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Novel Clinical Criteria Allow Detection of Short Stature Homeobox-Containing Gene Haploinsufficiency Caused by Either Gene or Enhancer Region Defects

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Short stature homeobox-containing gene deficiency · Genetic screening · Clinical features · Gene enhancer mutation

Abstract
Introduction: Short stature homeobox-containing gene (*SHOX*) haploinsufficiency is associated with short stature, Madelung deformity and mesomelia. Current clinical screening tools are based on patients with intragenic variants or deletions. However, recent discoveries showed that deletions of the enhancer elements are quite common. The majority of these patients show less body disproportion and respond better to recombinant human growth hormone treatment. We redefined clinical criteria for genetic analysis to facilitate detection of the full spectrum of *SHOX* haploinsufficiency. ***Methods:*** We analyzed 51 children with *SHOX* variants or deletions and 25 children with a deletion in its enhancer region. Data were compared to 277 children referred for suspicion of growth failure without endocrine or genetic pathology. ***Results:*** Only half of the patients with an enhancer region deletion fulfilled any of the current screening criteria. We propose new clinical criteria based on sitting height to height ratio >1 SDS or arm span ≥3 cm below height, with a sensitivity of 99%. When these criteria are combined with obligatory short stature, the sensitivity to detect *SHOX* haploinsufficiency is 68.1%, the specificity 80.6%, and the number needed to screen 21 patients. ***Conclusion:*** Novel clinical criteria for screening for *SHOX* haploinsufficiency allow the detection of patients within the full genetic spectrum, that is, intragenic variants and enhancer region deletions.

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Introduction

The short stature homeobox-containing gene (*SHOX*) on the pseudoautosomal region 1 of chromosomes X and Y encodes a transcription factor that regulates temporal and spatial expression of genes involved in linear growth [1]. *SHOX* function is dose dependent: a homozygous loss of *SHOX* expression results in a severe skeletal dysplasia (Langer mesomelic dysplasia [2–4]), a heterozygous loss of *SHOX* expression (*SHOX* haploinsufficiency) leads to a wide phenotypic spectrum [5–8], while duplication of *SHOX* leads to tall stature [9]. At one side of the spectrum of *SHOX* haploinsufficiency, there are patients with classical Leri-Weill dyschondrosteosis, typically presenting with short stature, Madelung deformity, mesomelia, cubitus valgus, bowing of the forearm, muscular hypertrophy, dislocation of the ulna, and typical radiological signs (e.g., triangulation of distal radial epiphyses, wedging of carpal bones, metaphyseal lucency). On the other side of the spectrum, one can encounter individuals with only mild body disproportions, and in some cases, even a height in the lower half of the height distribution of the population growth charts with normal body proportions. Even within the same family, considerable phenotypic and radiologic heterogeneity exist [10–15]. Causes of *SHOX* haploinsufficiency include heterozygous deletions or variants in the *SHOX* gene itself, heterozygous deletions of (part of) one of the 5′ or 3′ enhancer regions regulating *SHOX* expression, or duplications in these regions inhibiting proper expression [16]. *SHOX* haploinsufficiency has been found in 2–17% of children who initially were considered as having "idiopathic short stature," making it the most frequent monogenetic cause of short stature [12, 16–19]. Patients with *SHOX* haploinsufficiency benefit from treatment with recombinant human GH [7, 20–22] and from follow-up for development of Madelung deformity.

Several screening tools have been developed to guide clinicians in whom to screen for *SHOX* haploinsufficiency, such as extremities to trunk ratio (ETR) [23] or the sitting height to standing height (SH/H) ratio [8]. In 2007, Rappold et al. [10] suggested using a combination of body disproportions (arm span to height ratio <96.5% and SH/H ratio >55.5%) and cubitus valgus, bowing of the forearm, body mass index, dislocation of the ulna, muscular hypertrophy, and short forearm, with a reported sensitivity of 71% for a score of >4 out of 24 points. Hirschfeldova et al. [24] also concluded that the level of disproportion and typical signs of *SHOX* haploinsufficiency at clinical evaluation were most specific of intragenic *SHOX* defects. In 2013, Wolters et al. [25] tested several scoring systems in a cohort of 22 children with *SHOX* haploinsufficiency and reported a sensitivity of the Rappold cutoffs for arm span to height ratio and SH/H of 73 and 59%, respectively, for a Rappold score >4 points of 73%, and for a decreased ETR (as defined by Binder et al. [23]) of 59%.

Notably, most of these scoring systems were formulated based on patients with intragenic *SHOX* defects (although Wolters et al. [25] included 3 children with *SHOX* enhancer region deletions). However, in the past decade, it has become evident that *SHOX* enhancer region deletions are quite common in patients with *SHOX* haploinsufficiency, occurring in roughly 15–40% [16], and these patients usually show less body disproportion [7, 26–32] and a greater response to recombinant human growth hormone (rhGH) [7]. Recently, Genoni et al. [33] analyzed 19 patients with *SHOX* haploinsufficiency, with half of them having *SHOX* enhancer region deletions. They concluded that the sensitivity of the Rappold score was only 37% in the total group and suggested including low growth velocity (below –1.5 SDS) to increase the sensitivity to 89.5%.

In light of the recent widening of the phenotype of *SHOX* haploinsufficiency, and taking into consideration the decreasing costs of genetic analysis, the high prevalence of intragenic *SHOX* defects or deletions of its enhancer region in children with short stature and the positive effects of early rhGH treatment on adult height, we aimed at redefining screening criteria for genetic analysis for the full spectrum of *SHOX* haploinsufficiency based on our observations in a large group of patients with either intragenic *SHOX* defects or deletions of its enhancer region, in order to obtain a higher sensitivity. For this purpose, we reanalyzed the auxological data of the patients from our previous publication [7], as well as data from children referred to a pediatric outpatient clinic for suspected growth failure, in whom no endocrine or genetic pathology was found [34, 35].

Subjects and Methods

Patients

In this retrospective analysis, we included patients under the age of 18 with pathogenic defects of *SHOX* or its enhancer region, diagnosed between 2002 and 2014 in the Leiden University Medical Center, Erasmus Medical Center in Rotterdam, VU University Medical Center in Amsterdam, and University Medical Center Groningen in the Netherlands. None of the patients were on GH treatment at the time of evaluation. Patients were excluded in case of incomplete clinical data or comorbidity that could contribute to short stature. In a first analysis, the data were used to describe the clinical features of the various genetic subgroups and the response

to GH treatment [7]. Height and sitting height were measured using a wall-mounted stadiometer and weight with a digital floor scale. Arm span was measured by placing the patient against a wall with arms spread, marking the arm span on the wall, and subsequently using a measuring tape.

Control Subjects

In order to calculate the specificity of the clinical score, we analyzed auxological data from a database of 277 children referred to the pediatric outpatient clinic for suspected growth failure in the Tergooi Hospital, in whom no endocrine or genetic pathology was found. Referral criteria were short stature or decreased growth velocity. Patients were screened for *SHOX* haploinsufficiency in case of a Rappold score above 8 points or typical clinical and radiological features of Leri-Weil dyschondrosteosis. Excluded were adopted children and children with ethnicities for which no Dutch growth references are available (so those with another ethnicity than Dutch, North African, or South-Eastern European). A full description of this cohort, which includes head circumference, arm span, and SH/H ratio, has been reported previously [34, 35].

Genetic Analysis

DNA isolation and Sanger sequencing of the complete coding region, including intron-exon boundaries, were performed using standard procedures (PCR primers and conditions available upon request). Multiplex Ligation-dependent probe amplification was performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). See online supplementary Table 1 (see www.karger.com/doi/10.1159/000507215 for all online suppl. material) for characterization of the variants.

In 73 patients, variants were classified as pathogenic, and in 3 patients, a variant of uncertain significance (VUS) was detected. Two of these 3 patients harbor the same 5′ enhancer deletion, which has been previously associated with clinical features of *SHOX* haploinsufficiency [26, 29, 36, 37]. Both show phenotypical features of *SHOX* haploinsufficiency (as well as the affected father of one of them). The third patient carries a missense VUS based on the American College of Medical Genetics and Genomics/Association for Molecular Pathology classification, which has only been seen in 1 individual in a control population (MAF 0.00083%, for details see online suppl. Table 1); this patient and his affected father show a clinical phenotype consistent with *SHOX* haploinsufficiency. A recent report emphasizes that the clinical phenotype of a well-described disorder, as well as the frequency in publicly available data bases of normal individuals, should be taken into account when assessing the pathogenicity of variants, as they display a more realistic reflection of daily practice [38]. To allow the readership to compare the auxological data of these 3 cases to those carrying pathogenic variants, data from the 3 patients carrying likely pathogenic variants are marked with gray symbols in all relevant figures.

Statistical Analysis

Dutch nation-wide reference data were used to calculate SDS for height and SH/H ratio [39, 40] and body mass index [41]. Birth weight SDS was calculated using Niklasson et al. [42]. The ETR represents the sum of the subischial length and arm span, divided by sitting height [23]. The Binder et al. [23] criterion for decreased ETR was defined as being lower than 1.95 + 1/2 • height in meters. To assess whether information about height SDS of the parent carrying the *SHOX* (enhancer) defect and the noncarrier parent contributes to the decision which child should be tested for *SHOX* haploinsufficiency, we calculated the correlation between height SDS of the patient and their (non-)carrier parent.

Statistical significance of mean differences between groups was tested using the two-tailed independent Student *t* test or, in case the assumption of normality was not met (Shapiro-Wilk test), the Mann-Whitney U test. Categorical data were compared using the chi-square test or, when the expected cell count was <5, the Fisher's exact test. Associations were tested using Pearson's correlation. Linear regression was used to correct the associations between patient and affected parent features for variant type, gender, and height of unaffected parent, and to correct the difference between the ETR between patients and controls for height. Differences were considered statistically significant at $p < 0.05$. Receiver operation characteristic curves were calculated to assess the relationship between sensitivity and 1-specificity for various potential predictors of *SHOX* haploinsufficiency.

Results

Clinical Characteristics (Table 1)

We obtained data from 76 patients with either a variant ($n = 11$) or deletion ($n = 40$) in *SHOX*, or a deletion in the downstream ($n = 23$) or upstream ($n = 2$) enhancer of *SHOX* (Table 1). Average age at evaluation was 8.4 years (range 1.2–16.2 years). Typical clinical and radiological features of Leri-Weil dyschondrosteosis (e.g., Madelung deformity) were not routinely reported. Average height was –2.5 SDS (range –4.4 to –0.3 SDS), with height below –2 SDS in 71.1% of patients and below –1 SDS in 97.4%. In addition, data from 277 control subjects referred for a suspicion of growth failure to a non-academic growth clinic, in whom no endocrine or genetic pathology was found, were included (Table 1). Controls' average age was 10.4 years (range 3.1–18.0 years), average height –1.9 SDS (range –3.2 to 1.0 SDS), with height below –2 SDS in 43.7% and below –1 SDS in 89.9%. Controls differed significantly from patients in height SDS, target height SDS, body mass index SDS, arm span to height ratio, arm span minus height, and SH/H ratio SDS. Also, average age in controls was 2.0 years older, and the percentage of females was lower.

As can be observed in Figure 1, height SDS alone is a poor predictor of *SHOX* defects, with an area under the curve (AUC) of 0.717 and the optimal cutoff between –2.75 SDS and –1.75 SDS. A cutoff of height below –2.0 SDS was chosen because these children will likely benefit most from treatment with rhGH, yielding a sensitivity of 71.1% and specificity of 56.3%.

Average SH/H ratio of all patients was 2.8 SDS (range 0.2–5.5 SDS). Compared to patients with *SHOX* enhancer

Table 1. Clinical characteristics of patients and control subjects

	Number†	All patients with SHOX haploinsufficiency	Number†	SHOX variants or deletions	Number†	SHOX upstream or downstream enhancer deletions	Number†	Control subjects
Age, years	76	8.4±3.6	51	8.3±3.5	25	8.6±3.7	277	10.4±3.9*
Females, %	76	57	51	55	25	60	277	43*
Birth weight SDS	64	−0.4±1.3	41	−0.5±1.3	23	−0.3±1.3	277	−0.3±1.1
Height SDS	76	−2.5±0.8	51	−2.6±0.8	25	−2.3±0.8	277	−1.9±0.7*
Target height SDS	71	−1.0±0.6	47	−1.1±0.6	24	−0.9±0.5	277	−0.4±0.60*
BMI, kg/m^2 SDS	73	0.4±1.0	49	0.6±0.9	24	−0.2±1.0	277	−0.3±1.1*
Arm span/height ratio	32	0.95±0.02	20	0.95±0.02	12	0.96±0.03	254	0.99±0.03*
Arm span minus height, cm	32	−5.5±3.0	20	−5.7±3.0	12	−5.1±3.1	254	−1.0±3.6*
SH/H SDS	69	2.8±1.3	48	3.2±1.1	21	2.0±1.2**	261	0.4±0.9*
ETR‡	32	2.5±0.2	21	2.4±0.2	11	2.6±0.2**	250	2.8±0.2*

Data are presented as mean ± SD. † Number of persons with available data. ‡ Differences corrected for height using linear regression. * Difference between control subjects and patients with SHOX haploinsufficiency statistically significant at $p < 0.05$. ** Difference between patients with SHOX variants or deletions and SHOX enhancer deletions statistically significant at $p < 0.05$. SHOX, Short stature homeobox-containing gene; ETR, extremities to trunk ratio; SH/H, sitting height to height; BMI, body mass index.

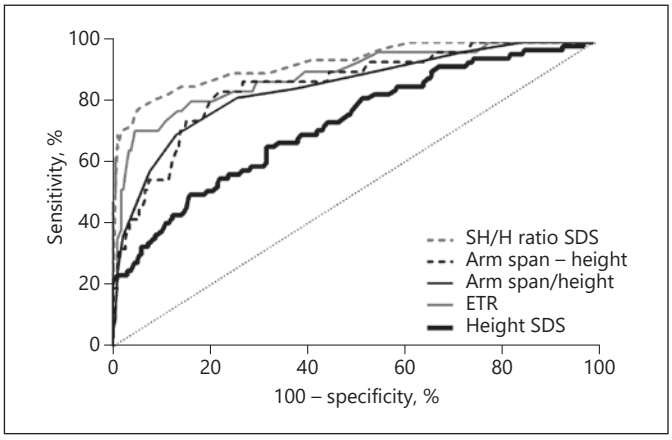

Fig. 1. Receiver operating characteristic curves illustrating the diagnostic ability of several parameters to detect *SHOX* gene defects. The ETR criterion was depicted as ETR −1.95 + 1/2 • height in meters, as used by Binder et al. [23] who suggested a cutoff at zero. SH/H, sitting height to height; ETR, extremities to trunk ratio.

region deletions, patients with intragenic *SHOX* defects had significantly higher SH/H ratio (mean difference ± SEM 1.17 ± 0.30, $p < 0.001$). All patients had a SH/H ratio >0 SDS, 94.2% a ratio >0.5 SDS, and 89.9% a ratio >1.0 SDS. SH/H ratio was strongly correlated with age in patients and controls ($p < 0.001$ in both). The Rappold score criterion of SH/H >55% has a sensitivity of 76.8% in patients with *SHOX* haploinsufficiency (83.3% in patients with intragenic *SHOX* defects, 61.9% in those with enhancer region deletions). SH/H ratio SDS was a good predictor of a *SHOX* defect, with an AUC of 0.913 (Fig. 1). We chose a cutoff of SH/H ratio >+1.0 SDS to optimize sensitivity (89.9%) with reasonable specificity (75.1%).

For 32 patients with sufficient data, Figure 2a shows that the arm span minus height is <0 in 90.6% and ≤−3 cm in 84.4%. When the arm span to height ratio is used (Fig. 2b), the Rappold score cutoff of <96.5% cm yields a sensitivity of 68.8% (80.0% in intragenic *SHOX* defects and 50.0% in enhancer region deletions). Although both the ratio and the arm span minus height are known to exhibit a slight association with age [43, 44], as was also present in our control cohort ($r = 0.327$ and $r = 0.281$, respectively), no statistical significant association was observed in our patients ($p = 0.175$ and $p = 0.849$, respectively). Figure 3 shows the ETR, which was significantly lower in patients with intragenic *SHOX* defects compared to those with enhancer region deletions (mean difference ± SEM 0.14 ± 0.06, $p = 0.028$). Using the Binder criterion, 71.0% of patients had a decreased ETR (85.0% in patients with intragenic *SHOX* defects, 45.5% in those with enhancer region deletions). Specificity of the Binder criterion was 89.6%. Figure 1 shows that, although the ETR performs better in the low sensitivity range, all 3 parameters that use arm span show equal AUC for sensitivities above 80%. Given the elaborate calculation of the ETR and easier interpretation of the arm span minus height compared to its ratio, we chose the arm span minus height as a screening criterion for *SHOX* haploinsufficiency. A cutoff of ≤−3 cm yields a high sensitivity of 84.4% with a reasonable specificity of 73.6%.

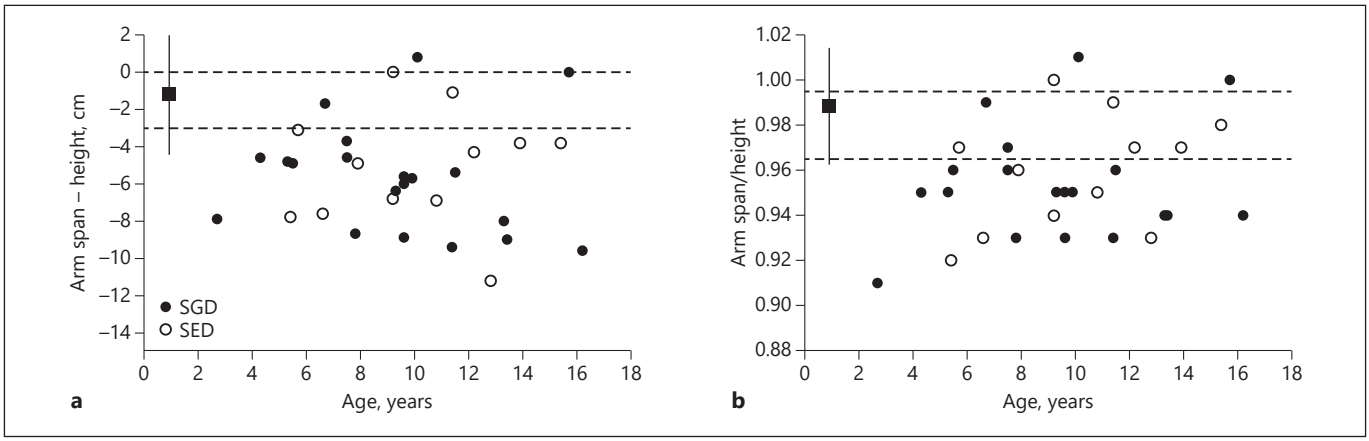

Fig. 2. Arm span minus height (**a**) or arm span divided by height (**b**) for patients with *SHOX* gene defect ($n = 21$) or SHOX enhancer region deletions ($n = 12$). Gray symbols denote patients with a genetic variant of unknown significance and phenotype suspect for *SHOX* haploinsufficiency. Dotted lines represent different cutoff points. The black square represents the control subjects' mean and SD. SGD, SHOX gene defect; SED, SHOX enhancer region deletions.

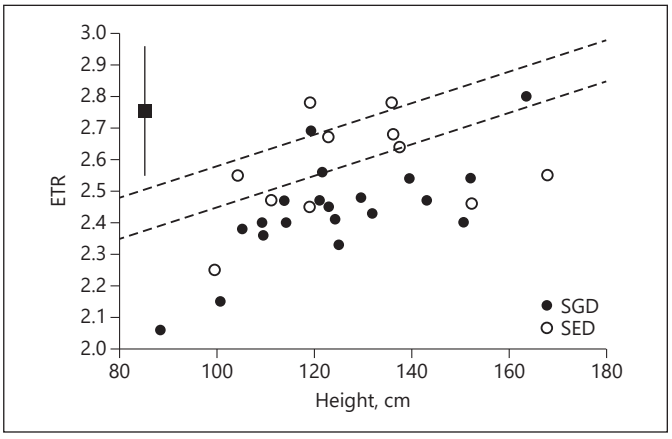

Fig. 3. ETR versus height for patients with *SHOX* gene defects ($n = 21$) or *SHOX* enhancer region deletions ($n = 10$). Gray symbols denote patients with a genetic variant of unknown significance and phenotype suspect for *SHOX* haploinsufficiency. Dotted lines represent different cutoff points. The black square represents the control subjects' mean and SD. ETR, extremities to trunk ratio; SGD, SHOX gene defect; SED, SHOX enhancer region deletions.

As can be appreciated in Figures 2–6, the 3 patients with variants that are classified as a VUS but considered as likely or possibly pathogenic (grey symbols) show similar characteristics as those classified as pathogenic (black symbols).

The affected parent's average height was –2.2 ± 0.9 SDS ($n = 49$, < –2 SDS in 57.1%), and median SH/H was 2.3 SDS ($n = 23$, interquartile range 1.5–4.2 SDS, >1 SDS in 82.6%). No significant correlation ($p = 0.655$) was observed between height SDS of the patient and the affected parent (Fig. 4a). Neither variant type nor gender significantly influenced this relation. Height of the unaffected parent was not significantly associated with patients' height ($p = 0.058$, Fig. 4b). Patients' height SDS significantly correlated with target height SDS ($p < 0.001$, Fig. 4c).

Combinations of Clinical Characteristics

Height <–2.0 SDS with an SH/H ratio >1.0 SDS is present in only 63.8% of patients (Table 2). Within the subgroup of patients with height <–2.0 SDS, however, all had an SH/H ratio >0 SDS, 88% >1 SDS, and 70% >2 SDS (Fig. 5).

Height <–2.0 SDS with arm span minus height ≤–3 cm is also present in only a small proportion of patients (56.3%, Table 2). Within patients with height <–2.0 SDS and with available arm span measurement ($n = 20$), 90% had an arm span minus height of ≤–3 cm and 55% of ≤–5 cm. The Binder criterion showed a sensitivity of 65% and a specificity of 90.8% within patients with height <–2.0 SDS.

Figure 6a and Table 2 show that a combined cutoff of SH/H ratio >1.0 SDS and arm span minus height ≤–3 cm yields a sensitivity of 77.4% if both criteria are met and 98.5% if either is met. Within patients with height <–2.0 SDS (Fig. 6b), sensitivity is 75% if both criteria are met and 95.0% if either are (specificity 84.4 and 55.9%, respectively). The combination of arm span to height ratio < 96.5% with SH/H ratio >55.5% as suggested by Rappold et al. [10] showed a sensitivity of 54.8% (36.4% in *SHOX* enhancer region mutations) and specificity of 95.2%.

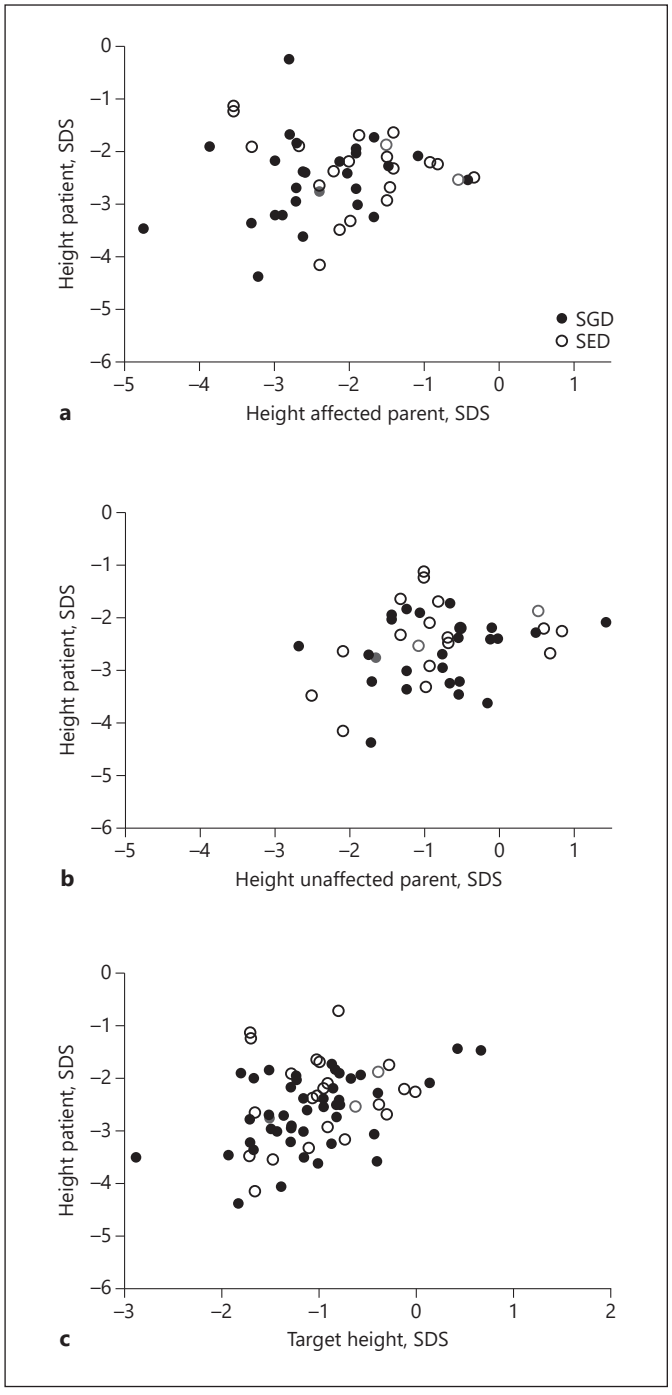

Fig. 4. Relation between patient height in SDS and height of the affected parent in SDS (**a**), height of the unaffected parent in SDS (**b**), and target height SDS (**c**). Gray symbols denote patients with a genetic variant of unknown significance and phenotype suspect for *SHOX* haploinsufficiency.

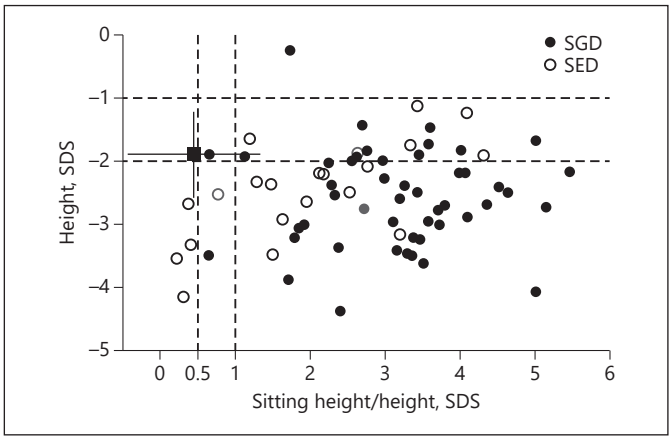

Fig. 5. SH/H in SDS versus height in SDS for patients with *SHOX* gene defects ($n = 48$) or *SHOX* enhancer region deletions ($n = 21$). Gray symbols denote patients with a genetic variant of unknown significance and phenotype suspect for *SHOX* haploinsufficiency. Dotted lines represent different cutoff points. The black square represents the control subjects' mean and SD. SH/H, sitting height to height; SGD, SHOX gene defect; SED, SHOX enhancer region deletions.

Proposed Criteria for SHOX Testing

We formulated revised selection criteria for testing for *SHOX* haploinsufficiency (Table 3). We propose to screen only those children with a height below –2 SDS (or in case of typical clinical features, see below), as these children will likely benefit most from rhGH treatment. Next, we chose a relatively high sensitivity for the cutoff of the 2 most distinctive auxological parameters, that is, SH/H SDS >1 or arm span minus height ≤–3 cm. When height <–2 SDS is combined with either SH/H SDS >1 or arm span minus height ≤–3 cm, sensitivity for the entire cohort is 68.1% (71.7% in intragenic *SHOX* defects and 56.5% in enhancer region deletions), specificity 80.6%, the positive likelihood ratio 3.5, and the negative likelihood ratio 0.4. Assuming a 2% pretest probability of a *SHOX* defect in children with a height SDS of <–2.0, the posttest odds using the total score would be 3.8%, resulting in a posttest probability of 6.7%, and a number needed to screen of 21 children. Sensitivity can be improved if also children with a height SDS in the lower half of the population range are investigated if either their arm span minus is ≤–3 cm or SH/H ratio is >1 SDS.

In addition, typical signs of Leri-Weill dyschondrosteosis present at physical examination or X-ray are considered highly specific and should warrant genetic analysis in all patients with a height below –1 SDS. Lastly, we advise to screen patients with short stature (height below –2

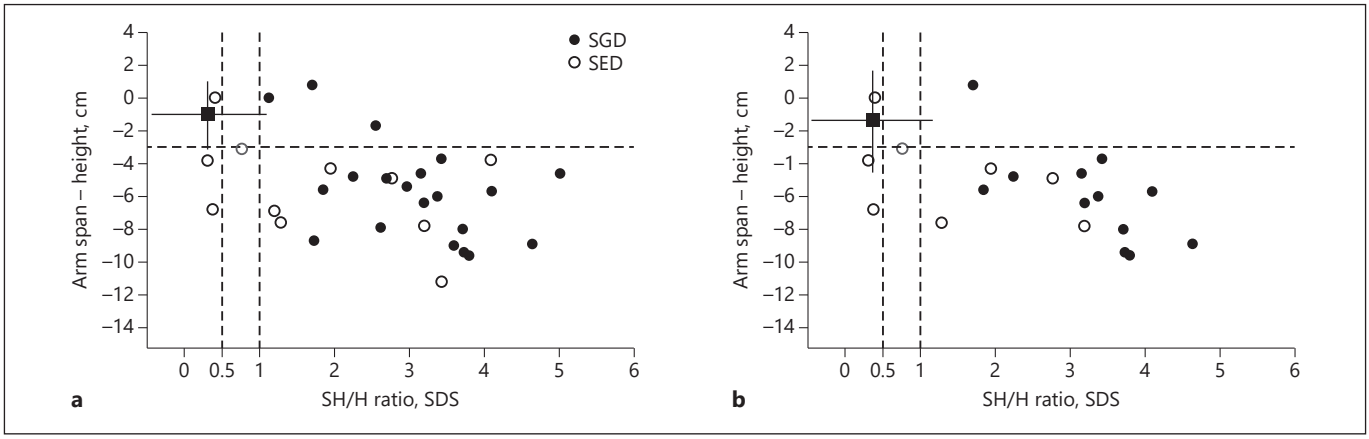

Fig. 6. Arm span minus height versus SH/H ratio SDS for patients with *SHOX* gene defects or *SHOX* enhancer region deletions in all patients (**a**, SGD $n = 20$, SED $n = 11$) and in patients with height below –2.0 SDS (**b**, SGD $n = 12$, SED $n = 8$). Gray symbols denote patients with a genetic variant of unknown significance and phenotype suspect for *SHOX* haploinsufficiency. Dotted lines represent cutoff points. The black square represents the control subjects' mean and SD. SH/H, sitting height to height; SGD, SHOX gene defect; SED, SHOX enhancer region deletions.

Table 2. Sensitivity, specificity and positive likelihood ratio of auxological criteria and their combinations in patients with *SHOX* haploinsufficiency and controls

Criterion	*SHOX* defects*	Controls*	Sensitivity	Specificity	LR+	LR–
Height SDS <–2.0	54 (76)	121 (277)	71.1	56.3	1.6	0.5
SH/H ratio SDS >+1	62 (69)	64 (261)	89.9	75.5	3.7	0.1
Arm span minus height ≤–3 cm	27 (32)	71 (254)	84.4	72.0	3.0	0.2
Either arm span minus height or SH/H ratio SDS criterion	65 (66)	103 (250)	98.5	58.8	2.4	0.03
Both arm span minus height or SH/H ratio SDS criterion	24 (31)	29 (250)	77.4	88.4	6.7	0.3
Height SDS <–2.0						
SH/H ratio SDS >+1	44 (69)	32 (261)	63.8	87.7	5.2	0.4
Arm span minus height ≤–3 cm	18 (32)	34 (254)	56.3	86.6	4.2	0.5
Both criteria	15 (31)	17 (250)	48.4	93.2	7.1	0.6
Either criterion	47 (69)	49 (253)	68.1	80.6	3.5	0.4

* Data represent number of patients that fulfil the criterion and in brackets the number of patients with available data for that criterion. SH/H, sitting height to length; LR+, positive likelihood ratio; LR–, negative likelihood ratio; SHOX, short stature homeobox-containing gene.

SDS) and a parent with either short stature (height below –2 SDS), typical signs of Leri-Weill dyschondrosteosis, or specific auxological signs, that is, SH/H >1 SDS or arm span ≥3 cm below height.

Discussion

We aimed to redefine criteria for genetic analysis that allow the discovery of pediatric patients with *SHOX* haploinsufficiency caused by either intragenic *SHOX* defects or deletions of its enhancer region. Based on data from 76 patients, the largest cohort to date that includes both types of *SHOX* haploinsufficiency, we formulated screening criteria that incorporate height SDS, SH/H SDS, arm span minus height, and typical clinical and radiological signs of Leri-Weill dyschondrosteosis (Table 3).

Previous criteria for genetic analysis were almost exclusively based on cohorts of patients with intragenic *SHOX* defects. However, patients with *SHOX* enhancer region deletions show a milder phenotype with respect to SH/H ratio SDS and ETR (Table 1). As a consequence, within the

Table 3. Clinical criteria for testing for variants in *SHOX* or its enhancer region

Test for variants in SHOX or its enhancer region if one of the following applies:
– Height below –2 SDS and either SH/H SDS >1 or arm span ≥3 cm below height
– Height below –1 SDS and typical signs of Leri-Weill dyschondrosteosis at either X-ray of hand/wrist/forearm or physical examination, that is, Madelung deformity, cubitus valgus, short forearm, muscular hypertrophy, dislocation of ulna
– Height below –2 SDS and a parent with either of the following: – Height SDS <–2 – SH/H SDS >1 and arm span ≥3 cm below height – Typical signs of Leri-Weill dyschondrosteosis (Madelung deformity, etc.,)

SHOX, short stature homeobox-containing gene; SH/H, sitting height to height.

group of patients with *SHOX* enhancer region deletions in our cohort, previous criteria such as the Rappold criteria for SH/H and arm span/height ratio are met in only 61.9 and 50.0%, respectively (and if both criteria are met, as required for the Rappold criterion of 4 points, in only 36.4%). The Binder criterion for ETR was met in only 45.5% [10, 23]. An additional disadvantage of the Rappold score is that it does not perform equally in different age groups, as the Rappold SH/H ratio criterion is not age dependent despite the strong correlation between SH/H ratio SDS and age ($p < 0.001$ in patients and controls).

In order to formulate novel screening criteria, several auxological parameters were studied in the total cohort of patients with intragenic *SHOX* defects and deletions of its enhancer region, aiming for high sensitivity with reasonable specificity. We used short stature (i.e., height < –2 SDS) as a starting point, as these children are most likely to be referred to the pediatric endocrinologist, and are expected to benefit most from treatment with rhGH. Within the 71.1% of patients fulfilling this criterion, we included the 2 parameters that were most distinctive and were irrespective of age; SH/H >1 SDS or arm span minus height ≤–3 cm. These parameters were present in 88 and 90% of patients with height < –2 SDS, respectively, and either one was present in 95%. Genoni et al. [33] reported a similar sensitivity of 89.5% in patients with short stature using a combination of growth velocity below –1.5 SDS and the Rappold score. However, accurate growth velocity might not be available at first presentation, as was the case in most of our patients who were referred to our tertiary centers. We used the difference between arm span and height instead of its ratio because the former seems more age independent than the latter [43, 45]. Instead of separate cutoff limits for SH/H and arm span minus height, the ETR can be used. However, this composite score does not diagnose *SHOX* haploinsufficiency better than the arm span to height ratio, as shown in the receiver operation characteristic curve, and is more elaborate to measure and calculate than the SH/H ratio SDS and arm span minus height. As our cohort also incorporated patients with height above –2 SDS, the sensitivity of the auxological criteria in our total cohort is 68.1%.

Next, we assessed the association between *SHOX* haploinsufficiency and parental height and found no correlation between height of the patient and affected parent. This implies that factors other than the specific *SHOX* defect explain the variability in height SDS within the group of patients with *SHOX* haploinsufficiency. One possible factor could be DNA polymorphisms inherited from the unaffected parent, although their height was also not significantly associated with that of the patient. Nevertheless, the affected parent often displayed short stature or increased SH/H, and we advise to screen for *SHOX* haploinsufficiency in patients with short stature and a parent with obvious auxological abnormalities such as height <–2 SDS, SH/H >1 SDS, or arm span ≥3 cm below height. Obviously, all patients with typical clinical or radiological signs of *SHOX* haploinsufficiency and height below –1 SDS should also be screened, as these features are considered highly specific [24].

Although this study is the first to date that combines data from a relatively large group of patients with intragenic *SHOX* defects and deletions of its enhancer region to formulate criteria for genetic analysis, there are several limitations that need to be addressed. First, using the height SDS below –2 SDS for all patients that should be screened excludes 28.9% of patients in our cohort. The reason we chose this criterion is that most patients referred to the pediatric endocrinologist for short stature will fulfill this criterion, these patients are expected to benefit most from rhGH, and within this group the criteria allow the detection of nearly all patients. Nevertheless, one must bear in mind that the excluded patients will not receive follow-up for the development of Madelung deformity (present in 59% of patients with height > –2 SDS), or genetic counselling in family planning. This implies that clinicians may consider testing for *SHOX* haploinsufficiency if height SDS is in the lower half of the population range, and there is a combination of relatively short arm span and relatively high SH/H ratio. Second, only

control subjects with a Rappold score of over 8 points or typical clinical and radiological features of Leri-Weil dyschondrosteosis were screened for *SHOX* defects. Therefore, we cannot completely exclude *SHOX* haploinsufficiency in the control group. Our estimation of the specificity may therefore be too conservative. Third, with the expanding use of growth-specific gene panels, multiplex ligation-dependent probe amplification, and whole exome sequencing, criteria for *SHOX* analysis may become less strict in the upcoming decennia, although one should realize that with current techniques whole exome sequencing based growth-specific gene panels cannot detect small deletions of the *SHOX* gene or its enhancers. Lastly, 2 variants in 3 patients were classified as likely or possibly pathogenic, but all 3 patients (and their affected family members) showed the typical disproportions associated with *SHOX* haploinsufficiency. A recent report calls for the appreciation of the clinical phenotype of patients suspected to have a well-described clinical syndrome when assessing the pathogenicity of their genetic variant [38]. This supports a more realistic reflection of daily practice, as was the case in our 3 patients that were considered *SHOX* haploinsufficient. As can be observed in Figures 2–6, omitting these cases does not significantly change the sensitivity and specificity of the proposed screening criteria.

In conclusion, we formulated novel criteria for screening for *SHOX* haploinsufficiency based on a cohort of 76 patients with either intragenic *SHOX* defects or deletions of its enhancer region and 277 controls referred for short stature. The criteria are highly sensitive to detect *SHOX* haploinsufficiency within patients with short stature and facilitate the diagnosis, follow-up, and treatment of these patients and their affected relatives.

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Statement of Ethics

Written informed consent was obtained from participants, and the study protocol was approved by the Medical Ethical Committee of the Leiden University Medical Center.

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Author Contributions

S.D.J.: analyzed and interpreted the data. S.H.D.: acquired the patient data. G.A.K. and S.E.S.: collected the control data. M.L.: was responsible for the genetic analyses. S.G.K., C.B., and W.O.: helped with acquisition and interpretation of data. J.M.W.: conceptualized the study. All authors aided in writing the manuscript, all approved the final version, and all agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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