, version 4.1.2 (14). The level of statistical significance was set to 0.05 throughout the analysis. In case of multiple comparisons adjustment (such as testing in various age-, sex- or diagnosis-specific categories), the Benjamini-Hochberg method was used.

3 Results

3.1 Comparison between automated and manual bone age assessment in children according to sex and age

Using the TW3 method, the BX2 version generally underestimated bone age in both sexes, whereas the BX3 version performed comparably to the manual assessment with mean of the differences close to zero (Table 1 – the data are given in years). On the other hand, BX3 performed significantly worse using the GP method compared to BX2 version in boys (Table 1). In particular, while BX2-assessed GP bone age did not differ from manually assessed GP bone age in boys, the BX3 version significantly overestimated GP bone ages. In girls, both BX2 and BX3 slightly underestimated GP bone age compared to manual evaluation.

The differences between automated and manual bone age results are presented in detail in Bland-Altman graphs in Figure 1. The best agreement was observed in the BX3 version using the TW3 method in both sexes (Figure 1B).

These findings were further supported by the RMSE analysis showing that the BX3 version has significantly better agreement with manual bone age assessment than the BX2 version in both sexes using the TW3 method and in girls using the GP method as well (Table 2 - the data are given in years). In contrast, the BX3 version performed worse than BX2 in boys using the GP method.

Sex- and age-specific RMSE for the BX2 version using the TW3 method showed that the largest differences between automated and manual bone age were present in girls aged 6-7 and 12-15 years (Figure 2). When using the BX3 version, the agreement between automated and manual bone age improved significantly in 8/10 age categories in girls, when compared to BX2. For the GP method, BX2 showed significantly larger RMSE than the BX3 version only in girls aged 7-8 years.

In boys, the BX3 version showed improvement of the TW3 method in 4 age categories (9-10, 11-12, 13-14 and 15-16 years), compared to BX2 (Figure 2). In contrast, the RMSEs between manual and automated bone age evaluation were larger when using the BX3 version compared to BX2 using the GP method in boys, in particular for ages 6-8 and 9-10 years. The RMSE numeric values (in years) are presented in Supplementary Table 1.

TABLE 1 Overall means of differences in years between automated and manual bone age assessment, separately for both sexes and software versions (BX2 and BX3).

		TW3				GP			
	N	BX2 – MAN		BX3 – MAN		BX2 – MAN		BX3 – MAN	
		mean (SD)	P	mean (SD)	P	mean (SD)	p	mean (SD)	P
Boys	657	-0.19 (0.54)	< 0.0001	-0.01 (0.51)	0.239	-0.00 (0.55)	0.924	0.39 (0.56)	< 0.0001
Girls	628	-0.47 (0.55)	< 0.0001	-0.02 (0.49)	0.635	-0.23 (0.55)	< 0.0001	-0.10 (0.51)	< 0.0001

P-values for one-sample t-test examining the difference from zero.
TW3, bone age assessment according to Tanner-Whitehouse 3 method; GP, bone age assessment according to Greulich-Pyle method; BX2, BoneXpert version 2.4.5.1.; BX3, BoneXpert version 3.0.3.; MAN, manual bone age assessment.

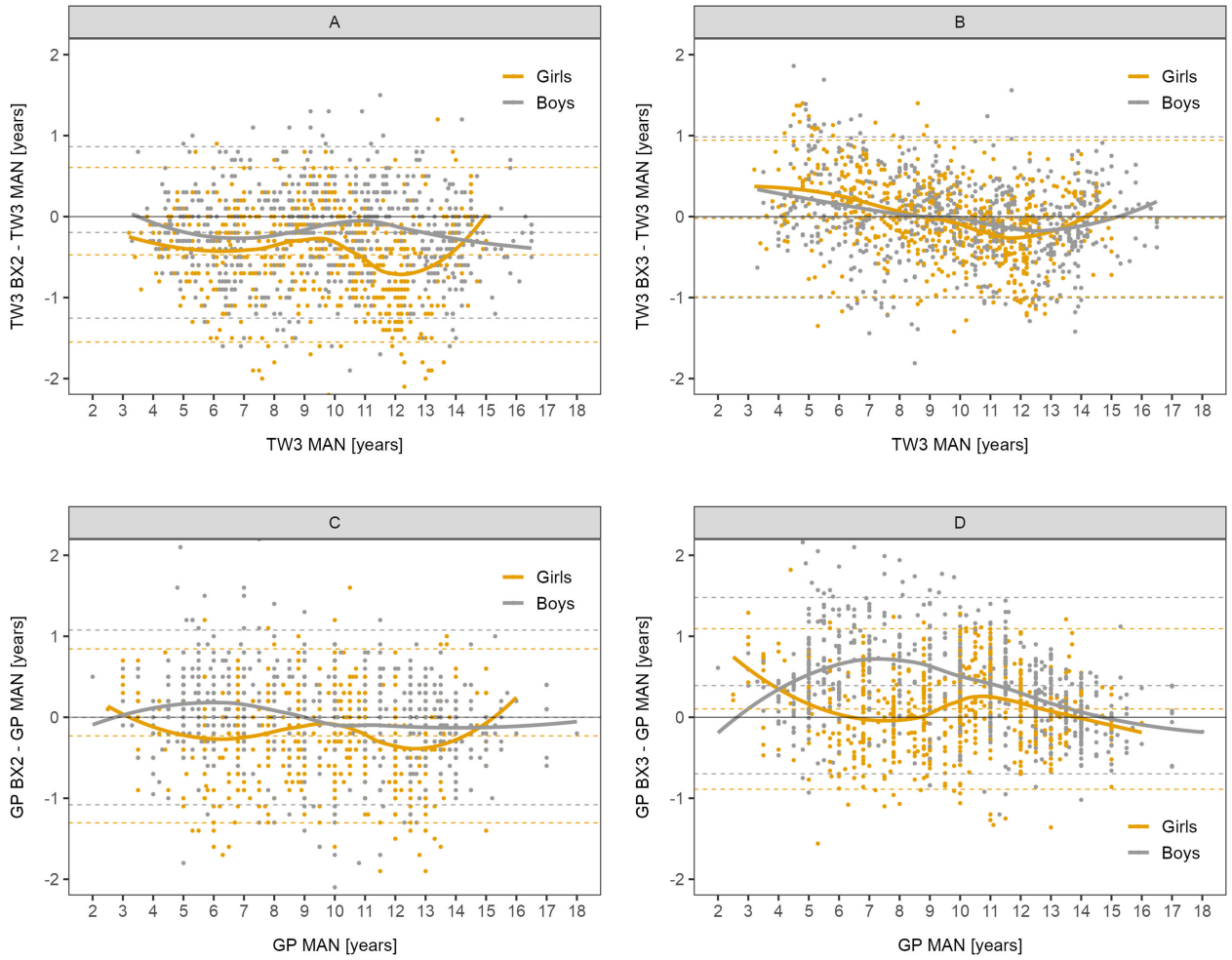

FIGURE 1
Bland-Altman analysis – difference in years between automated and manual bone age result plotted against the manual bone age values. Sex-specific smoothing lines computed by LOESS method. Bland-Altman analysis shows whether there is a systematic component to the differences between methods. Dashed lines show mean and upper and lower limit of accuracy for respective methods and sex. The closer the mean to 0 the less over/underestimating the method is, overall. **(A)** TW3, BX2 vs. manual, **(B)** TW3, BX3 vs. manual, **(C)** GP, BX2 vs. manual, **(D)** GP, BX3 vs. manual. TW3, bone age assessment according to Tanner-Whitehouse 3 method; GP, bone age assessment according to Greulich-Pyle method; BX2, BoneXpert version 2.4.5.1.; BX3, BoneXpert version 3.0.3.; MAN, manual bone age assessment.

The absolute difference in bone age result > 1.0 year was noted in 7.5% and 6.2% scans in boys and 16.4% and 8.4% scans in girls, for TW3 and GP respectively, when using the BX2 version. The BX3 version showed > 1.0 year difference in 6.3% and 12.8% scans in boys and 6.0% and 5.3% scans in girls for TW3 and GP, respectively.

3.2 Agreement between automated and manual bone age assessment in children with various diagnoses

The RMSE analysis confirmed that the best agreement between automated and manual bone age evaluation was reached when using

TABLE 2 Root mean square errors of automated vs. manual bone age assessment, separately for both sexes and software versions (BX2 and BX3).

		TW3			GP		
	N	BX2 vs MAN	BX3 vs MAN	p	BX2 vs MAN	BX3 vs MAN	P
Boys	657	0.57 (0.54-0.61)	0.51 (0.48-0.54)*	0.0007	0.55 (0.52-0.58)	0.68 (0.64-0.72) #	< 0.0001
Girls	628	0.72 (0.69-0.77)	0.49 (0.47-0.52)*	< 0.0001	0.59 (0.56-0.63)	0.52 (0.49-0.55)*	< 0.0001

Root mean square errors (and corresponding 95% confidence intervals) are shown (in years).
p-value: Diebold-Mariano test for method accuracy (* BX3 performs significantly better than BX2 α= 0.05, # BX3 performs significantly worse than BX2 at α =0.05).
TW3, bone age assessment according to Tanner-Whitehouse 3 method, GP, bone age assessment according to Greulich-Pyle method, BX2, BoneXpert version 2.4.5.1., BX3, BoneXpert version 3.0.3., MAN, manual bone age assessment.

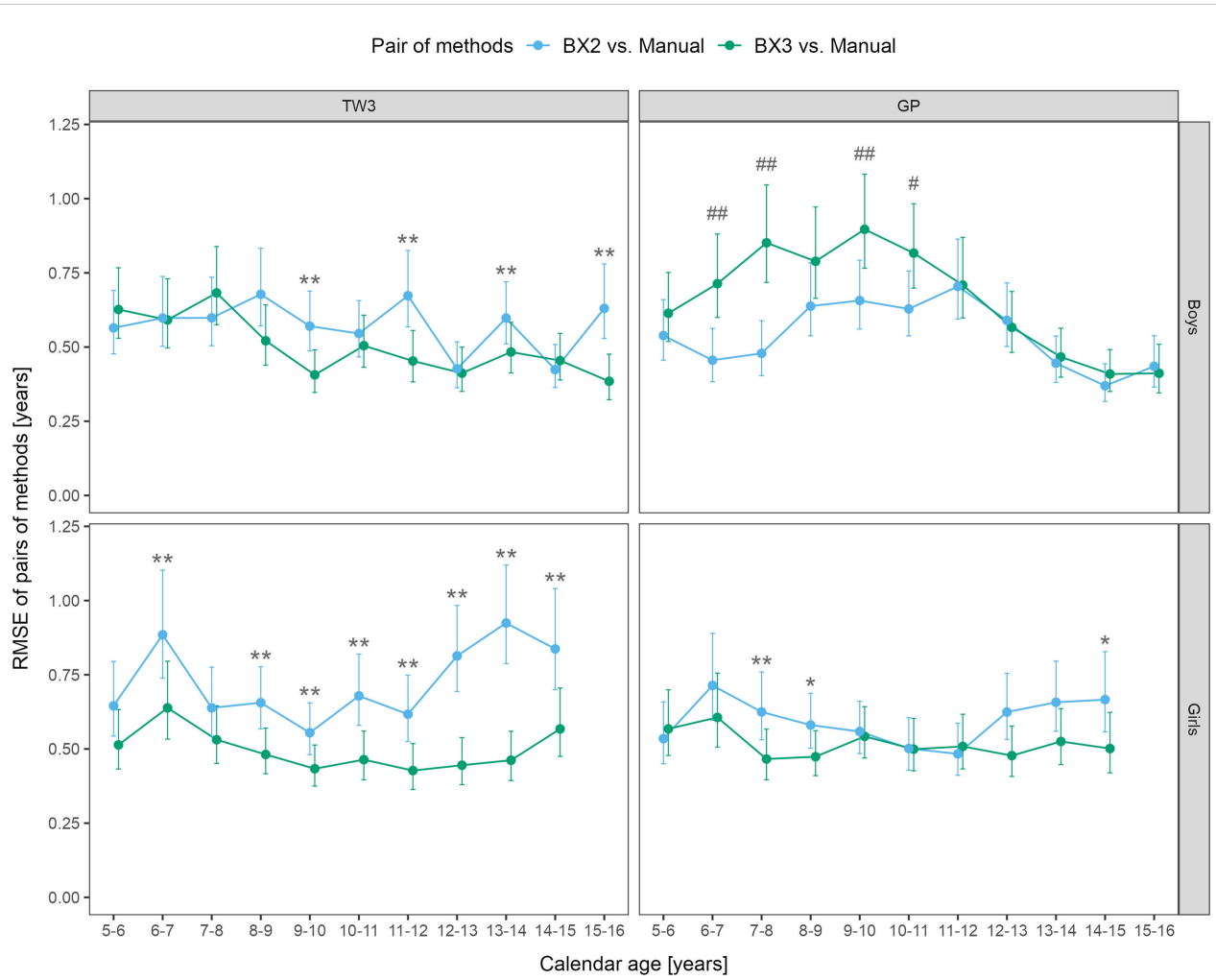

FIGURE 2
Root mean square errors (RMSE) and 95% confidence intervals for age and sex specific categories, separately for TW3 and GP methods and for BX2 and BX3 versions. RMSE is given in years. Before (*) and after (**) adjustment for multiple testing, BX3 performs significantly better, i.e. differs less from the manual assessment, than BX2 at $\alpha = 0.05$. Before (#) and after (##) adjustment for multiple testing, BX3 performs significantly worse, i.e. differs more from the manual assessment, than BX2 at $\alpha = 0.05$. TW3, bone age assessment according to Tanner-Whitehouse 3 method; GP, bone age assessment according to Greulich-Pyle method; BX2, BoneXpert version 2.4.5.1.; BX3, BoneXpert version 3.0.3.; MAN, manual bone age assessment.

the TW3 method in BX3, regardless of the patient's disease (Figure 3). Disease-specific RMSEs are shown in Supplementary Table 2.

The disease specific mean differences between automated and manual bone age values showed that the TW3 BX2 bone age differed significantly from manual evaluation in 16/24 disease groups. BX3 showed significant improvement, only children with growth hormone deficiency differed significantly from manual testing. The particular differences given in years are shown in Supplementary Figure 1.

3.3 Detailed analysis of the TW3 method: Differences of the automated and manual evaluation of particular bones and the effect on the outcome of the final bone age

A detailed analysis of the TW3 method was carried out on 1206 scans with detailed data on individual bones available. Out off these, 145 BX2 assessments (12.0%) differed by more than 1 year from the

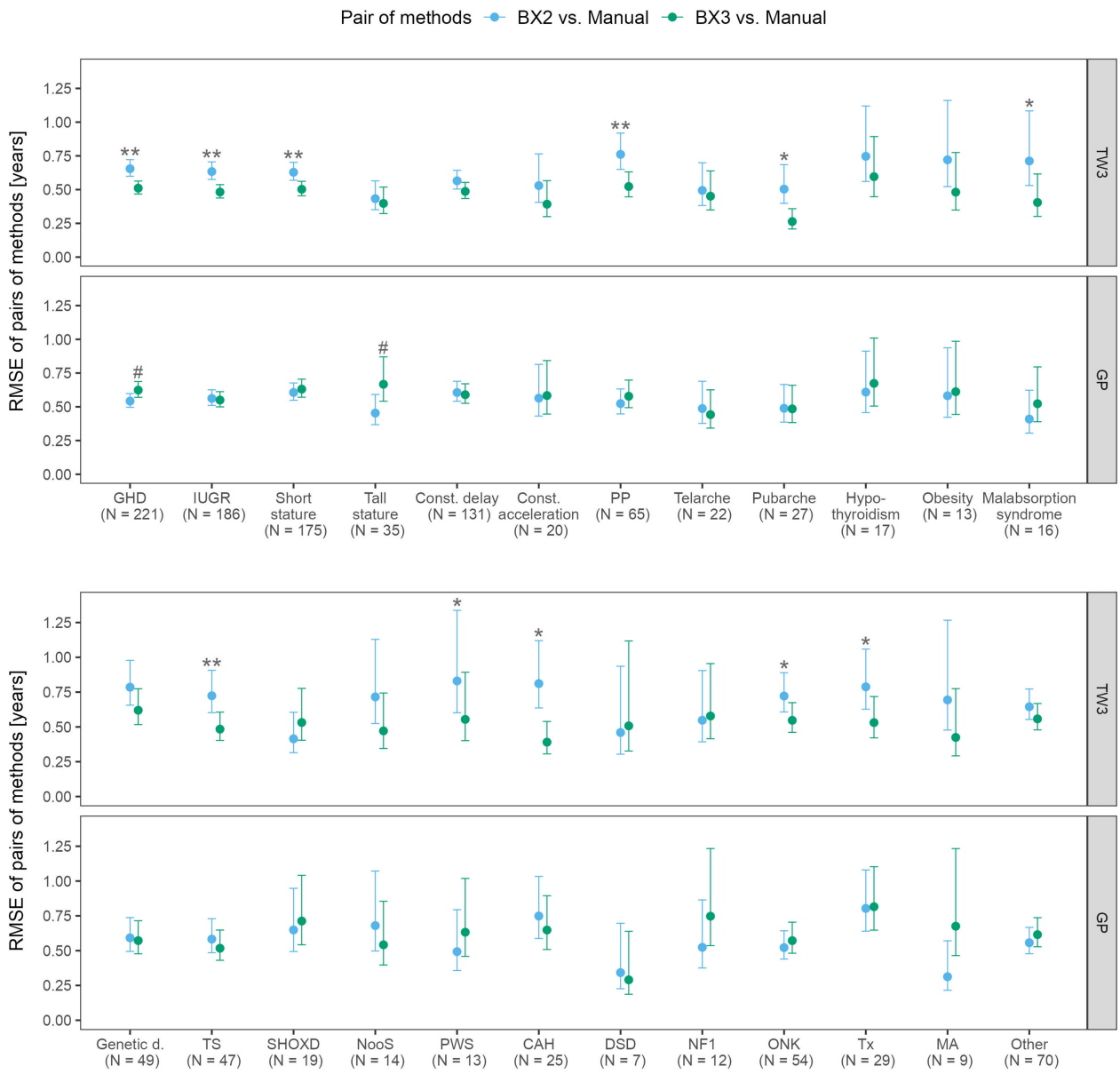

FIGURE 3
Root mean square errors (RMSE) and 95% confidence intervals for various diagnoses, separately for TW3 and GP methods and for both software versions (BX2 and BX3). RMSE is given in years. Before (*) and after (**) adjustment for multiple testing, BX3 performs significantly better, i.e. differs less from the manual assessment, than BX2 at α = 0.05, Diebold-Mariano test for method accuracy. Before (#) and after (##) adjustment for multiple testing, BX3 performs significantly worse, i.e. differs more from the manual assessment, than BX2 at a =0.05, Diebold-Mariano test for method accuracy. TW3, bone age assessment according to Tanner-Whitehouse 3 method; GP, bone age assessment according to Greulich-Pyle method; BX2, BoneXpert version 2.4.5.1.; BX3, BoneXpert version 3.0.3.; MAN, manual bone age assessment. GHD, growth hormone deficiency; IUGR, intra-uterine growth restriction; Const. delay, constitutional delay of growth; Const. acceleration, constitutional acceleration of growth; PP, precocious puberty; Genetic d., genetic disorders; TS, Turner syndrome; SHOXD, SHOX gene deficiency (all patients were treated with growth hormone); NooS, Noonan syndrome; CAH, congenital adrenal hyperplasia (16/25 were diagnosed with classical CAH); DSD, disorders of sex differenciation; NF1, neurofibromatosis type 1; ONK, oncology disorders; Tx, patients after liver; kidney or bone marrow transplant, MA, anorexia nervosa.

manual assessment, most of these (139) being lower than the manually estimated bone age. Seventy-four BX3 assessments (6.1%) differed by more than 1 year from the manual assessment (while being much more equally distributed: 47 were lower and 27 higher than the manually assessed bone age).

For each automated bone age software version and each group according to whether automated assessment resulted in the bone age being 1) > 1.0 year higher, 2) >1.0 year lower, or 3) less than one year different from the manually assessed bone age, differences in individual bone scores for each of the 13 bones were examined graphically (Supplementary Figure 2) and by using the ANOVA method with *post-hoc* pairwise comparisons. Out of these radius and ulna showed larger differences in assigned bone score among other bones (ANOVA F-test p< 0.001).

While focusing only on those x-rays where the ulna and/or radius scoring differed by more than 1 stage between automated and manual assessment,we have identified 90 such scans for the ulna with the BX2 version (85 underestimated and 5 overestimated scores) and 42 scans with BX3 (24 underestimated and 18 overestimated scores). For the radius, there were only 7 and 0 cases for BX2 and 3 and 0 cases for BX3, with under- and overestimated scores, respectively. In scans where BX3 under- or over-estimated the evaluation of the ulna, the mean difference between the automated (BX3) and manual bone age deviated significantly from 0 ($p< 0.001$) however the mean difference did not exceed 1 year (Figure 4 and Supplementary Table 3). The absolute difference in bone age exceeded 1 year (N = 15; median absolute difference 1.2 years; IQR 1.1-1.3 years) only in a minority of these cases and there was no discernable pattern in sex or diagnoses.

4 Discussion

The objective of this study was to explore the clinical utility of the BoneXpert automated bone age assessment on a large unselected cohort of children. We showed that the latest BoneXpert version (BX3) performed comparably to expert manual bone age reading in a large cohort of Caucasian children and that it performed better than the previous BoneXpert version (BX2). In particular, BX2-inherent underestimation of TW3 bone age, which was more pronounced in girls, was completely abolished in the newer BX3 version. The TW3 bone age assessed by the BX3 performed best among myriad of diseases as well, in which bone age is typically evaluated. Thus, this study encourages the use of automated TW3 bone age assessment in daily clinical practice.

Validation of automated bone age assessment is typically done by comparing the result to bone age assessed manually by a highly experienced individual. We showed that the BX2 version underestimated TW3 bone age especially in girls aged 6 to 7 and 12 to 15 years, when compared to manually-assessed TW3 bone age. Our results were similar to a previous study in participants of the First Zurich Longitudinal Study, where the differences between automated and manual TW3 bone age assessment (RMSEs) were reported to be 0.67 years in boys and 0.63 years in girls (10). The authors (10) noted considerable variability between individual age categories but did not show the data in extenso. Interestingly, our study showed that this inherent limitation of the BX2 version has been abolished in the latest software version (BX3).

There are no studies published comparing the TW3 bone age outcome between BX2 and BX3, only a single previous study explored the performance of the first (BX1) and third (BX3) software versions with regard to GP bone age (8, 15). In the Caucasian population a RMSE of 0.66 and 0.51 years in boys and 0.50 and 0.48 years in girls was reported, for BX1 and BX3 respectively. This was similar to our study, in which the BX3 version of GP bone age differed from the

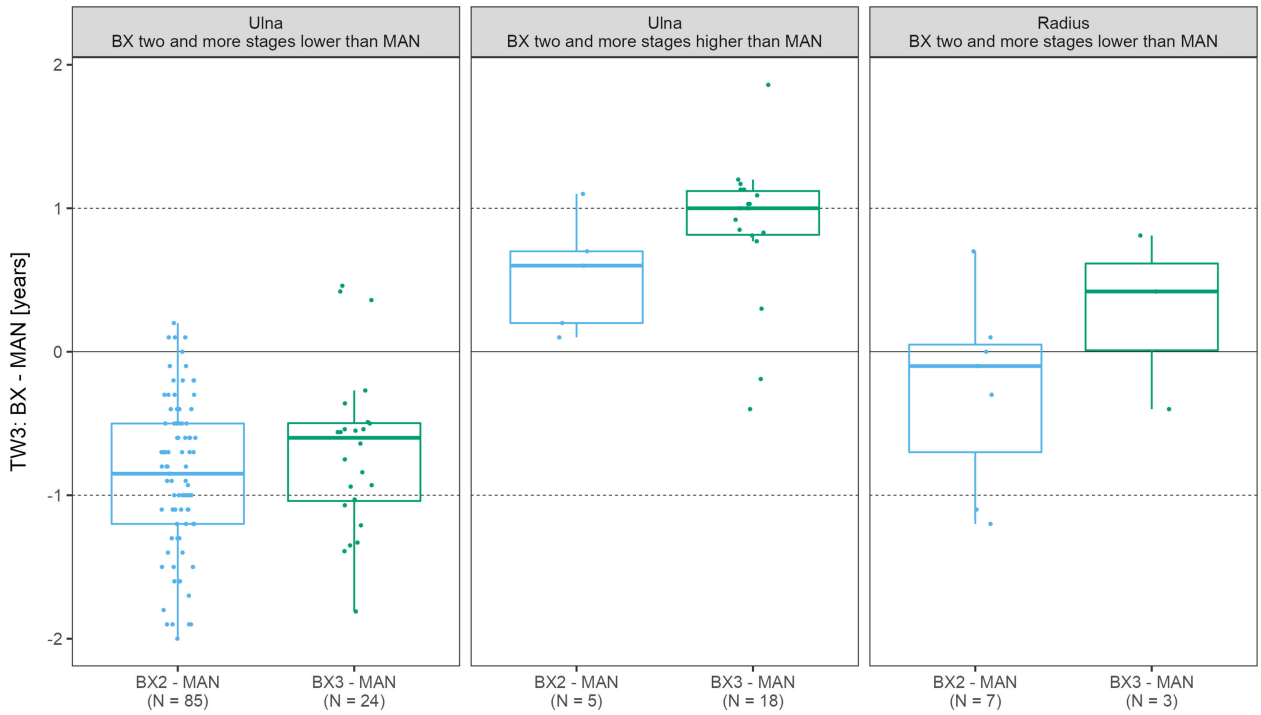

FIGURE 4
The boxplots depict the distribution and mean differences in years between automated and manual final bone age in scans where bone stage assigned to radius/ulna exceeded 2 stages. TW3, bone age assessment according to Tanner-Whitehouse 3 method; GP, bone age assessment according to Greulich-Pyle method; BX2, BoneXpert version 2.4.5.1.; BX3, BoneXpert version 3.0.3.; MAN, manual bone age assessment.

manual rating by 0.68 and 0.52 years in boys and girls respectively. Interestingly the GP results reported by Martin et al. (8) were in significantly worse agreement in girls of African descent (RMSE 0.75 years). On the other hand, a similar study on children of Indian ethnicity found the agreement between manual and automated GP bone age in girls to be 0.39 years (RMSE) (16). As both GP and TW3 methods are based on the Caucasian population, the causes are probably the differences in skeletal maturation among different ethnicities, geographical location and socioeconomic status (8, 17, 18) - in the Czech Republic the agreement between sexual maturation and bone age provided by the GP and TW3 methods has been well established (19).

To enhance clinical utility, automated bone age analysis needs proper validation in individual diseases. The BoneXpert software was introduced in 2009 (7) and the agreement of the first version with GP manual rating has been evaluated in children with a few common endocrine disorders (20–22). Our study explored the agreement between automated and manual bone age assessment in a large unselected group of disorders that can be commonly encountered in pediatric clinical practice. We showed that the BX3 version TW3 method performs consistently across various disorders. Interestingly, the RMSE for the TW3 method of the BX3 version were lower than the RMSE for GP in the first version of the software (22) in children with growth hormone deficiency or Turner syndrome (0.50 vs. 0.71 and 0.48 vs. 0.75, respectively). These results further support the use of the latest TW3 BoneXpert version in clinical practice.

In every automated analysis algorithm, systemic scoring errors should be excluded to avoid improper bone age assessment. The automated TW3 assessment by BoneXpert displays the scoring of individual bones, which allows for a more in-depth analysis. We showed that automated ulna scoring resulted in larger differences from the manual scores compared to the other bones. However, this did not have a significant influence on the TW3 bone age value. This eliminates the possibility that the differences between automated and manual TW3 bone age values may be due to systemic errors in the evaluation of a particular bone.

The strengths of this study are: 1) the large cohort of patients of Caucasian descent with various disorders, representing the common clinical situation, in whom we validated the latest version of automated GP as well as TW3 bone age assessment provided by BoneXpert, 2) the direct comparison between the latest software version (BX3) and the previous widely used version (BX2) and 3) the in depth analysis of the TW3 method.

As a limitation of this study we recognize: 1) the homogeneous cohort of children with Caucasian descent, therefore we recommend caution when applying our results to the non-Caucasian population, 2) that the disease-specific RMSEs were not further analyzed with regard to sex. This was due to relatively low number of children in certain groups with rare disorders and because we found no statistically significant difference between boys and girls in the overall RMSE analysis of the TW3 BX3 version.

The strengths of BoneXpert software include: 1) time efficiency - the number of specialists that spent more than 2 minutes evaluating an image decreased from 86 to 21% after installation of BoneXpert (23), 2) ease of use, 3) validation in different ethnicities (15) and various disorders (20–22), and 4) wide use (8). On the other hand 1) cost effectiveness in lower income countries may be an issue and 2) precision was not yet established.

5 Conclusion

Bone age analysis provided by the most recent BoneXpert software version showed clinically reliable agreement with manual evaluation among wide range of chronic diseases of children. BoneXpert is therefore a good alternative to manual rating. There are few relevant clinical implications for the use of BoneXpert in clinical practice. The major advantage is the ability to save time of the experienced evaluators. Manual bone age analysis could thus be reserved for cases where automated analysis performs improbably (i.e., discrepancy between bone age and sexual maturation) or is not feasible (i.e., skeletal dysplasia).On the other hand, bone morphology and structure, besides the bone age assessment, is routinely evaluated as part of the manual workup. The automated system does not provide such a feature. Thus, patients with mild to moderate skeletal dysplasia (which is clinically discrete) may escape the appropriate medical attention.

Data availability statement

All data generated and analyzed in this study are available from the corresponding author on reasonable request.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Motol University Hospital (Reference No.: EK-264/18). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

KM and DZ contributed equally to the conception, design and data collection. PS performed the independent reevaluation of selected bone

age scans. MP performed the statistical analysis. KM wrote the draft of the manuscript and ZS, OS, HK and SA were involved in data analysis and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1130580/full#supplementary-material

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Clinical and hormonal characteristics and growth data of 45,X/46,XY mosaicism in 38 Chinese patients

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Backgrounds: 45,X/46,XY mosaicism is the most common type of sex chromosomal abnormality in disorders of sex development (DSD). We investigated the clinical manifestations, serum sex hormone levels and growth data of 38 45,X/46,XY mosaicism patients, which provides better insight into this disease.

Methods: We prospectively evaluated 38 patients who were diagnosed with 45,X/46,XY mosaicism at the Department of Endocrinology of Shanghai Children's Hospital from 2010 to 2020. We analyzed clinical data from the patients, including hormone levels, height, weight, body mass index (BMI) and gonadal pathology results.

Results: Among the 38 cases of 45,X/46,XY mosaicism, 18 cases showed a female external genitalia phenotype (the female group) with an external masculinization score (EMS) of 1 (0–3) [median (range)], and 20 cases showed a male external genitalia phenotype (the male group) with an EMS of 7.63 (3–11) [median (range)]. The age at diagnosis ranged from 0.7 to 16.1 years. Under 2 years of age, the standard deviation scores of height (HtSDS) were in the normal range and then they gradually decreased. The inhibin B (INHB), anti-Mullerian hormone (AMH), and testosterone (T) levels after human chorionic gonadotropin (HCG) stimulation and the T:DHT ratio in the male group were significantly higher than those in the female group ($P < 0.001$). The basal luteinizing hormone (LH), basal follicle-stimulating hormone (FSH), peak LH and peak FSH in females were significantly higher than those in males ($P < 0.05$). Their height showed a positive correlation with T levels after HCG stimulation ($r = 0.636$, $P < 0.01$), T:DHT ratio ($r = 0.724$, $P < 0.01$), growth hormone (GH) ($r = 0.827$, $P < 0.05$), and insulin-like growth factor 1 (IGF-1) ($r = 0.067$, $P > 0.05$) and a negative correlation with gonadal pathology in ovarian tissue ($r = -0.663$, $P < 0.05$) and the number of chimaeric XY cells ($r = -0.533$, $P < 0.05$).

Conclusions: Patients with 45,X/46,XY mosaicism have specific growth patterns. Their HtSDS was in the normal range during 0–2 years of age and then they began to show a short stature after 2 years of age. The probability of short stature in females was higher than that in males. WtSDS were all in the normal range, but below the median. BMISDS was in the normal range, and there was no evidence of obesity. The gonads in the male group retained a certain androgen secretion function, while the gonadal damage is more severe in the female group.

KEYWORDS

height, weight, BMI, sex hormones, correlation, 45,X/46,XY mosaicism

Abbreviations

DSD, Disorders of sex development; HtSDS, height standard deviation scores; WtSDS, weight standard deviation scores; BMI, body mass index; BMISDS, body mass index standard deviation score; EMS, external masculinization score; INHB, Inhibin B; T, Testosterone; DHT, Dihydrotestosterone; E2, Oestradiol; SHBG, Sex hormone-binding globulin; LH, Luteinizing hormone; GH, growth hormone; FSH, Follicle stimulating hormone; IGF-1,insulin-like growth factor-1; IFGBP-3, insulin-like growth factor-binding protein-3; HCG, Human chorionic gonadotropin; AMH, Anti-Mullerian hormone; GnRH, Gonadotropin-releasing hormone.

Background

Disorders/differences of sex development (DSD) are congenital disorders of abnormal development of the internal and external genitalia, and these developmental disorders are usually caused by abnormal gonadal determination and differentiation (1). Among the various DSD conditions, there are sex chromosome DSDs due to numerical or structural abnormalities of the sex chromosomes (1). 45,X/46,XY mosaicism is a rare sex chromosome DSD associated with a broad spectrum of clinical phenotypes, from Turner females to phenotypically normal males with varying degrees of genital ambiguity (2). The sex of rearing may be male or female based on the appearance of the genitalia at birth (2). At present, there are few reports on Chinese 45,X/46,XY mosaicism. We retrospectively studied the clinical manifestations, hormone levels, and growth data of 38 patients with 45,X/46,XY DSD, which provides better insight into this disease.

Methods

Subjects

Informed consent from family members and patients was obtained before the study and approved by the Hospital Ethics Committee (2021R092-E01). In this study, 38 patients diagnosed with 45,X/46,XY mosaicism at the Department of Endocrinology of Shanghai Children's Hospital from 2010 to 2020 were selected as the research subjects. These patients were divided into a male phenotype group (20 cases) and a female phenotype group (18 cases) according to sex of rearing. Comparisons of height, weight and BMI were made against population norms.

Karyotyping

Blood sample (0.3–0.5 ml, heparin anticoagulation) was added into cell culture medium (Dubai Biomedical Co. Ltd., Shanghai, China). Thirty to 100 mitoses were examined to determine the percentage of cell line mosaicism. All karyotypes were evaluated by an experienced clinical geneticist, and according ISCN 2016. We use the ratio of the number of 46, XY mosaicism or 45,X mosaicism over the total number of mitoses to reflect the relative presence of the number of Y-chromosomes or 45,X monosomy.

External genital phenotype evaluation

We evaluated the external genital phenotypes according to the External Masculinization Score (EMS) (3), when they first visit in endocrinology, and all before surgery. The measurement method of the penis is manual measurement (4), measuring the length of the penis stretched while the subject is in a supine position. The measurer places the end of a ruler against the pubic symphysis, lifts the head of the penis with the thumb and index finger and gradually applies it along the length of the penis. Traction stretches the penis until the penis is stretched to its longest length. The length from the end of the ruler to the apex of the glans penis is the stretched length (the length of the foreskin is not counted). The length of the penis was compared with the data of normal Chinese children. The location of the testes was determined by physical examination and ultrasonography.

Hormonal analysis

To evaluate the hypothalamic-pituitary-gonadal (HPG) axis and testicular function, all patients underwent gonadotropin-releasing hormone (GnRH) stimulation and human chorionic gonadotropin (HCG) stimulation. Sex hormones, including basal testosterone (T), basal dihydrotestosterone (DHT), oestradiol (E2), basal luteinizing hormone (LH), basal follicle-stimulating hormone (FSH), anti-Mullerian hormone (AMH), inhibin B (INHB), sex hormone binding globulin (SHBG), insulin-like growth factor-1 (IGF-1), insulin-like growth factor-binding protein-3 (IFGBP-3), peak LH and peak FSH after gonadotropin-releasing hormone (GnRH) stimulation and T and DHT after human HCG stimulation, were detected. Detection methods: E2, T and DHT were tested by ELISA and measured with a USA Polar ELx800 microplate reader. Serum LH and FSH concentrations were tested with LH and FSH detection kits (Beckman Coulter) and measured with an automatic immunoluminescence analyser (UnicelDxI 800). Serum AMH and INHB were detected with solid-phase sandwich enzyme-linked immunosorbent assays (ELISAs) purchased from Guangzhou Kangrun Biotechnology Co., Ltd. IGF-1 and IGFBP-3 were detected by Siemens Medical Diagnostics Chemiluminescence Analyser IMMULITE 2000.

Statistical analysis

SPSS 25.0 software was used for the statistical analyses, and GraphPad Prism 9.0 was used for graphing. All detection indicators were tested for normality. The normally distributed data are expressed as the mean ± SD ($x \pm s$), and the data with a nonnormal distribution are expressed as the median (upper quartile to lower quartile). The nonparametric Mann–Whitney rank-sum test was used to compare two groups with a nonnormal distribution. Bivariate correlation analysis was performed using Pearson's method, and the correlation coefficient was denoted by r. Then, the correlations between height, weight and each index were analysed by multiple stepwise regression. $P < 0.05$ was considered statistically significant.

Growth curve plotting

The calculations for the growth curves were performed using LMS-Chartmaker Pro software, and the curves were drawn using Excel 2019. The internationally well accepted method (λ-median coefficient of variation, LMS) for generating standard curves was adopted to calculate the M, S, and L (after converting the data into a normal distribution, using Box-Cox transformation) (5), which described the growth index in each age group.

Results

External genitalia phenotype

Thirty-eight patients were scored for external genital virilization according to the EMS. The results showed that the EMS of patients raised as females was 1 (0–3) [median (range)] and that of the males was 7.63 (3–11) [median (range)] ($P = 0.0001 < 0.001$) (**Table 1**).

Growth pattern

Thirty-eight patients were divided into 6 groups according to age: 0–1 years old, 1–2 years old, 2–6 years old, 6–9 years old, 9–13 years old, and older than 13 years old. When the children were under 2 years old, their HtSDS was in the normal range (-0.9 ± 1.16, -1.79 ± 0.85) and then it gradually decreased. Their height began to appear short and was the lowest at the age of 9–13 (-3.17 ± 1.71) ($P = 0.014 < 0.05$). The lowest and highest weight standard deviation scores were at 6–9 years old and 0–1 years old (-1.94 ± 0.83, -0.51 ± 0.99) ($P = 0.026 < 0.05$). The lowest and highest BMIs were in the 2- to 6-year-old group and the older than 13-year-old group (15.16 ± 1.16, 20.13 ± 2.01) ($P < 0.0001 < 0.001$). The above results show that the growth of 45,X/46,XY mosaicism children appeared to decelerate around 2 years of age, and after that point, their height was significantly lower than that of normal children (**Figures 1**, **2**). Below −2 SD children with this disease were underweight but within the range of normal children with a normal BMI (**Table 2**).

Thirty-eight patients were divided into female (18 cases) and male (20 cases) groups according to the phenotype of their external genitalia. The HtSDS of females was -2.29 ± 1.42 and that of males was -1.85 ± 1.43, and the probability of short stature in females (67%) was higher than that in males (45%). The WtSDS of females and males was -1.26 ± 1.036 and -1.14 ± 1.14, respectively, both lower than the median but still within normal levels. The BMISDS of females was higher than that of males ($P = 0.029 < 0.05$) (**Table 3**). The above results suggest that 45,X/46,XY mosaicism children raised as girls have obvious androgen deficiency, which seriously affects their height growth and causes more severe short stature.

Hormones

The INHB, AMH, and T levels after HCG stimulation and the T:DHT ratio in the male group were significantly higher than those in the female group ($P < 0.001$), and the basal LH, basal FSH, peak LH and peak FSH in the female group were significantly higher than those in the male group ($P < 0.05$) (**Table 4**). This suggests that the male group has a certain level of testicular function and can secrete androgens after HCG stimulation, while the female group has the risk of manifesting hypergonadotropic hypogonadism with insufficient testosterone secretion (**Figure 3**).

Correlation analysis

The correlation analysis results of height, weight and BMI of the 45,X/46,XY children are shown in **Tables 5**, **6**. The 45,X/46,XY children's height showed a positive correlation with T (after HCG stimulation) ($r = 0.636$, $P < 0.01$), the T:DHT ratio ($r = 0.724$, $P < 0.01$), GH ($r = 0.827$, $P < 0.05$), and IGF-1 ($r = 0.067$, $P > 0.05$) and a negative correlation with gonadal pathology in ovarian tissue ($r = -0.663$, $P < 0.05$) and the number of XY chimaeras ($r = -0.533$, $P < 0.05$). WtSDS was positively correlated with IGF-1 ($r = 0.617$, $P < 0.05$) and negatively correlated with the number of X chimaeras ($r = -0.583$, $P < 0.05$); BMISDS had no significant correlation with any of the influencing factors. These data suggest that increased levels of T, T/DHT, GH, and IGF-1 can promote the growth of height.

Taking HtSDS and WtSDS as dependent variables and the related influencing factors as independent variables, multiple stepwise regression analysis was carried out (**Figure 4**). The results showed that the height of 45,X/46,XY children is affected by many factors. Under the control of other factors, T levels can affect the growth of height, and height increases as T levels increase. GH can affect height. Among 38 children, a total of 7 children received growth hormone stimulation tests. The higher the result of the stimulation test was, the taller the child. The pathological types were classified according to the pathological results, and twenty-nine cases underwent bilateral gonad biopsies. The results showed that children with testicular tissue in the bilateral biopsy results were the tallest, followed by the mixed type. The number of XY chimaeras was inversely proportional to height. As the number of XY cells in the serum increased, the height decreased, indicating that the degree of XY chimaerism in the blood of 45,X/46,XY children cannot determine their level of masculinization.

Discussion

45,X/46,XY mosaicism is the most common type of sex chromosomal abnormality DSD, with an incidence of 1.0/10,000 (6). The mechanism of the abnormality may be that the Y chromosome lags behind and does not segregate when the fertilized egg undergoes mitosis (7). Although some studies have

TABLE 1 EMS of 45,X/46,XY mosaicism patients.

Patient no.	Diagnosis		Sex	Karyotype	At last follow-up			EMS					
	Age (year)	Reason for referral			Age (month)	Height (cm)	Weight (kg)	Scrotal fusion	Micropenis	Urethral meatus	Right gonad	Left gonad	Total EMS
1	At birth	Abnormal genitals	M	45,X(15)/46,XY(15)	11	71	9.4	3	3	2	1	0	9
2	At birth	Abnormal genitals	M	45,X(15)/46,XY(15)	37	83.5	10.5	0	0	1	0.5	0.5	2
3	At birth	Abnormal genitals	M	45,X(6)/46,XY(24)	8	72	10	3	0	0	1.5	0	4.5
4	At birth	Abnormal genitals	M	45,X(2)/46,XY(18)	34	93	11.75	0	3	1	1	1	6
5	At birth	Abnormal genitals	M	45,X(45)/46,XY(15)	19	76.5	10	0	3	1	1	0.5	5.5
6	At birth	Abnormal genitals	M	45,X(10)/46,XY(25)	11	73	9.4	3	0	0	0.5	0.5	4
7	At birth	Abnormal genitals	M	45,X(16)/46,XY(34)	12	75	10.6	0	0	0	1.5	0.5	2
8	At birth	Abnormal genitals	M	45,X(15)/46,XY(35)	11	76	11.4	0	0	1	0.5	0	1.5
9	At birth	Abnormal genitals	M	45,X(56)/46,XY(6)	38	93	12.8	3	3	0	1	1	8
10	9	Growth retardation	M	45,X(8)/46,XY(91)	111	113	18.5	3	3	2	1.5	1.5	11
11	At birth	Abnormal genitals	M	45,X(23)/46,XY(77)	18	76	10	3	3	1	1	1	9
12	At birth	Abnormal genitals	M	45,X(7)/46,XY(20)	11	71	9	3	3	2	0.5	0.5	9
13	At birth	Abnormal genitals	M	45,X(40)/46,XY(10)	15	75	10	0	0	0	1	1.5	2.5
14	At birth	Abnormal genitals	M	45,X(38)/46,XY(12)	14	72	9.5	0	0	1	1	0.5	2.5
15	At birth	Abnormal genitals	M	45,X(16)/46,XY(30)	25	88	10.9	0	0	1	0.5	0.5	2
16	At birth	Abnormal genitals	M	45,X(39)/46,XY(44)	13	76	9.8	3	0	0	1	1	5
17	At birth	Abnormal genitals	M	45,X(17)/46,XY(83)	69	98	14.6	3	0	0	1	1.5	5.5
18	At birth	Abnormal genitals	M	45,X(7)/46,XY(13)	10	70	8.7	0	3	2	0.5	1	6.5
19	At birth	Abnormal genitals	M	45,X(19)/46,XY(81)	37	86	12.4	0	3	2	0	0.5	5.5
20	At birth	Abnormal genitals	M	45,X(9)/46,XY(41)	13	71	9	3	0	2	1	0.5	6.5
21	11	Growth retardation	F	45,X(5)/46,XY(17)	136	138	43	0	0	0	0	0	0
22	8	Growth retardation	F	45,X(31)/46,XY(29)	98	115	23.05	0	0	1	0	0	1
23	13	Growth retardation	F	45,X(5)/46,XY(55)	164	144	39.3	0	0	0	0	0	0
24	8	Growth retardation	F	45,X(22)/46,XY(8)	101	118.3	23	0	0	0	0	0	0
25	At birth	Abnormal genitals	F	45,X(14)/46,XY(16)	12	78	10	0	0	0	0	0	0
26	At birth	Abnormal genitals	F	45,X(15)/46,XY(35)	12	72	8.1	0	3	0	0	0	3
27	15	delayed puberty	F	45,X(19)/46,XY(31)	187	149	46.8	0	0	0	0.5	0.5	1
28	At birth	Abnormal genitals	F	45,X(33)/46,XY(17)	17	78	9.4	0	0	0	1	1	2
29	11	Growth retardation	F	45,X(74)/46,XY(26)	141	120	26.51	0	0	0	0	0	0
30	At birth	Abnormal genitals	F	45,X(30)/46,XY(70)	45	93	12.6	0	0	0	0	0	0
31	4	Growth retardation	F	45,X(11)/46,XY(89)	28	87	11.5	0	0	0	0	0	0
32	4	Growth retardation	F	45,X(60)/46,XY(40)	53	98	16.5	0	3	0	0	0	3
33	3	Growth retardation	F	45,X(72)/46,XY(28)	74	99	14.35	0	0	2	0.5	0.5	3
34	At birth	Abnormal genitals	F	45,X(17)/46,XY(83)	76	109	18.3	0	0	0	0.5	0.5	1
35	8	Growth retardation	F	45,X(34)/46,XY(16)	120	121	26.9	0	0	0	0	0	0
36	16	delayed puberty	F	45,X(42)/46,XY(18)	193	148	39.5	0	0	0	0	0	0
37	11	Growth retardation	F	45,X(15)/46,XY(25)	178	140	44	0	0	1	0	0	1
38	7	Growth retardation	F	45,X(6)/46,XY(24)	122	131	36.1	0	0	1	0	0	1

M, Male; F, female; EMS, External Masculinization Score;Scores (yes/no or gradient): scrotal fusion (3/0), micropenis (0/3), urethral meatus (normal, 3; glandular, 2; penile, 1; perineal, 0), right and left gonad (scrotal, 1.5; inguinal, 1; abdominal, 0.5; absent, 0).

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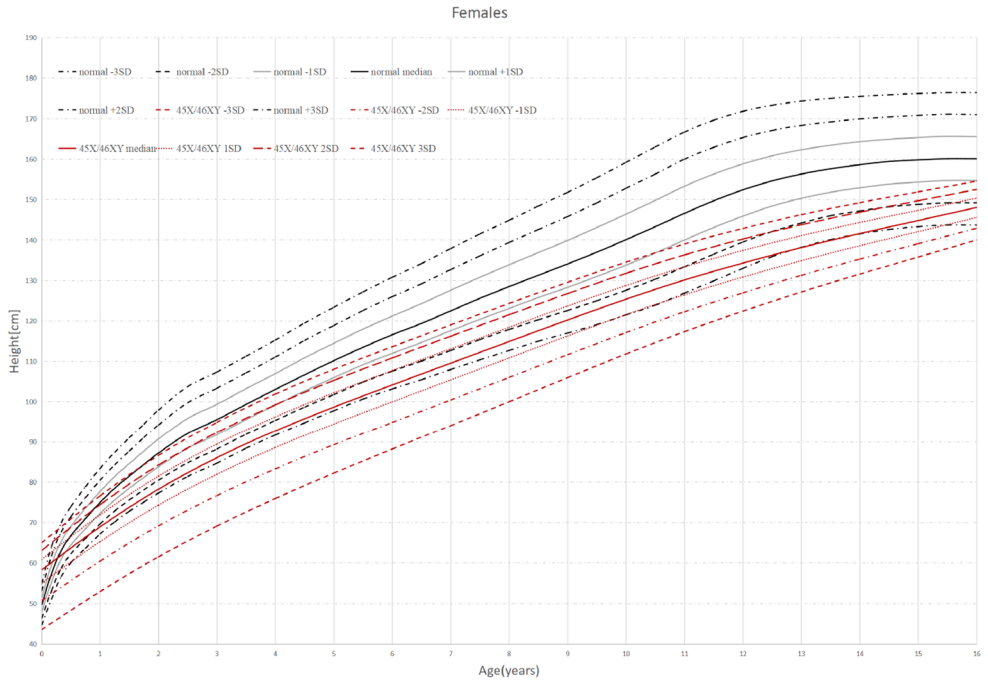

FIGURE 1
Standard deviation height curves of children with 45,X/46,XY mosaicism and normal Chinese girls.

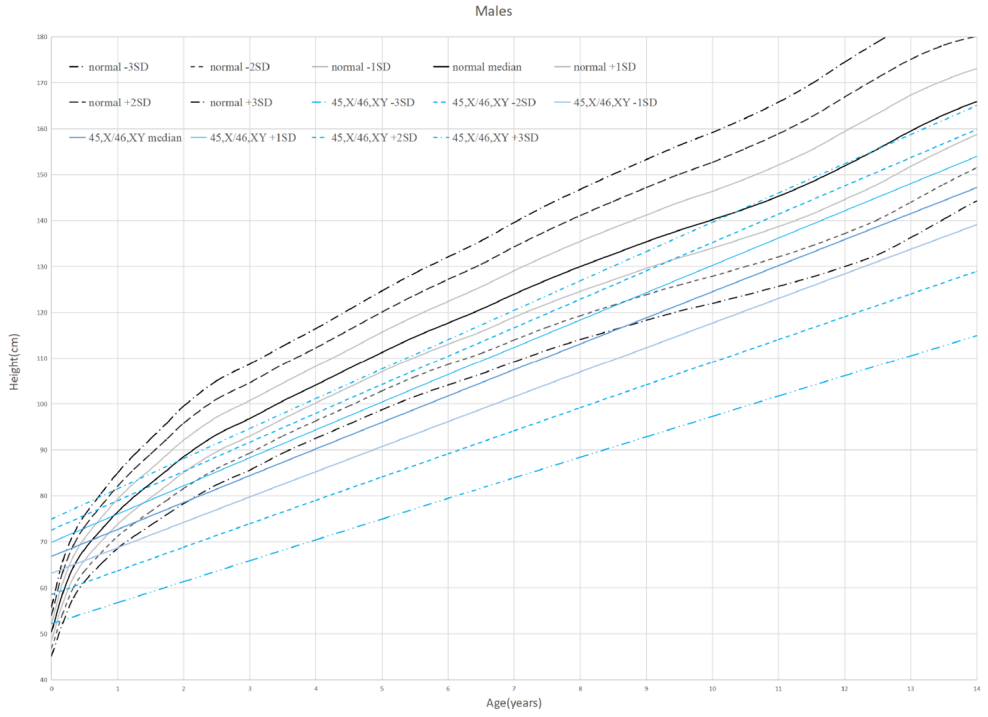

FIGURE 2
Standard deviation height curves of children with 45,X/46,XY mosaicism and normal Chinese boys.

TABLE 2 Growth of 45,X/46,XY mosaicism in different age groups.

Age	*N*	HtSDS△	WtSDS△	BMISDS△
0–1 year	10	−0.90 ± 1.16	−0.51 ± 0.99	0.30 ± 0.63
1–2 years	6	−1.79 ± 0.85	−0.9 ± 0.33	0.07 ± 0.56
2–6 years	9	−2.14 ± 1.38	−1.90 ± 0.86	−0.38 ± 1.19
6–9 years	4	−2.91 ± 0.59	−1.94 ± 0.83	−0.16 ± 1.81
9–13 years	5	−3.17 ± 1.71	−1.14 ± 1.75	0.77 ± 1.21
>13 years	4	−2.9 ± 1.34	−1.14 ± 0.63	0.29 ± 0.77
F		3.385	2.487	0.978
P		0.014*	0.052	0.446

N, number; HtSDS, height standard deviation scores; WtSDS,weight standard deviation scores; BMISDS, body mass index standard deviation score; *$P < 0.05$. △ indicates that according to the SK normality test, it follows a normal distribution.

TABLE 3 45,X/46,XY mosaicism female and male group height, weight, BMI levels.

	N	HtSDS△	WtSDS△	BMISDS△
Females	18	−2.29 ± 1.42	−1.26 ± 1.036	0.43 ± 1.17
Males	20	−1.85 ± 1.43	−1.14 ± 1.14	−0.8 ± 1.08
F		0.91	0.111	5.521
P		0.347	0.741	0.029*

N, number; HtSDS, height standard deviation scores; WtSDS,weight standard deviation scores; BMISDS, body mass index standard deviation score;*$P < 0.05$. △ indicates that according to the SK normality test the data follow a normal distribution.

shown that the prenatal sex chromosomes are 45,X/46,XY, 95% of cases have normal male sex organs after birth (8). In fact, the phenotype of the external genitalia of the disease is broad, including the appearance of female genitalia or mild clitoromegaly through ambiguous genitalia to hypospadias or a normal penis (9). In our study, a total of 38 children with sex chromosome karyotypes of 45,X/46,XY were included as research subjects. These patients were divided into a male phenotype group (20 cases) and a female phenotype group (18 cases) according to their external genital phenotype. The EMS of patients raised as female was 1 (0–3) [median (range)], and that of males was 7.63 (3–11) [median (range)]. EMS < 3 points indicates a female phenotype, and EMS > 3 points indicates a male phenotype.

In addition to external genital abnormalities, such children will also have short stature problems. At present, there are few reports on the growth of 45,X/46,XY individuals during childhood. A previous study (6) analyzed the height of 32 cases of 45,X/46,XY and found that patients with 45,X/46,XY might have normal heights until 2 years old, but growth decelerations after 2 years of age were common. In this study, we analysed the growth patterns of these children, and the results showed that these children have specific growth patterns. Their HtSDS was in the normal range from 0 to 2 years old, and after that, their age was negatively correlated with HtSDS since after 2 years of age, their growth began to slow. The probability of short stature in females (67%) was higher than that in males (45%). In this study, 7 children underwent growth hormone stimulation testing; 6 had short stature (HtSDS < 2 SD) and they were predominantly female (83%). The results of the two GH drug (clonidine, arginine) provocation tests in these 6 children were all less than 10 μg/L, suggesting the existence of growth hormone deficiency. The detection of IGF-1 and IGFBP-3 was conducted in 13 children, and 4 had lower IGF-1 than normal children, and the 6 children with bone age records all had bone age lag >2 SD. This shows that children with short stature 45,X/46,XY have growth hormone deficiency, a low bone age, and low levels of growth factors.

A previous report of 10 patients placed on growth hormone (GH) therapy found that although the HtSDS of the GH-treated patients was significantly higher than their mean HtSDS before GH treatment ($P = 0.013$), it was not significantly different from the HtSDS of the untreated group (7). This may be related to the short treatment time and the lack of the SHOX gene on chromosome X. In another study (2), in their male group, fourteen of 18 males had external masculinization scores consistent with normal virilization. Ten of 11 male patients experienced spontaneous puberty, gonadal function in most 45,X/46,XY males, even those with genital ambiguity, seems sufficient for spontaneous puberty. While 4 female patients in our study

TABLE 4 45,X/46,XY mosaicism male and female hormone levels.

	Females (*N* = 18)	Males (*N* = 20)	Z value	*P*-value
Basal T (nmol/L)[a]	0.35 (0.35–0.665)	0.35 (0.35–0.61)	−0.657	0.511
Basal DHT (pg/ml)[a]	102.3 (28.64–224.53)	73.86 (29.92–167.63)	−0.38	0.704
DHEAS (umol/L)[a]	0.9 (0.2–2.4)	0.1 (0.05–0.38)	−3.472	0.001**
INHB (pg/mL)[a]	12.85 (9.31–42.48)	89.43 (63.07–181.82)	−4.619	0.000***
AMH (ng/mL)[a]	1.26 (0.47–4.19)	54.94 (36.6–73)	−5.263	0.0008**
T after HCG stimulation (nmol/L)[a]	1.2 (0.645–2.38)	5.44 (3.195–9.7425)	−3.626	0.000***
DHT after HCG stimulation (pg/ml)[a]	156.7 (61.93–230.3)	111.59 (55.42–221.53)	−0.643	0.52
T:DHT ratio[a]	2.22 (1.01–8.4)	13.87 (8.58–28.68)	−3.625	0.000***
Basal LH (IU/L)[a]	0.87 (0.27–9.04)	0.39 (0.2–0.67)	−2.016	0.044*
Basal FSH (IU/L)[a]	17.72 (6.35–53.87)	2.96 (1.79–4.23)	−4.444	0.000***
Peak LH (IU/L)[a]	16.54 (7.14–37.15)	5.55 (3.65–7.55)	−3.459	0.001**
Peak FSH (IU/L)[a]	106.75 (43.51–119.95)	18.61 (14.84–24.27)	−4.785	0.000***

T, Testosterone; DHT, Dihydrotestosterone; INHB, Inhibin B; AMH, Anti-Mullerian hormone; DHEAS, Dehydroepiandrosterone sulfate; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; HCG, Human chorionic gonadotropin.

[a]Indicates that the SK normality test does not obey the normal distribution.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

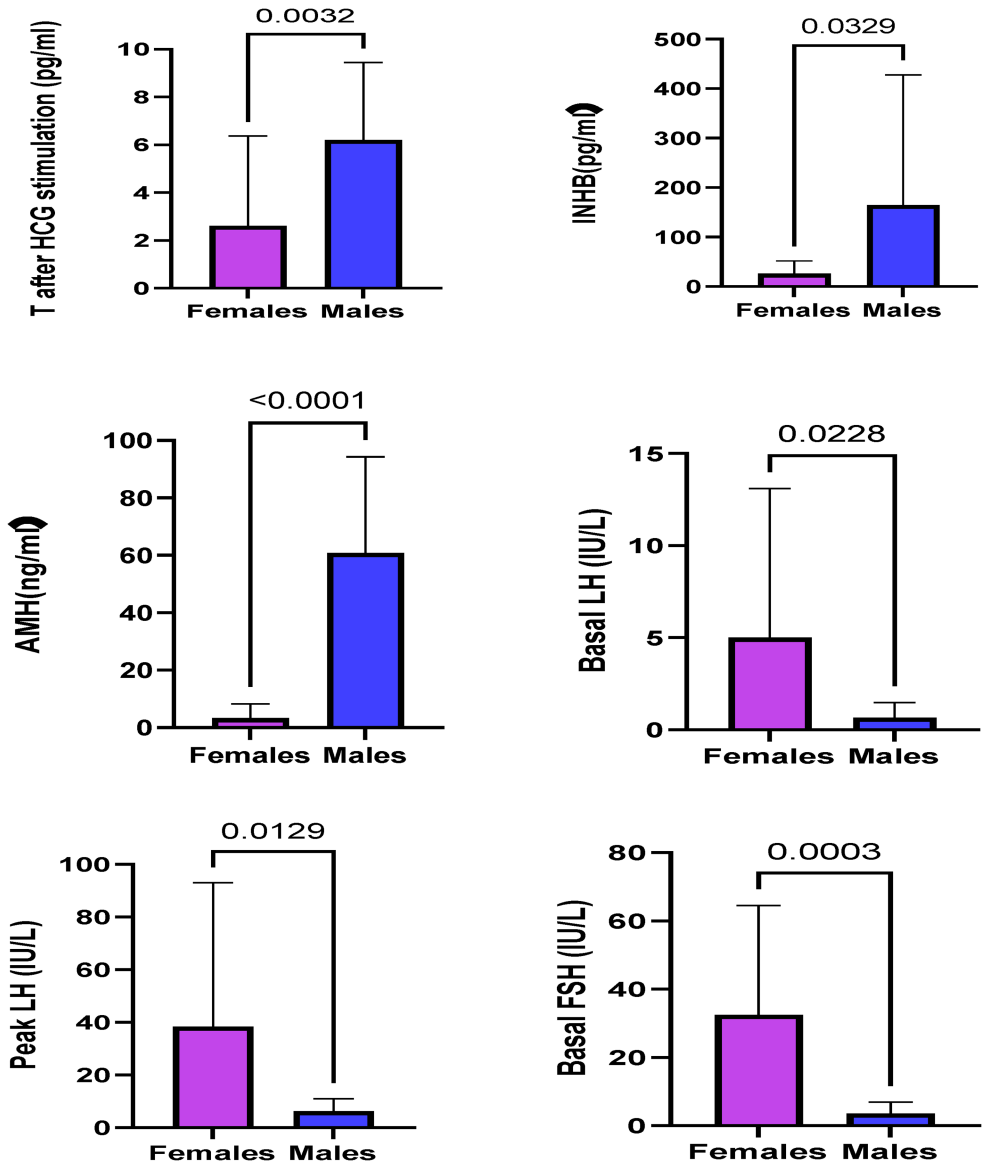

FIGURE 3
Hormones showing significant differences in the sex of 45,X/46,XY patients.

entered puberty but no development of secondary sexual characteristics, this further indicates that in such children, some testicular function is preserved in the male group, while the gonadal damage is more severe in the female group.

In this study, the WtSDS of 45,X/46,XY children in different age groups were all within the normal range but below the median, and there was no significant difference between males and females. The BMI of the different age groups was different, and it was positively correlated with age. The older the child, the higher the BMI, and the BMISDS was in the normal range with no manifestation of obesity. The BMISDS of females was higher than that of males. Due to the calculation method of BMI, this may be caused by males being taller than females.

AMH and INHB are markers suggesting the existence of Sertoli cells, and AMH and INHB play an important role in gonadal development and sex differentiation. Assessment of AMH and INHB helps determine the testicular presence and function. In patients with bilateral cryptorchidism, undetectable serum AMH and INHB suggest testicular tissue loss. The levels of AMH and INHB in the male phenotype group were significantly higher than those in the female phenotype group ($P < 0.05$). Compared with the reference range reported in the literature (10), the AMH of the male group was in the normal range, and that of the female group was lower than the normal range, suggesting that AMH and INHB are very important for evaluating testicular function and determining the sex of rearing.

All 38 children with 45,X/46,XY underwent an HCG stimulation test. The T level after the HCG challenge test was significantly higher in the male phenotype group than in the female phenotype group ($P < 0.001$). The male group had higher

TABLE 5 Correlation between height, weight, BMI and various indicators in 45,X/46,XY children.

Variable	HtSDS	WtSDS	BMISDS
T after HCG stimulation(nmol/L)	0.636**	0.268	−0.015
DHT after HCG stimulation(pg/ml)	−0.084	0.429	0.443
T:DHT ratio	0.724**	0.093	−0.282
DHEAS(μmol/L)	−0.384	0.201	0.766
INHB (pg/mL)	−0.200	0.311	−0.092
AMH (ng/mL)	0.104	−0.104	−0.162
Basal LH(IU/L)	−0.284	−0.001	0.520
Basal FSH(IU/L)	−0.385	0.063	0.610
Peak LH(IU/L)	−0.252	−0.046	0.396
Peak FSH(IU/L)	−0.351	−0.078	0.341
IGF-1 (ng/ml)	0.067	0.617*	0.907
IGF-3 (μg/ml)	−0.089	0.443	0.791
Peak GH(μg/L)	0.827*	0.602	−0.235
Gonadal pathology with female gonads tissue	−0.663*	−0.057	0.298
degree of 45,X mosaicism	−0.029	−0.583*	−0.329
degree of 46,XY mosaicism	−0.533*	0.015	−0.400

IGF-1,insulin-like growth factor-1; IFGBP-3, insulin-like growth factor-binding protein-3; GH, growth hormone; The values in the table are correlation coefficients; two variables are compared, *$P < 0.05$, **$P < 0.01$.

T:DHT ratio values than the female group ($P < 0.001$). This further shows that the testis function of the male group is better than that of the female group and also that there is insufficient function of 5α-reductase to convert T to DHT, indicating that the increase in the T:DHT ratio is not specific to 5α-reductase deficiency.

Thirty-seven cases of 45,X/46,XY children had a GnRH stimulation test, these data showed that the basal LH and FSH levels and the peak LH and FSH levels of the female group were higher than those of the male group. At the same time, they were significantly higher than that of normal children, and there was a phenomenon of hypergonadotropic hypogonadism in 45, X/46,XY children. Combined with the significant reduction of AMH and INHB in the female group, this further confirmed that the females had obvious hypogonadism. In the female group, 13 cases underwent bilateral gonad biopsy, for which the pathological results were2 cases of male gonad tissue on both sides, 1 case of bilateral female gonad tissue, 6 cases of mixed male and female gonad tissue, and 1 case of bilateral streak gonads (**Table 7**). These data all suggest that the female group has different types of hypogonadism and an increased tumour risk. In terms of gonad pathology and gonadectomy, In a previous study (11), they considered in girls, tumor risk is limited but gonads are not functional, making gonadectomy the most reasonable option, but in our study, in the female group, 9 children underwent bilateral dysplastic gonadectomy, and in the male group, 6 children underwent unilateral gonadectomy, not only because of the tumor, the extremely poor development of the gonads is also the reason for the gonadectomy, so evaluation for gonadectomy is necessary in both males and females.

In our study, a correlation analysis of height, weight, BMI and related factors was carried out. In this type of disease, the height of the children is related to testosterone, growth factors, and pathological types. T can directly stimulate the secretion of GH by interacting with the androgen receptor (AR) and oestrogen receptor (ER) located in the hypothalamus and pituitary and can also be converted to oestrogen through peripheral and central aromatization, indirectly affecting circulating IGF-1 (12). In addition, the perinatal surge of T can imprint the GH/IGF-1 axis, regulating pubertal GH secretion, body weight, and longitudinal bone growth (13). Our data indicate that height in 45,X/46,XY children is positively correlated with T.

The pituitary secretes pulsatile growth hormone (GH) and it acts directly or indirectly on peripheral tissues by stimulating the synthesis and secretion of IGF-1 (14). IGF-1 induces chondrocyte proliferation and endochondral ossification, leading to linear bone growth upon stimulation with GH (15). A total of 7 children underwent a growth hormone stimulation test, and the growth hormone stimulation test results were proportional to the height of the child.

The children were further classified according to their pathological results. Among them, 29 cases underwent bilateral gonad biopsies. The pathological types were testicular tissue, mixed ovarian and testis tissue, ovarian tissue, no gonad tissue, and germ cell tumour. We compared the pathological results with the T level. There was a correlation between the pathological types and height. The pathological type with the tallest children was testicular tissue with the highest T level, followed by the ovo-testis mixed type, and the shortest were those with a germ cell tumour.

IGF-1 binds to its receptor and plays an important role in growth and development. IGF-1 can bind to six types of IGFBP in the blood circulation to regulate the activity of IGF-1, of which the most abundant is IGFBP-3, which accounts for 80%–95% (16). Recent studies have shown that intrauterine IGF-1 levels can affect the birth weight of infants (17). It has been shown that children with this type of disease can be treated with GH to alter their GH-IGF-1 levels, thereby altering their growth.

TABLE 6 Regression analysis of HtSDS, WtSDS and various indicators in 45,X/46,XY children.

Dependent variable	Independent variable	Constant	*B*	SE	*β*	*t*	*P*
HtSDS	T after HCG stimulation	−2.915	0.24	0.073	0.636	3.3	0.005
	Peak GH	−5.45	0.365	0.124	0.827	2.943	0.042
	Gonadal pathology with female gonads tissue	−0.607	−0.61	0.208	−0.663	−2.933	0.014
	Chimaera XY number	−0.907	−0.028	0.01	−0.533	−2.674	0.015
WtSDS	IGF-1	−2.915	0.24	0.073	0.636	3.3	0.005
	degree of 45,X mosaicism	−0.907	−0.028	0.01	−0.533	−2.674	0.015

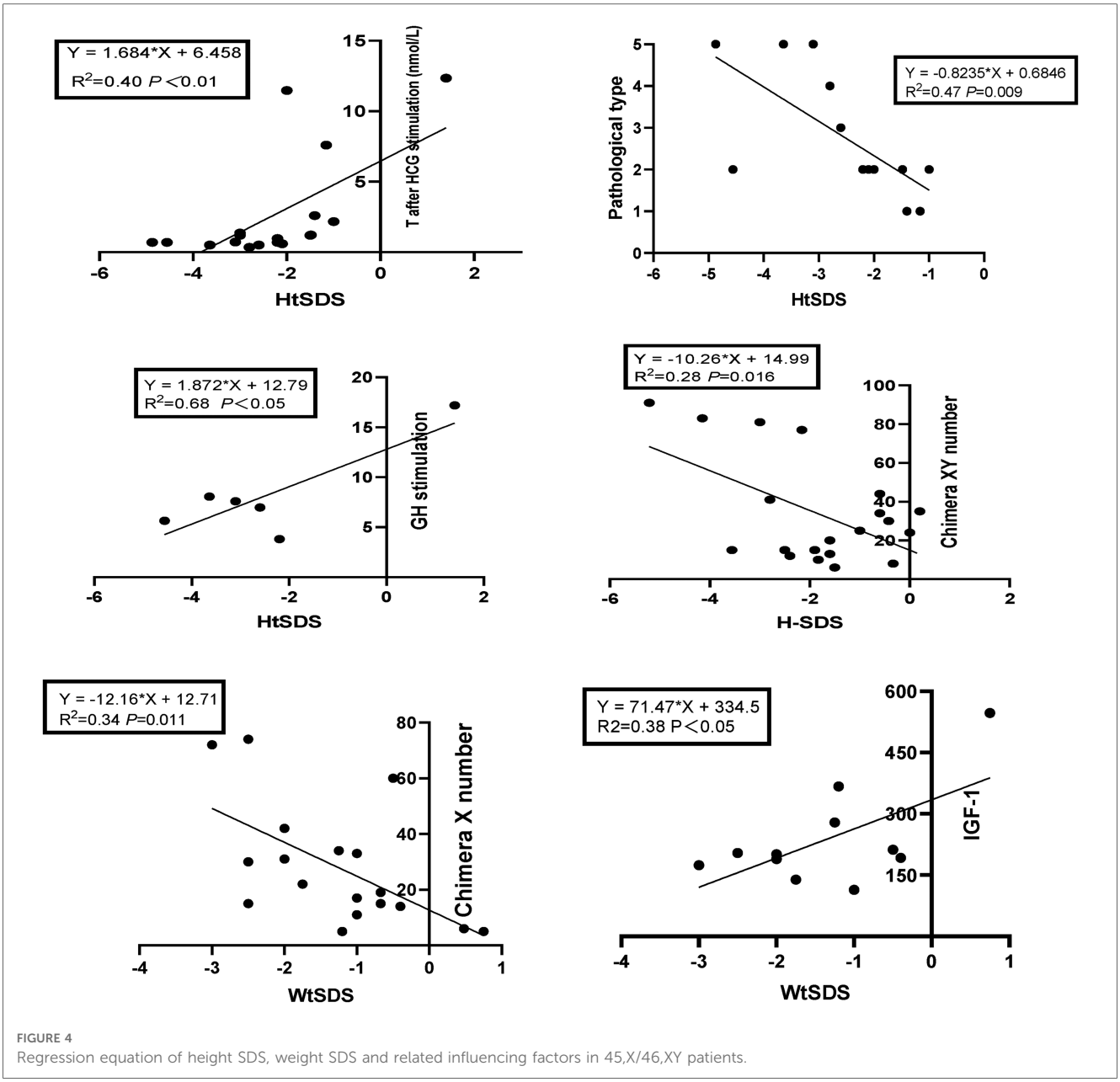

FIGURE 4
Regression equation of height SDS, weight SDS and related influencing factors in 45,X/46,XY patients.

TABLE 7 Histological findings in patients who underwent surgical exploration and/or gonadectomy.

Patient no.	Sex	Surgery	Histology	
			Left gonad	Right gonad
2	M	at 4 years B(right) O(left)		Streak gonad, no germ cells
3	M	at 1 years G(left)	epididymis and fallopian tube-like tissue	
4	M	at 23 months B(bilateral) O (bilateral)	Seminiferous tubule	Epididymis tissue, no seminiferous tubules
5	M	at 14 months B(bilateral) G (right)	Fibrous tissue, smooth muscle, lining columnar epithelium, oviduct-like structures	Testicular tissue
7	M	at 15 months B(bilateral) O (bilateral)	Seminiferous tubules, Sertoli cells, germ cells, and stromal cells	Seminiferous tubules, Sertoli cells, germ cells, and mesenchymal cells
8	M	at 13 months B(bilateral)	Seminiferous tubules, Sertoli cells and germ cells	Seminiferous tubules, Sertoli cells and germ cells
9	M	at 3.5 years B(bilateral)	Seminiferous tubule	Few seminiferous tubules
11	M		Streak gonad, no germ cells	Seminiferous tubule

(*continued*)

TABLE 7 Continued

Patient no.	Sex	Surgery	Histology	
			Left gonad	Right gonad
		at 21 months B(bilateral) O (bilateral)		
12	M	at 13 months B(bilateral) O (bilateral)	Few seminiferous tubules	Few seminiferous tubules
13	M	at 17 months B(bilateral) O (left) at19 months G(right)	Seminiferous tubule	Fibrovascular connective tissue
14	M	at 18 months B(bilateral) at 2.5 years 0 (left)	Seminiferous tubules	Few seminiferous tubules
15	M	at 27 months B(bilateral) G (left)	Fallopian tube and vas deferens structure	Few seminiferous tubules
16	M	at 15 months B(bilateral)	Streak gonad with some seminiferous tubules	Streak gonad with some seminiferous tubules
18	M	at 13 months B(bilateral) at 16 months G(left)	A small amount of ovarian stroma, no follicles and seminiferous tubules	Seminiferous tubules
19	M	at 22 months B(bilateral) at 4 years G(right)	Seminiferous tubule, epididymis	Streak gonad with a few tubules
20	M	at 16 months B(bilateral) O(left)	Few seminiferous tubules	Ovarian mesenchymal tissue
22	F	at 8 years B(bilateral)	Streak gonad, no germ cells	Streak gonad, no germ cells
23	F	at 13 years B(bilateral) G (bilateral)	Streak gonad with small glandular duct	Streak gonad with small glandular ducts and more calcifications
26	F	at 1.5 years B(bilateral)	Seminiferous tubules	Seminiferous tubules
27	F	at 15 years B(bilateral)	Seminiferous tubules	Streak gonad, no germ cells
28	F	at 19 minths B(bilateral)	A small amount of ovarian tissue is seen, and an immature follicle can be seen	Few seminiferous tubules
29	F	at 11 years B(bilateral) G (bilateral)	Streak gonad with few cavity structures	Epithelial nest structure
31	F	at 4.5 yr B(bilateral) G (bilateral)	Fibrovascular fatty nodules, Fallopian tube structure, epididymis and vas deferens structure	Fibrovascular fatty nodules, oviduct and vas deferens structures
32	F	at 55 months B(bilateral) G (bilateral)	Several glandular structures were seen in the ovarian-like stroma, but no obvious follicle tissue was seen	Fallopian tube structure and ovarian-like stroma, surrounded by lumen structure and a little vas deferens
33	F	at 68 months B(bilateral) G (bilateral)	Tumors of germ cell origin	Streak gonad, no germ cells
35	F	at 10 years B(bilateral) G (bilateral)	Gonadoblastoma	Slight ovarian-like stroma, vas deferens
36	F	at 16 years B(bilateral) G (bilateral)	A small amount of ovarian stroma and fallopian tubes	A small amount of ovarian stroma and fallopian tubes
37	F	at 13 years B(bilateral) G (bilateral)	Streak gonad with fallopian tube tissue	Gonadoblastoma
38	F	at 10 years B(bilateral) at 11 years G(bilateral)	Streak gonad, few glands	Streak gonad, no germ cells

Patient numbers are the same as in Tables 1, 2. M, Male; F, female; B, biopsy; G, gonadectomy; O, orchiopexy.

Conclusions

Patients with 45,X/46,XY mosaicism have specific growth patterns. Their HtSDS was in the normal range during 0–2 years of age, and then they began to show short stature after 2 years of age. The probability of short stature in females was higher than that in males. Short stature patients had growth hormone deficiency, retardation of bone age, and low IGF. Their WtSDS were all in the normal range but below the median. BMISDS was in the normal range, and there was no evidence of obesity.

The values of INHB, AMH, T (after HCG stimulation), and the T:DHT ratio in the male group were significantly higher than those in the female group, and the values of LH, FSH, peak LH and peak FSH in the female group were significantly higher than those in the male group. These data suggested that the gonads in the male group retained a certain androgen secretion function. The female group had impaired gonadal function, manifesting as hypergonadotropic hypogonadism. The hormone levels in the two groups can help us better understand this type of DSD disease, provide a basis for sex selection, and assist in the development of a personalized therapeutic schedule.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

Author contributions

YJ designed the experiments and analysed the data and presentation. LP contributed reagents/materials/analysis tools.YL gave revision suggestions during the process of revising the article and provided funding for the project. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2023.1135776/full#supplementary-material.

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Case Report: short stature, kidney anomalies, and cerebral aneurysms in a novel homozygous mutation in the *PCNT* gene associated with microcephalic osteodysplastic primordial dwarfism type II

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We report the case of a boy (aged 3 years and 7 months) with severe growth failure (length: -9.53 SDS; weight: -9.36 SDS), microcephaly, intellectual disability, distinctive craniofacial features, multiple skeletal anomalies, micropenis, cryptorchidism, generalized hypotonia, and tendon retraction. Abdominal US showed bilateral increased echogenicity of the kidneys, with poor corticomedullary differentiation, and a slightly enlarged liver with diffuse irregular echotexture. Initial MRI of the brain, performed at presentation, showed areas of gliosis with encephalomalacia and diffused hypo/delayed myelination, and a thinned appearance of the middle and anterior cerebral arteries. Genetic analysis evidenced a novel homozygous pathogenic variant of the pericentrin (PCNT) gene. PCNT is a structural protein expressed in the centrosome that plays a role in anchoring of protein complexes, regulation of the mitotic cycle, and cell proliferation. Loss-of-function variants of this gene are responsible for microcephalic osteodysplastic primordial dwarfism type II (MOPDII), a rare inherited autosomal recessive disorder. The boy died at 8 years of age as a result of an intracranial hemorrhage due to a cerebral aneurism associated with the Moyamoya malformation. In confirmation of previously published results, intracranial anomalies and kidney findings were evidenced very early in life. For this reason, we suggest including MRI of the brain with angiography as soon as possible after diagnosis in follow-up of MODPII, in order to identify and prevent complications related to vascular anomalies and multiorgan failure.

KEYWORDS

growth, microcephalic osteodysplastic primordial dwarfism, cerebral aneurysms, bone dysplasia, short stature, PCNT gene, MOPDII, intellectual disability

Background

The most common form of microcephalic primordial dwarfism (MPD) is MOPDII, which has several distinctive clinical features compared to the other forms, such as more severe growth impairment with skeletal dysplasia, and global vascular and metabolic disease with hypertension and insulin resistance. Microcephaly, a high forehead with receding hairline, a prominent beaked nose with ocular proptosis, micrognathia with relatively proportioned small mouth, dental dysplasia, and high squeaky voice represent the main phenotypic facial features (1, 2). Radiological abnormalities are also typically observed in MOPDII patients, with a tendency to worsen over time: at birth, children present with a high, narrow pelvis, small iliac bones, and flat acetabulum with subsequent femoral head subluxation or dislocation. As the child grows, skeletal changes become more severe, with possible proximal femoral epiphysiolysis, *coxa vara*, flared appearance of the metaphysis of distal long bones, progressive disharmony of the short stature due to mesomelic shortening of the limbs, and general bone age retardation as a result of delayed ossification (3, 4). Moreover, MOPDII is associated with increased vascular risk due to cerebral vessel anomalies that become responsible for early mortality (5, 6).

To date, at least 13 different genes responsible for specific disorders belonging to the MPD group have been identified. All these genes play a fundamental role in regulating centrosome activity, genome replication, and DNA damage response, with a strong overlap in function in the proteins encoded. Nevertheless, each disorder belonging to this group of skeletal dysplasias shows distinctive features depending on the gene involved (7). In addition to MOPDII and Seckel syndrome, MPD includes MOPD types I/III and Meier-Gorlin syndrome; despite distinctive molecular bases, these conditions share key clinical characteristics, such as extreme global growth impairment with severe short stature, microcephaly, and intellectual disability.

MOPDII (OMIM #210720) is a rare inherited autosomal recessive disorder caused by homozygous or compound heterozygous mutations in the pericentrin (*PCNT*) gene on chromosome 21q22 (8, 9).

PCNT mutations have previously been found to be associated with both Seckel syndrome and MOPDII, although the most recent analyses on larger case series have confirmed the specificity of pericentrin involvement in patients affected by MOPDII (10) (Figure 1). So far, over 150 individuals have been diagnosed with MOPDII (1), and 1147 variants of the *PCNT* gene have been described at present: 670 of these are classified as variants of uncertain clinical significance (VUS), 360 as benign, 90 as pathogenic, and 27 as probably pathogenic (11).

Here, we describe the case of a Moldovan child who moved to Italy at the age of three years, and who was diagnosed with MOPDII caused by a novel homozygous mutation in the *PCNT* gene (c.3019_3020del, p.Leu1007Serfs*50), which is responsible for an early termination site in protein synthesis; thus, premature truncation of protein synthesis was predicted, with a subsequent severe phenotype to be aware of.

Case report

The child was referred to our pediatric endocrinology outpatient clinic because of severe short stature and absence of catch-up growth since birth. All clinical features of MOPDII and of our patient are summarized in Table 1. He presented with intellectual disability and distinctive craniofacial features. At our first evaluation (when the patient was 3 years and 7 months old), his length was 62.4 cm (-9.53 SDS by WHO references), he weighed 5.15 kg (-9.36 SDS by WHO references), and he presented with microcephaly (43 cm, -4.8 SDS by WHO references). He had a high forehead with ocular proptosis, a prominent beaked nose, micrognathia, a relatively proportioned small mouth with multiple dental caries, brachymesophalangy,

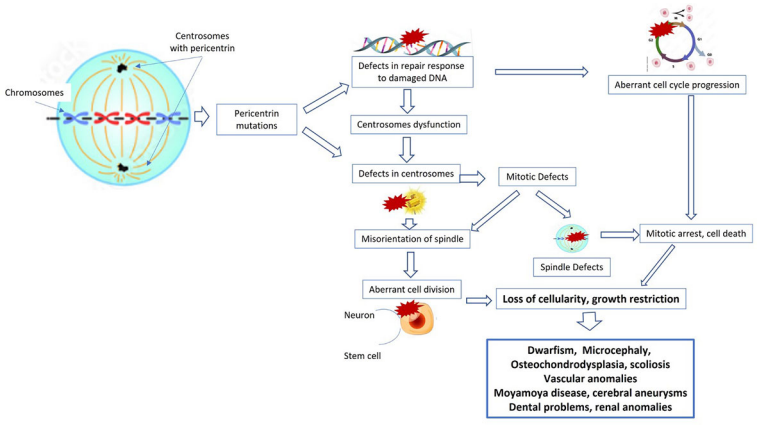

FIGURE 1
Cellular pathways and mechanisms implicated in primordial dwarfism. PCNT and other proteins are required for normal functioning of the centrosome, and therefore mutations in these genes also impair centrosome function. Moreover, pericentrin mutations can cause defects in the repair response to damaged DNA, with consequent aberrant cell cycle progression, mitotic arrest, and cell death. All these mechanisms lead to loss of cellularity, to growth restriction, and then to dwarfism, microcephalia, and bone, renal, and vascular anomalies.

TABLE 1 Features of Microcephalic Osteodysplastic Primordial Dwarfism Type II compared with the patient described.

Feature		Our patient
Extreme pre- & postnatal growth restriction: IUGR, severe short stature		**Present**
Microcephaly		**Present**
Skeletal dysplasia: hip deformity &/or scoliosis in addition to osteochondrodysplasia. Dysplasia may be difficult to recognize in newborn period.		**Present, with brachy-mesophalangy, arthrogryposis of the hands and equinus right foot**
Small, loosely rooted teeth: typically secondary teeth are more affected than primary teeth.		**Present, with multiple caries**
Hematologic	Anemia, Thrombocytosis	Absent
Cerebrovascular	Aneurysms: lifelong risk, median age 9.3 years	**Present**
	Moyamoya vasculopathy: mainly in younger ages	**Present**
Cardiovascular	Hypertension: Median age 13 yrs	Absent
	Hypercholesterolemia: Median age 18 yrs	Absent
	Cardiac malformations ASD, VSD, PFO	Absent
	Coronary artery disease w/premature Mis: Median age of MI 24 yrs	Absent
Renal	Chronic kidney disease Renal transplantation documented in 2 persons	Absent
	Accessory renal arteries: only described in males	Absent
	Renal vascular disease: Renal artery stenosis, aneurysm	Absent
	AR PKD-like features (hyperechoic thickening of adipose tissue of the perirenal space)	**In addition**
Liver	Liver enlargement, diffuse irregular echostructure	**In addition**
	Cryptorchidism / retractile testes	**Present**
Genital	Hypospadias	Absent
	Micropenis	**In addition**
Endocrine	Insulin resistance &/or diabetes mellitus: median age 11 yrs	Absent
Muscolo-skeletal	generalized hypotonia; tendon retraction	**In addition**
	Borderline/low-normal intellectual function: More impairment in those who have had strokes	Absent
Cognitive ability	ADHD Not yet definitively evaluated in large studies	Absent
	Intellectual disability	**In addition**

Modified from Duker et al (1) ADHD, attention-deficit/hyperactivity disorder; AR-PKD, Autosomal recessive polycystic kidney disease, ASD, atrial septal defect; IUGR, intraterine growth restriction; MI, myocardial infarction; PFO, patent foramen ovale; VSD, ventricular septal defect.

arthrogryposis of the hands, and equinus right foot. The boy also had a micropenis, hypotrophic-like right testis, and non-palpable left testis. On ultrasound the left testis was found to be intra-abdominal, with regular morphology and echotexture, while the right testis was observed within the inguinal canal, confirming clinical cryptorchidism. He also presented with generalized hypotonia and resistance to dorsiflexion of the right lower limb due to the presence of tendon retraction. Brisk and asymmetrical patellar osteotendinous reflexes were observed (Figure 2).

Medical history evidenced a pregnancy characterized by severe intrauterine growth retardation (IUGR). The mother reported that he was born around 28 weeks of gestational age, and that she discovered pregnancy late. Weight at birth was 890 **g** (he was born in Moldova, then moved to Italy). He was admitted for congenital CMV infection, but few data were available from the records brought by the mother. Karyotype was 46,XY. He was breastfed during the first weeks of age, and subsequently received formula milk. He was admitted to hospital again at 18 months of age for pneumonia.

At the first evaluation at our clinic, the patient was 3 years and 7 months old. He would eat only smooth foods and exhibited slow and difficult chewing. After dental eruption, fragmentation of the teeth was observed.

Considering the severe growth impairment, a Seckel's syndrome spectrum disorder was initially suspected, and the child underwent biochemical and radiological investigations. A total-body skeletal survey showed multiple and distinctive bone anomalies, including bone age delay (according to the Greulich and Pyle standards, this was 1 year and 3 months at a chronological age of 3 years and 10 months); dislocation of the humeral ossification center; convex shape of the radius bilaterally; and bilateral subluxation of the

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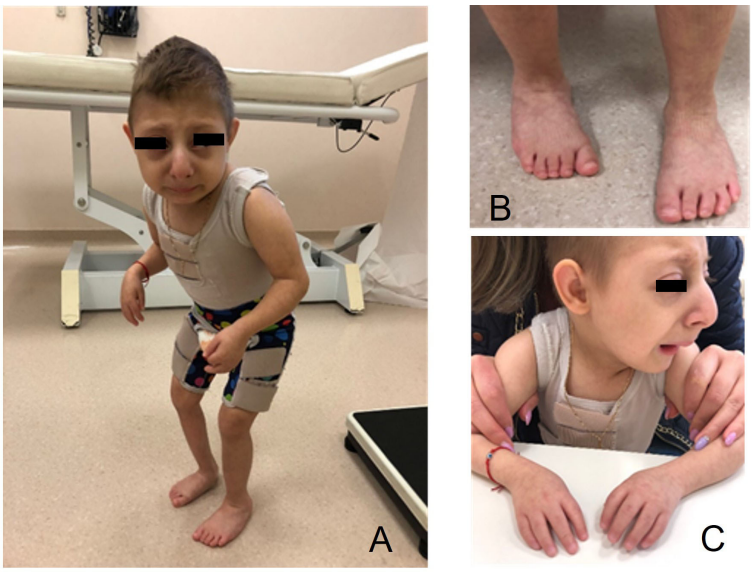

FIGURE 2
Features of the child with microcephalic osteodysplastic primordial dwarfism type II (MOPDII) at age 7 years. **(A)** Extreme short stature, arthrogryposis of the hands, and the forced posture can be observed; **(B)** equinus right foot; **(C)** high forehead with ocular proptosis, prominent beaked nose, and micrognathia.

femoral heads, more pronounced on the left (which evolved to luxation within three years), with wide acetabular angles and flared appearance of the distal metaphysis of both femurs as a consequence of severe skeletal dysplasia (Figure 3).

Abdominal ultrasound at 3 years of age showed normal echostructure and size of both kidneys, but at 6 years of age, bilateral increased echogenicity was present, with poor corticomedullary differentiation and hyperechoic thickening of

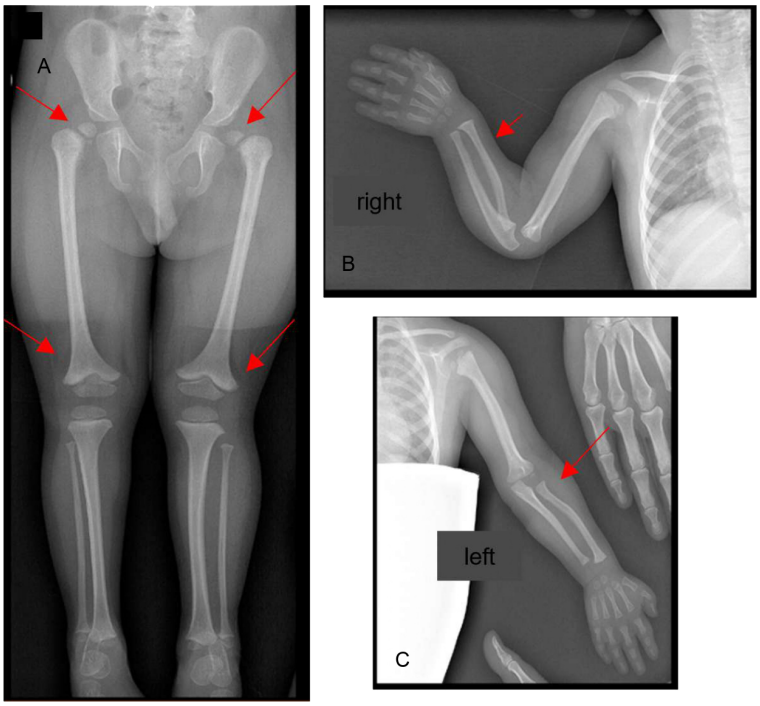

FIGURE 3
Bone Rx -ray anomalies at 3 years of age: **(A)** pelvis and lower limbs: bilateral subluxation of the femoral heads, more pronounced on the left with wide acetabular angles; flared appearance of the distal metaphysis of both femurs; **(B, C)** shoulders and upper limbs: dislocation of the humeral ossification center, convex shape of the radius bilaterally. The red arrows point at the anomalies described.

the adipose tissue of the bilateral perirenal space. This pattern is generally observed in autosomal recessive polycystic kidney disease. The remaining abdominal organs studied through ultrasound did not present significant abnormalities.

Insulin-like growth factor 1 (IGF-1) was 267.1 ng/mL (normal range: 56.2-267.1 ng/mL), and its binding protein 3 (IGFBP3) was 3941 ng/mL (normal range: 1995-4904 ng/dL); these levels were not suggestive of growth hormone deficiency.

Renal function and glucose metabolism were normal. Liver function tests initially showed elevation of transaminases, with negative infectious and immunologic liver tests, which progressively improved until normalization at 6 years of age, although liver enlargement was present on ultrasonography, with diffuse irregular echostructure. These findings have not been described previously in patients with MOPDII and remain of uncertain origin.

Following genetic counseling, genetic testing was carried out *via* next-generation sequencing on the PGM platform (Life Technologies), using an amplicon design covering all coding exons and exon–intron boundaries of the PCNT gene and following the DNA sample guidelines (https://varnomen.hgvs.org/). The DNA sample was obtained after the proband's mother provided informed consent to the analysis. The molecular analysis identified the c.3019_3020del in exon 15 predicted to lead to a premature codon termination fifty bases after codon 1007, p.(Leu1007Serfs*50) and production of truncated protein (Figures 4D, E). This could not be verified experimentally due to the small amount of peripheral blood available from the proband. However, based on the American College of Medical Genetics and Genomics (ACMG) criteria (12), the identified variant can be classified as pathogenic because loss-of-function mutation in the PCNT gene is a known mechanism of disease (13) (PVS1). The ClinVar database classifies this variant as pathogenic (PP5), and homozygotes for this variant are absent in the GnomAD (PM2) database. The pathogenic variant identified by NGS technology was validated by Sanger sequencing (Figures 4A–C) and reported using the Human Genome Variation Society nomenclature guidelines (https://varnomen.hgvs.org/). This genetic variant has not been described previously in the literature.

Initial magnetic resonance imaging (MRI) of the brain was performed at diagnosis, and showed areas of gliosis with encephalomalacia in the frontal cortico-subcortical and left parasagittal parietal area; a diffused hypo/delayed myelination with non-specific signal alteration, in particular on the left cerebral hemispheric side; irregularity of the subependymal surface; a thinned corpus callosum at the level of the trunk; dilation of the lateral ventricles, especially at the level of the trigons; a thinned appearance of the middle and anterior cerebral arteries; and triangular and flattened shape of the frontal bones with trigonocephaly (Figures 5A, B).

Based on previous recommendations, as possible cerebral accidents were described at a later age (14), the child did not undergo further brain MRI scans until he presented at the age of 8 years with a brain hemorrhage due to the rupture of an aneurysm located in the posterior inferior cerebellar artery. The MRI conducted after the stroke showed the presence of multiple aneurysms located in the internal carotid, communicating arteries, and the apical portion of the basilar artery that had been absent during the first neuroimaging evaluation conducted when the child was 3 years old. The MRI also highlighted an occlusion of the M1 segment of both middle cerebral arteries and an irregular and stenotic appearance of the A3-A4 segments of the anterior cerebral arteries associated with the development of a collateral vessel network (Moyamoya disease) (Figures 5C–F). Unfortunately, the child died as a result of this acute event.

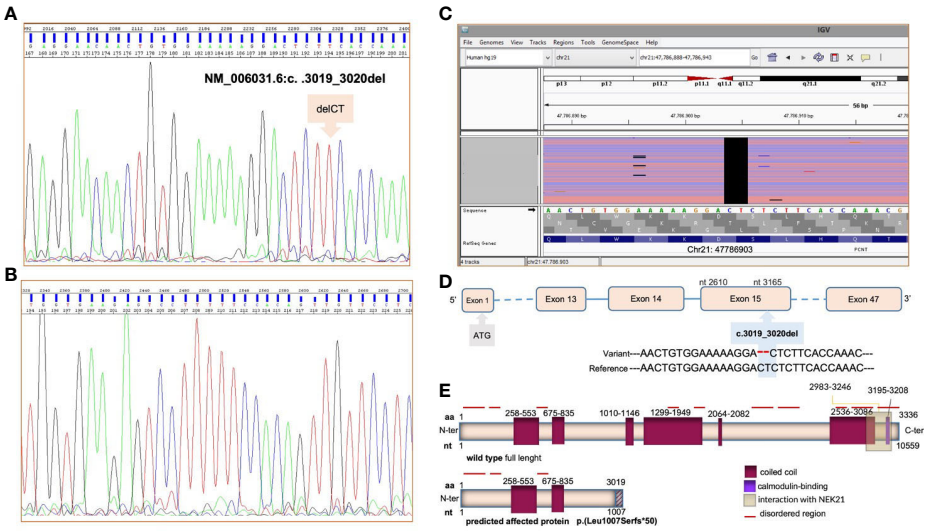

FIGURE 4
NGS and Sanger sequencing showed a novel homozygous c.3019_3020del deletion in the *PCNT* gene on genomic DNA. The forward DNA sequencing electropherogram of exon 15 and of the *PCNT* gene is reported in panel **(A)**, the reverse in **(B)**. Ref Seq (reference sequencing) was used for variant annotations: NM_006031.6 and NP_006022.3. The identified pathogenic variant in the *PCNT* gene, visualized using the Integrative Genome Viewer (IGV) software, is presented in **(C)**. In panels **(D, E)**, the identified mutation and the predicted consequence for the pericentrin protein are detailed. The pericentrin structure was derived from https://www.uniprot.org/uniprotkb/O95613/entry.

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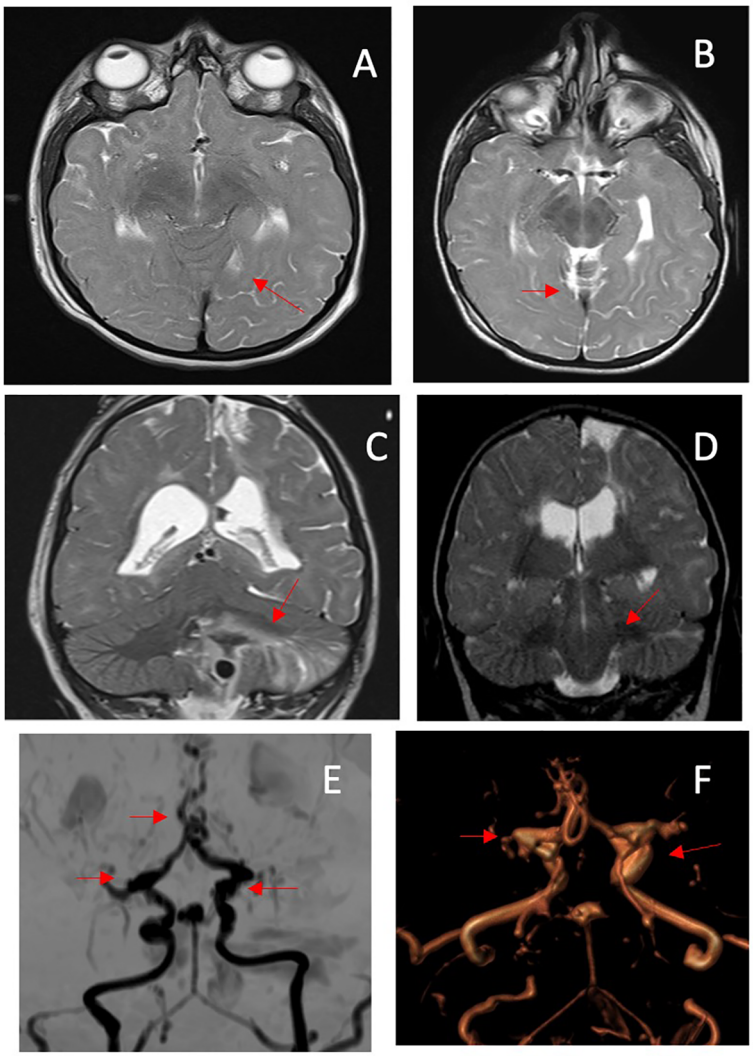

FIGURE 5
MRI performed at diagnosis. **(A)** Axial T2-weighted image (WI) showing a diffuse attenuation of the flow void of the middle cerebral arteries; **(B)** coronal T2WI shows multiple areas of prior infarction, seen as areas of high signal intensity. MRI performed at age 8 yr. **(C)** axial T2-WI shows a decrease in flow void of the anterior cerebral arteries; **(D)** an acute hemorrhagic infarction is also visible in the left cerebellar hemisphere on T2-WI coronal image. **(E, F)** MIP/3D TOF MRA: the coronal projection shows diffuse stenosis/dilatations of the vessels of the circle of Willis, with Moyamoya appearance, consisting in sub/occlusion of the middle cerebral arteries, stenosis of the anterior cerebral arteries (with a mouse-tail appearance), bilateral carotid siphons, basilar apex and tonsillar branch of the posterior-inferior cerebellar artery aneurysmal dilatation. The myriad of small collateral blood vessels including the rete mirabilis in the region of the perforating arteries is also visible. The red arrows point at the anomalies described.

Discussion

MOPDII is the most common form of microcephalic primordial dwarfism (MPD) and differs from other similar diseases, such as Seckel syndrome, in that it is characterized by more severe growth impairment, skeletal dysplasia, and less severe intellectual disability. *PCNT* gene variants are causative for this condition. Pericentrin is a multifunctional protein that plays both a structural role, as a major constituent of pericentriolar material, and a functional role in recruiting other proteins for cell cycle progression. Indeed, *PCNT* participates in the formation of the mitotic spindle as a core centrosomal component, but also regulates cellular proliferation through interactions with other signaling pathways that control cell cycle checkpoints and mitotic entry. Recent studies conducted in patients with MOPDII have additionally shown defective ataxia–telangiectasia and a Rad3-related protein (ATR)-dependent DNA damage response, as a consequence of an ectopic localization of checkpoint kinase 1 from the centrosome due to *PCNT* gene mutations. ATR was the first mutated gene discovered in patients with MPD: it encodes a protein kinase (PI3K) that regulates the DNA damage response pathway, and it is now also associated with Seckel syndrome (10).

Despite the heterogeneous molecular basis of these conditions, MOPDII shares some features with other disorders belonging to the MPD group (Seckel syndrome, MOPD I/III, and Meier-Gorlin syndrome), such as severe pre- and post-natal growth retardation

and marked microcephaly, in addition to the characteristic facies, skeletal dysplasia, abnormal dentition, diabetes/insulin resistance, and increased risk for neurovascular disease. In the case we describe, no metabolic issues were identified, possibly because of the patient's young age, but a close endocrinological follow-up was scheduled in order to identify these possible complications early.

The first brain MRI in our patient highlighted a thinned appearance of the middle and anterior cerebral arteries. Central nervous system vascular anomalies have been reported as an important cause of morbidity and mortality in patients with MOPDII (7), and Moyamoya disease has also been reported during childhood; however, aneurysmal disease has been described frequently, with a mean age of appearance of approximately 9.3 years (1, 15). Overall, nervous system vascular anomalies are reported to be an important cause of morbidity and mortality in patients with MOPDII (1).

This patient, however, died of a brain hemorrhage at the age of eight, highlighting the need for more careful and closer neuroimaging follow-up, which needs to be completed *via* specific angiographic studies.

The most recent review, published in May 2021 and covering 47 American patients, reported that 47% of these subjects were diagnosed with Moyamoya or intracranial aneurysms at a mean age of 9.3 years, and 19 underwent Moyamoya intravascular bypass surgery; 53% had aneurysms identified, but 36% had none of these vascular anomalies (9). The authors of this review recommended brain MRI evaluations early in childhood (16) (in particular, once yearly during the first decade of life), and thereafter according to previous findings, without exceeding 18-month intervals between investigations, for early identification of vessel anomalies in order to allow the use of both surgical and pharmacological approaches (anticoagulants) (9). More recent publications suggest that brain MRI should be carried out at the moment of diagnosis (1). This case report confirms the need for such close follow-up of brain MRI.

In this patient, kidney abnormalities were also identified on ultrasound. The findings were similar to those of autosomal recessive polycystic kidney disease. Renal involvement is not usually described in MOPDII, although rare cases of nephrolithiasis have been reported (15), and a very recent publication by Hettiarachchi et al. has described a child with a novel distinct mutation in the pericentrin gene associated with bilaterally small kidneys, without other renal anomalies (17).

It can be hypothesized that severe variants in the *PCNT* gene could lead to centrosome disruption, including mislocalization of the centrosome protein and proteins involved in cilia genesis, potentially contributing to cyst formation. Hall et al. reported observing unilateral cystic dysplasia of the kidney in a male patient with MOPDII, although the *PCNT* gene mutations were unknown at that time in that patient (18). Renal involvement with autosomal recessive polycystic kidney disease pattern, as observed in our patient, has never been described in children with MOPDII. However, further reports and studies are warranted to clarify this aspect, and to provide further understanding of liver function.

Conclusions

This case report identifies a novel *PCNT* gene mutation associated with MOPDII that is predicted to cause severe derangement in protein structure and function, explaining the severe features of the condition observed in the patient, who (in addition to characteristics described in previous reports on this syndrome) presented with early severe neurovascular disease, kidney abnormalities, and possible transient/relapsing changes in liver function. Considering the impact of neurovascular disease on morbidity and mortality in these patients, close follow-up *via* brain MRI associated with angiography is mandatory for early identification of vessel anomalies and determination of appropriate management.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

MPe wrote the first draft of the manuscript and was in charge of the patient's follow-up. EC, MG, GM and LB contributed to clinical activities and performed the literature analysis. VDP made scientific contributions. MPi and AP performed the genetic analysis and FO prepared, evaluated, and annotated the neuroimages. SMRE supervised the outpatient clinic activities. MS critically revised the text and made substantial scientific contributions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Growth response of syndromic versus non-syndromic children born small for gestational age (SGA) to growth hormone therapy: a Belgian study

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Introduction: A substantial proportion of SGA patients present with a syndrome underlying their growth restriction. Most SGA cohorts comprise both syndromic and non-syndromic patients impeding delineation of the recombinant human growth hormone (rhGH) response. We present a detailed characterization of a SGA cohort and analyze rhGH response based on adult height (AH).

Methods: Clinical and auxological data of SGA patients treated with rhGH, who had reached AH, were retrieved from BELGROW, a national database of all rhGH treated patients held by BESPEED (BElgian Society for PEdiatric Endocrinology and Diabetology). SGA patients were categorized in syndromic or non-syndromic patients.

Results: 272 patients were included, 42 classified as syndromic (most frequent diagnosis (n=6): fetal alcohol syndrome and Silver-Russell syndrome). Compared with non-syndromic patients, syndromic were younger [years (median (P10/P90)] 7.43 (4.3/12.37) vs 10.21 (5.43/14.03), p=0.0005), shorter (height SDS -3.39 (-5.6/-2.62) vs -3.07 (-3.74/-2.62), p=0.0253) and thinner (BMI -1.70 (-3.67/0.04) vs -1.14 (-2.47/0.27) SDS, p=0.0054) at start of rhGH treatment. First year rhGH response was comparable (delta height SDS +0.54 (0.24/0.94) vs +0.56 (0.26/0.92), p=0.94). Growth pattern differed with syndromic patients having a higher prepubertal (SDS +1.26 vs +0.83, p=0.0048), but a lower pubertal height gain compared to the non-syndromic group (SDS -0.28 vs 0.44, p=0.0001). Mean rhGH dose was higher in syndromic SGA patients (mg/kg body weight/day 0.047 (0.039/0.064) vs 0.043 (0.035/0.056), p=0.0042). AH SDS was lower in syndromic SGA patients (-2.59 (-4.99/-1.57) vs -2.32 (-3.3/-1.2), p=0.0107). The majority in both groups remained short (<-2 SDS: syndromic 71%, non-

syndromic 63%). Total height gain was comparable in both groups (delta height SDS +0.76 (-0.70/1.48) vs +0.86 (-0.12/1.86), p=0.41).

Conclusions: Compared to non-syndromic SGA patients, syndromic SGA patients were shorter when starting rhGH therapy, started rhGH therapy earlier, and received a higher dose of rhGH. At AH, syndromic SGA patients were shorter than non-syndromic ones, but their height gain under rhGH therapy was comparable.

KEYWORDS

short for gestational age, syndromic, growth hormone, growth, children, adult height, short stature

1 Introduction

Three percent of all children are born small for gestational age (SGA), of those 10-13% do not develop catch-up growth and remain short (1–3). Treatment with recombinant human growth hormone (rhGH) was reported to increase significantly adult height (AH) in short children born SGA (4–7). Based on these results, the European Medicines Agency (EMA) approved in 2003 rhGH therapy for children born SGA who are lacking catch-up growth at the age of 4 years.

Nonetheless, the response to growth hormone therapy is very variable and several studies have been published, trying to identify predicting factors for growth response in SGA patients (8, 9). One of the discussed reasons for the variable growth response, is that SGA patients are a heterogenous group including patients who suffered from an intrauterine growth restriction caused by a variety of reasons: maternal complication (preeclampsia, uterine anomalies, maternal drug use, including alcohol and tobacco), fetal complications (intrauterine infections, syndromes), placental abnormalities (reduced placental blood flow) and environmental insults (toxic substances, altitude) (10).

The reported cohorts often contain patients suffering from a severe or partial growth hormone deficiency (4, 5, 11), or patients who were additionally treated with GnRH analogues (12), which might further influence the variable growth hormone response of the studied cohort (13).

Syndromic patients are reported to respond worse to rhGH therapy than non-syndromic patients (12). In some studies, syndromic patients have been excluded (11, 14), while in others, only Silver-Russell Syndrome (SRS) patients were included (6, 15). Dahlgren et al. excluded SGA patients with chromosomal disorders, chondrodysplasia, fetal alcohol syndrome (FAS) and children with "serious malformations", but included a SRS patient (5). So, the published SGA cohorts are very heterogenous. A recent study examined rhGH response during the first two years of therapy in the following SGA subgroups: patients with dysmorphic features, FAS patients and SRS patients. This study revealed the best response to rhGH in the SRS subgroup and the highest rate of non-responders, defined as delta height SDS <0.3 after the first year of rhGH therapy, in the FAS subgroup (16). Data on rhGH response for syndromic patients on adult height are sparse. Few studies have published adult height after growth hormone therapy for SRS patients (17–19). They show a lower adult height, but an equal height gain compared to non-SRS SGA patients under rhGH therapy (18, 19).

We report on a large SGA cohort retrieved from the Belgian national registry for patients treated with growth hormone (BELGROW) held by BESPEED (BElgian Society for PEdiatric Endocrinology and Diabetology) and determined how many syndromic patients were included in this cohort and which syndromes had been diagnosed. We further compared the two SGA groups (syndromic versus non-syndromic SGA) and analysed their response to rhGH therapy.

2 Material and methods

2.1 Subjects

SGA patients who fulfilled the following criteria were extracted from BELGROW: birth length and/or birth weight <-2 SDS according to Niklasson (20) and for children born preterm <28.5 weeks of gestation according to Intergrowth (21, 22), who had a height <-2.5 SDS according to Roelants (23) when starting rhGH therapy, who were treated at least 2 years with daily subcutaneous rhGH injections and for whom an AH was documented in BELGROW (Figure 1).

Patients were excluded if they had been treated with aromatase inhibitors or GnRH analogues, if they were suffering from a chronic disease known to possibly interfere with growth, such as chronic intestinal diseases, cystic fibrosis, cardiac insufficiency, precocious puberty, 21 hydroxylase deficiency, immune deficiency syndromes, oncological disease, severe hypothyroidism and spastic paralysis, if they were diagnosed with or had symptoms of bone dysplasia, or if they had a genetically confirmed mutation in the IGF-1 receptor gene.

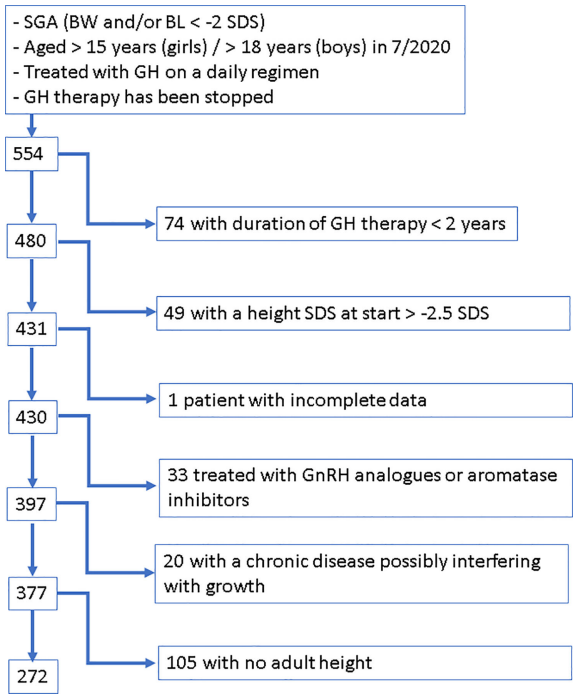

FIGURE 1
Flow Chart visualizing the applied procedure of patient selection.

After applying these criteria, our SGA cohort included 272 patients.

These patients were categorized into syndromic and non-syndromic patients. All patients who had a genetically confirmed syndrome, or a syndrome diagnosis based on a published clinical score (SRS, FAS) or who had in addition to their short stature at least two other symptoms (congenital heart defects, intellectual disability, dysmorphic features, …) suggesting a syndromic origin of their short stature were classified as syndromic SGA patients.

BELGROW is a database, which has been running since 1985 by BESPEED and includes almost all patients treated with rhGH in Belgium. This registry stores pseudonymized data. Informed consent of the registered patients has been obtained.

2.2 Methods

Variables retrieved from BELGROW were: diagnosis; gender; weight and length at birth; father's and mother's height; age, height, weight, pubertal stage at start of rhGH therapy, after 1 year, at start of puberty, at end of rhGH therapy and at adult height.

SDS calculations were performed applying reference values published by Niklasson (20) for birth parameters and Intergrowth data (21, 22) for preterm babies <28.5 weeks of gestation. For follow-up data we used reference data published by Roelants (23) to determine SDS.

AH was assumed if growth velocity was less than 2 cm/year and pubertal development was completed (Tanner stage 5 and/or min. 2 years after menarche in girls; min. testicular volume of 15 ml in boys) and/or bone age or estimated bone age (24) was min. 14 years in girls or min. 16 years in boys. As our applied growth velocity to define adult height was not 0 cm/year and as we expressed AH in SDS by applying the gender-adapted SDS of the age of 21 to the obtained AH, we are certainly underestimating adult height slightly.

Mean daily dose (mg/kg body weight/day) during the first year and during the whole treatment period was calculated using the dosage recorded at each visit.

Mid-parental height (MPH) was calculated by [father's height (cm) + mother's height (cm) + 13 cm for boys/- 13 cm for girls]/2 (25). Target height range was defined as MPH +/- 10 cm for boys and MPH +/- 9 cm for girls (26).

The response of growth hormone therapy was evaluated by the change from baseline height standard deviation score to AH standard deviation score (Δ-height SDS).

2.3 Statistical analysis

Results are expressed as median (P10-P90) or percentages. The percentage of subjects with an AH SDS <-2 and with an AH in their target height range was calculated. Continuous variables and percentages were compared across groups using Mann-Whitney U tests, or chi-square tests as appropriate. A p value <0.05 was considered statistically significant. Stata 15.1 was used for statistical analysis.

3 Results

3.1 Cohort characterization

We identified 42 syndromic patients in our SGA cohort (15%).

The most frequent diagnosis was FAS (six), and SRS (six), followed by patients with 3M syndrome (two). The diagnosis of a defined syndrome was mostly made before the start of treatment. Twenty-five of the 42 syndromic patients had a defined syndrome, hence 17 (40%) had no defined syndrome (Table 1). Genetic analysis in 8 of these 17 patients was not contributory. Genetic analysis included karytoype, CGH-array, specific gene analysis and whole exome sequencing. These methods were used either individually or in different combinations with or without a genetic consultation. As our data are based on a registry, which is used by different physicians from different Belgian centers, there was no uniform approach for the genetic work-up.

In 9 patients, no genetic analysis had been performed.

3.2 Syndromic versus non-syndromic SGA patients: Comparison of baseline characteristics

In the syndromic group there was a higher percentage of male patients (71%) compared to the non-syndromic group (55%), but this was not statistically significant (p=0.051). Gestational age did not differ between the two groups, but syndromic patients had lower birth weight (-2.83 versus -2.26 SDS, p=0.0011) and length (-3.09

TABLE 1 Description of the syndromic SGA group.

Syndromes	N =42
Silver-Russell syndrome	6
Fetal alcohol syndrome	6
3M syndrome	2
Becker dystrophia	1
Di George syndrome	1
Klinefelter	1
Mulvihill -Smith syndrome	1
Ohdo Blepharophimosis syndrome	1
Pierre Robin Sequence	1
Ring chromosome 11	1
Ring chromosome 7 in mosaicism	1
Renpenning syndrome	1
VATER syndrome	1
Seckel syndrome	1
Non defined syndromes	17

versus -2.48 SDS, p=0.0178) than non-syndromic patients at birth (Table 2A).

While mother's height was not significantly different, fathers of non-syndromic patients were shorter (-1.35 versus -1.13 SDS, p=0.0446) as was MPH (-1.4 versus -1.1 SDS, p=0.0232) (Table 2A).

Syndromic SGA patients were started on rhGH therapy at a younger age (7.43 versus 10.21 years, p=0.0005). At start of GH therapy, syndromic patients were shorter (height SDS -3.39 versus -3.07, p=0.0253), especially when taking into account their MPH (Height SDS – MPH SDS: -2.34 versus -1.74, p<0.0001). Syndromic SGA patients were lighter (weight SDS: -4.06 versus -2.91, p<0.0001) and had a lower BMI SDS (-1.70 versus -1.14, p=0.0054) at start of therapy (Table 2B.).

3.3 Syndromic versus non-syndromic SGA patients: Comparison of response to rhGH

After one year of therapy, syndromic patients remained shorter than non-syndromic SGA patients (height SDS -3.04 versus -2.51, p=0.0286; Table 3). The percentage of non-responders (delta-height SDS <0.3 after one year of therapy) was comparable (19% in syndromic versus 17% in non-syndromic patients, p=0.69; Table 3).

At the beginning of puberty, there was no longer a significant difference in height SDS between the two groups (-2.35 versus -2.46, p=0.59). Age at start of puberty was comparable (12.56 versus 12.39 years, p=0.66; Table 3). However, at the end of therapy, syndromic patients remained shorter (-2.07 versus -1.82 SDS, p=0.0188) and this difference was exacerbated at AH SDS (-2.59 versus -2.32, p=0.0107; Figure 2 and Table 4).

BMI SDS of syndromic patients remained lower after one year of rhGH therapy (-1.62 versus -1.03, p=0.0033), but was not different at other time points. Median BMI remained always below P50 throughout the follow-up for both groups (Tables 3, 4).

The age at the end of therapy was comparable in both groups (16.01 versus 16.16 years, p=0.90). Hence mean duration of the GH therapy was longer in the syndromic group (8.35 versus 5.5 years, p<0.0001). The syndromic group was treated with a higher median dose (47 mcg/kg/day versus 43 mcg/kg/day, p=0.0042) (Table 4).

As some patients were treated in the setting of studies before the EMA approval of SGA as an indication for rhGH therapy, some patients were treated with a discontinuous rhGH regime. After their study participation, rhGH was interrupted until they could be treated in a medical need program or eventually under the approved SGA indication. This was the fact for 12% of the syndromic patients and 6% of the non-syndromic patients. Median interruption time of their rhGH therapy was 3 and 1.8 years respectively. These differences were not statistically significant (Table 4).

Regarding height gain, there was no significant difference either after 1 year of therapy (delta height SDS: 0.54 versus 0.56, p=0.94), at the end of therapy (height SDS 1.3 versus 1.33 SDS, p=0.99), or at AH (height SDS 0.76 versus 0.86, p=0.41). However, before the start of puberty, the syndromic group had a greater height gain compared to the non-syndromic group (height SDS gain 1.26 versus 0.83, p=0.0048) (Figure 3). The pubertal height gain was hence lower in the syndromic group (-0.28 versus 0.44 SDS, p=0.0001; Table 4).

The majority of patients in both groups remained short (<-2 SDS) at AH (71% versus 63%, p=0.27). The syndromic SGA group remained slightly shorter than the non-syndromic group (height SDS at 21 years -2.59 SDS versus -2.32, p=0.0107), but when comparing final heights for males and females separately, there was no significant difference in males (median final height: 165.5 cm for syndromic and non-syndromic males). Non-syndromic females were taller than syndromic SGA females at final height (median final height: 148.3 cm for syndromic and 152.3 for non-syndromic females, p=0.0291; Table 4).

More than 75% of non-syndromic patients reached an AH in their target height range (79%). In the syndromic group, only 42% reached an AH in their target height range (p=<0.0001; Table 4).

4 Discussion

Our cohort is so far the largest SGA cohort with published information about AH.

The absolute AH we report, is shorter than in some cohorts (4, 5), but similar to some other reports (15) (see Table 5).

This holds true as well for AH SDS (4, 5, 14, 28). As we are applying SDS for the age of 21 years to calculate AH SDS, we are probably underestimating AH SDS. Other studies calculate AH SDS on the basis of chronological age at final height and hence tend to overestimate AH. But even if we are applying chronological SDS for AH in our study cohort, our AH SDS are below some published data (4, 5, 11, 28), but similar to other reports (6, 15, 27) (see Table 5). As syndromic patients reach a shorter AH in our cohort and as they

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TABLE 2 Syndromic versus non-syndromic SGA patients: Comparison of baseline characteristics.

A: Syndromic versus non-syndromic SGA patients: Birth parameters and parental heights							
	Syndromic SGA			Non-Syndromic SGA			Comparison of the 2 groups
	n=42			n=230			
	median or n (%)	P10	P90	median or n (%)	P10	P90	p
Gender Male/Female	**30 (71%)/12 (29%)**			**127 (55%) /103 (45%)**			p=0.051
Birth weight SDS	**-2.83**	-4.36	-1.34	**-2.26**	-3.44	-1.39	p=0.0011
Birth length SDS	**-3.09**	-4.72	-1.20	**-2.48**	-3.72	-1.63	p=0.0178
gestational age (wks)	**38.0**	34.0	40.0	**39.0**	33.8	40.0	ns p=0.71
Father's ht SDS	**-1.13**	-2.70	0.45	**-1.35**	-2.55	-0.15	p=0.0446
Mother's ht SDS	**-0.98**	-2.75	0.29	**-1.35**	-2.80	-0.10	ns p=0.12
MPH SDS	**-1.10**	-2.15	0.03	**-1.40**	-2.25	-0.52	p=0.0232

B: Syndromic versus non-syndromic SGA patients: Anthropometric parameters and pubertal status before start of rhGH therapy							
	Syndromic SGA			Non-Syndromic SGA			Comparison of the 2 groups
	n = 42			n = 230			
	median or n (%)	P10	P90	median or n (%)	P10	P90	p
At start GH							
Age yrs	**7.43**	4.30	12.37	**10.21**	5.43	14.03	p=0.0005
In puberty, n (%)	**4 (9.5%)**			**59 (25.6%)**			p=0.023
Height SDS	**-3.39**	-5.60	-2.62	**-3.07**	-3.74	-2.62	p=0.0253
Height SDS minus MPH SDS	**-2.34**	-4.45	-1.38	**-1.74**	-2.85	-0.83	p<0.0001
weight SDS	**-4.06**	-6.42	-2.13	**-2.91**	-4.38	-1.89	p<0.0001
BMI SDS	**-1.70**	-3.67	0.04	**-1.14**	-2.47	0.27	p=0.0054

ns = not significant.

TABLE 3 Syndromic versus non-syndromic SGA patients: Comparison of response to rhGH after the first year of therapy and at onset of puberty.

	Syndromic SGA			Non-syndromic SGA			Comparison of the 2 groups
	n = 42			n=230			
	median or n (%)	P10	P90	median or n (%)	P10	P90	p
After 1 year GH							
Age yrs	**8.60**	5.33	13.30	**11.24**	6.45	15.04	p=0.0006
Height SDS	**-3.04**	-5.04	-1.85	**-2.51**	-3.24	-1.93	p=0.0286
Height SDS minus MPH SDS	**-1.79**	-3.91	-0.89	**-1.22**	-2.30	-0.25	p<0.0001
Weight SDS	**-3.38**	-5.65	-1.46	**-2.37**	-3.54	-1.27	p=0.0001
BMI SDS	**-1.62**	-3.13	-0.02	**-1.03**	-2.20	0.20	p=0.0033
Delta ht SDS 1st yr	**0.54**	0.24	0.94	**0.56**	0.26	0.92	ns p=0.94
Delta ht SDS 1st yr>0.5, n (%)	**22 (52%)**			**134 (58%)**			ns p=0.48
Delta ht SDS 1st yr>0.3, n (%)	**34 (81%)**			**192 (83%)**			ns p=0.69

(Continued)

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TABLE 3 Continued

	Syndromic SGA			Non-syndromic SGA			Comparison of the 2 groups
	n = 42			n=230			
	median or n (%)	P10	P90	median or n (%)	P10	P90	p
At start of puberty							
Age yrs	**12.56**	10.86	13.98	**12.39**	10.41	14.30	ns p=0.66
Age yrs in males	**13.03**	11.45	14.86	**13.09**	11.64	14.57	ns p=0.68
Age yrs in females	**11.41**	10.35	12.56	**11.55**	9.77	13.56	ns p=0.75
Height SDS	**-2.35**	-4.01	-0.99	**-2.46**	-3.47	-1.48	ns p=0.59
Height SDS minus MPH SDS	**-1.43**	-2.86	0.32	**-1.06**	-2.31	0.13	ns p=0.081
Weight SDS	**-2.21**	-3.58	-0.83	**-2.22**	-3.34	-0.92	ns p=0.49
BMI SDS	**-1.01**	-3.13	-0.12	**-1.11**	-2.33	0.36	ns p=0.38
Delta ht SDS before puberty*	**1.26**	0.27	2.50	**0.83**	0.16	1.75	p=0.0048

*Only including prepubertal patients under rhGH therapy.
ns = not significant.

were excluded from several studies (11, 14), this might contribute to our lower reported AH. Several other studies included only prepubertal SGA patients (4, 5, 28), while others reported a better rhGH response when therapy was started before puberty (11, 14). Our cohort comprises quite an important proportion of patients who started rhGH during puberty (9.5% syndromic and 25.6% non-syndromic SGA), this could again contribute to our lower reported AH. On the other hand, in our study, although the syndromic patients were younger at rhGH treatment start, they did not note reach a better AH, so further studies are needed to elucidate the effect of timing of the rhGH treatment start.

Furthermore, some patients in our study cohort were treated with a discontinuous rhGH regimen as they had been included in clinical trials before official EMA approval of SGA as an indication for rhGH therapy, then stopped rhGH at the end of the trial and reinitiated it in a medical need program or eventually after EMA approval. This might have compromised AH in our cohort, although de Zegher et al. have shown, that discontinuous rhGH regimens are equally effective, if a higher rhGH is used (29) (this was the case in the study setting before EMA approval).

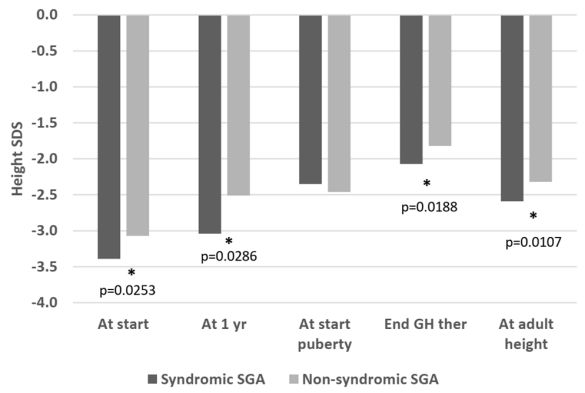

FIGURE 2
Height SDS, syndromic versus non-syndromic SGA patients: Differences in Height SDS between syndromic and non-syndromic patients before, throughout and after rhGH therapy. * statistically significant.

The majority of our cohort remained short. More than 75% of the non-syndromic patients, but only 42% of the syndromic patients reached a height within their target height range. This might be due to the fact, that MPH was significantly shorter in the non-syndromic group. The difference to MPH (AH SDS – MPH SDS) of the non-syndromic group, if applying AH for chronological age rather than for 21 years of age (in order to compare our results to other publications), is very similar to most published results (14, 15, 28) and to the meta-analysis published by Maiorana et al. (30). This underlines the importance of a careful evaluation of published results regarding rhGH treatment outcome in SGA cohorts (inclusion or not of growth hormone deficient patients, syndromic patients and patients suffering from bone dysplasia, the number of included patients, MPH) as well as the expression of outcome (AH SDS based on chronological age or on SDS for 21 years) in order to interpret correctly the effect of rhGH therapy. Table 5 provides an overview of the results of the so far published SGA cohorts with documented AH and their inclusion criteria.

However, in terms of height gain, our study showed that no significant difference in total height gain was observed between syndromic and non-syndromic SGA patients. Syndromic patients were more severe SGA, were shorter and lighter before growth hormone therapy, and ended up shorter after growth hormone therapy, but the height gain was comparable.

This contrasts with the results of Adler et al, who describe in a multivariant analysis a worse response to growth hormone in their syndromic SGA subcohort. This study included a significant number of SGA patients suffering from a bone dysplasia in their syndromic subgroup, which might have caused the lower height gain (12).

TABLE 4 Syndromic versus non-syndromic SGA patients: Comparison of response to rhGH at the end of therapy and at AH, and details on applied rhGH therapy.

	Group 1: Syndromic			Group 2: Non-syndromic			Comparison of the 2 groups
	n=42			n=230			
	median or n (%)	P10	P90	median or n (%)	P10	P90	p
At end of GH therapy							
Age yrs	**16.01**	14.43	17.83	**16.16**	13.78	18.00	ns p=0.90
Height SDS	**-2.07**	-4.59	-0.87	**-1.82**	-2.77	-0.93	p=0.0188
Height gain SDS	**1.30**	0.35	2.04	**1.33**	0.57	2.05	ns p=0.99
At near adult height							
Age yrs	**17.52**	15.29	20.75	**17.32**	14.95	22.77	ns p=0.85
Height SDS for CA	**-2.18**	-4.95	-1.43	**-1.98**	-2.94	-1.07	p=0.0173
Height SDS 21 yr	**-2.59**	-4.99	-1.57	**-2.32**	-3.30	-1.20	p=0.0107
Height. cm male	**165.5**	150.2	170.6	**165.5**	158.0	173.8	ns p=0.0812
Height. cm female	**148.3**	132.0	155.2	**152.3**	147.2	158.3	p=0.0291
Height SDS 21 yr <-2, n (%)	**30 (71%)**			**144 (63%)**			ns p=0.27
Height SDS minus MPH	**-1.24**	-3.59	0.27	**-0.52**	-1.78	0.49	p<0.0001
Ht SDS 21 yr minus MPH	**-1.70**	-3.79	-0.02	**-0.78**	-2.17	0.12	p<0.0001
Height (cm) in MPH range n (%)	**16 (42%)**			**173 (79%)**			p<0.0001
Total height gain SDS 21 yr	**0.76**	-0.70	1.48	**0.86**	-0.12	1.86	ns p=0.41
Total height gain SDS >1, n (%)	**25 (60%)**			**143 (62%)**			ns p=0.75
Total height gain SDS 21 yr >1, n (%)	**16 (38%)**			**99 (43%)**			ns p=0.55
Total pubertal height gain SDS	**-0.28**	-1.14	1.11	**0.44**	-0.55	1.48	p=0.0001
Duration GH	**8.35**	4.10	11.20	**5.50**	3.10	9.75	p<0.0001
Treatmt interruption, n (%)	**5 (12%)**			**14 (6%)**			ns p=0.17
Total interruption time yr	**3.00**	1.00	3.10	**1.80**	1.00	2.90	ns p=0.10
Mean dose mg/kg day	**0.047**	0.039	0.064	**0.043**	0.035	0.056	p=0.0042

ns = not significant.

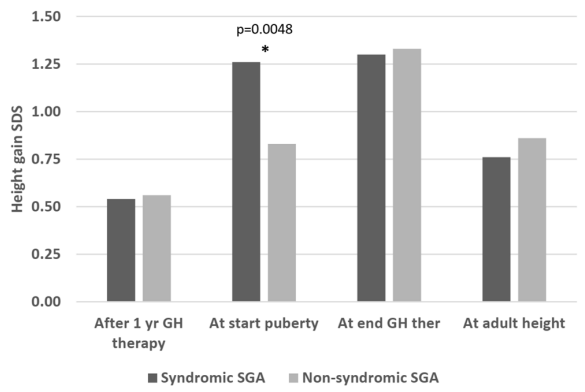

FIGURE 3
Height gain (delta height SDS compared to start of therapy) in syndromic versus non-syndromic SGA patients: Differences in height gain between syndromic and non-syndromic patients before, throughout and after rhGH therapy. * statistically significant.

In our study, the height gain after one year was equivalent in both groups, as was the percentage of non-responders. The percentage of syndromic SGA patients with a delta height gain of more than 0.3 SDS during the first year (81%) was comparable to published results regarding syndromic SGA (16).

However, the growth pattern was different in the two groups. Following a comparable height gain in the first year of therapy, syndromic patients grew better before puberty. Height and age at start of puberty were comparable in syndromic and non-syndromic patients. So, as syndromic patients started rhGH treatment at a younger age than non-syndromic SGA patients, they already had a longer treatment period before reaching puberty, which might have resulted in the greater prepubertal height gain.

The pubertal height gain of syndromic patients was lower, thus they ended up shorter than non-syndromic patients. This could be due to the fact, that SRS patients accounted for 15% of the syndromic patients and that SRS patients present an earlier pubertal onset (18) and an earlier adrenarche than other SGA

TABLE 5 Overview of published SGA cohorts with documented AH after rhGH therapy.

Publications	Number of SGA patients	Mean/ Median adult height [cm]	Mean/ Median adult height [SDS*]	Mean/ Median MPH [SDS]	Mean/ Median height gain [SDS*]	Inclusion/ exclusion of syndromic patients	Exclusion of patients who started rhGH after onset of puberty
Coutant et al. (27)	70		*mean* -2.0	*mean* -0.8	*mean* +1.0	exclusion	no
Van Pareren et al. (4)	54	*mean* ♀160.1 ♂169.3	*mean* -1.1 (33 mcg/ kg/d)	*mean* -0.9	*mean* +1.8	inclusion of SRS, other syndromic patients excluded	yes
Carel et al. (6)	102	*mean* ♀151 ♂162	*mean* -2.1	*mean* -1.2	*mean* +1.1	inclusion	prepubertal or early pubertal stage included
Dahlgren et al. (5)	77	*mean* ♀159 ♂172	*mean* -1.2	*mean* -1.2	*mean* +1.3	exclusion, except 1 SRS patient	yes
Ranke et al. (15)	161	*median* ♀148.5 ♂161.9	*median* -2.2	*median* -0.8	*median* +1.4	inclusion (55 SRS patients)	yes (min. 2 years prepubertal rhGH)
Renes et al. (14)	136	*mean* ♀159 ♂171.6	*median* ♀-1.9 ♂-1.8	*median* -0.6	*median* +1.1	exclusion	Prepubertal or early pubertal stage included
Beisti Ortego et al. (11)	80		*mean* -1.63	*mean* -1.41	*mean* +0.96	exclusion	no
Becker et al., 2023	272	*median* ♀152 ♂165.5	*median* ♀-2.07 ♂-1.95	*median* -1.36	*median* +1.13	Inclusion, analysis in separated groups	no

*SDS based on chronological age.
♀ = female; ♂ = male.

patients (31). SRS boys with an early adrenarche are known to be taller at gonadarche but to end up as short as the boys with normal adrenarche (32). However, in our cohort, age at start of puberty was not younger in syndromic patients compared to the non-syndromic ones. We lack data on adrenarche in our cohort.

Concerning adult height, if applying the chronological SDS for adult height, the AH SDS outcome of our syndromic group corresponds to most published AH SDS of SRS patients treated with rhGH (19, 32).

For the second largest group of patients (FAS patients) in the syndromic group, no data regarding AH after rhGH and timing of puberty in a larger cohort have been published. Sparse data (based on seven patients) are available on rhGH response in the first two years indicating a worse rhGH response than observed in SRS patients (16).

SGA patients were treated with a higher rhGH dose than the EMA- approved dose. This is due to the fact, that some patients have been treated with higher rhGH doses in the setting of clinical trials preceding EMA approval. An analysis of adult height in large SGA cohorts including only patients started on a rhGH therapy after 2003 in order to evaluate the effect of the currently applied dose recommendation has not yet been published.

Our syndromic group comprised 17 patients (40%) with no defined syndrome. In 47% of these patients a genetic analysis has not been contributory. Of note, in the majority of patients no update of the genetic analysis was carried out and most patients only had a karyotype and/or a SNP analysis performed at the time of their rhGH treatment start. As the genetic field is developing fast and more and more genetic disorders are unraveled (33, 34), it is quite possible, that a molecular genetic analysis performed today in these patients would substantially decrease the number of undefined syndromes. In 9 syndromic patients, no genetic analysis has been performed. This might be due to the fact, that our cohort comprises patients who were treated more than 20 years ago, when genetic analysis was not that widely and easily available or as the patients completed the SGA criteria and had access to rhGH therapy, a further diagnostic work-up might have not been regarded as indispensable.

Another weakness of our study is, that although we have established and applied criteria to divide the patients into the syndromic and non-syndromic group, it is not excluded, that there might still be some syndromic patients in the non-syndromic group. Some syndromes have only very subtle clinical signs which might be overlooked. Further, as this study is a retrospective study based on a registry, if symptoms have not been documented in our database, patients might have been falsely classified as non-syndromic.

In conclusion, we report, that syndromic SGA patients have a similar height gain after rhGH therapy, as non-syndromic SGA patients. Hence, syndromic SGA patients should not be excluded from a rhGH therapy, nor do they have to be excluded from a SGA

cohort analysis of rhGH response. Syndromic patients were significantly shorter before rhGH therapy and remained significantly shorter in stature after rhGH therapy. An AH in the normal range was achieved only in ca. 1/3 of all patients, but 73% reached an AH within their target height range.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comissie Medische Ethiek, VUB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

MB, MT and DB designed the study. MB, MT, JDS and DB contributed to the data collection. MT contributed to the data analysis. MB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MB has received a travel grant form Pfizer and participated in advisory boards of Novonordisk and Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

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A novel link between chronic inflammation and humanin regulation in children

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Objective: Children with inflammatory bowel disease (IBD) often suffer from poor bone growth and impaired bone health. Humanin is a cytoprotective factor expressed in bone and other tissues and we hypothesized that humanin levels are suppressed in conditions of chronic inflammation. To address this, humanin levels were analyzed in serum samples from IBD patients and in *ex vivo* cultured human growth plate tissue specimens exposed to IBD serum or TNF alone.

Methods: Humanin levels were measured by ELISA in serum from 40 children with IBD and 40 age-matched healthy controls. Growth plate specimens obtained from children undergoing epiphysiodesis surgery were cultured *ex vivo* for 48 hours while being exposed to IBD serum or TNF alone. The growth plate samples were then processed for immunohistochemistry staining for humanin, PCNA, SOX9 and TRAF2 expression. Dose-response effect of TNF was studied in the human chondrocytic cell line HCS-2/8. *Ex vivo* cultured fetal rat metatarsal bones were used to investigate the therapeutic effect of humanin.

Results: Serum humanin levels were significantly decreased in children with IBD compared to healthy controls. When human growth plate specimens were cultured with IBD serum, humanin expression was significantly suppressed in the growth plate cartilage. When cultured with TNF alone, the expression of humanin, PCNA, SOX9, and TRAF2 were all significantly decreased in the growth plate cartilage. Interestingly, treatment with the humanin analog HNG prevented TNF-induced bone growth impairment in cultured metatarsal bones.

Conclusion: Our data showing suppressed serum humanin levels in IBD children with poor bone health provides the first evidence for a potential link between chronic inflammation and humanin regulation. Such a link is further supported by the novel finding that serum from IBD patients suppressed humanin expression in *ex vivo* cultured human growth plates.

KEYWORDS

IBD, TNF, growth plate, humanin, chondrocyte

Introduction

Children with chronic inflammatory diseases, such as inflammatory bowel disease (IBD), often experience impaired bone health including bone growth retardation (1, 2). Although many of these children are effectively treated with glucocorticoids and other anti-inflammatory therapies, bone growth retardation often remains or even worsen due to undesired side-effects of the treatment. A better understandings of how chronic inflammatory disorders affect bone health may facilitate the development of new bone-protective treatment strategies.

Longitudinal bone growth occurs at the growth plate, a hyaline cartilage layer situated in the metaphysis of long bones. In the growth plate, chondrocytes undergo proliferation and hypertrophy while producing cartilage matrix. During these steps, new cartilage tissue is formed which is subsequently remodeled into trabecular bone (3). This process is tightly controlled by a variety of growth factors, cytokines and ubiquitin/proteasome system (4), and any disturbance of this will result in growth retardation. Inflammation is characterized by active immune cells and elevated production of cytokines including tumor necrosis factor (TNF) which has been associated with disease activity in IBD. Furthermore, TNF has been linked to growth retardation and anti-TNF treatment has been found to have the capacity to rescue bone growth in patients with IBD (5, 6). Experimental data have suggested that circulating TNF may impair the growth hormone–insulin-like growth factor-1 axis (7, 8). Locally at the growth plate level, TNF and IL-1beta have been shown to act in synergy suppressing chondrocyte proliferation and bone growth in *ex vivo* cultured metatarsal bones (9). TNF is also known to trigger the production of other pro-inflammatory cytokines, such as IL6 and IL-1beta (10), suggesting that TNF is a master player in the inflammatory process. Whether TNF alone may impair chondrogenesis in the human growth plate has so far not been studied.

Humanin, a 24-amino acid peptide first identified in surviving neurons from an Alzheimer disease patient, has been found to be a potent neurosurvival and anti-apoptotic factor (11). Humanin is also expressed in growth plate cartilage where it was shown to protect chondrocytes from undergoing undesired apoptosis (12). Interestingly, humanin itself was also found to exert anti-inflammatory effects (12). Recently, it was reported that humanin can improve metabolic health and increase lifespan in mice (13).

To identify any association between chronic inflammation and systemic humanin regulation, we measured humanin levels in serum samples obtained from IBD patients with known poor bone health. By applying a unique organ culture system of human growth plate tissues from children, we also investigated the local effects of IBD serum and TNF alone on local humanin expression and chondrogenesis within the growth plate.

Materials and methods

Serum collection from IBD patients and controls

We used previously collected serum samples from IBD children (boys and girls, median age 14.9 years) with known history of low bone mineral density and decreased height Z score, and gender-matched healthy children which served as controls (2).

Serum humanin ELISA

Circulating humanin levels were measured in the serum of IBD patients and healthy controls by an ELISA kit (CSB-EL015084HU; CUSABIO, Houston, USA) according to the manufacturer's instruction. We used the serum samples obtained from 40 IBD patients and 40 age matched healthy controls to measure humanin levels. Briefly, standards, or 10-fold diluted serum samples were added to the appropriate wells and incubated for 2 hours at 37°C. After removing the liquid of each well, 100 µl of Biotin-antibody was added and then incubated for 1 hour at 37°C. After washing, 100 µl of HRP-avidin was added to each well and incubated for 1 hour at 37°C followed by five times washing. Then, 90 µl of TMB Substrate was added to each well and incubated 25 minutes at 37°C followed by adding 50 µl Stop Solution. The absorbance was read at 450 nm on a microplate reader.

Collection and culture of human growth plate tissues

Human growth plate tissues were collected during epiphysiodesis surgeries performed at Karolinska University Hospital. After informed consent, human growth plate samples were obtained from 2 children (1 boy, 1 girl) diagnosed with constitutional tall stature. Growth plate biopsies from the distal femur or proximal tibia were harvested by using a biopsy needle (8 gauge; Gallini Medical Products and Services, Modena, Italy) as earlier described by us (14). The biopsies were collected in DMEM, high glucose (21063-029; Thermo Fisher Scientific, Waltham, Massachusetts, USA) with 10 µg/ml gentamicin (11530506; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and then kept on ice while being transported from the operating room to the laboratory for culture. Under aseptic conditions, the human growth plate biopsies were cut into 1-2 mm slices and each slice was then individually cultured in a 24-well plate with 0.5 ml culture media per well. The culture media was DMEM, high glucose (21063-029; Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with of 0.2% bovine serum albumin, 1 mM β-Glycerophosphate and 0.05 mg/ml ascorbic acid as described earlier (14). The human growth plate biopsies were subsequently treated and cultured in media with 10% serum, or 30 ng/ml TNF (510-RT-010; Bio-Techne, Minneapolis, Minnesota, USA) combined or not combined with 1 µM HNG for 48 hours, in a 5% CO_2 incubator. Thereafter, the growth plates were fixed for 24 hours in 4% paraformaldehyde (HL96753.1000; Histolab, Askim, Sweden) followed by decalcification in EDTA buffer for 24 hours before dehydration and paraffin embedding.

Immunohistochemistry and quantification

To analyze protein expression in the growth plate, immunohistochemistry was performed in serial sections of human

growth plates as earlier described (12). Briefly, the sections were first deparaffinized and rehydrated. Antigen retrieval was then performed in sodium citrate buffer (10 mM pH 6.0) for 10 min at 75°C followed by washing with distilled water. Thereafter, sections were quenched with 3% hydrogen peroxide (1072090250; Burlington, Massachusetts, USA) in methanol (1060092511; Burlington, Massachusetts, USA) for 10 minutes and blocked with 2% goat serum for 1 hour. Next, slides were incubated with anti-humanin antibody (NB100-56877; Novus Biologicals, Littleton, Colorado, USA), anti-proliferating cell nuclear antigen (PCNA) antibody (ab-18197; Abcam, Cambridge, United Kingdom), anti-SOX9 antibody (ab-5355; Sigma-Aldrich, Burlington, MA, USA), and anti-TRAF2 antibody (NB100-56173SS; Novus Biologicals, Littleton, Colorado, USA) overnight at 4°C, 1:300 diluted for all antibodies. After primary antibody incubation, sections were washed with PBS with 0.05% Tween 20 (P1379; Sigma-Aldrich, Burlington, MA, USA) for 15 minutes followed by incubation with secondary antibody (1:300; BA-9500 Vector Laboratories; 1:500, ab 97049 Abcam) for 1 hour at room temperature. Sections were then incubated with an avidin-peroxidase complex (Vectastain ABC-kit PK-6100) and visualized with 3,3' diaminobenzidine (DAB) (Dako K3468) development for 1-3 minutes. Finally, the slides were counterstained with Alcian Blue for 5 minutes and dehydrated. Image J software (NIH) was used to quantify the percentages of positive stained cells per mm^2 in the growth plates. Each growth plate slice was regarded as one observation.

Bone growth analysis in cultured bones

The metatarsal bones were dissected from the hind paws of 17-18 days old fetal Sprague-Dawley rats (E17/18) as previously described (9). Only the middle three metatarsal bones were collected. Thereafter, the bones were cultured in 24-well plates with 0.5 ml/well of culture media. The culture media used was MEM (31095029; Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 50 µg/ml ascorbic acid (A5960-100G; Sigma-Aldrich, Burlington, MA, USA), 1 mM sodium glycerophosphate (G9422-10G; Sigma-Aldrich, Burlington, MA, USA), 0.2% bovine serum albumin (A8806-5G; Sigma-Aldrich, Burlington, MA, USA), and 20 µg/ml gentamicin. The metatarsals were treated with 100 ng/ml TNF (510-RT-010; Bio-Techne, Minneapolis, Minnesota, USA), 1 µM humanin analog HNG, or both for 12 days at 37°C with 5% CO_2. Images of the bones were captured on days 0, 2, 5, 7, 9, and 12 of culture using a Hamamatsu C4742–95 digital camera mounted on a Nikon SMZ-U microscope. The bone length was measured with the Infinity Analyze software (Lumenera Corporation).

Cell culture

The human chondrocytic cell line, HCS-2/8 was cultured as described previously (15). Briefly, the cells were maintained in DMEM/F-12 (11320033; Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 20% fetal bovine serum (FBS) and 20 µg/ml gentamycin at 37°C with 5% CO_2. The cells were sub-cultured every week and the culture medium was changed every 2-3 days. For quantitative realtime PCR and Western blot analysis, the cells were seeded in 6-well cell culture plates in DMEM/F12 medium containing 20% FBS. When cells were approximately 80% confluent, they were washed with 1× PBS and the medium was changed to test medium (DEME/12 without FBS) with TNF (510-RT-010; Bio-Techne, Minneapolis, Minnesota, USA) for 72 hours.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from harvested HCS-2/8 cells using TRIzol reagent (15596026; Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instruction. iScript™ cDNA Synthesis Kit (1708890; Bio-Rad, Hercules, California, USA) was used for the reverse transcription of total RNA into cDNA. SsoAdvanced Universal SYBR Green Supermix (1725271; Bio-Rad, Hercules, California, USA) was used for quantitative real-time PCR (qPCR) reactions. The primers of PCNA (Assay ID: qHsaCID0012792), humanin (Assay ID: qHsaCED0019576), SOX9 (Assay ID: qHsaCED0021217), and Beta-actin (Assay ID: qHsaCED0036269) were purchased from Bio-Rad. qPCR reactions were performed with Bio-Rad 96 CFX RT-PCR System. Beta-actin was used as an internal reference gene to normalize the target genes. Relative levels of target genes were calculated using the ΔΔCT method.

Western blot analysis

Western blot analysis was performed as previously described (16). The antibody against SOX9 (1:500; ab-5355) was purchased from Sigma-Aldrich, Burlington, MA, USA). The antibody against PCNA (1:1000; ab-18197) was purchased from Abcam (Cambridge, United Kingdom). Anti-GAPDH antibody (1:2000; 10494-1-AP) and Goat anti-Rabbit IgG (H+L) Secondary Antibody (1:3000; 65-6120) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Image J software (NIH) was used to quantify the images obtained from Western blots.

Immunofluorescence

HCS-2/8 cells were seeded on Falcon 8-well Culture Slide (354108; Corning, USA), treated with TNF (510-RT-010; Bio-Techne, Minneapolis, Minnesota, USA) at 100 ng/ml concentration for 72 hours. Thereafter, the cells were washed with PBS and fixed in pure methanol (1060092511; Burlington, Massachusetts, USA) for 10 minutes at −20°C. After washing with PBS, fixed cells were blocked in 5% bovine serum albumin (A8806-5G; Sigma-Aldrich, Burlington, MA, USA) for 1 h at room temperature and incubated overnight at 4° C with anti-humanin antibody (1:500; NB100-56877; Novus Biologicals, Littleton, Colorado, USA). After primary antibody incubation, cells were washed with PBS with 0.05% Tween 20

(P1379; Sigma-Aldrich, Burlington, MA, USA) for 15 minutes followed by incubation with Cy3 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L) secondary antibody (1:500; 711-166-152; Jackson ImmunoResearch, United Kingdom) for 1 hour at room temperature. Nuclei were stained with Hoechst 33342 (1:500; H3570; Thermo Fisher Scientific, Waltham, Massachusetts, USA) together with secondary antibody incubation. The coverslips were mounted in Prolong Gold antifade reagent (P36930; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Images were captured on an LSM 700 confocal microscope (ZEISS, Jena, Germany). Quantification of the fluorescence intensity in each image was performed using ZEN Microscopy Software (ZEISS, Jena, Germany).

Statistical analysis

All statistical analyzes and receiver operating characteristics (ROC) analysis were performed in GraphPad Prism. 2-tailed unpaired t test and Wilcoxon rank-sum test were used to evaluate statistical significance between 2 groups. 2-way ANOVA was used for the analysis in the fetal metatarsal experiment. All data are shown as mean ± SE, and a p-value less than 0.05 was considered significant.

Results

Decreased humanin levels in serum from IBD patients

We first measured humanin levels in serum samples obtained from 40 patients with IBD (median age 14.9 years) and 40 age-matched healthy controls. Humanin levels were found to be significantly decreased in IBD patients (p=0.0053 vs controls) (Figures 1A, B) suggesting a link between inflammation and systemic humanin regulation. To investigate the sensitivity and specificity of circulating humanin in the serum samples, a receiver operating characteristics (ROC) curve was constructed (Figure 1C). The ROC curve analysis revealed that the area under the curve (AUC) value for circulating humanin is 0.68 (95% confidence interval: 0.56 to 0.80).

Decreased humanin expression in human growth plate cartilage exposed to IBD serum

To test if serum obtained from children with IBD may locally affect humanin expression in the growth plate, tissue specimens obtained from a human growth plate were cultured with serum from IBD patients and healthy controls for 48 hours (Figure 2A). Immunohistochemistry analysis showed that humanin levels were significantly decreased in growth plate specimens exposed to serum obtained from IBD patients when compared to healthy controls (p=0.0483) (Figures 2B, C and Supplementary Figure 1A).

TNF suppressed humanin expression in cultured human growth plate cartilage and chondrocytes

To test if the important pro-inflammatory cytokine TNF may have a direct suppressive effect on humanin expression in growth plate cartilage, growth plate tissue specimens obtained from 2 different children were cultured with TNF (30 ng/ml) or control medium for 48 hours. Our data showed that TNF significantly suppressed humanin expression within the human growth plate cartilage (p=0.0226) (Figures 3A, B and Supplementary Figure 1B). A similar, although not significant, trend was found when data from the 2 patients were analyzed separately (Figures 4A, E).

To investigate the dose-response effect of TNF on humanin expression in human chondrocytes, we treated the HCS-2/8 chondrocytic cell line with TNF at 10, 30, 100, and 300 ng/ml for 72 hours. The qPCR results showed that humanin was significantly suppressed by TNF at 10, 100, and 300 ng/ml vs control (p=0.0075, 0.0066 and 0.0104, respectively) (Figure 3C). To further validate this data, we performed immunofluorescence and found that humanin was significantly suppressed in the HCS-2/8 chondrocytic cell line treated with TNF (p=0.0149) (Figures 3D, E).

TNF suppressed SOX9 and PCNA in cultured human growth plate cartilage and chondrocytes

To investigate if TNF alone may suppress chondrogenesis in the human growth plate, tissue specimens were further analyzed for expressions of SOX9 and PCNA. When treated with TNF alone, SOX9 expression was decreased by 73.4% vs control (p=0.0325) (Figures 5A, B and Supplementary Figure 2A). Similarly, PCNA, a marker of cell proliferation, was suppressed by TNF (p=0.0497) (Figures 5D, E, Supplementary Figure 2B). When analyzing data from patient 1 and patient 2 individually, a similar trend was noticed in both patients (Figures 4B, F, C, G).

We next performed dose-response studies in the HCS-2/8 cell line with qPCR and observed that TNF significantly decreased SOX9 gene expression at 10, 30, 100, and 300 ng/ml vs control (p=0.0005, 0.0003, 0.0048 and 0.0032, respectively) (Figure 5C). Similarly, PCNA expression was suppressed by TNF at 100 and 300 ng/ml vs control (p=0.002 and 0.0018, respectively) (Figure 5F). Western blot analysis also showed that SOX9 and PCNA were significantly decreased in the HCS-2/8 chondrocytic cell line treated with TNF (p=0.0089 and 0.0126, respectively) (Figures 5G–I).

TNF suppressed TRAF2 in cultured human growth plate cartilage

Since TRAF2 plays an important role in TNF-induced inflammatory signaling, TRAF2 expression was measured in human growth plate tissue specimens treated with TNF. Our data showed that TNF suppressed TRAF2 expression by 70.1% vs

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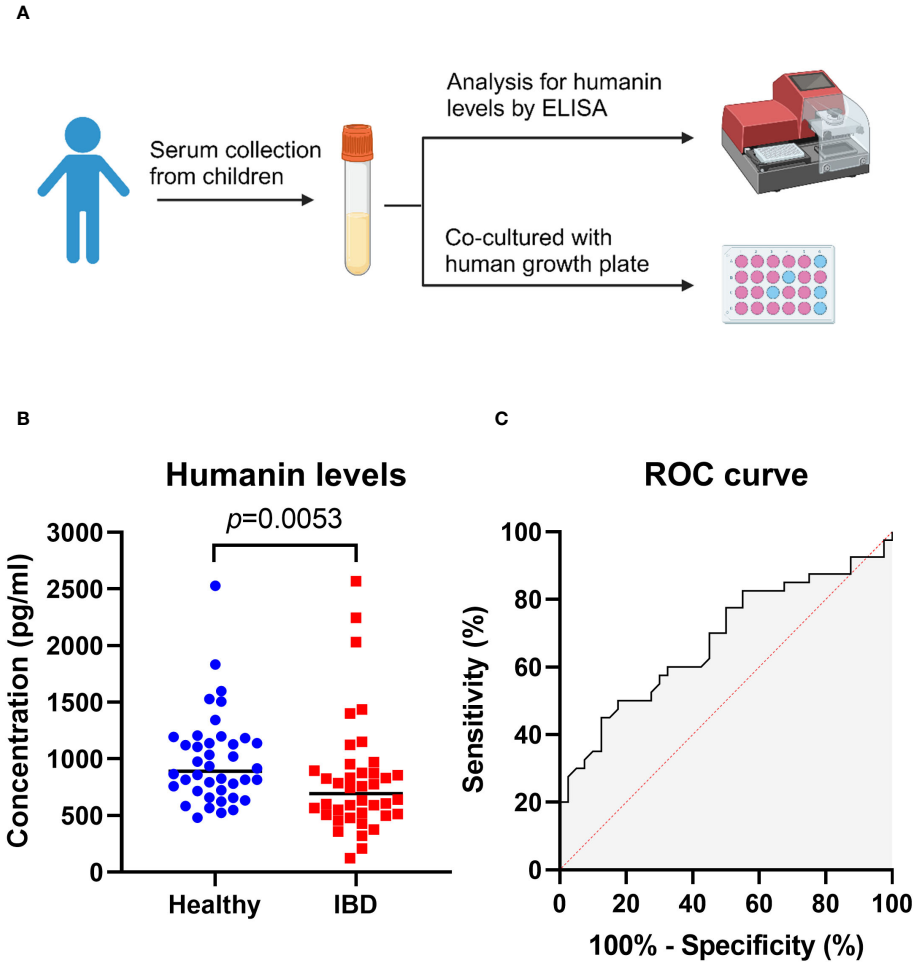

FIGURE 1
Humanin levels were decreased in serum of IBD patients. **(A)** Graphical illustration (created with BioRender.com) of the experimental overview, showing serum samples were obtained from children for ELISA analysis and human growth plate co-culture. **(B)** Humanin levels analyzed by using ELISA in IBD patients (n=40) and healthy controls (n=40). Wilcoxon rank-sum test was used to analyze differences between groups. **(C)** Receiver operating characteristic (ROC) curve for circulating humanin in IBD patients (n=40) and healthy controls (n=40).

control (p=0.0198) (Figures 5J, K and Supplementary Figure 2C). When analyzing the two patients separately, a similar trend was noticed in both patients (Figures 4D, H).

Treatment with humanin analog prevents TNF-induced growth impairment in cultured bones

To investigate whether humanin could be a therapeutic target to prevent bone growth impairment caused by chronic inflammation, cultured rat metatarsal bones were treated with TNF or the humanin analog HNG, or in combination for 12 days. We observed that TNF alone impaired bone growth (p<0.0001 vs control), whereas co-treatment with HNG rescued bone growth (p= 0.0436 vs TNF) (Figures 6A, B), confirming the therapeutic effect of humanin.

Discussion

We here report a novel direct link between chronic inflammation and humanin regulation in children. Such link is supported by our findings of decreased serum humanin levels in IBD patients and suppressed humanin expression in *ex vivo* cultured human growth plate tissues exposed to IBD serum or TNF alone.

To our knowledge, there is no previous evidence supporting any link between chronic inflammation and humanin regulation. To address this gap of knowledge, we measured humanin levels in serum samples from 40 IBD patients and 40 age and gender matched healthy controls. Our data showed that humanin levels were significantly decreased in children with IBD. Humanin is a mitochondrial-derived polypeptide, first identified in survived neuronal cells from Alzheimer disease patients (11). Under physiological conditions, humanin is produced by various tissues

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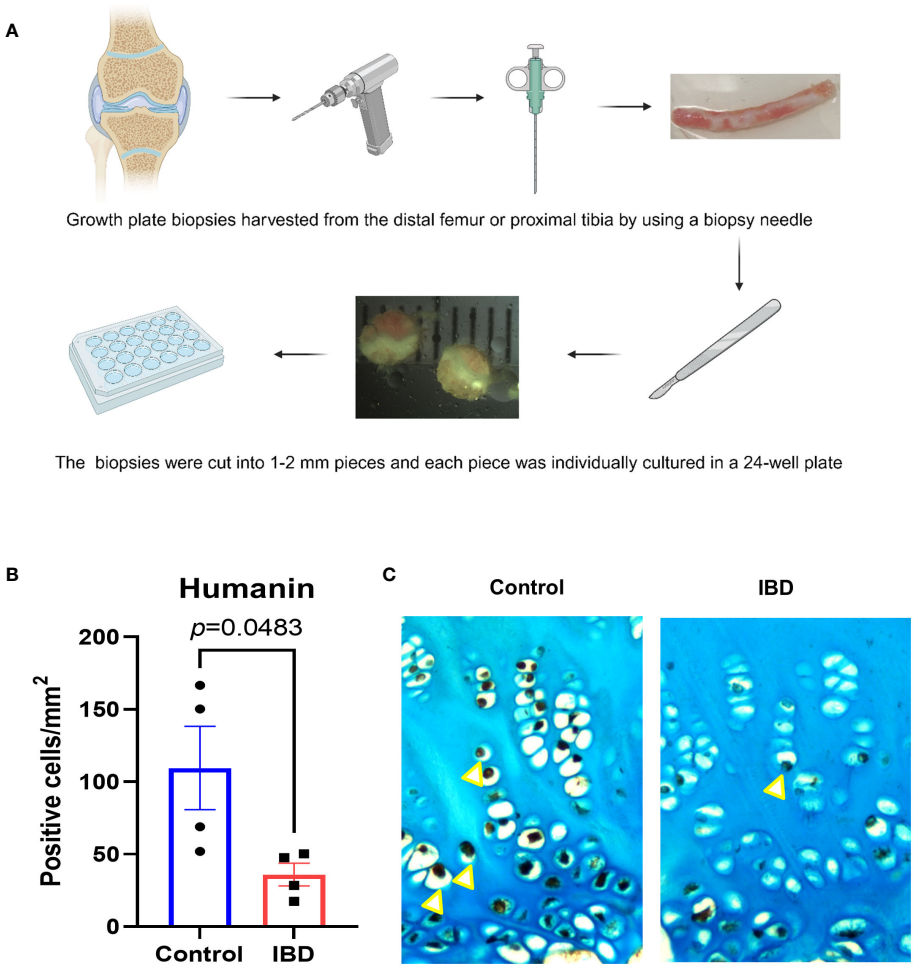

FIGURE 2
Humanin levels were decreased in human growth plate exposed to IBD serum. **(A)** Graphical illustration (created with BioRender.com) of the methods using to collect and culture human growth plate biopsies obtained from children undergoing epiphysiodesis surgeries. **(B, C)** Quantitative analysis of humanin staining (yellow arrows), quantified as number of positive cells per mm^2 (n=4 pieces of biopsies, from 1 patient). Error bars indicate mean ± SE. Students t-test was used to analyze differences between groups.

such as skeletal muscle, brain, and liver (17–19). After production, it is circulated in the blood and plays a protective role in targeted cells (11). In the cytoplasm, humanin is known to protect cells from apoptosis by interacting with pro-apoptotic proteins (20). Similarly, humanin can also interact with extracellular receptors such as G protein-coupled formylpeptide receptor-like-1 to exert its cell-protective effects (21). The kinetic half-life of humanin is 30 minutes in the plasma of mice and greater than 4 hours in rats (22). Previous studies have reported that in multiple species including human, circulating humanin levels gradually decrease with age (13). In addition, systemic humanin levels are suppressed in patients with Alzheimer's disease and coronary endothelial dysfunction (13, 23). The mechanism of how humanin is decreased by chronic inflammation is still unclear. However, it has been reported that when mitochondrial DNA copy number decreased, humanin levels were also decreased (13). Interestingly, inflammation is known to decrease the mitochondrial DNA copy number (24). These observations may explain a possible link between inflammation and humanin regulation.

We have previously reported that humanin is expressed in the growth plate and that humanin has the capacity to prevent glucocorticoid-induced bone growth impairment (12). Furthermore, glucocorticoids have been reported to suppress humanin expression in the growth plate (12). However, so far it has been unknown if chronic inflammation *per se* also may suppress endogenous humanin produced within the growth plate. Considering the protective roles of humanin in different diseases reported earlier (17, 25, 26), we hypothesized that humanin might be a novel biomarker of poor bone health in patients with chronic inflammation. To investigate this, growth plate cartilage was collected from children with constitutional tall stature undergoing epiphyseal surgery performed to limit their further bone growth. A unique human growth plate culture system (14) was then applied which allowed tissue specimens to be exposed to serum obtained from IBD patients or healthy controls. Interestingly, we found suppressed humanin expression in growth plates exposed to IBD serum suggesting a direct link between chronic inflammation and local humanin regulation within the growth plates.

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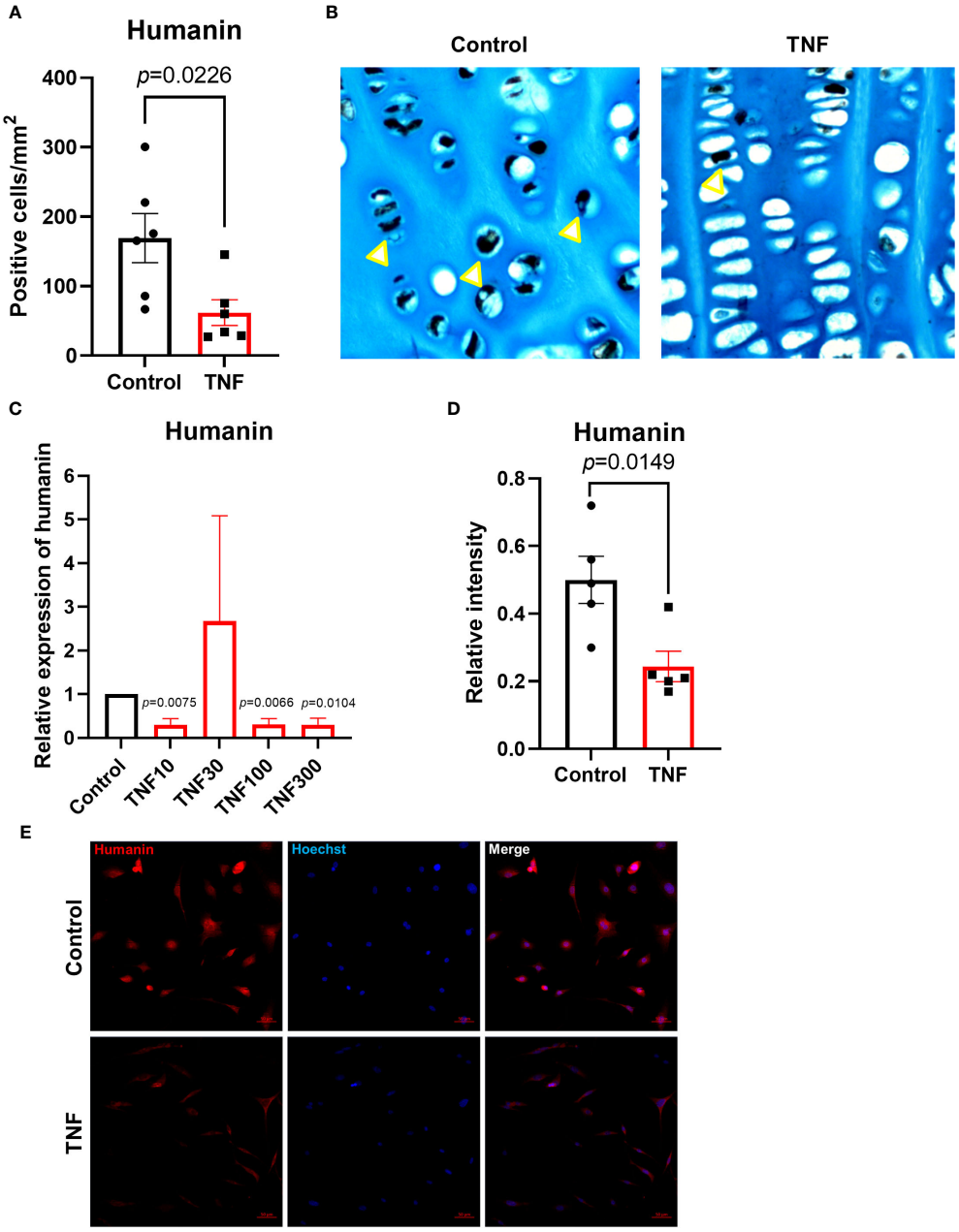

FIGURE 3
TNF suppressed humanin expression in human growth plate tissue specimens (n=6) obtained from 2 children or human chondrocytes. **(A, B)** Quantitative analysis of humanin staining (yellow arrows), calculated as number of positive cells per mm^2. **(C)** Relative expression of humanin assessed by qPCR in HCS-2/8 cell line treated for 72 hours with TNF at 10, 30,100, 300 ng/ml concentrations (n=3-4). **(D)** Quantification of humanin expression by immunofluorescence in HCS-2/8 cells treated with TNF (100 ng/ml). **(E)** Representative images of humanin staining (red) in HCS-2/8 cells treated with TNF and untreated controls. Error bars indicate mean ± SE. Students t-test was used to analyze differences between groups.

In an attempt to clarify a possible underlying mechanism behind inflammation-induced humanin suppression, we focused on TNF, a key player in chronic inflammation triggering the release of other pro-inflammatory cytokines such as IL-6 and IL-1beta (10). Although TNF overexpression was recently reported by us to suppress chondrocyte proliferation and chondrogenesis within the mouse growth plate, any such effects have not yet been reported in humans (27). In this study, we observed a strong inhibition of humanin expression in human growth plates exposed to TNF for 48 hours. Further studies showed that the proliferation marker PCNA and the important controller of chondrocyte proliferation, SOX9, were both markedly decreased in growth plates exposed to TNF. Heterozygous mutations of SOX9 have been described in patients with campomelic dysplasia, a skeletal dysplasia characterized by bowed long bones and defects in cartilage formation (28). Furthermore, several animal studies have demonstrated that intact SOX9 is essential for proliferative chondrocyte columns and keeping the growth plates open (29, 30). Consequently, our data showing suppressed SOX9 in growth plates exposed to TNF may explain why this pro-inflammatory cytokine exerts growth

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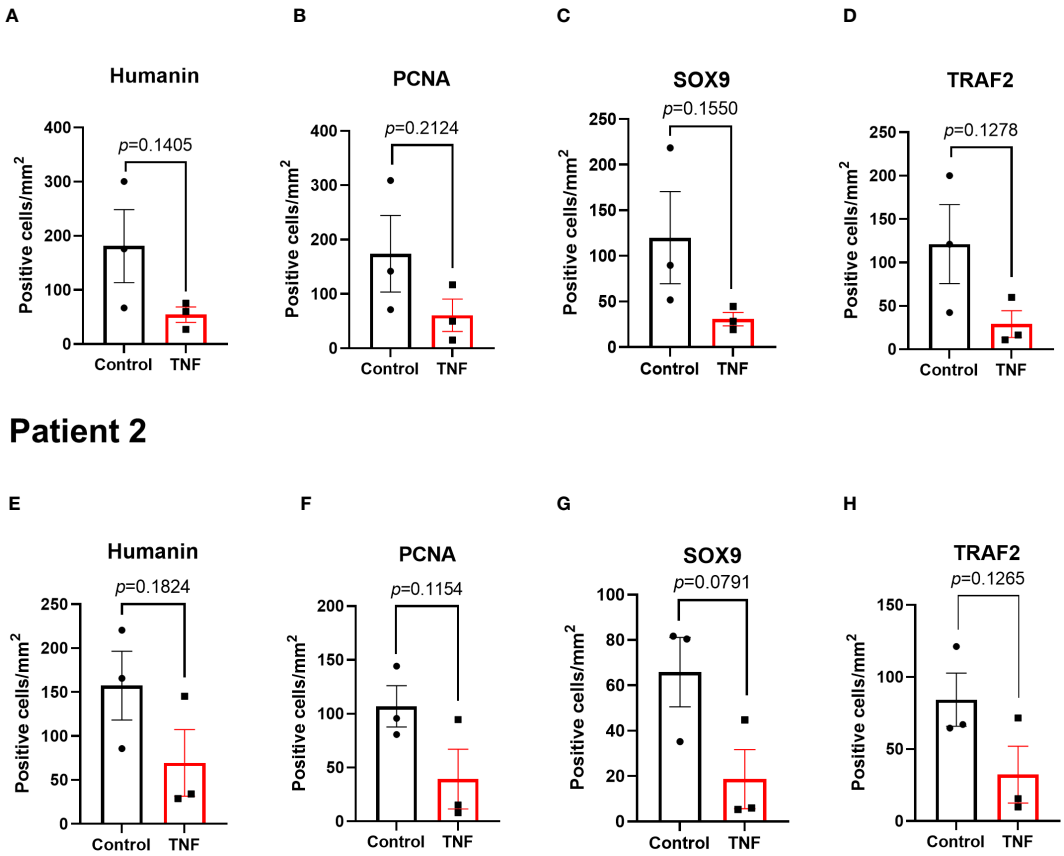

FIGURE 4
Local effects of TNF on humanin, PCNA, SOX9 and TRAF2 expressions in growth plates tissue specimens (n=3) in patient 1 and patient 2 analyzed separately. Quantitative analysis of **(A, E)** humanin staining, **(B, F)** PCNA staining, **(C, G)** SOX9 staining, and **(D, H)** TRAF2 staining. Error bars indicate mean ± SE. Students t-test was used to analyze differences between groups.

suppressive effects. Interestingly, we also found that TNF Receptor Associated Factor 2 (TRAF2) was suppressed in human growth plates exposed to TNF. These novel findings suggest a direct link between chronic inflammation and humanin regulation, both systemically and locally within human growth plates.

To validate the data collected from human growth plates, we performed dose-response studies using the human chondrocytic cell line HCS-2/8. The rationale to choose this cell line was that HCS-2/8 cells resemble primary human chondrocytes and have been widely used to study chondrocyte proliferation and differentiation since established (31). As expected, the expressions of humanin, SOX9, and PCNA in HCS-2/8 cells were suppressed when exposed to TNF. These findings are in line with the data collected in cultured human growth plates.

Since humanin was suppressed under chronic inflammation, we next investigated whether exogenous treatment with humanin can rescue from inflammation-induced bone growth impairment. In an *ex vivo* model of cultured bones where direct effects on bone growth can be monitored (32), we found that humanin can effectively protect from TNF-induced bone growth impairment. This observation further implies a potential link between humanin and poor bone growth under chronic inflammation. The clinical significance of our finding is underscored by the fact that existing treatments are associated with various side effects. Biological drugs such as etanercept (TNF-inhibitor) and anakinra (IL-1 receptor antagonist) has been reported to cause infections and injection-site reactions (33). Similarly, the long-term safety of recombinant human growth hormone is associated with increased mortality in certain patient groups and the cost of this therapy is also very high (34). Therefore, new treatment strategies are highly desired to prevent bone growth impairment caused by chronic inflammation. Moreover, glucocorticoids (GCs) are also widely used in the management of IBD and several attempts have been made to reduce the toxicity of GCs (35–37). Interestingly, it has been reported that the combination of the humanin analog HNG with a GC does not interfere with the desired anti-inflammatory effects of the GC (12, 38). Thus, it would be of great clinical interest to further expand the scope of the present study regarding the therapeutic effects of humanin.

There are several limitations of this study. Firstly, growth plate cartilage was obtained from 2 children due to the rarity of these samples. However, as these biopsies could been sectioned into

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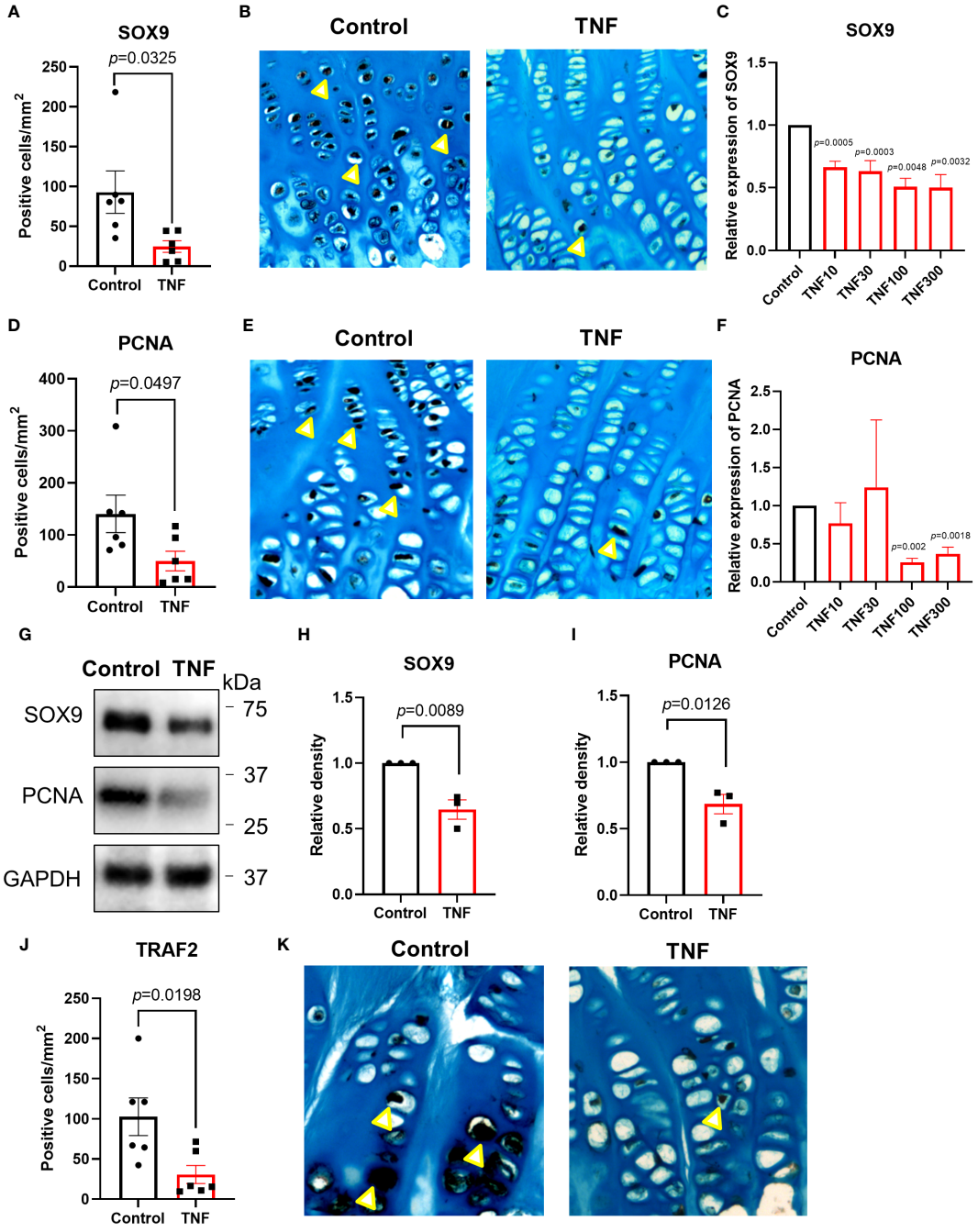

FIGURE 5
TNF suppressed SOX9, PCNA and TRAF2 expressions in human growth plate tissue specimens (n=6) obtained from 2 children or human chondrocytes. **(A, B)** Quantitative analysis of SOX9 staining (yellow arrows), calculated as number of positive cells per mm^2. **(C)** Relative expression of SOX9 assessed by qPCR in HCS-2/8 cells treated for 72 hours with TNF at 10, 30, 100, 300 ng/ml concentrations (n=3). **(D, E)** Quantitative analysis of PCNA staining (yellow arrows), calculated as number of positive cells per mm^2. **(F)** Relative expression of PCNA assessed by qPCR in HCS-2/8 cells treated for 72 hours with TNF at 10, 30, 100, 300 ng/ml concentrations (n=3). **(G)** Western blot analysis of SOX9 and PCNA expressions in HCS-2/8 cells treated with TNF (100 ng/ml). **(H, I)** Quantification of SOX9 and PCNA expressions by three independent Western blot experiments. **(J, K)** Quantitative analysis of TRAF2 staining (yellow arrows), calculated as number of positive cells per mm^2. Error bars indicate mean ± SE. Students t-test was used to analyze differences between groups.

several slices being cultured and treated individually the number of replicates could be increased. A second limitation was that we could not perform dose-response studies, again due to the scarcity of human growth plate tissues. Thirdly, the serum samples from IBD patients in our study were obtained only at one time point, therefore, more detailed studies are required to reveal whether circulating humanin could potentially act as a biomarker of poor bone health in children with IBD. The last limitation was that we did not explore the role of other pro-inflammatory cytokines than TNF and it is therefore possible that other pathways may also be

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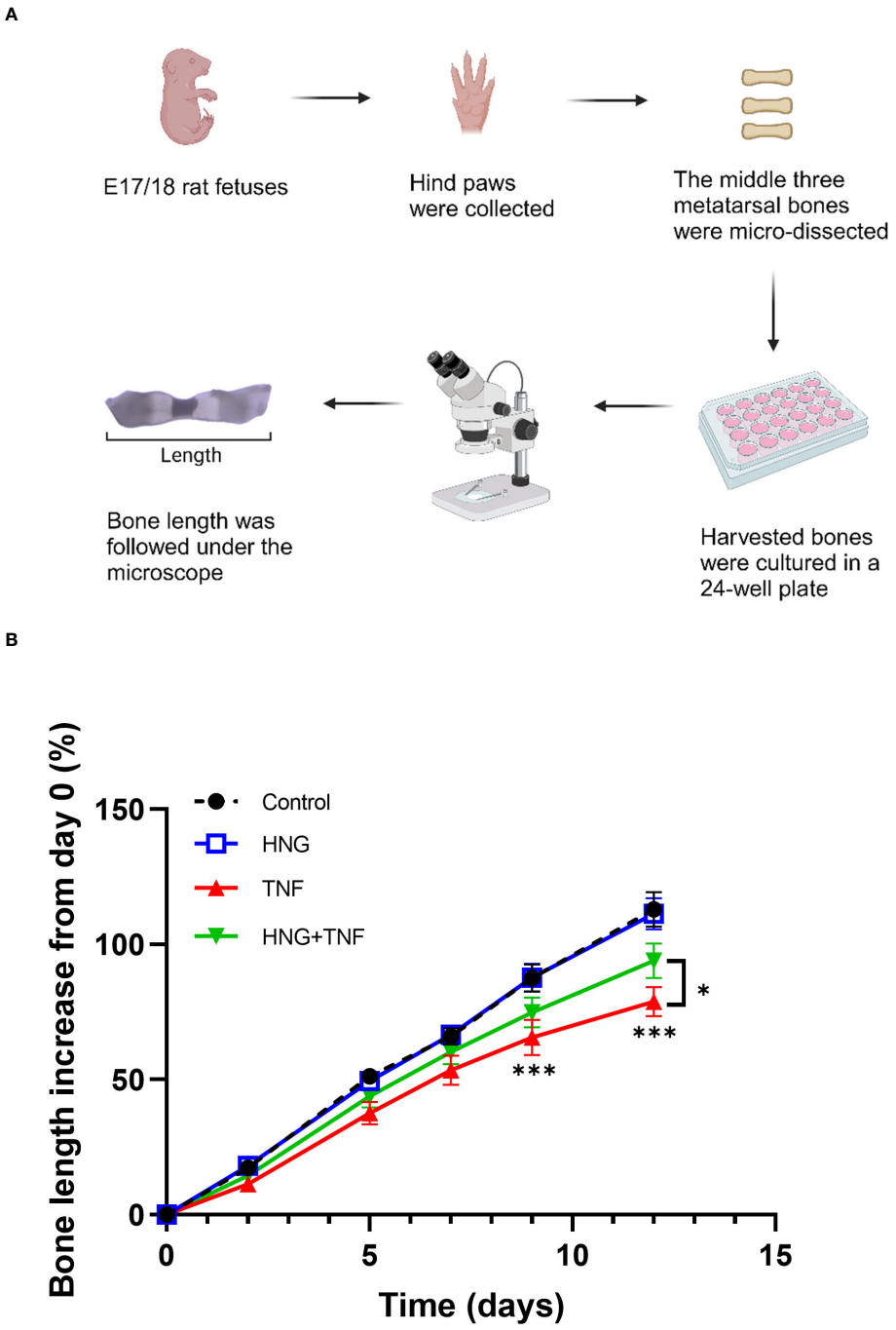

FIGURE 6
Treatment with humanin analog HNG prevents TNF-induced growth impairment in cultured bones. **(A)** Microscopic image of *ex vivo* cultured fetal rat metatarsal bones. **(B)** *Ex vivo* cultured fetal rat metatarsal bones treated with HNG (1 μM), TNF (100 ng/ml) or in combination for 12 days (n=12). Bone length was measured on day 0, 2, 5, 7, 9 and 12. Error bars indicate mean ± SE. 2-way ANOVA was used to analyze differences between groups. *$p < 0.05$ (TNF versus HNG+TNF), ***$p < 0.001$ (Control versus TNF).

involved in mediating the local effects of chronic inflammation on humanin regulation.

Conclusion

We report that systemic humanin levels are decreased in IBD children with poor bone health. Mechanistic studies in *ex vivo* cultured human growth plate cartilage and human chondrocytes showed that serum from IBD patients or TNF alone suppressed endogenous humanin expression. Furthermore, treatment with the humanin analog HNG prevented growth retardation caused by TNF in cultured bones. Altogether, our study provides evidence of a link between chronic inflammation, bone health and humanin regulation, which is a novel finding of potential clinical significance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Helsingin ja Uudenmaan sairaanhoitopiiri, Finland, and Karolinska Institutet Research Ethics Committee North at the Karolinska University Hospital, Stockholm, Sweden. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal study was approved by the local ethical committee (Stockholm North Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ participated in study design, experimental work, statistics, data analysis, manuscript writing. OM provided samples, reviewed manuscript. SL provided samples, reviewed manuscript. VF participated in experimental work, data analysis and manuscript writing. LS participated in study design, statistics, data analysis, manuscript writing, study concept, manuscript revision. FZ participated in study design, experimental work, statistics, data analysis, manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1142310/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Humanin levels were decreased in human growth plate tissue specimens exposed to IBD serum and TNF. **(A)** Representative images of immunohistochemistry for humanin (dark brown staining) in human growth plate tissue specimens exposed to serum from IBD patients and healthy controls. **(B)** Representative images of immunohistochemistry for humanin (dark brown staining) in human growth plate tissue specimens treated with TNF and untreated control. 10x magnification.

SUPPLEMENTARY FIGURE 2
TNF suppressed SOX9, PCNA and TRAF2 expressions in human growth plate tissue specimens (n=6) obtained from 2 children or human chondrocytes. **(A–C)** Representative images of immunohistochemistry for SOX9, PCNA and TRAF2 (dark brown staining) in human growth plate tissue specimens treated with TNF and untreated controls. 10x magnification.

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Clinical and laboratory characteristics but not response to treatment can distinguish children with definite growth hormone deficiency from short stature unresponsive to stimulation tests

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Introduction: It has been proposed that not all children with short stature displaying an inadequate response to tests for growth hormone (GH) secretion truly suffer from GH deficiency (GHD). Only children with a monogenic cause of GHD or an identifiable combined hormonal deficiency or anatomical anomaly in the hypothalamic-pituitary axis should be considered definite GHD (dGHD). The remaining patients can be defined as a separate group of patients, "short stature unresponsive to stimulation tests" (SUS). The aim of this proof-of-concept study, was to assess whether SUS patients treated with rhGH exhibit any differences compared to GHD patients undergoing the same treatment.

Methods: Retrospective analysis on 153 consecutive patients with short stature and pathological response to two GH stimulation tests. Patients with dGHD were defined as those with a clear genetic or anatomical hypothalamic-pituitary anomaly, as well as those with combined pituitary hormone deficiencies and those with a known insult to the hypothalamic-pituitary axis (i.e. total brain irradiation) (n=38, 25%); those without any of the previous anomalies were defined as SUS (n=115, 75%).

Results: At diagnosis, dGHD and SUS populations did not differ significantly in sex (F 32% vs 28%, p=0.68), age (11.9 vs 12.1, p=0.45), height SDS at diagnosis (-2.2 vs. -2.0, p=0.35) and prevalence of short stature (height <-2 SDS) (56% vs 51%, p=0.45). IGF-1 SDS were significantly lower in dGHD (-2.0 vs -1.3, p<0.01). After 1 year of treatment, the prevalence of short stature was significantly reduced in both groups (31% in dGHD vs. 21% in SUS, p<0.01) without any significant differences between groups (p=0.19), while the increase in IGF-1 SDS for bone age was greater in the dGHD category (+1.9 vs. +1.5, p<0.01), with no further difference in IGF-1 SDS between groups. At the last available follow-up, 59 patients had reached the near adult height (NAH) and underwent retesting for GHD. No differences in NAH were found (-0.3 vs. -0.4 SDS, 0% vs. 4% of short stature). The prevalence of pathological

retesting was higher in dGHD (60% vs. 10%, p<0.01) as well as of overweight and obesity (67% vs. 26%).

Conclusion: Stimulation tests and the equivalent benefit from rhGH therapy, cannot distinguish between dGHD and SUS populations. In addition, lower IGF-1 concentrations at baseline and their higher increase during treatment in dGHD patients, and the lack of pathological retesting upon reaching NAH in SUS patients, are facts that suggest that deficient GH secretion may not be the cause of short stature in the SUS studied population.

KEYWORDS

endocrinologic diseases, stimulation tests, epidemiology, growth hormone deficiency, short stature

Introduction

Childhood growth hormone deficiency (GHD) is a rare endocrine disorder characterized by inadequate secretion of growth hormone (GH) from the pituitary gland, with an estimated prevalence between 1 in 3,500 and 1 in 10,000 children (1). It usually results in short stature and can lead to significant physical and psychosocial challenges for affected children. Conventionally, the diagnosis of GHD is confirmed through stimulation tests that evaluate the ability of the pituitary gland to produce an appropriate GH response (2).

However, the accurate diagnosis of GHD in children who present with short stature or slow growth remains a diagnostic dilemma for clinicians, since several issues have been raised regarding the reliability of stimulation tests (3, 4). As a matter of fact, it has been calculated that the probability of a true positive result for a stimulation test in a child with short stature is about 1 in 36 cases (5).

It has been previously proposed that not all children with short stature displaying an inadequate response to tests for GH secretion truly suffer from GHD. Instead, amongst these, solely children with an identifiable monogenic cause of GHD or an identifiable functional or anatomical anomaly in the hypothalamic-pituitary axis should be considered definite GHD (dGHD). The remaining patients could be defined as having a "Short stature Unresponsive to Stimulation tests" (SUS) (4), rather than "idiopathic GHD" (6). In other words, SUS patients may well benefit from GH treatment even if GH deficiency is not the certain cause of their growth failure, which could be related to other causes, e.g. genetic short stature.

This study aims to present a proof of concept for the definition of SUS by providing a comprehensive analysis of the clinical and laboratory features in children diagnosed with GHD and treated with recombinant human GH (rhGH). To the best of our knowledge, no other authors have analyzed the differences between the two populations.

Materials and methods

We conducted a retrospective study at the Institute for Maternal and Child Health IRCCS "Burlo Garofolo" in Trieste, Italy, a tertiary hospital and research institute that serves as a pediatric referral center for the province of Trieste, and as a national reference hospital.

All records of children and adolescents diagnosed with GHD from July 5^{th,} 2014 to March 31^{st,} 2022 were reviewed. Since July 5th, 2014, according to Italian regulation (7), GHD is defined by at least one of the following clinical-auxological parameters:

- *Criterion a)* Height ≤ -3 SDS (standard deviation score); or
- *Criterion b)* Height ≤ -2 SDS and growth velocity/year ≤ -1.0 SDS for age and sex, assessed at a distance of at least 6 months, or a decrease in height of 0.5 SDS/year in children older than two years; or
- *Criterion c)* Height ≤ -1.5 SDS compared to the genetic target and growth velocity/year ≤ -2 SDS or ≤ -1.5 SDS after 2 consecutive years; or
- *Criterion d)* Growth velocity/year ≤ -2 SDS or ≤ -1.5 SDS after two consecutive years, even in the absence of short stature and after excluding other pathological conditions as the cause of growth deficiency; or
- *Criterion e)* Hypothalamic-pituitary malformations/lesions demonstrated by neuro-radiological imaging;

and a GH response <8 ng/mL in two pharmacological tests performed on different days. One of the two tests can be growth hormone-releasing hormone (GHRH)+arginine, and in this case, GHD is defined as a GH response <20 ng/mL.

Definite GHD (dGHD) was defined (4) when at least one of the following criteria was present: genetic diagnosis of isolated GHD (pathogenic mutation in *GH1*, *GHRHR* or *RNPC3* genes); combined pituitary hormone deficiencies (CPHD); presence of

abnormalities within the hypothalamus-pituitary axis at magnetic resonance imaging (MRI); acquired damage (such as brain trauma, central nervous system infection, tumors of the hypothalamus or pituitary, radiation therapy, infiltrative diseases). All the other patients were considered as SUS.

The height and BMI SDS were determined by employing Growth Calculator 3 Software using WHO reference charts (8, 9), which were chosen over national reference charts to avoid the underestimation of overweight and obesity (10). Short stature was defined as height <-2 SDS. Overweight was defined as BMI SDS between 1 and 2 SDS and obesity as BMI SDS >2. The IGF-1 SDS were determined according to bone age (11). Differences (Δ) in variables were calculated compared to baseline values.

When near adult height (NAH) [defined as a height velocity of <2 cm/year, an individual growth curve showing asymptotic growth toward adult height, and bone age of ≥15 years of age (12)] was reached, retesting of GH secretion was performed with growth hormone-releasing hormone (GHRH)+arginine test, after at least 1 month of rhGH treatment suspension. Persistent GHD was defined as a GH response <19 ng/mL. Stimulation tests were performed according to protocols, as previously described (13).

Brain MRI was performed in all individuals before rhGH treatment started. Anomalies of the pituitary gland at MRI were defined as such by expert pediatric radiologists and through comparison with relevant pediatric literature (14, 15). Pituitary stalk agenesis, ectopic posterior pituitary, and pituitary stalk interruption syndrome are indeed highly specific findings for GHD (16). Empty sella and pituitary hypoplasia instead, while also found in the general population, were considered relevant when in association with hormonal deficit (17).

Since normal MRI imaging reasonably excludes GH1, GHRHR, or RNPC3 mutations (18–20), genetic testing for these genes was not performed routinely in our cohort. According to our clinical practice, indications for genetic testing were: familial short stature, disproportionate short stature, facial dysmorphisms, and skeletal abnormalities (e.g. Madelung deformity, brachydactyly). When performed, genetic analysis was limited to next-generation sequencing of genes known to be associated with short stature: SHOX, NPR2, CNP, IHH, ACAN, PAPPA2, FGFR3, STAT5B, GHR, GH1, IGF1, IGF1R, IGFALS, GHSR.

The "G2 clinico" platform (management system specialist activities) was employed to access all patients' data. Information retrieved included gender, target height, criteria to perform GH stimulation tests, type of tests performed and GH peaks, presence of genetic mutations, presence of other pituitary deficiencies, anomalies at MRI, presence of acquired pituitary damage; at diagnosis, after 1 year of treatment and at last follow-up visits: age, IGF-1, bone age (according to Greulich & Pyle) (21), rhGH dose.

Ethical Committee approval was not requested since General Authorization to Process Personal Data for Scientific Research Purposes (Authorization no. 9/2014) declared that retrospective archive studies that use ID codes, preventing the data from being traced back directly to the data subject, do not need ethics approval (22). According to the Research Institute policy informed consent was signed by parents at the first visit, in which they agreed that "clinical data may be used for clinical research purposes, epidemiology, study of pathologies and training, with the objective of improving knowledge, care and prevention".

All statistical analyses were conducted with JMP™ (version 16.1.0, SAS Institute Inc., Cary, NC, United States). Data are presented as median and interquartile ranges (IQRs). Mann-Whitney rank-sum tests and Two-tailed Fisher exact tests were performed to evaluate the relations between variables. Wilcoxon signed-rank test was performed to check whether the differences between paired data were statistically significant. Single-linear regression and multivariate logistics regressions were carried out to study associations between a dichotomous outcome and one or more independent variables. A p-value <0.05 was considered statistically significant.

Results

We collected data on 153 consecutive patients (44 females) with a median age at diagnosis of GHD of 12.0 years (IQR 9.6;13.5), 59% pre-pubertal. The first stimulation tests were performed with arginine (n=149), GHRH+arginine (n=3), or glucagon (n=1); the second stimulation tests were performed with insulin (n=115), arginine (n=26), clonidine (n=11) or GHRH+arginine (n=1). Overall, 38 individuals were identified as having dGHD (n=32 with MRI abnormalities [n=18 reduced pituitary volume; n=7 empty sella; n=4 pituitary stalk interruption syndrome (PSIS); n=2 pituitary stalk thickening; n=1 pituitary agenesis]; n=5 with CPHD, 3 of whom had MRI abnormalities; n=2 with genetic diagnosis [pathogenetic mutations in *GH1* gene]; n=2 with acquired damage secondary to brain radiotherapy). The remaining 115 individuals were identified as SUS (Figure 1). 16 children (10%) with a known genetic abnormality (unrelated to GHD) were included in the SUS category (4 with muscular dystrophies; 3 with metabolic disorders such as Kearns-Seyre syndrome, GLUT1 deficiency, and hyperinsulinism/hyperammonemia syndrome; 4 with isolated genetic abnormalities such as Xp22.3 duplication, 13q21.2 deletion, 20q13.33 deletion and 2q37 deletion; 1 with tricho-entero-hepatic syndrome and 4 with syndromes known to affect growth such as ACAN mutation, Mazzanti syndrome and MIRAGE syndrome). All

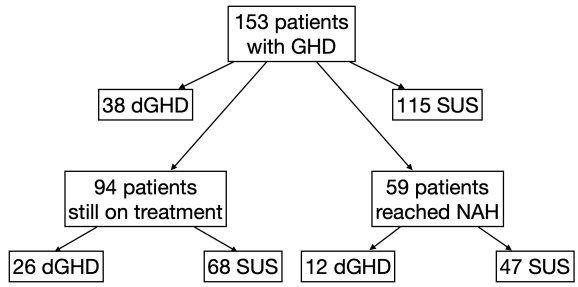

FIGURE 1
Flow-chart explaining the division to different groups of the study. dGHD, definite growth hormone deficiency; GHD, growth hormone deficiency; SUS, short stature unresponsive to stimulation tests.

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other patients were otherwise healthy. Clinical-auxological parameters for GHD diagnosis and dGHD criteria are presented in Table 1.

The clinical and laboratory characteristics of the entire cohort and the 2 groups at baseline are reported in Table 2. While no difference was found in height at diagnosis between dGHD and SUS (-2.2 vs. -2.0 SDS, p=0.35), target height in SDS was lower in SUS than in dGHD (-0.3 vs. -0.1, p=0.04) and the difference between height and target height in SDS was higher in dGHD than in SUS (-2.1 vs. -1.4, p=0.01) (Figure 2).

There was a higher prevalence of pre-pubertal children in dGHD than in SUS (74% vs. 55%, p=0.03), although no significant differences were found in age at diagnosis (11.9 vs. 12.1 years, p=0.45). Individuals with dGHD had lower GH peaks at the first stimulation tests with arginine (3.8 vs. 4.8 ng/mL, p<0.01), with a higher prevalence of GH peak <3 ng/mL (37 vs. 19%, p=0.02), although SUS had a higher prevalence of GH peak <3 ng/mL at second stimulation tests with insulin (70% vs. 41%, p<0.01).

At diagnosis, dGHD had lower IGF-1 in SDS (-2.0 vs -1.3, p<0.01) (Figure 2), with a higher prevalence of IGF-1 <-1.5 SDS (58% vs. 35%, p=0.02) and <-2 SDS (48% vs. 25%) (Table 2). However, the IGF-1 <-1.5 SDS cut-off had a sensitivity was 66% and specificity 58% for dGHD, and the IGF-1 <-2 SDS cut-off had a sensitivity was 0%, and specificity 100%.

All patients reached at least 1 year of follow-up, and both GHD and SUS benefitted from rhGH therapy. Indeed, median increase in height SDS after 1 year of treatment was 0.5 in both groups. The difference between height and target height in dGHD compared to SUS persisted (-1.6 vs. -0.9 SDS, p=0.02). Although the height gain was similar in the two groups, dGHD exhibited a greater increase in IGF-1 compared to baseline was found in SUS (+1.9 vs. +1.5 SDS, p<0.01) (Supplementary Table 1).

Among the entire cohort, 94 individuals (61%) were still on treatment (26 dGHD; 68 SUS) (Figure 1); the median age at the last available follow-up was 13.5 years (IQR 11.1;15.1) with a median length of treatment of 3.2 years (IQR 1.4;4.1). At the last follow-up visit, a greater difference between height and target height was still found in dGHD compared to SUS (-1.2 vs. -0.4 SDS, p=0.02), and a higher rhGH dose was used in dGHD (29.8 vs. 27.8 mcg/kg/day, p=0.03) (Supplementary Table 2).

Overall, 59 individuals reached NAH and ended rhGH treatment (39%; 12 dGHD; 47 SUS) (Figure 1), at a median age of 17.2 years (IQR 16.0;18.1) with a median length of treatment of 4.1 years (IQR 3.1;4.7). Those with dGHD had a higher prevalence of pathological retesting (40% vs. 10%, p<0.01) with a lower GH peak at GHRH+Arginine test (23.4 vs. 52.7 ng/mL, p<0.01) (Figure 3, Table 3). Moreover, dGHD individuals with pathological retesting (2 with PSIS and 2 with CPHD) had significantly lower IGF-1 SDS (median -2.4 SDS [IQR -4.1;-1.5]) compared to those with normal retesting (median -1.0 [-1.9;-0.6]) (3 with empty sella, 2 with pituitary hypoplasia and 1 with a pathogenic mutation in the GH1 gene). A greater BMI SDS was found in dGHD compared to SUS (1.3 vs. -0.1, p<0.01) with a higher prevalence of overweight and obesity (67% vs. 26%, p<0.01) (Figure 3, Table 3). No increased prevalence of CPHD, hypothalamus-pituitary axis abnormalities at MRI, or acquired damage were found in dGHD individuals with overweight and obesity at the last follow-up; moreover, no limited subjects' mobility was reported. Although these children had a higher BMI already at baseline (1.22 vs 0.14 SDS) this difference did not reach statistical significance (p=0.06). All dGHD with pathological retesting were overweight/obese (100%) compared to 50% of those with normal retesting and 26% of SUS both with or without pathological retesting (p<0.01). Peak GH in pathological retesting was correlated with IGF-1 SDS (r^2 0.50, p=0.04), but not with BMI

TABLE 1 GHD diagnostic criteria according to the Italian regulation: comparison of the whole cohort, GHD and SUS population and prevalence of the proposed dGHD diagnostic criteria in the dGHD population.

	Total	dGHD	SUS	p
GHD diagnosis - clinical-auxological parameters				
Height ≤ -3 SDS	8%	13%	7%	0.23
Height ≤ -2 SDS and growth velocity/year ≤ -1.0 SDS, or a decrease in height of 0.5 SDS/year	53%	45%	56%	0.24
Height ≤ -1.5 SDS compared to the genetic target and growth velocity/year ≤ -2 SDS or ≤ -1.5 SDS after 2 consecutive years	29%	18%	32%	0.10
Growth velocity/year ≤ -2 SDS or ≤ -1.5 SDS after two consecutive years	77%	71%	79%	0.30
Hypothalamic-pituitary malformations/lesions at imaging	20%	82%	0%*	**<0.01**
dGHD criteria				
Genetic diagnosis of isolated GHD	1%	5%	0%	
Multiple pituitary combined deficiencies	3%	13%	0%	
Hypothalamus-pituitary axis abnormalities at MRI	21%	84%	0%	
Acquired damage	1%	5%	0%	

SDS, standard deviation score. Significant p values in bold.
* "Hypothalamic-pituitary malformations/lesions at imaging" were one of the criteria that defined dGHD.

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TABLE 2 Clinical and laboratory characteristics at baseline.

	Total	dGHD	SUS	p
N (%)	153 (100%)	38 (25%)	115 (75%)	
Sex (female, %)	29%	32%	28%	0.68
Target height (SDS)	-0.3 (-0.8;0.2)	-0.1 (-0.6;0.2)	-0.3 (-1.0;0.1)	**0.04**
Age (years)	12.0 (9.6;13.5)	11.9 (8.0;13.4)	12.1 (9.9;13.6)	0.45
Pre-pubertal (%)	59%	74%	55%	**0.03**
Height (SDS)	-2.0 (-2.5;-1.4)	-2.2 (-2.8;-1.2)	-2.0 (-2.5;-1.4)	0.35
Height – TH (SDS)	-1.5 (-2.2;-1.0)	-2.1 (-2.7;-1.2)	-1.4 (-2.0;-0.9)	**0.01**
Short stature (%)	52%	56%	51%	0.45
BMI (SDS)	-0.1 (-1.0;1.1)	0.2 (-0.8;1.4)	-0.1 (-1.1;1.0)	0.43
Overweight/obese (%)	18%/9%	26%/13%	17%/8%	0.19
Bone age (years)	11.0 (8.0;12.5)	11.0 (6.0;12.6)	11.0 (8.5:12.5)	0.92
Bone age-chronological age (years)	-1.4 (-2.3;-0.5)	-1.1 (-1.8;-0.3)	-1.5 (-2.4;-0.7)	0.08
GH peak 1st test (ng/ml)	4.5 (3.1;6.0)	3.8 (2.1;5.2)	4.8 (3.5;6.1)	**<0.01**
GH peak 1st test – arginine only (ng/ml)	4.6 (3.0;6.0)	3.8 (2.1;5.2)	4.8 (3.6;6.0)	**<0.01**
GH peak 1st test <3 ng/mL –arginine only (%)	23%	37%	19%	**0.02**
GH peak 2nd test (ng/ml)	2.8 (1.6;4.5)	3.2 (1.8;4.5)	2.5 (1.5;4.4)	0.35
GH peak 2nd test – insulin only (ng/ml)	2.3 (1.3;4.0)	3.5 (1.3;5.2)	2.2 (1.3;3.9)	0.06
GH peak 2nd test <3 ng/mL – insulin only (%)	63%	41%	70%	**<0.01**
IGF-1 (SDS)	-1.4 (-2.2;-0.9)	-2.0 (-2.8;-1.4)	-1.3 (-2.1;-0.8)	**<0.01**
IGF-1 <0 SDS (%)	94%	100%	92%	0.07
IGF-1 <-1.5 SDS (%)	52%	58%	35%	**0.02**
IGF-1 <-2 SDS (%)	31%	48%	25%	**0.01**
Genetic analysis performed	29%	50%	21%	**<0.01**
rhGH dose (mcg/kg/day)	25.1 (24.0;26.6)	25.4 (24.0;27.0)	25.0 (23.8;26.5)	0.17

BMI, body mass index; GH, growth hormone; IGF-1, insulin-like growth factor 1; rhGH, recombinant human growth hormone; SDS, standard deviation score; TH, target height. Significant p values in bold.

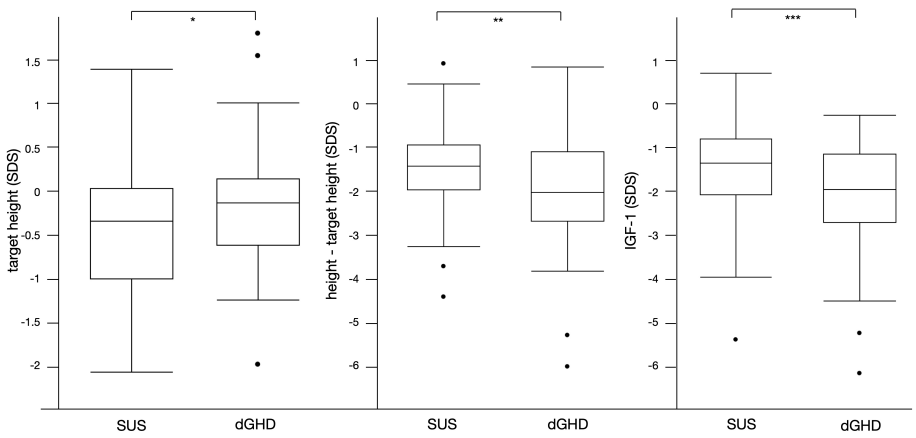

FIGURE 2
Significant differences between SUS and dGHD at baseline: target height SDS (* p=0.04), height-target height SDS (**p=0.01), IGF-1 SDS (***p<0.01).

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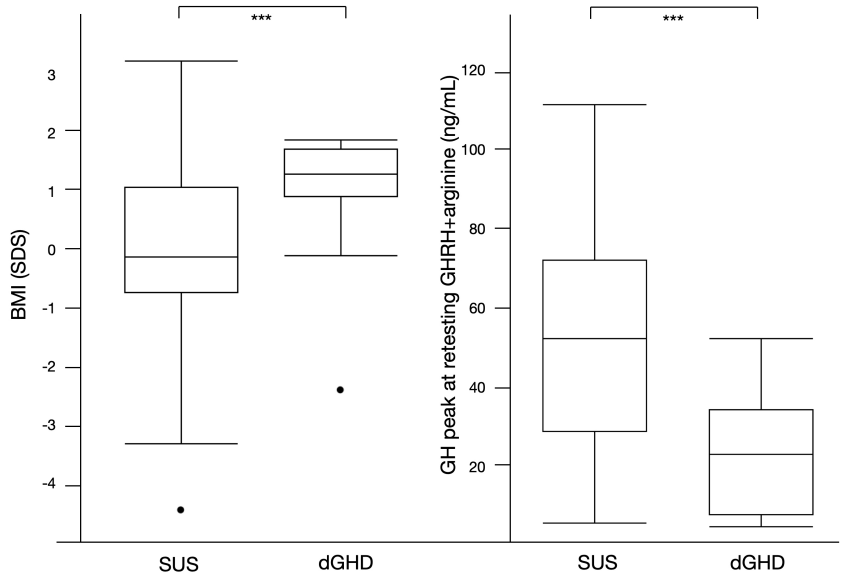

FIGURE 3
Significant differences between SUS and dGHD at the last follow-up visit for individuals who reached near adult height (n=59): BMI SDS and GH peak at retesting with GHRH+arginine (*** p<0.01).

TABLE 3 Clinical and laboratory characteristics at the last follow-up visit for individuals who reached near adult height (n=59).

	Total	dGHD	SUS	p
N (%)	59 (100%)	12 (20%)	47 (80%)	
Sex (female, %)	20%	20%	20%	0.96
Age (years)	17.2 (16.0;18.1)	17.4 (16.1;18.3)	17.1 (16.0;18.1)	0.64
Length of treatment (years)	4.1 (3.1;4.7)	4.2 (3.4;4.5)	3.8 (3.1;4.7)	0.52
NAH (SDS)	-0.5 (-1.0;0.3)	-0.3 (-1.0;0.3)	-0.4 (-1.0;0.3)	0.70
NAH – TH (SDS)	0.0 (-0.6;0.4)	-0.5 (-1.2;0.3)	0.0 (-0.3;0.4)	0.11
Short stature (%)	3%	0%	4%	0.46
Δ Height (SDS)	1.4 (0.9;1.9)	1.5 (0.8;1.8)	1.3 (0.9;1.9)	0.93
BMI (SDS)	0.3 (-0.6;1.3)	1.3 (-0.9;1.7)	-0.1 (-0.7;1.0)	**<0.01**
Overweight/obese (%)	27%/7%	67%/0%	17%/9%	**<0.01**
Δ BMI (SDS)	-0.1 (-0.6;0.5)	0.1 (-0.4;0.7)	-0.2 (-0.7;0.5)	0.35
Bone age (years)	16.0 (15.0;16.5)	16.3 (15.3-17.0)	15.5 (15.0-16.5)	0.15
Bone age-chronological age (years)	-1.3 (-2.2;-0.1)	-1.1 (-2.0;0.0)	-1.3 (-2.4;-0.1)	0.57
Δ bone age/Δ chronological age	0.9 (0.7;1.3)	0.9 (0.6;1.2)	0.9 (0.7;1.3)	0.48
IGF-1 (SDS)	-1.2 (-1.8;-0.7)	-1.2 (-2.3;-0.3)	-1.2 (-1.6;-0.7)	0.75
Δ IGF-1 (SDS)	0.2 (-0.4;1.3)	0.6 (0.1;1.4)	0.2 (-0.5;1.3)	0.27
rhGH dose (mcg/kg/day)	28.4 (26.0;32.9)	29.1 (27.3;32.6)	28.3 (25.0;32.9)	0.39
Pathological retesting (%)	16%	40%	10%	**<0.01**
GH peak at retesting test (ng/ml)	49.1 (25.4;71.7)	23.4 (7.8;34.6)	52.7 (29.9;72.6)	**<0.01**

BMI, body mass index; GH, growth hormone; IGF-1, insulin-like growth factor 1; NAH, near adult height; rhGH, recombinant human growth hormone; SDS, standard deviation score; TH, target height; Δ BMI, difference between BMI at last follow-up compared to baseline; Δ bone age/Δ chronological age, difference between bone age at last follow-up compared to baseline over difference between chronological age at last follow-up compared to baseline; Δ height, difference between height at last follow-up compared to baseline; Δ IGF-1, difference between IGF-1 at last follow-up compared to baseline. Significant p values in bold.

SDS (r^2 0.01, p=0.81). The median IGF-1 of dGHD patients with pathological retesting was -2.4 SDS (-4.1;-1.5).

While at baseline 52% had short stature (<- 2 SDS), after 1 year of treatment the rate decreased to 23% and 18% at the last follow-up visit or 3% at the end of rhGH treatment (p<0.01), with no significant differences between dGHD and SUS (Figure 4). The increase in height SDS was significant from baseline to 1 year of treatment and to the end of treatment both in dGHD (baseline -1.7 SDS [-2.3;-1.0]; 1 year -1.2 SDS [-1.5;-0.4]; end -0.3 SDS [-1.0;0.3]; p<0.01) and in SUS (baseline -1.9 SDS [-2.3;-1.3]; 1 year -1.2 SDS [-1.9;-0.7]; end -0.5 SDS [-1.0;0.3]; p<0.01).

Overall, 44 individuals performed NGS for genes causative of short stature, 25/115 among SUS (21%), and 19/38 among dGHD (50%) (p<0.01).

Discussion

In this retrospective study, we comprehensively analyzed 153 patients diagnosed with GHD according to auxological and laboratory parameters and treated with rhGH to identify possible differences supporting the definition of SUS as a proof of concept. As a matter of fact, this study highlighted some significant differences between patients who had a definite and identifiable monogenic, functional, or anatomical cause of GH deficiency (dGHD) and those who did not (SUS).

The main findings of this retrospective study were that individuals identified as dGHD had lower IGF-1 concentrations at baseline, with a higher increase after 1 year of treatment, and had a higher prevalence of pathological retesting and overweight/obesity at the end of treatment. Individuals labeled as SUS had a lower target height and a greater difference between height and target height at diagnosis, after 1 year of treatment, and at the last available follow-up visit for those still on treatment. Nevertheless, results in terms of first-year and final responses were similar between SUS and dGHD.

As could be expected (4), GH stimulation tests were found to be of no aid in discriminating the two categories. Indeed, while GH peak was lower in dGHD at the first stimulation test, with a higher prevalence of "severe" GHD, the opposite was found at the second stimulation test. GH stimulation tests, though useful for the clinician to find some bearings in the *mare magnum* of the causes of short stature, have in fact long been known to be an imperfect means of diagnosis of GHD, displaying problems with reproducibility and yielding potential false positive or false negative results (3, 23, 24). For instance, pathological stimulation tests have been found in conditions where a problem in GH secretion is not supposed to be the cause of short stature (such as Turner syndrome, Noonan syndrome, or *SHOX* deficiency) (25–27). Similarly, we did not find any significant difference in height SDS at diagnosis between the two groups that could help in differentiating a true GHD from other causes of short stature (28, 29).

On the contrary, some major differences were in line with the etiopathogenesis of GHD: lower IGF-1 at baseline and greater increase during treatment, higher prevalence of persistent GHD at retesting, and higher BMI when they reached NAH.

With regards to IGF-1, their concentrations were lower at baseline in dGHD compared to SUS; however, due to a significant increase in dGHD patients after the first year of treatment, differences in IGF-1 SDS did not persist at 1 year or the end of treatment. Since IGF-1 is secreted by the liver when stimulated by GH, lower concentrations of IGF-1 at baseline and normalization after treatment are consistent with a differentiation between dGHD and SUS. Many IGF-1 SDS cut-offs have been proposed for the diagnosis of GHD (30, 31) and dGHD had a higher prevalence of IGF-1 <-1.5 or <-2 SDS in our cohort; however, we do not propose these cut-offs as diagnostic criteria in discriminating dGHD from SUS, but more as supportive factors. Indeed, it is already known that they suffer from low sensitivity and specificity in GHD diagnosis (13, 32), therefore their values should always be interpreted in combination with other clinical and biochemical parameters.

The majority of our patients (59%) were pre-pubertal at diagnosis, with a higher prevalence of pre-pubertal children in dGHD than in SUS. This may be partially explained by the

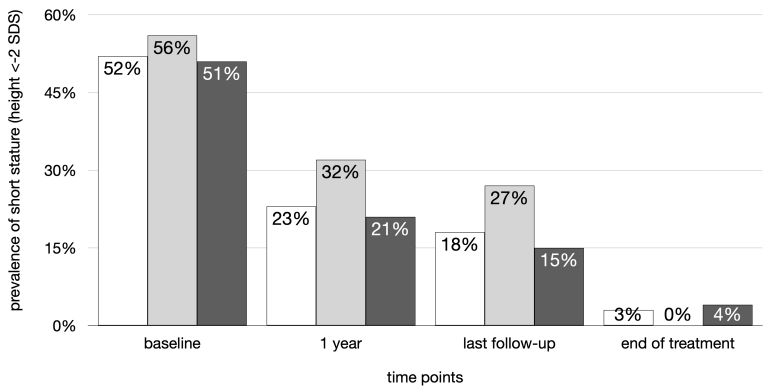

FIGURE 4
Prevalence of short stature (height < -2 SDS) in the overall cohort (white), dGHD (light grey) and SUS (dark grey) at different follow-up timepoints: at baseline, after 1 year of follow-up, at last visit and at the end of rhGH therapy.

presence, in the dGHD cohort, of patients with CPHD, which is known to be associated with a delay in pubertal development (33). Amongst all pre-pubertal patients, solely 6 (4%) had delayed puberty, defined as the absence of thelarche by 13 years of age in females or the absence of an increase in testicular volume >4 mL by 14 years of age in males. Interestingly, all patients with delayed puberty were males. It has been suggested that sex steroid priming may be useful in pre-pubertal patients of peri-pubertal age, reducing the overdiagnosis of children with constitutional delay of growth and puberty (CDGP) as GHD patients. However, presently it is not recommended by our national guidelines and there are no standardized protocols for sex steroid administration in this type of patient, particularly concerning sex steroid dose and timing of supplementation (34, 35). Furthermore, some authors have suggested that sex steroid priming may lead to an underdiagnosis of GHD patients, due to a temporary and unsustained increase in GH secretion (36). For these reasons, sex steroid priming was not performed.

Childhood-onset GHD is usually retested after the achievement of NAH to verify whether they need to continue rhGH treatment, and a rate ranging from 20 to 87% percent of individuals have been found to have normal GH secretion (37). While GH stimulation tests remain an imperfect means of defining pediatric GH deficiency, a deficient response to insulin or arginine+GHRH stimulus is highly specific for GHD in adults (38–40). The wide difference in positive retesting in pediatric GHD in the literature suggests that the GHD definition in the pediatric age is a sort of umbrella term, including different conditions. Remarkably, in our cohort, we found that almost all of SUS had normal retesting. On the contrary, more than half of dGHD had the confirmation of a pathological GH secretion and continued rhGH treatment. These findings are consistent with the reported rates of persistent GHD in patients with known pituitary abnormalities (such as ectopic posterior pituitary or pituitary hypoplasia) ranging from 27 to 66% (41, 42). For example, in some genetically determined GHD (such as GHRHR mutations) GH secretion may be reduced and not completely absent (43). Indeed, one of our dGHD individuals with normal retesting had a pathogenic mutation in the GH1 gene. We therefore believe that normal retesting does not exclude the dGHD diagnosis.

It has been reported that GHD is associated with mild to moderate truncal obesity, mostly in adults (44, 45), and mild to moderate overweight is usually thought to be a feature of GHD also in children (46); however, children with GHD have been shown to have average BMI, with no differences between organic and idiopathic GHD (46, 47), therefore only the pattern of fat distribution should be considered in the clinical diagnosis of GHD. In our cohort, we did not evaluate fat mass; however, no significant differences were noticed in BMI between dGHD and SUS at baseline or after 1 year of treatment. However, at the end of treatment, dGHD had a greater BMI compared to SUS and a higher prevalence of overweight/obese adolescents. Interestingly, all dGHD with pathological retesting were overweight/obese, compared to half of those with normal retesting or a quarter of those with SUS. While it has been suggested that the GHRH+arginine stimulation test is highly dependent on BMI (48), in our cohort peak GH was correlated with IGF-1 SDS in pathological retesting, with no correlation with BMI SDS.

A significant increase in BMI in non-overweight children after 2 years of rhGH treatment (47) and in adults after 3 years of therapy (49) has been reported, however, no studies have ever evaluated BMI at the end of childhood GHD so far. It should be noted that 40% of dGHD with pathological retesting presented multiple pituitary hormone deficits requiring hydrocortisone supplementation. A single patient instead presented multiple pituitary hormone deficiency with central hypothyroidism. In all cases, however, hormone supplementation was optimized and unlikely to contribute to weight gain. A possible explanation could be that the increase in BMI is a characteristic of GHD that is evident only after childhood and only in severe GHD (i.e. persistent GHD after childhood), although further studies are necessary to explore this issue.

An interesting finding was that target height SDS was significantly lower in children with SUS than in those with dGHD. Moreover, the difference between height and target height in SDS was higher in dGHD than in SUS at baseline as well as after 1 year of treatment and at the last follow-up visit, but not at the end of treatment. These data suggest that the mechanism underlying short stature in SUS patients might not be GH deficiency, nor CDGP (50), but autosomal dominant short stature (51, 52), with mutations in genes known to influence the growth plate independently of GH, such as paracrine signaling and the composition of extracellular matrix and chondrocytes (53–55). A recent study found that among children with GHD and a family history of short stature a causative genetic mutation was found in 29%, none of them were in genes related to isolated GHD (*GH1*, *GHRHR*, or *RNPC3*) and only 13% had a genetic variant affecting GH secretion or function (*GSHR* and *OTX2)*, while mostly had a primary growth plate disorder; therefore, genetic results frequently did not correspond with the clinical diagnosis of GHD, even with faltering response to stimulation tests (56). Moreover, the same study showed that genetic causes for short stature may have an excellent response to rhGH treatment, contrary to what was thought so far about short genetic stature (56). While these results may be influenced by our still incomplete knowledge of genetic defects affecting the GH-IGF1 axis, they emphasize the hypothesis that the low response of GH during stimulation tests may be an epiphenomenon rather than the cause for short stature. Therefore, whenever feasible, pediatric endocrinologists should consider performing genetic studies as part of the routine diagnostic work-up for short stature (57).

Besides these differences, dGHD and SUS groups did not differ at baseline for sex, age, height SDS, bone age, the difference between bone and chronological age, and prescribed rhGH dose. Moreover, no significant advance in bone age during rhGH treatment has been found in dGHD or SUS (58). In particular, our data confirm the effectiveness of rhGH treatment in both groups. The first-year response to rhGH treatment, a critical determinant of the total treatment height outcome in growth disorders (59), was good in both groups, and both groups reached the target height and a normal height. Therefore, a good response to rhGH treatment is non-specific and should not be used to define the etiology of short stature (5) or to decide to whom treatment should be offered.

Indeed, rhGH therapy has been found to improve both short-term and long-term height gain even in children with idiopathic short stature (ISS) (60); however, usually, supraphysiological doses of rhGH are required in ISS (61), with lower gain in height SDS compared to GHD (62), which is not the case for SUS patients in our study.

This study has limits. It is based on retrospective data collection from a single center, therefore results may be related to the local population. Moreover, although a normal MRI realistically excludes *GH1*, *GHRHR*, or *RNPC3* mutations, genetic analyses were not performed in 90 out of 115 SUS patients and not all known genes associated with GHD (for example *GHRHR* and *RNPC3*) were included in the NGS analysis, therefore some patients may have been inappropriately considered as SUS. In addition, amongst the SUS patients, 10% had a genetically determined syndrome which may have impacted GH secretion. Lastly, we had data at the end of treatment only for 59 individuals (39% of the cohort).

On the other hand, to our knowledge, this is the first study comparing dGHD and SUS children from their diagnosis to the end of rhGH treatment, showing that they are two distinct groups with differences in IGF-1 concentrations, target height, distance from target height at baseline and BMI SDS and positive retesting at the end of treatment.

In our view, SUS is not a definitive diagnosis, and a strategy to increase the rates of correct and precise diagnosis should be developed. We have suggested the distinction between the SUS and dGHD populations to help in defining the different etiologies of short stature, in a way that may keep the field open to other relevant diagnoses, such as genetic short stature, and applications of research. Moreover, it may avoid labeling children with a diagnosis that is not entirely confirmed and that entails multiple clinical sequelae over the years (e.g., the evolution of subsequent multiple pituitary hormone deficiencies, altered body composition, decreased bone mineral density, persistent GHD at retesting. The findings of this study are proof of concept of the definition of SUS: not all children with abnormal responses to GH stimulation tests have GHD. However, this does not mean that rhGH treatment is not advisable in SUS patients. On the contrary, rhGH treatment should continue to be offered to children with SUS, since results in terms of first-year and final response are similar to those of children with dGHD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical Committee approval was not requested since General Authorization to Process Personal Data for Scientific Research Purposes (Authorization no. 9/2014) declared that retrospective archive studies that use ID codes, preventing the data from being traced back directly to the data subject, do not need ethics approval. Informed consent was signed by parents at the first visit, in which they agreed that "clinical data may be used for clinical research purposes, epidemiology, study of pathologies and training, with the objective of improving knowledge, care and prevention".

Author contributions

ML: Data curation, Investigation, Writing – original draft. ED: Data curation, Investigation, Writing – original draft. GTa: Conceptualization, Supervision, Validation, Writing – review & editing. VV: Investigation, Writing – review & editing. GV: Investigation, Writing – review & editing. EF: Validation, Writing – review & editing. EB: Resources, Validation, Writing – review & editing. GTo: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1288497/full#supplementary-material

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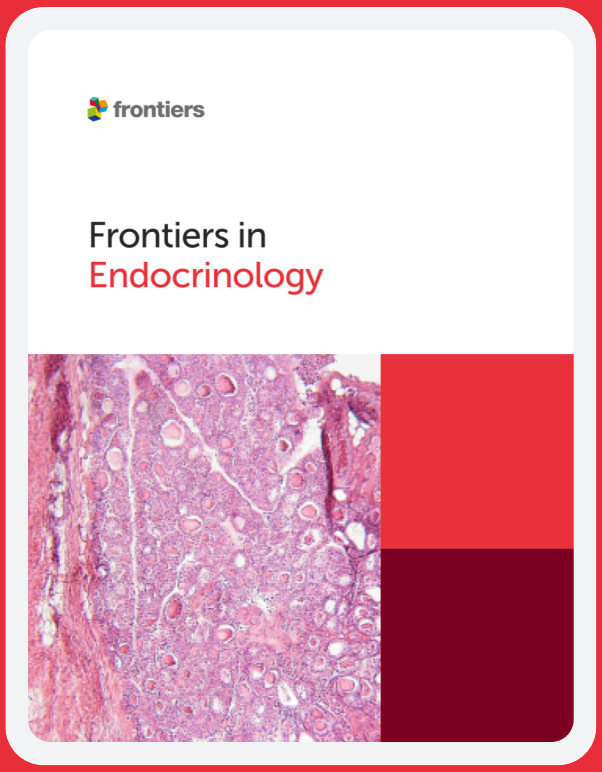

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