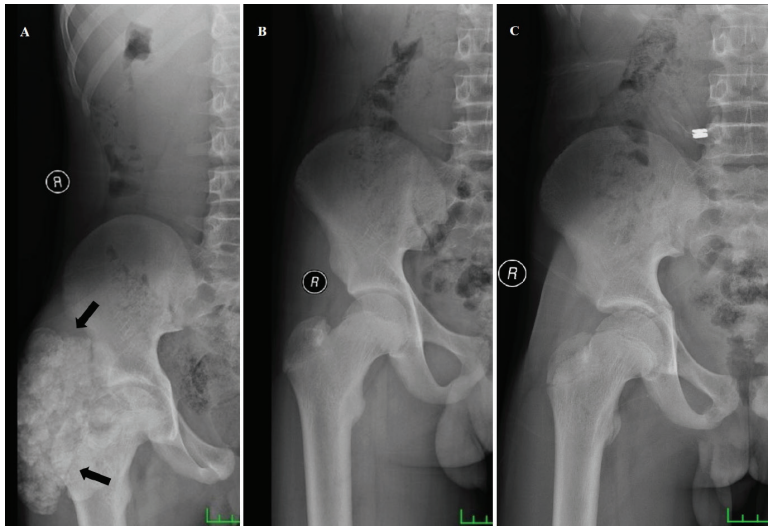
JCRPE

Journal of Clinical Research in Pediatric Endocrinology

June 2022 | volume 14 | issue 2 | www.jcrpe.org | ISSN: 1308-5727

E-ISSN: 1308-5735



(A) Soft tissue calcification on the anteroposterior radiograph of the right hip before the treatment (black arrows). (B) 3 months after topical STS and acetazolamide. (C) 36 months after acetazolamide and topical STS treatments were stopped

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239-243

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Date of printing: June 2022
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JCRPE Journal of Clinical Research in Pediatric Endocrinology
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DOI: 10.4274/jcrpe.galenos.2021.2021.0169

J Clin Res Pediatr Endocrinol 2022;14(2):131-144

Efficacy and Safety of Letrozole in the Management of Constitutional Delay in Growth and Puberty: A Systematic Review and Meta-analysis

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Abstract

No meta-analysis is available which has analysed the role of letrozole in constitutional delay in growth and puberty (CDGP). Electronic databases were searched for randomized controlled trials (RCTs) involving children with CDGP receiving letrozole. Primary outcomes were changes in predicted adult height (PAH) and pubertal progression. Secondary outcomes were alterations in bone age (BA), hormonal markers of puberty, bone mineral density and side-effects. One hundred-thirty articles were reviewed, from which seven RCTs which fulfilled all criteria were analysed. Letrozole was superior to placebo [mean difference (MD) 4.63 cm (95% confidence interval (CI): 3.90-5.36); $p < 0.01$; I2 = 0%] but not testosterone [MD: 2.21 cm (95% CI: -1.71-6.16); $p = 0.27$; $I^2 = 98\%$] with regards to improvement in PAH after 12-months use. Letrozole was superior to both placebo [MD: 4.80 mL (95% CI: 0.57-9.03); $p = 0.03$] and testosterone [MD: 3.36 mL (95% CI: 0.58-6.75); $p = 0.02$; $I^2 = 0\%$] with regards to improvement in testicular volume after 12-months use. Letrozole tended to be superior to testosterone [MD: -0.84 years (95% CI: 2.83-8.18); $p = 0.06$; $I^2 = 0\%$] with regards to slowing in BA progression after 12-months use. Serum luteinizing hormone, follicle stimulating hormone, testosterone and inhibin-B were significantly higher after 6-months letrozole use compared to active as well as passive controls. No increased occurrence of adverse events, including spinal deformities, were noted with letrozole. Letrozole is safe and effective for improving height and pubertal outcomes in CDGP, and is better than testosterone with regards to improvement in testicular volume and may be better at delaying bone-age progression.

Keywords: Letrozole, meta-analysis, safety, constitutional delay in growth and puberty, short stature

Introduction

Constitutional delay in growth and puberty (CDGP) is perhaps the most common cause of short stature in both the sexes; and for not yet determined reasons, is much more common in boys than girls (1). The diagnosis of CDGP is often a diagnosis of exclusion (2). Children with CDGP have delayed pubertal growth spurt and usually have catch-up growth with the late onset of puberty, by 18 years age (2). Although reassurance and watchful waiting is recommended in CDGP, many children with CDGP in their teenage years have associated significant psychosocial stress, negative interaction with peers, anxiety or depression, warranting medical intervention (3). Suggested interventions include medications which will promote sexual maturation (4). Traditionally, low dose testosterone injections/oxandrolone and ethinyl estradiol/estradiol patches have been tried to accelerate puberty in boys and girls, respectively, for many decades now (4). Recently a few trials have been published which have suggested that letrozole may have a role in activation of the hypothalamic-pituitary-gonad (HPG) axis and faster testicular growth, resulting in accelerated height growth and pubertal progression in CDGP (5,6).

Theoretically, aromatase inhibitors are uniquely suited to manage different aspects of CDGP. Letrozole would delay bone maturation by inhibiting conversion of testosterone to estradiol and thereby lowering blood estradiol concentrations

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Conflict of interest: None declared
Received: 01.07.2021
Accepted: 02.09.2021

(7). Letrozole also has the potential to induce maturation of the HPG axis by decreasing the negative feedback loop from estradiol to the hypothalamus. Some safety concerns include reduced bone density, which have primarily been documented in adult cancer survivors (8).

However, to date, no meta-analysis is available which has holistically analysed and summarized the clinical efficacy and safety of letrozole in CDGP. Hence the aim of this meta-analysis was to evaluate the efficacy and safety of letrozole in the management of CDGP.

Methodology

The meta-analysis was carried out according to the recommendations of the Cochrane Handbook for Systematic Reviews of Interventions (9). The predefined protocol has been registered in PROSPERO, having the registration number CRD42021250345. All randomized controlled trials (RCTs) published up to March 2021 were considered for this meta-analysis. This meta-analysis has been reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria, the filled checklist of which can be found at the end of the manuscript (9). Since ethical approval already exists for the individual studies included in the meta-analysis, no separate approval was required for this study.

The PICOS criteria was used to screen and select the studies for this meta-analysis with patients (P) being children diagnosed with CDGP; intervention (I) being the use of letrozole for managing CDGP; control (C) being patients either on placebo or any other approved medication for managing CDGP, which included testosterone, oxandrolone or estradiol; outcomes (O) being evaluated were impact on predicted adult height (PAH), height standard deviation score (Ht-SDS), bone age (BA), clinical and hormonal measures of puberty and any adverse effects noted; and (S) being studies included which were RCTs. Only children with CDGP were considered for this meta-analysis. Children with other forms or causes of short stature, such as familial short stature, growth hormone deficiency, panhypopituitarism, syndromic short stature, and idiopathic short stature were excluded. Only those studies were included in this meta-analysis which had at least two treatment groups of children with CDGP, with one of the groups receiving letrozole and the other group receiving either placebo or any other medication in place of letrozole.

The primary outcomes were to evaluate the changes in PAH and pubertal progression, as determined by testicular volume. The secondary outcomes were to evaluate the alterations in Ht-SDS, final height, BA, hormonal markers of puberty [including testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol, inhibin-B, and anti-Müllerian hormone], bone mineral density (BMD), body composition changes (lean mass, fat mass), and any side effects reported. Analysis of the growth and puberty outcomes was based on whether the control group received an active comparator (like testosterone, oxandrolone or any other approved medication for use in CDGP) - labelled here as the active control group (ACG) or a placebo/nothing - labelled as passive/placebo control group (PCG).

Search Method for Identification of Studies

A detailed search was done of electronic databases of Medline (Via PubMed), Embase (via Ovid SP), Cochrane central register of controlled trials (CENTRAL) (for trials only), ctri.nic.in, clinicaltrials.gov, global health and Google scholar using a Boolean search strategy: (letrozole) AND [(delayed puberty) OR (short stature)].

Data Extraction and Study Selection

Data extraction was carried out independently by two authors using standard data extraction forms. In cases where more than one publication of a single study group were found, results were grouped and relevant data from each report were used in the analyses. Data on the primary and secondary outcomes, as stated above, was extracted. Patient characteristics (including demographic information and comorbidities) from the different studies included and excluded from the analysis were noted in a tabular form (Table 1, 2). All disagreements were resolved by the third and fourth authors.

Assessment of Risk of Bias in Included Studies

Three authors independently assessed the risk of bias using the risk of bias assessment tool in Review Manager (Revman) Version 5.3 (The Cochrane Collaboration, Oxford, UK, 2014) software. The following points were taken into consideration when assessing if there was adequate sequence generation to rule out selection bias. We examined if the patient group allocation reconcealed adequately to rule out selection bias. We also looked for whether researcher knowledge of the allocated interventions was adequately prevented during the study or not. Participants and personnel blinding was specifically looked for to rule out performance bias. We also looked for the blinding of the outcome assessors to rule out detection bias. We looked for whether incomplete outcome data issue was adequately addressed or not to rule out attrition bias. We also looked at if the reports of the study

were free of suggestion of selective outcome reporting to rule out reporting bias. Lastly we also looked for whether the study was apparently free of other problems that could put it at risk of bias. Any disagreements were resolved by the fourth author. The risk of bias has been elaborated in Supplementary Table 1.

Measures of Treatment Effect

For continuous variables, the outcomes were expressed as mean differences (MD). International system (SI) units were used for analysis, and all studies reporting results in conventional units were converted to SI units for analysis. For dichotomous outcomes (treatment success) results were expressed as risk ratios (RR) with 95% confidence intervals (CI). For adverse events, results were expressed as post treatment absolute risk differences. RevMan 5.3 was used to compare MD of the different primary and secondary outcomes between letrozole and the control groups of the included studies.

Assessment of Heterogeneity

Heterogeneity was initially assessed by studying the forest plot generated for the primary and secondary outcomes of this study. Subsequently, heterogeneity was analysed using a chi^2 test on N-1 degrees of freedom, with an alpha of 0.05 used for statistical significance and with the I^2 test (10). The

Table 1. Characteristics of patients in the different randomized controlled trials evaluated in this meta-analysis on use of letrozole in constitutional delay in growth and puberty

Study details	Number of patients in letrozole & control groups	Patient characteristics and nature of controls	Duration of study
Hero et al (2006) (13)	Lz + T (n = 9) T + Placebo (n = 8) Testosterone was used at a dose of 1 mg/kg i.m. every 4 weeks for 6 months	Children with testis volume < 4 mL after 13.5 years of age.	Period of intervention 52 weeks; follow up ~ 4 years
Hero et al (2010) (14)	Lz + T (n = 6) T + Placebo (n = 6) Testosterone was used at a dose of 1 mg/kg intramuscularly every 4 weeks for 6 months	Children with testis volume < 4 mL after 13.5 years of age.	52 weeks
Kohva et al (2020) (15)	Lz (n = 15), T (n = 13)	The inclusion criteria were testicular volume between 2.5 and 4 mL and serum T < 5 nmol/L or serum T ≥1 nmol/L, if the mean testicular volume was < 2.5 mL, or Tanner genital stage 2 and serum T < 3 nmol/L. At the start of the trial, the boys were above 14 years of age. Controls were similar to patients	52 weeks
Rohani et al (2019) (16)	Lz (n = 8) Placebo (n = 8)	PAH < 1 SD MPH and Tanner pubic hair stage delayed by > SD or TV < = 3 mL	Follow up ~ 8 years
Salehpour et al (2010a) (6)	Lz (n = 31) Oxandrolone (n = 30)	12.6-14.6 years old boys with PAH < 1 SD MPH and Tanner pubic hair stage delayed by > SD or TV < = 3 mL	Intervention 104 weeks; follow up 260 weeks
Salehpour et al (2010b) (6)	Lz (n = 31) Placebo (n = 30)	12.6-14.6 years old boys with PAH < 1 SD MPH and Tanner pubic hair stage delayed by > SD or TV < = 3 mL	Intervention 104 weeks; follow up 260 weeks
Varimo et al (2019) (5)	Lz (n = 15) T (n = 15) (testosterone (Sustanon 250; Aspen Nordic, Ballerup, Denmark) was injected intramuscularly every 4 weeks for 6 months (six injections in total)	The inclusion criteria were testicular volume between 2.5 and 4 mL and serum T < 5 nmol/L or serum T ≥1 nmol/L, if the mean testicular volume was < 2.5 mL, or Tanner genital stage 2 and serum T < 3 nmol/L. At the start of the trial, the boys were above 14 years of age. Controls were similar to patients	Intervention 26 weeks; follow up 52 weeks
Wickman et al (2001) (17)	Lz + T (n = 10) T + Placebo (n = 12) (testosterone enanthate (Testoviron- Depot-250, Schering, Berlin, Germany) six times at a dose of 1 mg/kg intramuscularly every 4 weeks, and placebo orally once daily for 12 months) Placebo (n = 10)	Children with testis volume < 4 mL after 13.5 years of age.	18 months

Lz: letrozole, T: testosterone, LH: luteinizing hormone, FSH: follicle stimulating hormonel, IGF: insulin like growth factor, IGFBP: insulin like growth factor binding protein, HDL: high density lipoprotein, BMD: bone mineral density, MPH: mid parental height, SD: standard deviation, TV: testicular volume, PAH: predicted adult height; letrozole was used at a dose of 2.5 mg/day for the duration of the study in all the above RCTs; Oxandrolone was used at dose of 2.5 mg/day in the study by Salehpour et al (6)

interpretation of I^2 values was as follows: 0% to 40%: might not be important; 40% to 60%: may represent moderate heterogeneity; 60% to 90%: may represent substantial heterogeneity; 90% to 100%: considerable heterogeneity. The importance of the observed value of I^2 depends on the magnitude and direction of treatment effects and the strength of the evidence for heterogeneity (e.g. p value from the chi^2 test, or a CI for I^2) (10).

Grading of the Results

An overall grading of the evidence (certainty of the evidence) related to each of the primary and secondary outcomes of the meta-analysis was done using the GRADE (Grades of Recommendation, Assessment, Development and Evaluation) approach (11). The GRADE approach defines the quality of a body of evidence as the extent to which one can be confident that an estimate of effect or association is close to the true quantity of specific interest. The quality of a body of evidence involves consideration of within-trial risk of bias (methodological quality), directness of evidence, heterogeneity, precision of effect estimates and risk of publication bias (11). The GRADEpro Guideline Development Tool software (McMaster University and Evidence Prime Inc, 2015) was used to create the Summary of Findings (SoF) table in this meta-analysis (Table 3). The "certainty of evidence" has been graded into four categories, namely "high" (there is a lot of confidence that the true effect lies close to that of the estimated effect), "moderate" (there is moderate confidence in the estimated effect: The true effect is likely to be close to the estimated effect, but there is a possibility that it is substantially different), "low" (there is limited effect in the estimated effect: The true effect might be substantially different from the estimated effect) and, "very low" (there is very little confidence in the estimated effect: The true effect is likely to be substantially different from the estimated effect) (11).

Publication bias was assessed by plotting the Funnel Plot, which specifically targets small study bias, in which small studies tend to show larger estimates of effects and greater variability than larger studies (9). Presence of one or more of the smaller studies outside the inverted funnel plot was taken as evidence of presence of significant publication bias (Supplementary Figure 1) (12).

Data Synthesis

Data was pooled as a random effect model for the analysis of primary and secondary outcomes. The outcomes were expressed as 95% CI. Forrest plots were plotted with the left side of the graph favouring letrozole and the right side of the graph favouring control using RevMan 5.3 software. A $p < 0.05$ was considered statistically significant.

Results

A total of 357 articles were found after the initial search (Figure 1). Following the screening of the titles, and abstracts, the search came down to 173 articles. Thirty eight duplicates were removed. One hundred and thirty articles were reviewed in details from which seven RCTs which fulfilled all inclusion and exclusion criteria were included in the meta-analysis (Figure 1) (5,6,13,14,15,16,17). One study was removed due to lack of a valid control group (18). Three studies evaluating impact on growth of letrozole in children with precocious puberty were excluded (19,20,21).

Of the seven studies included in this meta-analysis, analysis was done based on the nature of the control group. The control was testosterone in the studies by Kohva et al (2020) (15) and Varimo et al (2019) (5) and hence the results from these studies have been analysed in the ACG. The controls were placebo in the studies by Hero et al (2006) (13), Hero et al (2010) (14), and Wickman et al (2001) (17), and hence

Table 2. Characteristics of patients in the different studies evaluated but excluded this meta-analysis

Study details	Number of patients in study & control groups	Patient characteristics and nature of controls	Duration of study	Reasons for exclusion
Karmazin et al 2005 (19)	6 children on androgen + Lz. No control group	Definition of case not clearly congruent with CDGP. There is also mention of GH replacement in 3 patients.	12 months	Lack of matched control group
Zhao et al 2014 (18)	22 children. No control group	Study done in children with idiopathic central precocious puberty	6 months	Study not done in children with CDGP
Neely et al 2014 (20)	Lz 17 Anastrazole 22	Short stature with reduced PAH in prepubertal boys. ISS and CDGP not differentiated	24 months	Lack of a matched control group Not a randomized controlled trial
Xu et al 2021 (21)	GH + Lz single arm	Short stature with reduced PAH in prepubertal boys. ISS and CDGP not differentiated	12 months	Lack of a matched control group Not a randomized controlled trial

Lz: letrozole, GH: growth hormone, PAH: predicted adult height, ISS: idiopathic short stature, CDGP: constitutional delay in growth and puberty

the results from these studies have been analysed in the PCG. In the study by Rohani et al (2019) (16), the control group received nothing and hence its results have been analysed in the PCG). The study by Salehpour et al (2010) (6) has three groups comparing the outcomes of children with CGDP receiving letrozle, oxandrolone or placebo. Hence the results of comparison between children receiving letrozole vs oxandrolone in this study has been presented as Salehpour et al 2010a (6) under the ACG. The results of comparison between children receiving letrozole vs placebo in this study has been presented as Salehpour et al 2020b (6) under the PCG.

The details of the studies included in this meta-analysis have been elaborated in Table 1. The studies which were evaluated but were excluded have been summarized in Table 2.

Risk of Bias in the Included Studies

The summaries of risk of bias of the seven studies included in the meta-analysis have been elaborated in Figure 2a, Figure 2b and Supplementary Table 1. Random sequence generation, reporting bias and other bias were judged to be at low risk of bias in all seven studies (100%). Source of funding, especially pharmaceutical, authors from the pharmaceutical organizations and conflict of interests were looked into in the "other bias" section. Allocation concealment bias (selection bias) were judged to be low risk in two studies (28.57%). Performance bias (blinding of participants and investigators) and detection bias (blinding of outcome assessors) were judged to be at low risk of bias in four out of seven studies (57.14%). Attrition bias was judged to be at low risk in six out of seven studies (85.71%).

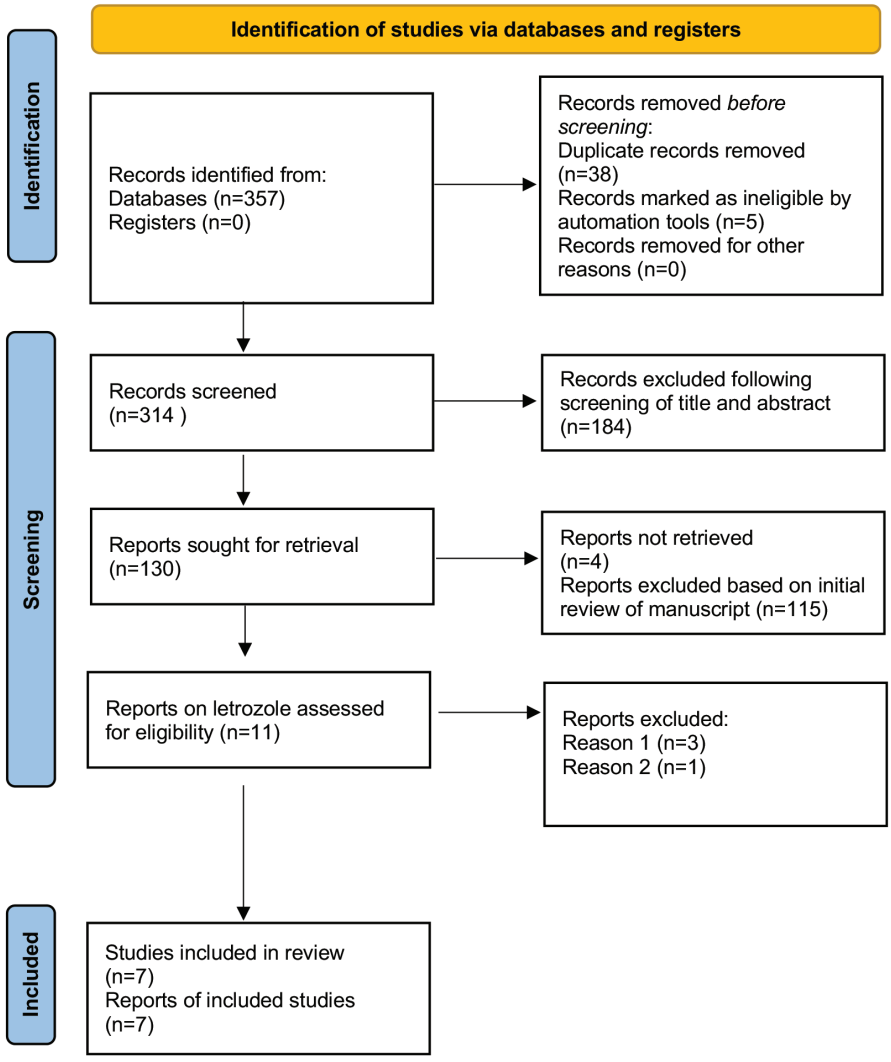


Figure 1. Flowchart elaborating on study retrieval and inclusion in the meta-analysis

RCT: randomized controlled trial

Effect of Letrozole on Primary Outcomes

Predicted Adult Height

Data from two studies involving 88 children with CDGP was analysed to find out the impact of letrozole on PAH after at least 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). Individuals receiving letrozole had a greater improvement in PAH but was statistically not significant when compared to those in the ACG [MD: 2.21 cm (95% CI: -1.71-6.16); $p = 0.27$; $I^2 = 98\%$ (considerable heterogeneity); Figure 3a; moderate certainty of evidence (MCE); Table 3]. Data from three studies involving 84 children with CDGP was analysed to find out the impact of letrozole on PAH after at least 12 months of treatment, when compared to those receiving placebo (PCG). Individuals receiving letrozole had a significantly greater improvement in PAH when compared to PCG [MD: 4.63 cm (95% CI: 3.90-5.36); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 3b; high certainty of evidence (HCE); Table 3].

Testicular Volume

Data from two studies involving 57 children with CDGP was analysed to find out the impact of letrozole on testicular volume after 6 and 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). After 6 months of treatment, children receiving letrozole had a significantly greater increase in testicular volume when compared to those in the ACG [MD 5.51 mL (95% CI: 2.83-8.18); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 3c; HCE; Table 3], which persisted even after 12 months of treatment [MD: 3.36 mL (95% CI: 0.58-6.75); $p = 0.02$; $I^2 = 0\%$ (low heterogeneity); Figure 3d; HCE; Table 3].

Data from only one study [Wickman et al (2001) (17)] involving 19 children with CDGP was analysed to find out the impact of letrozole on testicular volume after 12 and 18 months of treatment, when compared to those receiving placebo (PCG). Children receiving letrozole had significantly higher testicular volume after 12 months [MD: 4.80 mL (95% CI: 0.57-9.03); $p = 0.03$] but not after 18 months of therapy [MD: 1.90 mL (95% CI: -2.61-6.41); $p = 0.41$], when compared to those receiving placebo.

Effect of Letrozole on Secondary Outcomes

Height Standard Deviation Score

Data from one study [Salehpour et al (6)] involving 61 children was available comparing the changes in Ht-SDS after at least 12 months of therapy in ACG. Data from two studies [Salehpour et al (6) and Rohani et al (16)] involving 65 children were available comparing the changes in Ht-SDS after at least 12 months of therapy in PCG. Children receiving letrozole had a significantly greater improvement in Ht-SDS when compared to those receiving placebo in

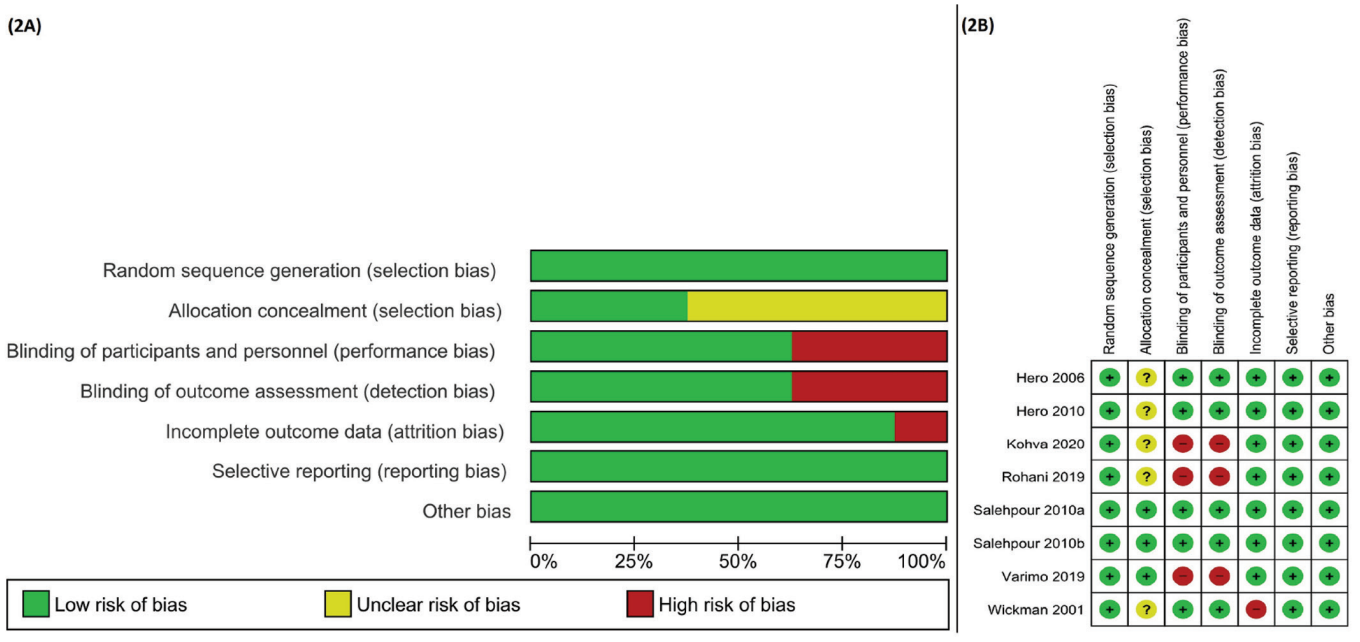


Figure 2. a) Risk of bias graph: review authors' judgements about each risk of bias item presented as percentages across all included studies. b) Risk of bias summary: review authors' judgements about each risk of bias item for each included study

PCG [MD: +0.63 (95% CI: 0.52-0.74); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity)], but not when compared to those receiving testosterone (ACG) [MD: 0.00 (95% CI: -0.16-0.16); $p = 1.00$].

Bone Age

Data from two studies involving 88 children was analysed comparing the changes in BA after 12 months of therapy with letrozole, as compared to those receiving testosterone in the ACG. When compared to ACG, children receiving letrozole has a slower progression in BA, which approached statistical significance [MD: -0.84 years (95% CI: 2.83-8.18); $p = 0.06$; $I^2 = 0\%$ (low heterogeneity); Figure 3e; low certainty of evidence; Table 3].

Data from three studies involving 88 children was analysed comparing the changes in BA after 12 months of therapy with letrozole, as compared to those receiving placebo (PCG). When compared to PCG, children receiving letrozole has similar progression in BA [MD: 0.06 years (95% CI: -0.88-0.99); $p = 0.91$; $I^2 = 90\%$ (considerable heterogeneity); Figure 3f].

Luteinizing Hormone

Data from two studies involving 55 children with CDGP was analysed to find out the impact of letrozole on serum LH after 6 and 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum LH was significantly higher after 6 months [MD: 5.28 IU/L (95% CI: 3.16-7.40); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 4a], but not after 12 months [MD: 0.05 IU/L (95% CI: -0.69-0.79); $p = 0.90$; $I^2 = 0\%$ (low heterogeneity); Figure 4b] of treatment with letrozole, when compared to ACG.

Table 3. Summary of findings: Letrozole compared to control in the management of constitutional delay in growth and puberty a systematic review and meta-analysis

Patient or population: Managing constitutional delay in growth and puberty A systematic review and meta-analysis
Setting:
Intervention: Letrozole
Comparison: Control

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	No of participants (studies)	Certainty of the evidence (GRADE)
	Risk with control	Risk with letrozole			
PAH ACG	The mean PAH ACG was **175.5** cm	MD **2.21 cm higher** (1.71 lower to 6.13 higher)	-	88 (2 RCTs)	⊕⊕⊕◯ MODERATE[a]
PAH PCG	The mean PAH PCG was **173.85** cm	MD **4.71 cm higher** (3.97 higher to 5.45 higher)	-	72 (2 RCTs)	⊕⊕⊕⊕ HIGH
Progression in bone age ACG	The mean progression in bone age ACG was **13.65** years	MD **0.84 years lower** (1.73 lower to 0.05 higher)	-	88 (2 RCTs)	⊕⊕◯◯ LOW[b,c]
Testicular volume 6 months ACG	The mean testicular volume 6 months ACG was **5.65** mL	MD **5.51 mL higher** (2.83 higher to 8.18 higher)	-	57 (2 RCTs)	⊕⊕⊕⊕ HIGH
Testicular volume 12 months ACG	The mean testicular volume 12 months ACG was **9.6** mL	MD **3.66 mL higher** (0.58 higher to 6.75 higher)	-	57 (2 RCTs)	⊕⊕⊕⊕ HIGH
Testosterone 12 months ACG	The mean testosterone 12-24 months ACG was **19.6** nmol/l	MD **0.6 nmol/l lower** (2.35 lower to 1.15 higher)	-	118 (3 RCTs)	⊕⊕⊕⊕ HIGH
Testosterone 12 months PCG	The mean testosterone 12 months PCG was **16.36** nmol/l	MD **32.37 nmol/l higher** (10.58 higher to 54.16 higher)	-	89 (3 RCTs)	⊕⊕⊕◯ MODERATE[c]
TAEs	90 per 1,000	**57 per 1,000** (17 to 177)	**OR 0.61** (0.17 to 2.17)	235 (8 RCTs)	⊕⊕⊕⊕ HIGH
SAEs	54 per 1,000	**65 per 1,000** (16 to 232)	**OR 1.22** (0.28 to 5.28)	235 (8 RCTs)	⊕⊕⊕⊕ HIGH

*__The risk in the intervention group__ (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: confidence interval, MD: mean difference, OR: odds ratio, ACG: active control group, PCG: passive/placebo control group, PAH: predicted adult height, TAEs: total adverse events, SAEs: severe adverse events.
GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.
Explanations
[a]: Due to large variation in effect, CIs do not overlap, p value for heterogeneity is < 0.01 and I^2 is more than 90%.
[b]: Due to large variation in effect, CIs do not overlap, I^2 is more than 80%.
[c]: High publication bias suspected as evidenced by the funnel plot (Supplementary Figure 1).

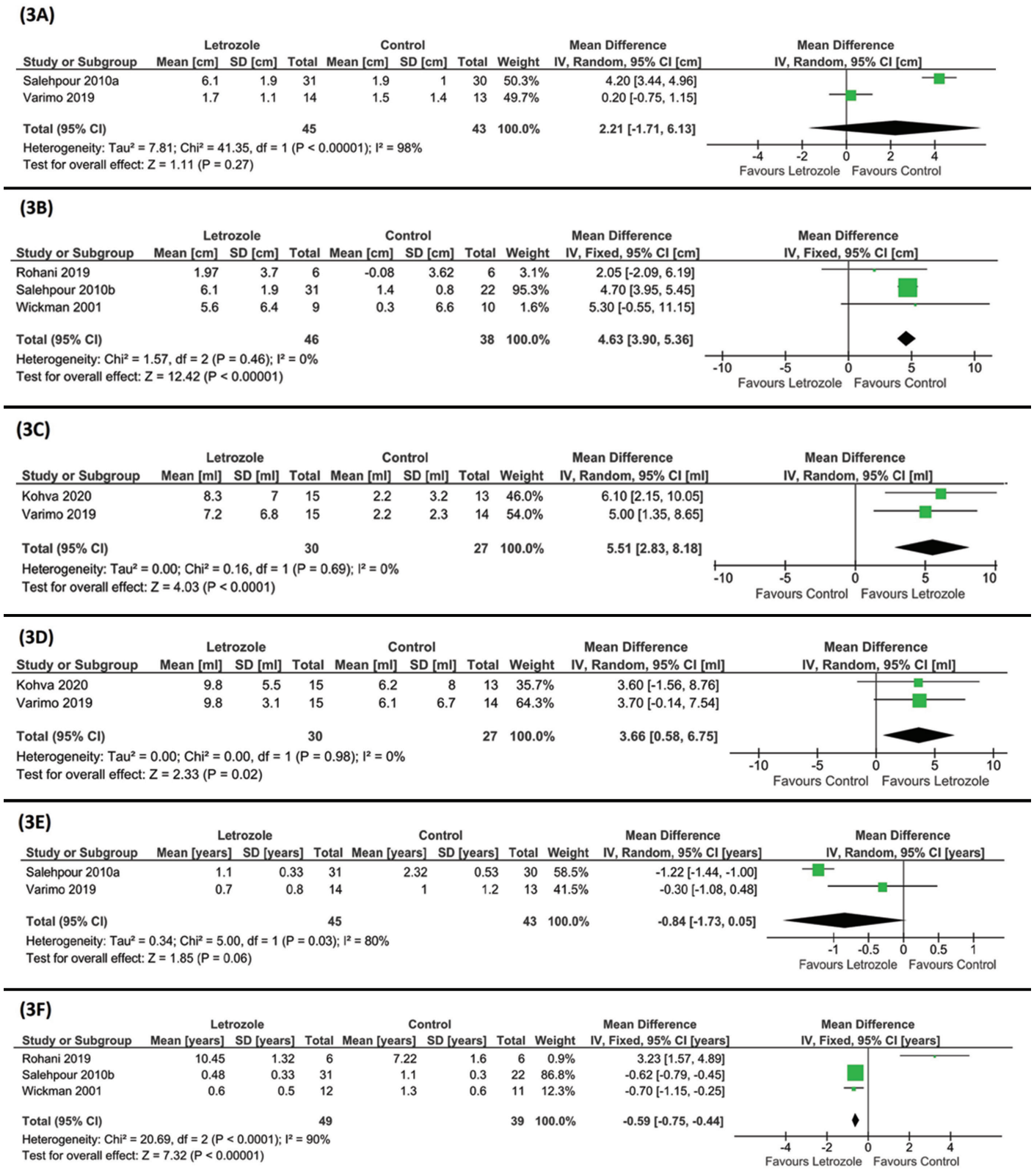


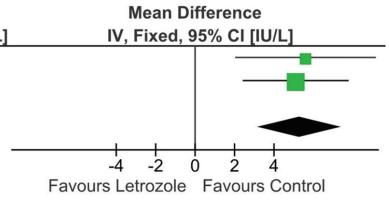
Figure 3. Forest plot highlighting the impact of letrozole on (a) Predicted adult height (PAH) in the ACG; (b) PAH in the PCG; (c) Testicular volume at 6 months in the ACG; (d) Testicular volume at 12 months in the ACG; (e): Bone age progression in ACG; (f): Bone age progression in PCG

ACG: active control group, PCG: passive/placebo control group

(4A)

Study or Subgroup	Letrozole Mean [IU/L]	Letrozole SD [IU/L]	Letrozole Total	Control Mean [IU/L]	Control SD [IU/L]	Control Total	Weight	Mean Difference IV, Fixed, 95% CI [IU/L]
Kohva 2020	5.5	6.7	15	-0.1	1.8	13	36.0%	5.60 [2.07, 9.13]
Varimo 2019	5	4.7	14	-0.1	1.8	13	64.0%	5.10 [2.45, 7.75]
Total (95% CI)			**29**			**26**	**100.0%**	**5.28 [3.16, 7.40]**

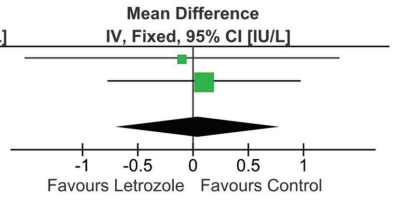
Heterogeneity: Chi² = 0.05, df = 1 (P = 0.82); I² = 0%
Test for overall effect: Z = 4.88 (P < 0.00001)



(4B)

Study or Subgroup	Letrozole Mean [IU/L]	Letrozole SD [IU/L]	Letrozole Total	Control Mean [IU/L]	Control SD [IU/L]	Control Total	Weight	Mean Difference IV, Fixed, 95% CI [IU/L]
Kohva 2020	0.8	1.8	15	0.9	2	13	27.2%	-0.10 [-1.52, 1.32]
Varimo 2019	0.3	1.2	14	0.2	1.1	13	72.8%	0.10 [-0.77, 0.97]
Total (95% CI)			**29**			**26**	**100.0%**	**0.05 [-0.69, 0.79]**

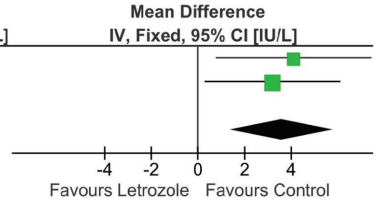
Heterogeneity: Chi² = 0.06, df = 1 (P = 0.81); I² = 0%
Test for overall effect: Z = 0.12 (P = 0.90)



(4C)

Study or Subgroup	Letrozole Mean [IU/L]	Letrozole SD [IU/L]	Letrozole Total	Control Mean [IU/L]	Control SD [IU/L]	Control Total	Weight	Mean Difference IV, Fixed, 95% CI [IU/L]
Kohva 2020	4.5	6.1	15	0.4	2.2	13	43.1%	4.10 [0.79, 7.41]
Varimo 2019	3.4	4.6	14	0.2	2.9	13	56.9%	3.20 [0.32, 6.08]
Total (95% CI)			**29**			**26**	**100.0%**	**3.59 [1.42, 5.76]**

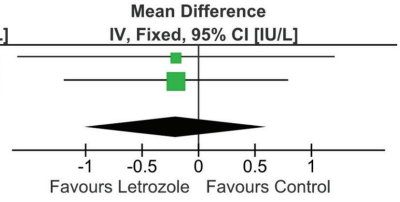
Heterogeneity: Chi² = 0.16, df = 1 (P = 0.69); I² = 0%
Test for overall effect: Z = 3.24 (P = 0.001)



(4D)

Study or Subgroup	Letrozole Mean [IU/L]	Letrozole SD [IU/L]	Letrozole Total	Control Mean [IU/L]	Control SD [IU/L]	Control Total	Weight	Mean Difference IV, Fixed, 95% CI [IU/L]
Kohva 2020	0.6	1.6	15	0.8	2.1	13	33.2%	-0.20 [-1.60, 1.20]
Varimo 2019	0.1	1.2	14	0.3	1.4	13	66.8%	-0.20 [-1.19, 0.79]
Total (95% CI)			**29**			**26**	**100.0%**	**-0.20 [-1.01, 0.61]**

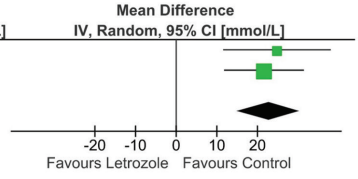
Heterogeneity: Chi² = 0.00, df = 1 (P = 1.00); I² = 0%
Test for overall effect: Z = 0.49 (P = 0.63)



(4E)

Study or Subgroup	Letrozole Mean [mmol/L]	Letrozole SD [mmol/L]	Letrozole Total	Control Mean [mmol/L]	Control SD [mmol/L]	Control Total	Weight	Mean Difference IV, Random, 95% CI [mmol/L]
Kohva 2020	28.3	25.1	15	3.5	5.7	13	35.3%	24.80 [11.73, 37.87]
Varimo 2019	28	18.6	15	6.4	4.1	14	64.7%	21.60 [11.95, 31.25]
Total (95% CI)			**30**			**27**	**100.0%**	**22.73 [14.96, 30.50]**

Heterogeneity: Tau² = 0.00; Chi² = 0.15, df = 1 (P = 0.70); I² = 0%
Test for overall effect: Z = 5.74 (P < 0.00001)



(4F)

Study or Subgroup	Letrozole Mean [mmol/L]	Letrozole SD [mmol/L]	Letrozole Total	Control Mean [mmol/L]	Control SD [mmol/L]	Control Total	Weight	Mean Difference IV, Random, 95% CI [mmol/L]
Kohva 2020	8.2	7.5	15	9.3	8.1	13	9.1%	-1.10 [-6.91, 4.71]
Salehpour 2010a	15.66	4.88	31	17.13	5.11	30	48.6%	-1.47 [-3.98, 1.04]
Varimo 2019	8.1	3.2	15	7.6	4.1	14	42.3%	0.50 [-2.19, 3.19]
Total (95% CI)			**61**			**57**	**100.0%**	**-0.60 [-2.35, 1.15]**

Heterogeneity: Tau² = 0.00; Chi² = 1.13, df = 2 (P = 0.57); I² = 0%
Test for overall effect: Z = 0.68 (P = 0.50)

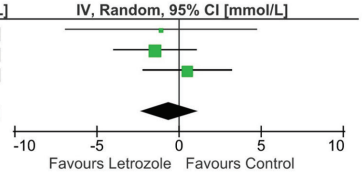


Figure 4. Forest plot highlighting the impact of letrozole vs the active control group on (a) Luteinizing hormone at 6 months; (b) Luteinizing hormone at 12 months; (c) Follicle stimulating hormone at 6 months; (d) Follicle stimulating hormone at 12 months; (e): Testosterone at 6 months; (f): Testosterone at 12 months

Data from one study [Hero et al 2006 (13)] involving 17 children with CDGP was analysed to find out the impact of letrozole on serum LH after 6 and 12 months of treatment, when compared to those receiving placebo (PCG). Serum LH was significantly higher after 6 months [MD: 6.50 IU/L (95% CI: 3.40-9.60); $p < 0.01$], and 12 months [MD: 5.10 IU/L (95% CI: 2.14-8.06); $p < 0.01$] of treatment with letrozole.

Follicle Stimulating Hormone

Data from two studies involving 55 children with CDGP was analysed to find out the impact of letrozole on serum FSH after 6 and 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum FSH was significantly higher after 6 months [MD: 3.59 IU/L (95% CI: 1.42-5.76); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 4c], but not after 12 months [MD: -0.20 IU/L (95% CI: -1.21-0.61); $p = 0.63$; $I^2 = 0\%$ (low heterogeneity); Figure 4d] of treatment with letrozole, when compared to ACG.

Data from one study [Hero et al 2006 (13)] involving 17 children with CDGP was analysed to find out the impact of letrozole on serum FSH after 6 and 12 months of treatment, when compared to those receiving placebo (PCG). Serum FSH was significantly higher after 6 months [MD: 7.70 IU/L (95% CI: 4.37-11.03); $p < 0.01$], and 12 months [MD: 2.70 IU/L (95% CI: 0.42-4.98); $p = 0.02$] of treatment with letrozole.

Testosterone

Data from two studies involving 57 children with CDGP was analysed to find out the impact of letrozole on serum total testosterone after 6 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum total testosterone was significantly higher after 6 months [MD: 22.73 mmol/L (95% CI: 14.96-30.50); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 4e], treatment with letrozole, when compared to ACG. Data from three studies involving 118 children with CDGP was analysed to find out the impact of letrozole on serum total testosterone after 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum total testosterone was not significantly different after 12-24 months [MD: -0.60 mmol/L (95% CI: -2.35-1.15); $p = 0.50$; $I^2 = 0\%$ (low heterogeneity); Figure 4f; HCE; Table 3], treatment with letrozole, when compared to ACG.

Data from one study [Hero et al 2006 (13)] involving 17 children with CDGP was analysed to find out the impact of letrozole on serum total testosterone after 6 months of treatment, when compared to those receiving placebo (PCG). Serum testosterone was significantly higher after 6 months [MD: 49.20 mmol/L (95% CI: 22.05-76.35); $p < 0.01$], of treatment with letrozole. Data from three studies [Hero et al 2006 (13), Salehpour et al (6) and Wickman et al (2001) (17)] involving 89 children with CDGP was analysed to find out the impact of letrozole on serum total testosterone after 12 months of treatment, when compared to those receiving placebo (PCG). Serum total testosterone was significantly higher after 12 months [MD: 32.37 mmol/L (95% CI: 10.58-54.16); $p < 0.01$; $I^2 = 97\%$ (considerable heterogeneity); MCE; Table 2] treatment with letrozole, when compared to PCG.

Inhibin-B

Data from two studies involving 57 children with CDGP was analysed to find out the impact of letrozole on serum inhibin-B after 6 and 12 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum inhibin-B was significantly higher after 6 months [MD: 62.97 ng/L (95% CI: 24.50-101.43); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity); Figure 5a], and 12 months [MD: 25.40 ng/L (95% CI: 1.51-49.29); $p = 0.04$; $I^2 = 0\%$ (low heterogeneity); Figure 5b] of treatment with letrozole, when compared to ACG.

Data from one study [Wickman et al (2001) (17)] involving 19 children with CDGP was analysed to find out the impact of letrozole on serum inhibin-B after 5, 12 and 18 months of treatment, when compared to those receiving placebo (PCG). Serum inhibin-B was significantly higher only after 5 months [MD: 64.00 ng/L (95% CI: 27.84-100.16); $p < 0.01$], but not 12 month [MD: 35.00 ng/L (95% CI: -6.01-76.01); $p = 0.09$], and 18 months [MD: -5.00 ng/L (95% CI: -43.46-33.46); $p = 0.80$] of treatment with letrozole, when compared to PCG.

Insulin Like Growth Factor-1

Data from two studies [Kohva et al (2020) (15) and Varimo et al (2019) (5)] involving 57 children with CDGP was analysed to find out the impact of letrozole on serum IGF-1 after 6 months of treatment, when compared to those receiving testosterone in the control group (ACG). Serum IGF-1 was significantly lower after 6 months [MD: -11.86 nmol/L (95% CI: -18.08 – -5.64); $p < 0.01$; $I^2 = 0\%$ (low heterogeneity)], of treatment with letrozole, when compared to ACG. Data from three studies [Kohva et al (2020) (15) Salehpour et al (6) and Varimo et al (2019) (5)] involving 118 children with CDGP was analysed to find out the impact of letrozole on serum IGF-1 after 12-24 months of treatment, when compared

to those receiving testosterone in the control group (ACG). Serum IGF-1 was not significantly different after 12-24 months [MD: -1.24 nmol/L (95% CI: -8.70-6.22); p = 0.74; I^2 = 0% (low heterogeneity)] of treatment with letrozole, when compared to ACG.

Data from one study [Wickman et al (2001) (17)] involving 19 children with CDGP was analysed to find out the impact of letrozole on serum IGF-1 after 6 and 12 months of treatment, when compared to those receiving placebo (PCG). Serum IGF-1 was significantly lower after 6 months [MD: -11.30 nmol/L (95% CI: -19.39 – -3.21); p < 0.01], and 12 months [MD: -9.00 nmol/L (95% CI: -14.52 – -3.48); p < 0.01] of treatment with letrozole, when compared to PCG.

Safety

Data from seven studies (235 children) was analysed to evaluate the impact of letrozole on the occurrence of adverse events [(total adverse events (TAEs) and severe adverse events (SAEs)], over 1-4 years of treatment. The occurrence of TAEs [RR 0.61 (95% CI: 0.17-2.17); p = 0.45; I^2 = 0% (low heterogeneity); Figure 6a; HCE; Table 3] and SAEs [RR 1.22 (95% CI: 0.28-5.28); p = 0.79; I^2 = 0% (low heterogeneity); Figure 6b; HCE; Table 3] was not statistically different in children receiving letrozole as compared to the control group.

Vertebral deformity and end-plate deformity was investigated in the study by Hero et al 2010 (14). In that study data from 12 children (6 receiving letrozole with testosterone vs 6 receiving placebo with testosterone) was analysed after 4.2 years follow up. The occurrence of vertebral deformity [RR 0.28 (95% CI: 0.01-8.42); p = 0.47] and end-plate deformity [RR 0.50 (95% CI: 0.05-5.15); p = 0.56] was not statistically different in children receiving letrozole as compared to the control group.

Changes in the BMD Z-scores, before and after therapy was noted in the study by Salehpour et al (6). A significantly lower (improvement) in lumbar spine BMD Z-score [MD: -2.59 (95% CI: -2.88 – -2.30); p < 0.01; n = 61] and femoral neck BMD Z-score [MD: -2.2 (95% CI: -2.55 – -1.85); p < 0.01; n = 61] was noted in children receiving letrozole as compared to those receiving testosterone in the control group (ACG). Changes in lumbar spine BMD Z-score [MD: 0.11 (95% CI: -0.35-0.57); p = 0.64; n = 61] and femoral neck BMD Z-score [MD: 0.10 (95% CI: -0.25-0.45); p = 0.58; n = 61] was not significantly different when comparing children receiving letrozole to those receiving placebo (PCG).

A small decline in high density lipoprotein-cholesterol (HDL-C) was noted in children receiving letrozole as compared to those receiving placebo in a cohort of 69 patients from two studies [MD: -0.77 mmol/L (95% CI: -2.00-0.47); p = 0.22; I^2 = 99% (considerable heterogeneity); Figure 6c], which was however statistically not significant.

Discussion

This is the first meta-analysis to highlight the efficacy and safety of letrozole in children with CDGP. An important observation from this meta-analysis is letrozole use in children with CDGP is associated with a significantly greater improvement in PAH when compared to receiving placebo. This improvement in PAH with letrozole is comparable to the improvements seen with the use of testosterone in CDGP. In accordance with the previous observation, children receiving letrozole had a significantly greater improvement

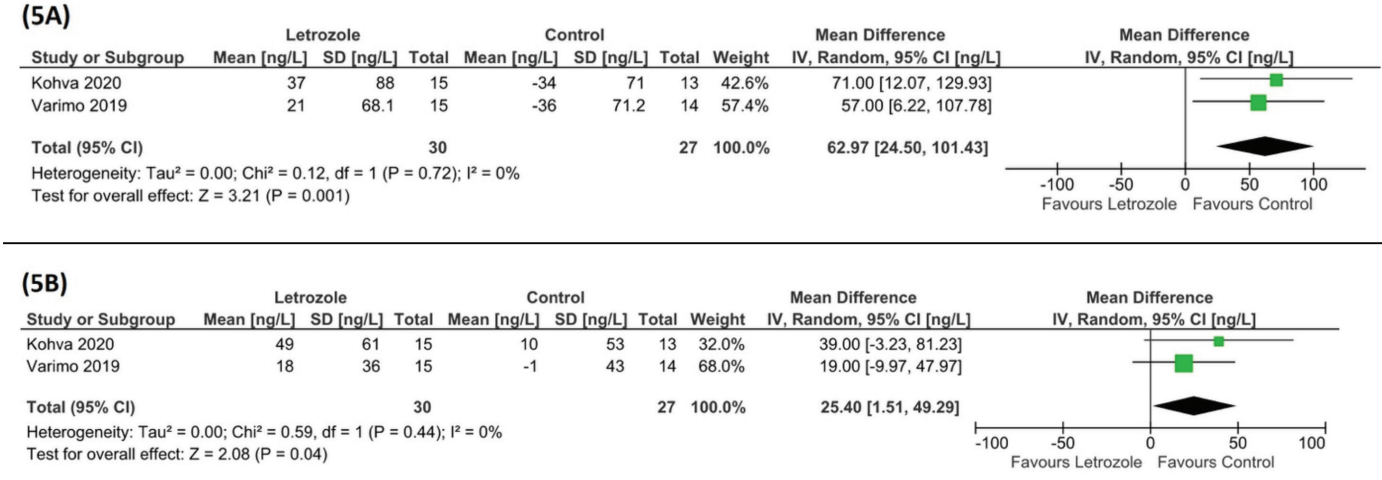


Figure 5. Forest plot highlighting the impact of letrozole vs the active control group on (a) Inhibin-B at 6 months; (b) Inhibin-B at 12 months

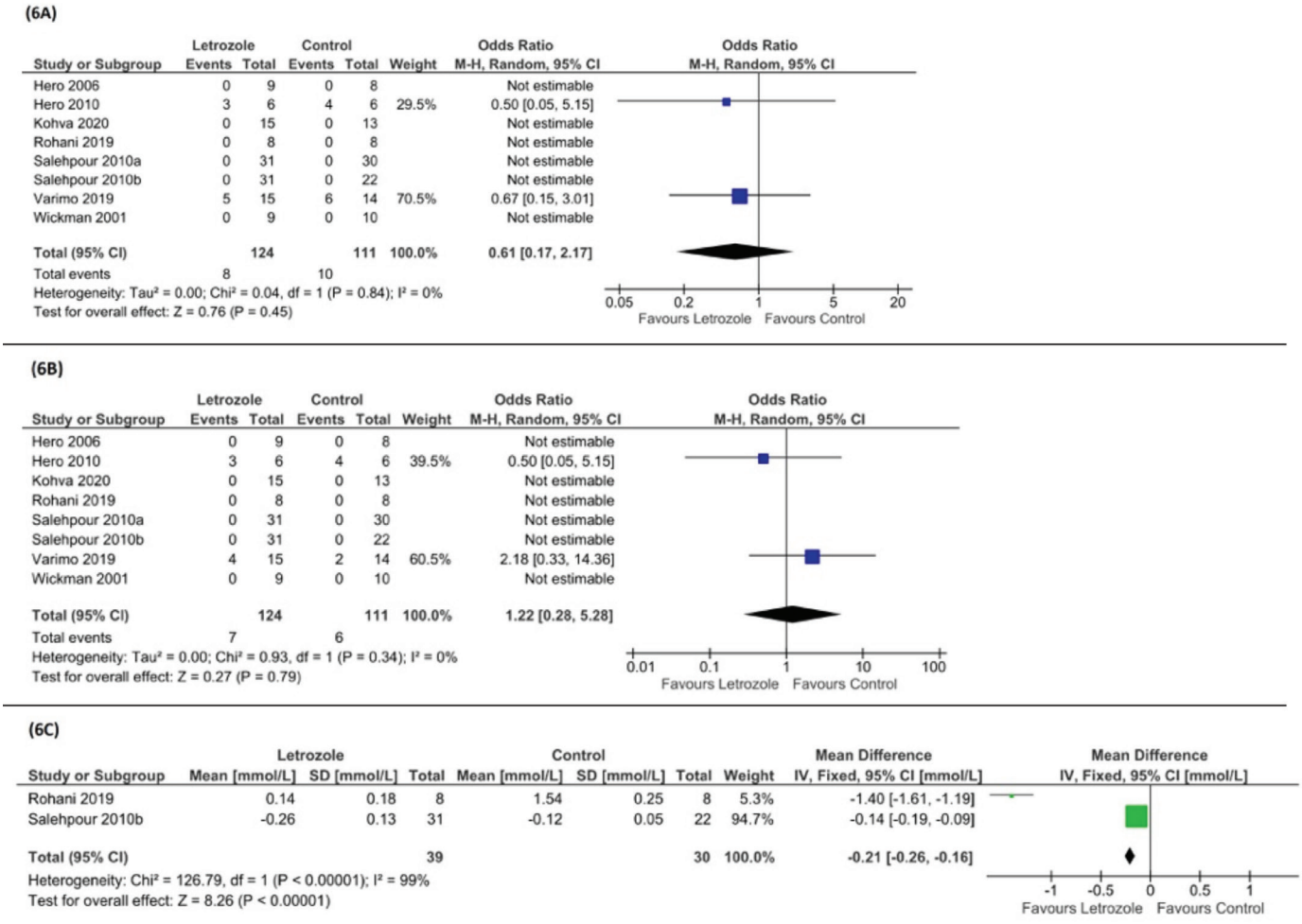


Figure 6. Forest plot highlighting the side effect profile of the use of letrozole as compared to controls focussing on (a): Total adverse events (TAEs); (b): Severe adverse events (SAEs); (c): High density lipoprotein cholesterol

in Ht-SDS when compared to those receiving placebo but not testosterone. Six months and 12 months letrozole use was associated with a significantly greater improvement in testicular volume in CDGP when compared to both placebo and testosterone. All studies used letrozole in dose of 2.5 mg/day for the duration of the study.

Use of letrozole in CDGP was associated with a slower progression in BA when compared to those receiving placebo or testosterone. Serum LH, FSH, total testosterone and inhibin-B were significantly higher after 6 months of use of letrozole in children with CDGP, when compared to those receiving placebo or testosterone. After 12 months of use, the difference for LH, FSH and testosterone persisted only when compared to placebo, but not with regards to those receiving testosterone. Inhibin-B continued to be significantly higher in children receiving letrozole as compared to testosterone after 12 months of use. Serum IGF-1 was significantly lower after 6 months use of letrozole when compared to those receiving placebo or testosterone. Oestrogen has a trophic impact on growth hormone through paracrine effects, which indirectly has a trophic impact on IGF-1 levels (22). Letrozole is an aromatase inhibitor associated with lower oestrogen levels. Testosterone in contrast is aromatized to oestrogen to some extent in the body, having a trophic impact on GH release from pituitary, explaining the higher IGF-1 levels at 6 months (22). Pubertal BA progression is associated with higher IGF-1 levels (23). A lower BA progression may contribute to the marginally lower IGF-1 levels in the first 6 months use of letrozole. It is important to note that this observation is transient and the difference did not persist after 12 months of clinical use. After 12 months use, IGF-1 levels were comparable in children receiving letrozole or testosterone.

This meta-analysis provided reassuring data regarding the long term safety of letrozole use in CDGP. No increased occurrence of TAEs and SAEs were noted with the use of letrozole. The occurrence of vertebral deformities was not significantly increased. No significant decline/change in BMD Z-scores were noted in children receiving letrozole as compared to placebo. Bone health outcomes were better in children receiving testosterone as compared to letrozole because of the anabolic impact of testosterone on BMD. Testosterone is aromatized to estrogen in the body which has a direct impact on increased bone formation (24). A mild statistically not-significant decline in the good cholesterol HDL-C was noted in children receiving letrozole. This is an observation and its impact on long term cardiovascular outcomes needs further evaluation. Advantages of letrozole over testosterone also includes its oral administration in contrast to monthly injections with regards to testosterone.

Conclusion

To conclude, it may be said that this first meta-analysis on the efficacy and safety of letrozole in CDGP, provides us with reassuring data on the good efficacy and tolerability of this molecule on height outcomes and pubertal progression. Letrozole in comparable to testosterone and superior to placebo with regards to improving height outcomes in CDGP. Letrozole tended to show a better slowing effect on BA progression, when compared to both testosterone and placebo. This may have an additional impact on improving height outcomes. Letrozole is superior to both testosterone and placebo with regards to improvement in testicular volume (an important marker of pubertal progression in boys). Letrozole has a better short term impact on hormonal markers of pubertal progression (LH, FSH, testosterone and inhibin-B).

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Deep Dutta, Design: Deep Dutta, Meha Sharma, Data Collection or Processing: Deep Dutta, Rajiv Singla, Vineet Surana, Meha Sharma, Analysis or Interpretation: Deep Dutta, Rajiv Singla, Vineet Surana, Meha Sharma, Literature Search: Deep Dutta, Rajiv Singla, Vineet Surana, Meha Sharma, Writing: Deep Dutta, Rajiv Singla, Vineet Surana, Meha Sharma.

Financial Disclosure: The authors declared that this study received no financial support.

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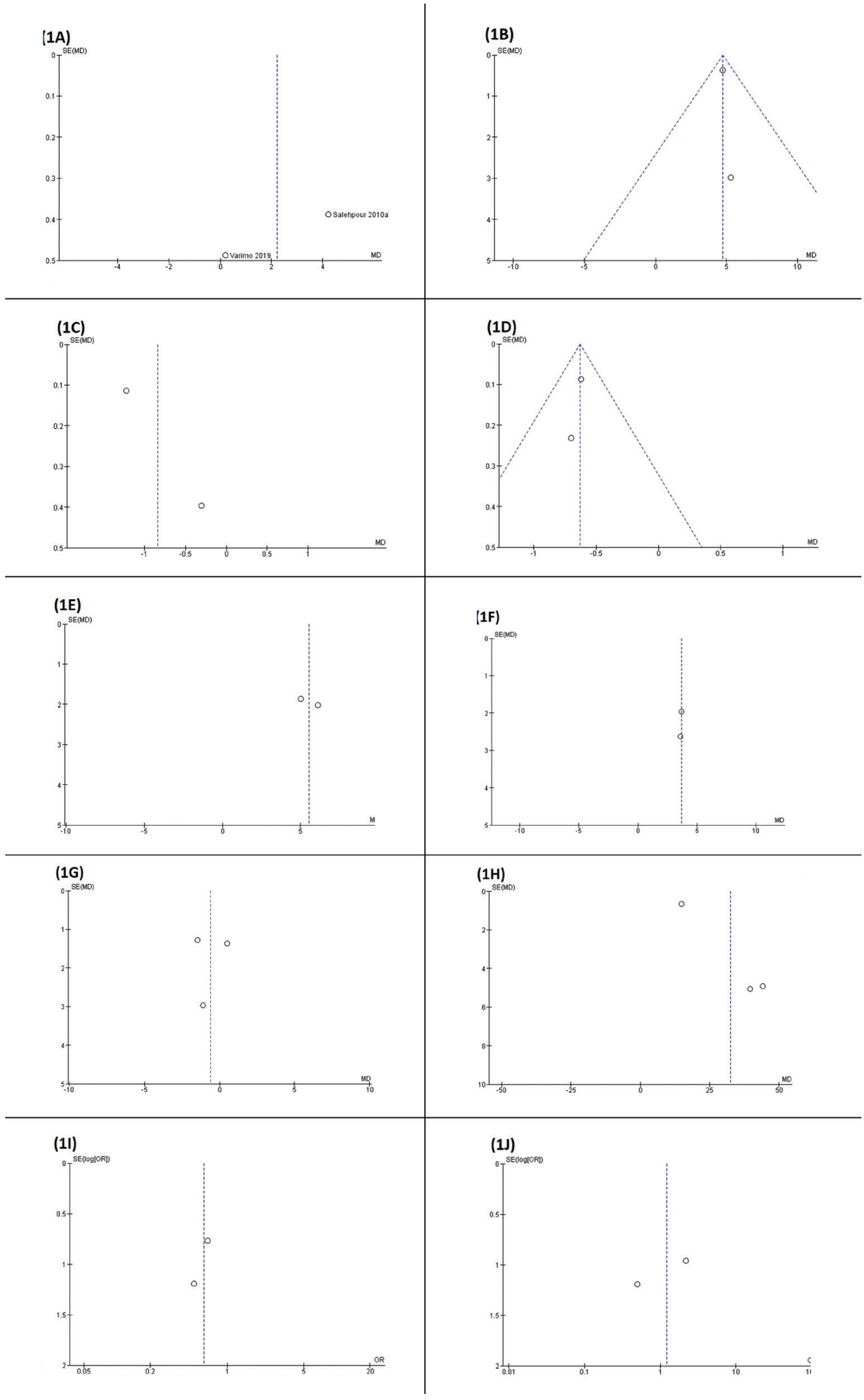
Click for Supplementary Figure 1, Supplementary Table 1 access link: http://glns.co/qvqp6



Supplementary Figure 1. Funnel plot of all the included studies in the meta-analysis (assessing the publication bias) of the main outcomes assessed (a) PAH ACG; (b) PAH PCG; (c) Bone age progression ACG; (d): Bone age progression PCG; (e): Testicular volume at 6months in ACG; (f): Testicular volume at 12 months in ACG; (g) Serum testosterone at 12 months in ACG; (h): Serum testosterone at 12 months in PCG; (i): Total adverse events; (j): Severe adverse events.

ACG: active control group, PCG: passive/placebo control group, PAH: predicted adult height

Supplementary Table 1. Risk of bias assessment table

Hero 2006 (13)	**Risk of bias**	**Author judgement**
Random Sequence Generation (Selection Bias)	Low risk	Randomised, double-blind, placebo controlled study
Allocation Concealment (Selection Bias)	Unclear risk	Details not available
Blinding Of Participants & Personal (Performance Bias)	Low risk	Yes, double blinded RCT
Blinding Of Outcome Assessment (Detection Bias)	Low risk	Yes, double blinded RCT
Incomplete Outcome Data (Attrition Bias)	Low risk	Of the 19 boys initially evaluated in this study, 17 boys completed the study
Selective Reporting (Reporting Bias)	Low risk	All pre-specified outcomes were reported
Other Biases	Low risk	This work was supported by the Foundation for Paediatric Research, Helsinki, Finland, and the Hospital District of Helsinki and Uusimaa.
Hero 2010 (14)	**Risk of bias**	**Author judgement**
Random Sequence Generation (Selection Bias)	Low risk	Randomised, double-blind, placebo controlled study
Allocation Concealment (Selection Bias)	Unclear risk	Details not available
Blinding Of Participants & Personel (Performance Bias)	Low risk	Yes, double blinded RCT
Blinding Of Outcome Assessment (Detection Bias)	Low risk	Yes, double blinded RCT
Incomplete Outcome Data (Attrition Bias)	Low risk	No drop-outs; all patients completed the study
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	This study was supported by the Foundation for Paediatric Research, Helsinki, Finland
Kohva et al 2020 (15)	**Risk of bias**	**Author Judgement**
Random Sequence Generation (Selection Bias)	Low risk	Randomised, controlled, open-label multicentric trial
Allocation Concealment (Selection Bias)	Unclear risk	Details not available in the manuscript
Blinding Of Participants & Personel (Performance Bias)	High risk	Open labelled study
Blinding Of Outcome Assessment (Detection Bias)	High risk	Open labelled study
Incomplete Outcome Data (Attrition Bias)	Low risk	All patient outcomes reported. NO drop-outs
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	The Academy of Finland, The Foundation for Pediatric Research, The Emil Aaltonen Foundation, Sigrid Juselius Foundation, Helsinki University Hospital Research Funds.Takeda Development Center
Rohani 2019 (16)	**Risk of bias**	**Author Judgement**
Random Sequence Generation (Selection Bias)	Low risk	Randomized, open-label, parallel-group study
Allocation Concealment (Selection Bias)	Unclear risk	Details not available
Blinding Of Participants & Personel (Performance Bias)	High risk	Open labelled study
Blinding Of Outcome Assessment (Detection Bias)	High risk	Open labelled study
Incomplete Outcome Data (Attrition Bias)	Low risk	Outcomes of al the 16 randomized patients have been presented.
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	Nothing significant was noted
Salehpour 2010 (6)	**Risk of bias**	**Author Judgement**
Random Sequence Generation (Selection Bias)	Low risk	A prospective, double-blind, randomized, placebo-controlled clinical trial
Allocation Concealment (Selection Bias)	Low risk	Stratified randomization was done
Blinding Of Participants & Personel (Performance Bias)	Low risk	Yes, double blinded RCT
Blinding Of Outcome Assessment (Detection Bias)	Low risk	Yes, double blinded RCT
Incomplete Outcome Data (Attrition Bias)	Low risk	83 out of the 91 randomized children completed the study. Hence attrition rate was 8.79%
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	This study was supported by the Genomic Research Center of Shaheed Beheshti University of Medical Sciences

Supplementary Table 1. Risk of bias assessment table

Varimo et al 2019 (5)	**Risk of bias**	**Author Judgement**
Random Sequence Generation (Selection Bias)	Low risk	Multicenter, open-labeled, randomized, placebo-controlled, parallel-group
Allocation Concealment (Selection Bias)	Low risk	Patients randomly assigned in blocks of ten to receive either letrozole or testosterone for 6 months. The randomisation sequence was generated with a computer
Blinding Of Participants & Personel (Performance Bias)	High risk	Open labelled study
Blinding Of Outcome Assessment (Detection Bias)	High risk	Open labelled study
Incomplete Outcome Data (Attrition Bias)	Low risk	30 children were randomized of which 29 completed the treatment and the study. Hence attrition rate was 3.33%
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	The study was funded by Helsinki University Hospital, Academy of Finland, and Finnish Foundation for Pediatric Research
Wickman et al (2001) (17)	**Risk of bias**	**Author Judgement**
Random Sequence Generation (Selection Bias)	Low risk	Randomised, double-blind, placebo controlled study
Allocation Concealment (Selection Bias)	Unclear risk	Details not available
Blinding Of Participants & Personel (Performance Bias)	Low risk	Yes, double blinded RCT
Blinding Of Outcome Assessment (Detection Bias)	Low risk	Yes, double blinded RCT
Incomplete Outcome Data (Attrition Bias)	High risk	23 children were randomized of which 19 children completed the study. Hence attrition rate was (17.39%). An attrition rate of more than 15% was considered to be significant
Selective Reporting (Reporting Bias)	Low risk	All Pre-Specified Outcomes Were Reported
Other Biases	Low risk	This study was supported by the Foundation for Paediatric Research, Helsinki, Finland

RCT: randomized controlled trials

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021-9-17

J Clin Res Pediatr Endocrinol 2022;14(2):145-152

A 4-hour Profile of 17-hydroxyprogesterone in Salt-wasting Congenital Adrenal Hyperplasia: Is the Serial Monitoring Strategy Worth the Effort?

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What is already known on this topic?

Clinicians need to consider various indicators, such as growth velocity, weight gain, and 17-hydroxyprogesterone (17-OHP) and androstenedione levels to avoid over- and under-treatment in children with congenital adrenal hyperplasia (CAH). However, no gold standard exists.

What this study adds?

A 4-hour 17-OHP profile is not useful in predicting hyperandrogenemia. Sex hormone-binding globulin can be considered as an indicator of hyperandrogenemia in CAH in pubertal children.

Abstract

Objective: Since there is no gold standard laboratory variable for adjustment of treatment in congenital adrenal hyperplasia (CAH), the aim was to assess the use of a 4-hour profile of serum 17-hydroxyprogesterone (17-OHP) to determine the most appropriate sample time and level of 17-OHP in predicting the metabolic control and evaluate the role of sex hormone-binding globulin (SHBG) in hyperandrogenemia.

Methods: This study included children with salt-wasting CAH. Measurements for 17-OHP and cortisol were made from samples obtained before and 1, 2, and 4 hours after the morning dose of hydrocortisone. Patients were designated to have poor metabolic control when androstenedione levels according to age and sex-specific reference intervals were high and annual height standard deviation score (SDS) changes were ≥0.5.

Results: The study cohort was 16 children (9 girls) with a median age of 7-years old. Premedication 17-OHP levels were strongly correlated with 17-OHP levels 1, 2, and 4 hours after the morning dose ($r_s = 0.929$, $p < 0.01$; $r_s = 0.943$, $p < 0.01$; $r_s = 0.835$, $p < 0.01$, respectively). 17-OHP profiles (0, 1, 2, 4 hours) of poor (n = 6) and good (n = 10) metabolically controlled cases were similar. Among the patients with poor metabolic control, two cases had 17-OHP levels < 2 ng/mL at all times. The remaining patients with poor metabolic control had median 17-OHP levels above 104 ng/mL, 82 ng/mL, 14 ng/mL, and 4 ng/mL, for baseline and 1, 2, and 4 hours, respectively. Differences between the poor and well-controlled group were androstenedione levels with respect to upper limit of normal [1.8 (1.5) and 0.5 (1.5) ng/mL, respectively p = 0.03], annual change in height SDS [0.7 (0.2) and -0.03 (0.8) SDS, respectively, p = 0.001], and daily hydrocortisone doses [7 (6) and 16 (8) mg/m^2/day, respectively, p = 0.02]. Androstenedione and SHBG levels were negatively correlated in the pubertal children ($r_s = -0.7$, $p = 0.04$).

Conclusion: We conclude that: (i) a 4-hour 17-OHP profile is not useful in predicting hyperandrogenemia; (ii) suppressed levels of 17-OHP do not always indicate overtreatment; (iii) reference intervals of 17-OHP for different time periods might be of importance; (iv) low hydrocortisone doses should be avoided; and (v) SHBG could be used in pubertal children as an indicator of hyperandrogenemia.

Keywords: CYP21A2, androgens, adrenocorticotropic hormone, steroid

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Conflict of interest: None declared
Received: 01.10.2021
Accepted: 06.11.2021

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessively inherited group of disorders characterized by cortisol deficiency (1,2,3,4). Neonatal screening programs report the incidence of CAH to be around 1:14,000 to 1:18,000 worldwide, and as of 2018, the approximate incidence is 1:15,000 in Turkey (3,5). CAH often develops due to pathogenic variants in the *CYP21A2* gene encoding the 21-hydroxylase enzyme. Impairment of cortisol synthesis, with or without aldosterone deficiency, causes increased secretion of adrenocorticotropic hormone with subsequent accumulation of androgen precursors (1,2).

Hydrocortisone, the mainstay of treatment, needs to be adjusted to suppress the accumulation of adrenal androgens while avoiding overtreatment and cushingoid side effects (6). 17-hydroxyprogesterone (17-OHP), androstenedione, 21-deoxycortisol, 11-oxysteroids, plasma renin activity, bone age, and height velocity are among the various indicators which are evaluated in disease control (2,3). Age and sex-specific reference ranges are accepted as targets for androstenedione (2). As for 17-OHP, another traditional marker for disease control, other than avoiding complete suppression, a consensus regarding the optimal sampling timing or reference ranges has not been made. In addition, factors limiting the accuracy of the test, such as prematurity, sickness, stress or methods of measurements also add ambiguity to the interpretation of results (2). Various strategies on sampling time including early in the morning or later in the evening, 3-4 hours after the morning dose, and even 24-hour monitoring have been suggested and assessed with other laboratory markers in the literature (6,7,8,9,10,11). Progesterone, sex hormone binding globulin (SHBG) and several other backdoor pathway metabolites measured in urine were also suggested as possible novel biomarkers (9,12). In the present study, we evaluated the efficacy of a laboratory marker, using a previously unassessed definition of metabolic control: a clinical indicator, change in height standard deviation score (SDS) over a year [annual delta height SDS (Δh SDS)] combined with a traditional laboratory marker (androstenedione). We aimed to assess the use of a 4-hour profile of serum 17-OHP to determine the most appropriate sample time and level of 17-OHP in predicting metabolic control, and evaluate the role of SHBG in response to hyperandrogenemia in terms of both clinical and laboratory parameters.

Methods

Patients

We designed a cross-sectional study with pediatric CAH patients who were followed in our pediatric endocrinology department between 2003-2021. Among 20 patients with classical CAH, aged between 2-17 years of age, one patient with inflammatory bowel disease, one receiving dexamethasone, and two patients with simple virilizing forms of CAH were excluded.

Methods

Clinical records of the patients over the last 1-year period were retrospectively collected. All patients with the salt-wasting form were diagnosed from the neonatal period, and regularly followed up in a single center. Patients had been evaluated every three months regularly, auxological measurements were obtained at each visit, and treatment adherence was encouraged by a phone call one month before the scheduled visit for the study. All patients received fludrocortisone replacement with a daily dose of 0.1 mg/d. None of the subjects had hypo- or hyperthyroidism, or hyperinsulinemia. The data collected regarding anthropometric characteristics included: age (years); sex; height [measured with a sensitivity of 0.1 cm, using a Harpenden stadiometer for those who could stand or crown-heel length was measured using a portable infantometer (Seca 417, Hamburg, Germany) for those who could not stand (cm)]; weight [measured using a scale with a sensitivity of 0.1 kg (Seca, Hamburg, Germany), (kg)]; body mass index (BMI) (kg/m^2); the respective SDSs [calculated with an online calculator for pediatric endocrinologists according to Turkish standards (13)]; height gain (Δh SDS), change in height SDS calculated by final height SDS minus height SDS measured 1-year earlier]; predicted adult height [SDS, calculated according to the Roche-Wainer-Thissen method (14)]; target height [SDS (mother's height + father's height)/2 ± 6.5]; and pubertal staging [evaluated according to Tanner and Whitehouse (15)]. Short stature was defined when height was < -2 SDS and obesity was defined if the BMI was ≥95th percentile for age and gender-specific reference ranges (13,16,17). Left hand and wrist radiographs of the patients were assessed using the Greulich-Pyle radiographic atlas and the SDSs were calculated using the tables of the atlas (18). Average hydrocortisone dose for the last six months, as well as current doses, were recorded.

Blood samples were obtained between 07.00 and 08.00 a.m., after an overnight fast, before morning doses of hydrocortisone and fludrocortisone. Serum levels of 17-OHP (ng/mL), cortisol (mcg/dL), androstenedione (ng/mL), sodium (mmol/L), potassium (mmol/L), glucose (mg/dL), SHBG (nmol/L), free T4 (ng/dL), TSH (mIU/L), and insulin (mU/L) were taken. Samples for 17-OHP and cortisol were also obtained 1, 2, and 4 hours after the morning dose. 17-OHP and cortisol were measured using

a commercial kit (catalog no LC72315, Euroka, Italy) with liquid chromatography-tandem/mass spectrometry (LC/MS) by an MS device (Shimadzu triple quadrupole, LC/MS-MS 8030, Japan). Androstenedione and SHBG were analyzed by Immulite 2000 systems (Siemens Inc, Germany). Age and sex-specific reference intervals (Immulite 2000 systems) of androstenedione (19,20) and SHBG (20) are provided in Supplementary Table 1.

Subjects were evaluated for hyperandrogenic state according to age- and sex-specific androstenedione levels (19) and annual change in height SDS (≥0.5 & <0.5) (21). Bone age was not considered as a criterion because advancements may be related to exposure to hyperandrogenic state at some point since the time of diagnosis.

Institutional approval was granted by the Ethics Committee of Dokuz Eylül University Faculty of Medicine (ethics approval number: 2021/16-25, date: 27.05.2021). When applicable, both patients and parents were required to sign the informed consent form to participate in the study.

Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences application for Windows, version 24.0 (IBM Inc., Armonk, NY, USA). Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The data did not comply with normal distribution. Descriptive results were presented as median (interquartile range) (IQR) or as median (minimum-maximum) according to the distribution of the variables. Comparisons among groups of good and poor control were made using the Mann-Whitney U test for numeric variables and x^2-test or Fisher's exact test for categorical variables. Correlations were analyzed with Spearman correlation analysis. A p value of <0.05 was considered statistically significant.

Results

A total of 16 children [9 girls, 7 boys; median age 7 (7) years] with salt-wasting CAH due to 21-hydroxylase deficiency, all followed-up since the neonatal period, were included in the study. Median (IQR) values of chronological age, height, BMI, MPH, PAH SDSs were 7.1 (7), 0.3 (1.8), 1.1 (1.7), -0.6 (1.1), and -0.4 (1.7) respectively. Average hydrocortisone dose was 12 (11) mg/m^2/d. Clinical and laboratory data of each patient are presented in Table 1.

The serum levels of 17-OHP and cortisol of all patients over 4 hours are shown in Figure 1. Median (IQR) 17-OHP levels for baseline, 1, 2, and 4-hours after the medication were 22

Table 1. Auxological and laboratory data of the patients

Pt no.	Sex	Age, yrs	HC	Tanner stages	Height SDS	BMI SDS	MPH SDS	PAH SDS	Δh SDS	Bone age SDS	17-OHP, ng/mL				A4/ULN	SHBG/ ULN
											Baseline	1-hour	2-hour	4-hour		
1	M	16.9	19	5	-0.34	-0.19	-0.68	-1.10	-0.42	2.6	0.14	0.05	0.14	0.3	0.3	0.9
2	M	10.2	20	3	1.35	1.67	-0.36	0.37	0.17	3.9	0.54	0.87	0.22	8.96	0.3	0.3
3	M	3.3	24	1	-0.24	3.36	-0.90	-0.13	0.10	6.5	0.21	0.57	0.3	0.1	0.1	0.4
4	F	10.2	11	3	0.28	-0.02	-1.29	-0.87	-0.34	2	26.15	17.15	21.58	22.24	0.9	0.7
5	F	7.4	19	1	0.34	-0.58	-0.61	-0.21	0.40	3.1	6.7	1.61	1.09	0.61	0.1	0.5
6	M	6	16	1	1.13	1.01	0.21	1.47	0.45	6.8	41.43	30.2	7.14	3.35	0.7	0.6
7	M	3.4	6	1	-0.24	0.94	0.21	0.72	0.47	1	29.65	5.49	2.51	1.3	0.1	1.0
8	F	13.4	15	4	-2.15	2.96	-2.31	-3.28	-0.70	2.4	5.07	4.10	2.2	1.39	1.3	0.1
9	F	9.4	11	4	1.58	1.58	-2.06	-1.03	-0.31	3.4	38.86	23.76	9.03	18.31	2.6	0.2
10	F	10.4	15	4	1.80	1.20	-0.27	-0.56	0.8	3.4	134.56	91.30	92.26	77.35	2.8	0.02
11	F	13.6	12	5	-1.22	1.89	-1.04	-2.44	-0.16	1.8	18.4	0.27	1.16	1.38	2.3	0.3
12	F	6.9	11	2	1.20	-1.30	-0.60	0.40	0.78	3.2	0.35	0.46	0.69	0.39	1.9	0.01
13	M	2.3	6	1	-0.74	1.19	-1.98	-0.90	0.56	1.3	135.6	98.17	94.13	124.19	1.6	0.7
14	M	2.8	7	1	0.39	-0.02	-0.68	1.53	0.50	6.9	104.65	82.41	14.11	4.27	1.2	1
15	F	5.9	6	1	0.85	1.30	0.15	2.21	0.77	1.8	225.72	199.48	165.15	160.21	2.6	1.2
16	F	2.8	8	1	-2.24	0.68	-1.46	-2.06	0.70	0.8	0.83	1.05	0.31	0.49	1.1	1.2

Data are presented as numbers. Rows highlighted in grey indicate patients with poor control.
Pt no: patient number, F: female, M: male, yrs: years, SDS: standard deviation score, BMI: body mass index, PAH: predicted adult height, Δh SDS: annual change in height SDS (current height SDS-height SDS measured 1-year earlier). MPH: midparental height, HC: average hydrocortisone dose in the last 6-months (mg/m^2/day), SHBG: sex-hormone binding globulin. A4: androstenedione, 17-OHP: 17-hydroxyprogesterone, ULN: upper limit of normal.
Normal ranges for 17-OHP, <2 ng/mL

(88), 5 (69), 2 (19), and 2 (21) ng/mL, respectively. Median (IQR) cortisol levels for baseline, 1, 2, and 4-hours after the medication were 0.9 (1), 29 (16), 15 (25), and 9 (11) mcg/dL respectively. Median androstenedione level with respect to upper limits of normal was 1.1 (2).

Premedication 17-OHP levels were strongly correlated with levels measured 1, 2, and 4-hours after the morning dose ($r_s = 0.929$, $p < 0.01$; $r_s = 0.944$, $p < 0.01$; $r_s = 0.835$, $p < 0.01$, respectively). There was no correlation between the hydrocortisone dose and the reduction rate of 17-OHP ($r_s = -0.1$, $p = 0.5$).

With respect to indicators of hyperandrogenemia, nine patients had increased androstenedione levels, while growth was accelerated in six of them; and 17-OHP levels were over 10 ng/mL in seven, six, four, and four of these subjects at baseline, 1, 2, and 4 hours after, respectively. Among four patients with 17-OHP levels over 10 ng/mL at all consecutive measures, androstenedione levels were increased in three, and those who grew fast were also these three patients.

The groups of good and poor control based on the indicators of hyperandrogenemia are presented in Table 2. The 17-OHP profiles (0, 1, 2 and 4 hours) of good (n = 10) and poor (n = 6) metabolically controlled cases were similar ($p = 0.1$, $p = 0.08$, $p = 0.1$, and $p = 0.2$, respectively). Among the patients with poor metabolic control, two (33%) had 17-OHP levels < 2 ng/mL at all of the time points. When these two cases were excluded, those with 17-OHP levels measured above 104 ng/mL, 82 ng/mL, 14 ng/mL, and 4 ng/mL measured at 0, 1, 2, and 4 hours, respectively had poor metabolic control. In the poor control group, higher androstenedione levels [with respect to upper limit of normal, 1.8 (1.5) and 0.5 (1.5), respectively, $p = 0.03$], higher annual change in height SDS [0.7 (0.2) and -0.03 (0.8) SDS, respectively, $p = 0.001$], and lower daily hydrocortisone doses [7 (6) and 16 (8) mg/m^2/day, respectively, $p = 0.02$] were observed.

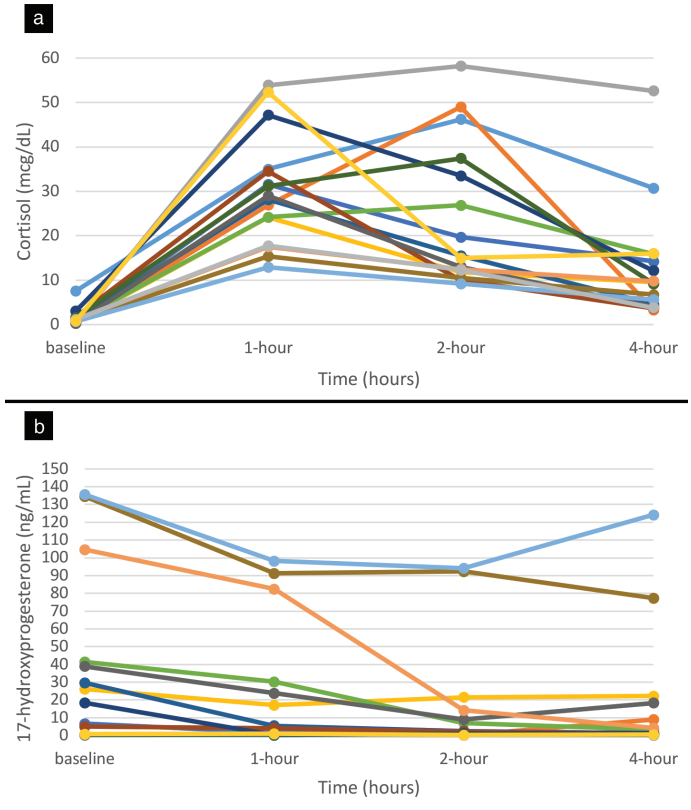

Figure 1. 17-hydroxyprogesterone (ng/mL) and cortisol (mcg/dL) concentrations after hydrocortisone dose in 16 patients over 4-hours are presented in (a) and (b)

When subjects were further divided according to their pubertal status and metabolic control was defined accordingly [poor vs good control, median (minimum-maximum)], annual change in height SDS was significantly higher in both prepubertal (n = 7) and pubertal (n = 8) children with poor control [prepubertal, 0.6 (0.5-0.8) vs 0.5 (0.1-0.5), $p = 0.03$; and pubertal 0.8 (0.78-0.8) vs -0.3 (-0.7-0.4), $p = 0.04$). Androstenedione levels with respect to upper limit of normal were increased only in the prepubertal group with poor control (0.1 (0.1-0.7) vs 1.4 (1.1-2.6), $p = 0.03$), whereas SHBG levels with respect to upper limit of normal was significantly decreased in the pubertal children with poor control (0.02 (0.01-0.02) vs 0.3 (0.1-0.9), $p = 0.04$). Androstenedione and SHBG levels with respect to upper limits of normal were negatively correlated in the pubertal children ($r_s = -0.7$, $p = 0.04$) but there was no correlation in the prepubertal group ($r_s = 0.5$, $p = 0.2$).

Discussion

This is the first study investigating the 4-hour serum profile of 17-OHP and the use of SHBG as a monitoring parameter in the context of changes in growth over a 1-year period in patients classical CAH. Several studies (7,9,22,23) evaluated the pharmacokinetic and pharmacodynamic actions of hydrocortisone. The utility of SHBG in CAH was explored with respect to other laboratory markers, such as 17-OHP which is already subject to debate in terms of reference ranges and sampling time (12). In contrast to these studies, we introduced another definition of metabolic control; in terms of a clinical indicator (annual change in height SDS) combined with a traditional laboratory marker (androstenedione) to evaluate other laboratory markers. There is a need for novel approaches since the interpretation of traditional disease markers can be challenging (2,7,9,24).

Previous studies suggested that single blood measurement of 17-OHP can be misleading, instead recommended serial samples, especially for cases of inadequate control (6,8).

Performing a 24-hour profile study for traditional markers, as recommended by Hindmarsh and Geertsma (11), would be ideal, but requires in-patient stay, increases infection risk, and may not be possible in most centers. Hence, we obtained a 4-hour profile and evaluated the laboratory results accordingly. In our study, median baseline 17-OHP concentrations were higher than the samples obtained after hydrocortisone administration. Even though serial monitored levels of 17-OHP were divergent for each patient, concentrations measured at different time points strongly correlated with the baseline values. Higher values obtained at any time point, most likely indicated that the other measurements were higher as well. On the other hand, treatment decisions based solely on the baseline measurements of 17-OHP would falsely result in overtreatment in more than half of the subjects, since either androstenedione and/or consecutive 17-OHP levels were in normal ranges for most of these patients. It is also noteworthy that Bizzarri et al (25) reported similar 17-OHP levels, mean height velocity, and androstenedione concentrations between groups of which adequacy of hydrocortisone therapy was adjusted according to samples taken prior to morning dose and 2-3 hours after the morning dose. Acknowledging these exceptions, excluding measurements taken before medication, irrespective of the sampling time, any single high value of 17-OHP may be

Table 2. Comparison of clinical characteristics and laboratory measurements between good and poor control groups

	Good control (n = 10)	Poor control* (n = 6)	p value
Age, years	9.8 [8]	4.3 [5.2]	0.06
Height SDS	0.02 [1.8]	0.6 [2.5]	0.6
BMI SDS	1.3 [2.2]	0.9 [1.6]	0.3
MPH SDS	-0.8 [1.3]	-0.6 [1.4]	0.8
Bone age SDS	2.9 [2.7]	2.5 [3.1]	0.5
Patients with advanced bone age, n (%)	8 (80%)	3 (50%)	0.3[i]
PAH SDS	-0.5 [1.9]	-0.08 [2.9]	0.3
Corrected height SDS	-0.5 [1.4]	-1 [2.5]	0.2
Annual delta height SDS	-0.03 [0.8]	0.7 [0.2]	0.001
Number of fast growing patients	0 (0%)	6 (100%)	< 0.001[i]
17-OHP (ng/mL)			
Patients with increased 17-OHP, n (%)	3 (30%)	6 (100%)	0.5[i]
Baseline	13 [31]	120 [157]	0.1
1-hour after morning dose	2.8 [18]	87 [123]	0.08
2-hours after morning dose	2 [7]	53 [111]	0.1
4-hours after morning dose	1.4 [11]	41 [133]	0.2
Cortisol (mcg/dL)			
Baseline	0.7 [1.7]	1 [0.5]	0.4
1-hour after the morning dose	30 [12]	18 [22]	0.08
2-hours after the morning dose	23 [34]	12 [10]	0.1
4-hours after the morning dose	11 [16]	8 [6]	0.7
A4 with respect to ULN	0.5 [1.5]	1.8 [1.5]	0.03
Number of patients with increased androstenedione	3 (30%)	6 (100%)	0.01[i]
SHBG with respect to ULN	0.5 [0.5]	0.9 [1.2]	0.4
Hydrocortisone dose (mg/m^2)			
6 months average of daily dose	16 [8]	7 [6]	0.02
Current daily dose	16.5 [8.4]	7 [6]	0.006
Current morning dose	6.6 [4.6]	3.8 [3.7]	0.05
Current afternoon dose	4.2 [2.5]	2 [1.7]	0.04
Current night dose	4.2 [2.4]	1.8 [1.1]	0.02

*Poor control defined when androstenedione is high and delta height SDS ≥0.5. Data are presented as median (interquartile range) or n (%).
[i]Difference between groups were calculated by Mann-Whitney U test; and [i]Fisher's exact test. All statistical assessments were considered significant at $p < 0.05$.
IQR: interquartile range, SDS: standard deviation score, BMI: body mass index, PAH: predicted adult height, Δh SDS: annual change in height SDS (current height SDS-height SDS measured 1-year earlier), MPH: midparental height, HC: average hydrocortisone dose in the last 6-months. SHBG: sex-hormone binding globulin, ULN: upper limit of normal

equally informative, only when it is evaluated with other indicators of hyperandrogenemia.

The guidelines (2) do not provide specific reference intervals for 17-OHP but complete normalization is related to overtreatment. In line with a previous report by Sarafoglou et al (7), suppressed values of 17-OHP, do not always show overtreatment as were demonstrated by patients #8, #11, #12, and #16 in our study. One alternative approach could be defining age and sex specific SDSs or time specific normal ranges for 17-OHP, as suggested by Clausen et al (26) and Neumann et al (27), but standard norms for SDSs has not been established yet.

As a monitoring parameter in disease control, we also investigated the role of SHBG, which is associated with several conditions, such as hyperinsulinemia, hyperandrogenism, and hyperthyroidism (28,29,30,31). To our knowledge, only a single report by Zamrazilová et al (12), explored the significance of SHBG in CAH in a retrospective study, irrespective of long term clinical control status, but found no relation with respect to 17-OHP levels, which are already changeable. Similarly, when we stratified groups according to indicators of hyperandrogenemia, we also found no difference in SHBG levels between the two control groups. However, SHBG levels depend on factors such as age, gender, puberty, obesity, and diet (20,32,33). When patients were further grouped according to their pubertal status, there was an inverse relationship between SHBG and androgens in pubertal children, while in pre-pubertal children, regardless of their androgen levels, SHBG levels were either at or above reference ranges. Due to the regulation of the hypothalamopituitary axis, neuroendocrine control, and the effect of insulin resistance, SHBG levels physiologically decline at puberty (30,33,34). This decline is more evident in pubertal children in the presence of hyperandrogenemia. However, high levels of SHBG seen in prepubertal children might be indicative of an age-specific protective mechanism, since SHBG determines the fraction of circulating testosterone by decreasing the metabolic clearance of testosterone, suppressing the conversion of testosterone to androstenedione, and reducing androgen availability to target cells by decreasing circulating testosterone (33,35). Further, as reported by Wallace et al (36), the administration of glucocorticoids lowers SHBG values too. This suggests that under the influence of both androgens and steroids, SHBG could be used in pubertal children as an indicator of hyperandrogenemia or a monitor of treatment adherence. However, the exact role of SHBG in prepubertal children is an area of research.

The use of the lowest effective hydrocortisone regimen in CAH is recommended for optimal long-term growth trajectory (25,37). Even though, the recommended daily dose range is 10-15 mg/m^2 in CAH patients, a recent retrospective multicenter study which included 11 countries demonstrated that up to 57% of cases used doses below 10 mg/m^2/day, whilst 75% of patient visits were above these ranges (38). Similarly, our patients received hydrocortisone doses between 6-24 mg/m^2/day. Although Pijnenburg-Kleizen et al (39) recommend higher hydrocortisone doses in early childhood, Bonfig et al (40) and Thilén et al (41) highlight the relative androgen insensitivity in growth patterns during the first 1.5 years of life. We also try to use the lowest possible hydrocortisone doses, and the growth patterns of children in our study were similar to the literature. In comparison to a meta-analysis (42) including 35 eligible studies, which showed that the corrected final height SDS of CAH patients to be 1.38 SDS lower than the population, the patients in our cohort were estimated to achieve a predicted adult height SDS of -0.4 (1.7). Although linear growth was not complete in our cohort, we believe that the clinical reasoning behind this better growth trajectory might be related to the use of lowest possible therapeutic doses, better nutritional status, and regular follow-up visits. However, in short term growth trajectory, the comparison between poor and good control groups indicates that doses lower than 7 mg/m^2/day should be avoided in CAH patients.

Study Limitations

Our study was weakened due to the retrospective nature of our data which consisted of only a small number of patients. Since our small cohort was heterogeneous with pre-, and post-pubertal children with varying ages, all factors affecting the levels of SHBG, age-defined reference ranges based on pubertal status for SHBG in CAH could not be concluded. However, interpretation of our results provides insights into SHBG in CAH but warrants further multicenter studies with a higher number of patients to unravel the underlying mechanisms. Our small numbered, salt wasting CAH cohort in a single center was also a strength, since we could evaluate the final follow-up year with standardized laboratory methods, and confounding factors, such as additional medications, concomitant diseases, or treatment adherence could be carefully monitored in a short, close period of time. As for changes in growth, to minimize the problems of pubertal height spurt, we also included a traditional laboratory marker (androstenedione) to define metabolic control status, and defined poor control when both indicators were elevated. Further, we used a change of 0.5 instead of 0.3 SDSs in height assessment, since both are recommended for monitoring growth velocity (43).

Conclusion

We conclude that: (i) the 4-hour 17-OHP profile is not useful in predicting hyperandrogenemia; (ii) normal reference intervals for different time periods should be developed for 17-OHP; (iii) contrary to the guidelines, a suppressed levels of 17-OHP does not always indicate overtreatment; (iv) low hydrocortisone doses should be avoided; and (v) SHBG can be considered as an indicator of hyperandrogenemia in pubertal children.

Ethics

Ethics Committee Approval: Institutional approval was granted by the Ethics Committee of Dokuz Eylül University Faculty of Medicine (ethics approval number: 2021/16-25, date: 27.05.2021).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Özge Besci, Ayhan Abacı, Design: Özge Besci, Ayhan Abacı, Ece Böber, Korcan Demir, Data Collection or Processing: Özge Besci, İbrahim Mert Erbaş, Kübra Yüksek Acinikli, Ayhan Abacı, Ece Böber, Korcan Demir, Analysis or Interpretation: Özge Besci, İbrahim Mert Erbaş, Tuncay Küme, Kübra Yüksek Acinikli, Ayhan Abacı, Ece Böber, Korcan Demir, Literature Search: Özge Besci, Korcan Demir, Writing: Özge Besci, Ayhan Abacı, Korcan Demir.

Financial Disclosure: The authors declared that this study received no financial support.

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Click for Supplementary Table 1 access link: http://glns.co/o97f3

Supplementary Table 1. Normal reference ranges of androstenedione and SHBG serum levels according to age and sex (18)

Age (years)	Androstenedione (nmol/L)		SHBG (nmol/L)	
	Female	Male	Female	Male
	Median (central 95% range)			
2.1-4	< 1.0 (< 1.0-11.1)	< 1.0 (< 1.0-10.3)	53.9 (33.2-135.0)	52.1 (26.7-110.0)
4.1-6	< 1.0 (< 1.0-11.3)	< 1.0 (< 1.0-5.8)	68.0 (23.0-100.0)	61.0 (37.4-147.6)
6.1-8	2.3 (< 1.0-8.7)	1.3 (< 1.0-6.5)	61.2 (29.7-121.0)	72.6 (19.8-114.0)
8.1-10	2.5 (< 1.0-5.3)	2.0 (< 1.0-4.5)	73.4 (26.3-127.6)	70.8 (37.9-132.0)
10.1-12	3.4 (< 1.0-12.4)	3.0 (< 1.0-7.8)	46.4 (16.4-112.2)	47.3 (21.4-149.7)
12.1-14	6.7 (1.7-11.6)	5.3 (< 1.0-9.5)	41.5 (18.5-89.3)	43.5 (13.0-101.5)
14.1-16	8.7 (2.4-15.4)	6.2 (1.6-12.2)	40.2 (14.7-91.2)	29.5 (10.1-73.8)
16.1-18	10.3 (1.4-17.3)	9.4 (3.4-14.6)	38.1 (20.0-85.9)	24.2 (11.5-45.2)

SHBG: sex hormone-binding globulin

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2022.2021-9-19

J Clin Res Pediatr Endocrinol 2022;14(2):153-171

Mutations in *AR* or *SRD5A2* Genes: Clinical Findings, Endocrine Pitfalls, and Genetic Features of Children with 46,XY DSD

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What is already known on this topic?

Androgen insensivity syndrome and 5α-reductase deficiency are the most common causes of 46,XY disorders of sexual development. They can present as indistinguishable phenotypes that usually necessitate molecular analyses for the definitive diagnosis in the prepubertal period.

What this study adds?

Testosterone to dihydrotestosterone ratio may lead to diagnostic confusion. Genetic analysis for actual diagnosis seems to be essential. Four novel androgen receptor variants were identified in this Turkish pediatric population.

Abstract

Objective: Androgen insensivity syndrome (AIS) and 5α-reductase deficiency (5α-RD) present with indistinguishable phenotypes among the 46,XY disorders of sexual development (DSD) that usually necessitate molecular analyses for the definitive diagnosis in the prepubertal period. The aim was to evaluate the clinical, hormonal and genetic findings of 46,XY DSD patients who were diagnosed as AIS or 5α-RD.

Methods: Patients diagnosed as AIS or 5α-RD according to clinical and hormonal evaluations were investigated. Sequence variants of steroid 5-α-reductase type 2 were analyzed in cases with testosterone/dihydrotestosterone (T/DHT) ratio of ≥20, whereas the androgen receptor (*AR*) gene was screened when the ratio was < 20. Stepwise analysis of other associated genes were screened in cases with no causative variant found in initial analysis. For statistical comparisons, the group was divided into three main groups and subgroups according to their genetic diagnosis and T/DHT ratios.

Results: A total of 128 DSD patients from 125 non-related families were enrolled. Birth weight SDS and gestational weeks were significantly higher in 5α-RD group than in AIS and undiagnosed groups. Completely female phenotype was higher in all subgroups of both AIS and 5α-RD patients than in the undiagnosed subgroups. In those patients with stimulated T/DHT < 20 in the prepubertal period, stimulated T/DHT ratio was significantly lower in AIS than in the undiagnosed group, and higher in 5α-RD. Phenotype associated variants were detected in 24% (n = 18 AIS, n = 14 5α-RD) of the patients, revealing four novel *AR* variants (c.94G > T, p.Glu32*, c.330G > C, p.Leu110 = ; c.2084C > T, p.Pro695Leu, c.2585_2592delAGCTCCTG, p.(Lys862Argfs*16), of these c.330G > C with silent status remained undefined in terms of its causative effects.

Conclusion: T/DHT ratio is an important hormonal criterion, but in some cases, T/DHT ratio may lead to diagnostic confusion. Molecular diagnosis is important for the robust diagnosis of 46,XY DSD patients. Four novel *AR* variants were identified in our study.

Keywords: 46,XY disorders of sex development, 5α-reductase deficiency, androgen insensitivity syndrome, androgen receptor gene mutations, *SRD5A2* gene mutations

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Conflict of interest: None declared
Received: 12.11.2021
Accepted: 12.12.2021

Introduction

Acompletelyvirilized male phenotype requires a 46,XY chromosome, adequate testosterone (T) and dihydrotestosterone (DHT) which is formed from T by the enzyme steroid 5-α-reductase type 2 (SRD5A2) and, fully functionally active androgen receptors (AR) and post receptor pathways (1,2,3). A disturbance at any of those last stages can lead to 46,XY disorders of sex development (DSD), characterized by a range of female phenotype to incompletely virilized external genitalia (1,2,3). Although a significant proportion of 46,XY DSD cases may remain aetiologically unclarified, the most common identifiable cause in the reported series is androgen insensitivity syndrome (AIS) and 5α-reductase deficiency (5α-RD) is the second most common cause (1,2,3). The diagnosis of AIS requires the exclusion of other aetiologies of 46,XY DSD, which are gonadal differentiation defects, and T biosynthesis and metabolism. In the prepubertal period, phenotypes of AIS, 5α-RDor unknown aetiologies of 46,XY DSD are indistinguishable because of the similarities intheir clinical findings. Although adequate serum T concentrations rule out a defect in T biosynthesis, a low T value at baseline or following human chorionic gonadotrophin (hCG) stimulation does not always rule out AIS (2). On the other hand, phenotype at birth varies widely in 5α-RD, according to the levels of residual enzymatic function, so a very similar clinical picture to AIS at prepubertal ages with normal testicular T production can be observed in these patients. Although serum T/DHT ratio is an important screening tool for identifying patients with possible 5α-RD, cut-off values for diagnosis remain uncertain. Cases with a molecular diagnosis of 5α-RD and T/DHT ratio below the suggested cut-off values have also been reported (4). So, there are overlaps in the diagnosis of AIS or 5α-RD based on clinical or hormonal data and to distinguish them from each other or to differentiate these two main or most common diagnoses from unknown aetiologies, molecular analyses is necessary.

The *AR* gene is located on the X-chromosome inthe Xq12 region, composed of eight exons and encodes a protein with 920 aa in length (NP_000035), that function as a transcription factoractivated via binding of steroid hormones. The peptide chain of AR consists of three domains:residues at the N-terminal region between 6-449 aa encodes androgen receptor domain (NTD; N terminal domain); 558-627 aa zinc finger C4 type domain (DBD, DNA binding domain); and 690-881 ligand binding domain (LBD) of nuclear hormone receptor (EMBL-EBI; P10275, Pfam) (PubMed: 16381856) (5,6). Loss-of-function mutation of *AR* is responsible for X-linked androgen insensitivity (MIM#300068) and hypospadias (MIM# 300633) in humans. The *SRD5A2* gene is located at 2p23, comprises five exons encoding a 254 aa peptide chain that encircles 3-oxo-5-alpha-steroid 4-dehydrogenase domain, encoded by residues between 105-254 (EMBL-EBI; P31213, Pfam) (PubMed: 16381856). Bi-allellic pathogenic alterations of *SRD5A2* are associated with pseudovaginal perineoscrotal hypospadias (MIM# 264600) caused by steroid 5α-RD.

Since previous studies have indicated *AR* and *SRD5A2* gene mutationsas the most common culprits behind 46,XY DSD for the most part (1,2,3), we focused on these two genes in patients with 46,XY DSDs with normal testicular development. Here, a relatively large cohort of Turkish children is reported to present the clinical, hormonal and genetic features of 46,XY DSD patients who were considered as AIS or 5α-RD and to analyze the accordance between the clinical and laboratory results withgenetic analysis.

Methods

Participants

A retrospective medical chart review of 46,XY DSD patients was performed to collect data from the Pediatric Endocrinology Outpatient Clinic of İstanbul University, İstanbul, Turkey. DSD patients who were diagnosed as AIS or 5α-RD according to the clinical, hormonal or molecular evalutions, were included in the study. Since the study was performed respectively, patient-informed consent forms were not needed. The study has been reviewed by the Ethics Committee of İstanbul Faculty of Medicine, İstanbul University, and has therefore been performed in accordance with the ethical standards laid down in an appropriate version of the Declaration of Helsinki. Initially, we sought all cases with AIS, 5α-RD or undiagnosed groups according to their molecular diagnosis. In addition, the participants were also subclassified according to their T/DHT for statistical analysis.Clinical diagnosis of AIS or 5α-RD was based on normal T secretion without Mullerian duct structures. Criteria suggesting DSD included overt genital ambiguity, apparent female genitalia with or without clitoromegaly, posterior labial fusion or inguinal/labial mass, and apparent male genitalia with non-palpable testes, micropenis, isolated perineal hypospadias or mild hypospadias with undescended testis (7). Also, file records of older children and adolescents who hadincomplete or delayed puberty, lack of breast development or primary amenorrhea or virilization at puberty were retrospectively evaluated with respect to DSD. A detailed history including age at presentation, main complaints, sex of rearing, and parental consanguinity were recorded for each patient. A clinical examination

consisting of anthropometry, assessment of pubertal stage, severity of ambigious genitalia, penile length and associated anomalies or dysmorphic features were evaluated for each patient. Quigley scale for grading AIS was used to determine the degree of external virilization in 46,XY DSD patients (8,9,10). Grades 2 through 5 quantify four degrees of increasingly feminized genitalia that correspond to partial AIS (PAIS). Grades 1 and 6/7 correspond to mild AIS (MAIS) and complete AIS (CAIS), respectively (10). The external masculinization score (EMS, range 0-12) was also assessed in patients (11,12). The standard deviation score (SDS) of height and weight were calculated according to the reported data of Neyzi et al (13) for Turkish children and adolescence, whereas birth weight SDS based on gestation week, were calculated according to reported national data for Turkish newborns (14). Patients were subdivided into three groups according to birth weight SDS: small for gestational age [SGA ($<$ -2 SDS)], large for gestational age [LGA ($>$ + 2 SDS)] and appropriate for gestational age [AGA (between -2 and + 2 SDS)].

Laboratory Monitoring

As a part of routine evaluation of DSD, we performed hormonal measurements, karyotype analysis, abdominopelvic and scrotal ultrasound and, if required, magnetic resonance imaging. Basal level of T, DHT, T/DHT ratio and gonadotropins were measured at pubertal age or in mini puberty. A short-term hCG test was applied in the appropriate cases who were in the prepubertal period. The hCG stimulation test was carried out by administering 1500 IU/m^2/dose of hCG daily IM for three consecutive days to determine the ability of the gonads to produce T and DHT. Blood samples were obtained before the first dose and 24 h after the last (15). An increment in plasma T (ΔT) of more than 0.8 ng/mL or an absolute level greater than 0.9 ng/mL after hCG treatment was considered to be indicative of the presence of functioning testicular tissue and was defined as normal (16). Laboratory diagnosis of AIS was identified as normal-sized testes, absent Mullerian structures, normal follicle-stimulating hormone (FSH), normal/mildly elevated luteinizing hormone (LH) and normal/elevated baseline or hCG-stimulated T level, and normal T/DHT ratio (T-to-DHT ratio $<$ 20). A T/DHT ratio ≥20 was accepted as suggestive of 5α-RD (17,18). Also for exclusion of 46,XY DSD causes related to congenital adrenal hyperplasia,cortisol, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEA-S), and androstenedione (A) were also measured. A T-to-A ratio (T/A) $<$ 0.8 was accepted as suggestive of 17β-hydroxysteroid dehydrogenase (17β-HSD) deficiency (11,12) and they were excluded.For statistical comparisons, the groups were divided into subgroups according to their genetic diagnosis and T/DHT ratios.

Hormone Assays

Adrenocorticotropic hormone, cortisol, DHEA-S, A and 17-OHP were measured using the IMMULITE 2000 system (immunochemiluminescence assay; ICMA; Siemens AG, Berlin and Munich, Germany) while LH, FSH, and T were analyzed by electrochemiluminiscense immunoassay (Cobas, Roche Diagnostics, Mannheim, Germany). Enzyme-linked immunosorbent assay kits were also used for the direct quantitative determination of DHT although different laboratory results using different reagents were included in the study as a result of retrospective design.

Molecular Analysis

Conventional karyotyping analysis were performed before molecular genetic investigations. Sequence variants of the *SRD5A2* gene (NM_000348.3) screened in the cases who had T/DHT ratio ≥20 whereas the *AR* gene (NM_000044.4) was investigated by Sanger sequencing in patients who had the ratio $<$ 20 for pathogenic alterations. If no pathogenic alteration was detected in the first analysis, then the second genetic analysis was applied for *SRD5A2* or *AR*, whichever was not analysed in the first run (Figure 1). Those who did not have a mutation in either *AR* or *SRD5A2* constituted the undiagnosed group. Each group were further subgrouped according the T/DHT value. Pathogenic variants were confirmed by database search (Human Genome Mutation Database, ClinVar) and literature search and classified according to ACMG guideline (19,20,21). Human splice finder analysis was used for variants with silent status (22). Segregation in family members were performed whenever available.

Statistical Analysis

The data were analyzed using Statistical Package for the Social Sciences (SPSS), version 21 (SPSS Inc., Chicago, IL, USA). Data were analyzed using descriptive statistical methods [mean, standard deviation, median, frequency, rate, ranges (minimum-maximum)] as well as some methods for comparing quantitative data. The results are given as median (minimum-maximum values) according to the distribution of data or as percentages, where appropriate. Mann-Whitney U test was used in the two-group comparisons of parameters without normal distribution. In comparison of three or more parameters without normal distribution Kruskal-Wallis test was used. A p value of less than 0.05 were considered statistically significant.

Results

Clinical Findings

A total of 128 DSD patients from 125 unrelated families were enrolled in the study. The flowchart of the distribution of the patients is given in Figure 1.

On admission, the median age of AIS patients (n = 18) was 1.0 [minimum (min): 0.01, maximum (max): 17.4] years, in 5α-RD (n = 14) it was 6.7 years (min: 0.01, max: 17.5), and 0.3 years (min: 0.01, max: 14.3) in the undiagnosed group (n = 96) ($p < 0.05$). Review of patient charts indicated that Quigley scale grade and EMS on admission, reared gender, consanguinity, birth weight (BW) SDS and gestational age (GA, weeks) of patients were different between the three groups. BW SDS and GA were significantly higher in 5α-RD patients than the other two groups. Median (range) BW SDS in 5α-RD patients was 0.4 (-1.5 to 2.4) whereas median BW SDS were -1.0 (-4.2 to 2.0) in AIS and -1.5 (-7.7 to 3.3) in the undiagnosed group. Median values of GA in groups were also 40 (38-40) weeks in 5α-RD, 38 (28-40) weeks in AIS and 38 (27-42) weeks in the undiagnosed group. SGA rate was significantly different between groups. None of the 5α-RD patients had SGA whereas the SGA rates in AIS and undiagnosed groups were 27.8% and 40.2%, respectively. Consanguinity rate was higher in 5α-RD patients than in both AIS and in the undiagnosed patients (Tables 1,2,3,4).

Comparisons of AIS and 5α-RD groups with the undiagnosed group according to T/DHT ratios are detailed in Tables 1,2,3,4, respectively. Although age at presentation did not differ between any subgroup of AIS and undiagnosed patients, 5α-RD patients presented later than undiagnosed patients (Tables 1,2,3,4). BWSDS and GA of undiagnosed patients were significantly lower than 5α-RD patients, whereas it did not differ between the subgroups of AIS and undiagnosed patients (Tables 1,2,3,4). However, the percentages of SGA, AGA, and LGA were same between the subgroups. Symptoms on admission,additional findings, current anthropometry and pubertal status of all subgroups are also shown in Tables 1,2,3,4. Additional findings or diagnoses, including SGA or prematurity, were significantly lower in 5α-RD subgroups than in the undiagnosed subgroups whereas it was not different between AIS and undiagnosed subgroups. However, other disease comorbidities (kidney disease, congenital heart disease, anal atresia and autism) were detected only in the undiagnosed group (Tables 1,2,3,4). According to Quigley scale, CAIS grade rate was higher in all subgroups of both AIS and 5α-RD patients than in the undiagnosed subgroups (Tables 1,2,3,4). Comparison of EMS within subgroups differed significantly except between genetically proven AIS and undiagnosed patients with a normal T/DHT value (< 20) (Tables 1,2,3,4). Reared gender was also different between the subgroups (Tables 1,2,3,4).

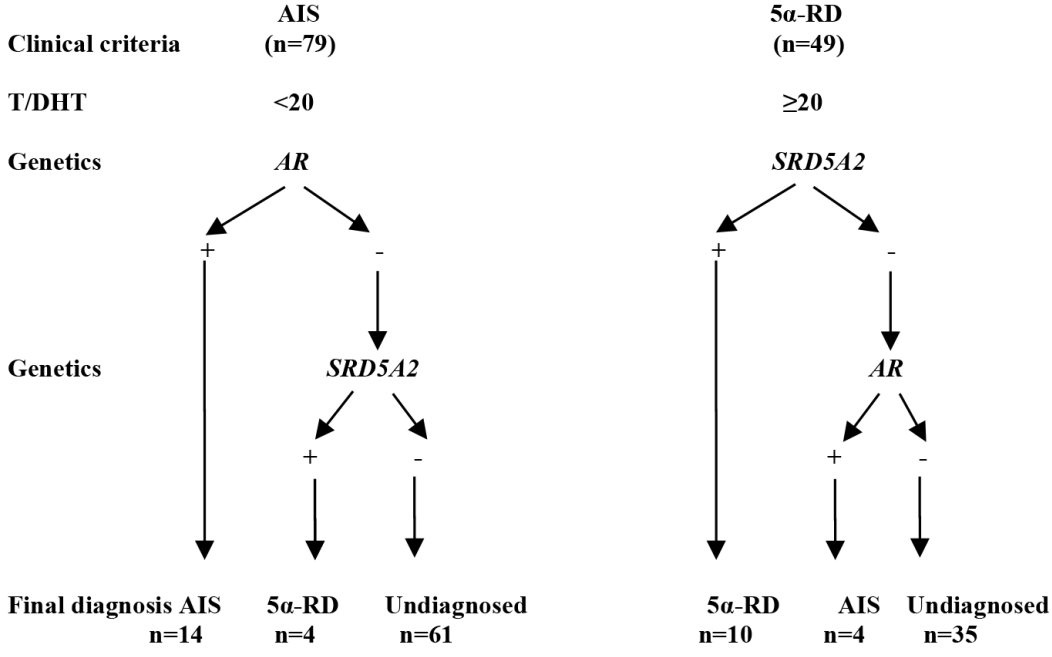

Figure 1. Analysis of the patients with 46,XY DSD

5α-RD: 5α-reductase deficiency

Table 1. Comparison of clinical and hormonal features of genetically proven AIS and undiagnosed patients with a normal T/DHT value (< 20)

	Mutation (+)	Mutation (-)	p
T/DHT: Normal	n = 14	n = 61	
Age at presentation (years)	1.8 ± 3.1	1.3 ± 2.0	0.98
Consanguinity, n (%)	25% (n = 3)	23% (n = 14)	0.87
Birth weight SDS	-0.8 ± 1.9	-1.7 ± 2.0	0.19
Gestational age (weeks)	37 ± 4.1	36.6 ± 3.7	0.46
Gestational age groups (weeks), n (%)			0.4
< 37	21.4% (n = 3)	39.3% (n = 24)	
≥37	78.5% (n = 11)	60.6% (n = 37)	
BW groups, n (%)			0.6
AGA (-2 to +2 SDS), n (%)	64.3% (n = 9)	54% (n = 33)	
SGA (< -2 SDS), n (%)	28.6% (n = 4)	42.6% (n = 26)	
LGA (> +2 SDS), n (%)	7.1% (n = 1)	3.3% (n = 2)	
Symptom on admission			0.07
Ambiguous genitalia	78.5% (n = 11)	93.4% (n = 57)	
Same diagnosis siblings	-	1.6% (n = 1)	
Mass in groin	14.2% (n = 2)	1.6% (n = 1)	
Undescended testes	-	1.6% (n = 1)	
Micropenis	-	1.6% (n = 1)	
Finding testis during inguinal hernia surgery	7.1% (n = 1)	-	
At current			
Age (years)	6.7 ± 0.9	6.5 ± 5.4	0.40
Weight SDS	-0.4 ± 1.6	-0.7 ± 1.2	0.69
Height SDS	-0.3 ± 1.4	-1.0 ± 1.1	0.16
Puberty status			0.21
Tanner stage 1	78.5% (n = 11)	77% (n = 47)	
Tanner stage 2	7.1% (n = 1)	8.2% (n = 5)	
Tanner stage 3	7.1% (n = 1)	1.6 (n = 1)	
Tanner stage 4	7.1% (n = 1)	-	
Tanner stage 5	-	9.8% (n = 6)	
Mini puberty	-	1.6% (n = 1)	
Estrogen replacement after gonadectomy	-	1.6% (n = 1)	
Reared gender, n (%)			**< 0.01***
Male	71.4% (n = 10)	96.7% (n = 59)	
Female	28.5% (n = 4)	1.6% (n = 1)	
Female reared, changed identity after diagnosis	-	1.6 % (n = 1)	
Ouigley scale			0.06
2	7.1% (n = 1)	18% (n = 11)	
3	35.7% (n = 5)	57.4% (n = 35)	
4	14.2% (n = 2)	11.5% (n = 7)	
5	7.1% (n = 1)	4.9% (n = 3)	
6/7	28.5% (n = 4)	1.6% (n = 1)	
Applied after operation	7.1% (n = 1)	6.5% (n = 4)	
EMS			0.23
1	5.5% (n = 1)	1.6% (n = 1)	
2	16.7% (n = 3)	0% (n = 0)	
4	11.1% (n = 2)	16.4% (n = 10)	
5	0% (n = 0)	23% (n = 14)	
6	22.2% (n = 4)	19.7% (n = 12)	
7	11.1% (n = 2)	27.9% (n = 17)	
8	5.5% (n = 1)	4.9% (n = 3)	
Applied after operation	5.5% (n = 1)	6.5% (n = 4)	

Table 1. Continued

	Mutation (+)	Mutation (-)	p
AIS groups, n (%)			**0.02***
PAIS	71.4% (n=10)	98.4% (n=60)	
CAIS	28.5% (n=4)	1.6% (n=1)	
Additional findings			0.19
None	57.1% (n=8)	36% (n=22)	
Prematurity	21.4% (n=3)	34.4% (n=21)	
IUGR	-	9.8% (n=6)	
Multicystic dysplastic kidney	-	3.3% (n=2)	
Congenital heart disease	-	4.9% (n=3)	
Anal atresia	-	3.3% (n=2)	
Nephrolithiasis	-	1.6% (n=1)	
Autism	-	1.6% (n=1)	
Gonadoblastoma	-	1.6% (n=1)	
Infant of diabetic mother	-	1.6% (n=1)	
Klinefelter syndrome	7.1% (n=1)	1.6% (n=1)	
47,XYY	7.1% (n=1)	-	
Agenesis of the corpus callosum	7.1% (n=1)	-	
Target height SDS	-0.8±0.2	-0.9±0.09	0.97
Laboratory results according to the age			
0-6 months	n=7	n=26	
Age (months)	2.1±1.5	1.6±1.6	0.38
LH (mIU/mL)	4.9±4.3	4.1±3.7	0.62
FSH (mIU/mL)	1.7±1.2	2.2±1.6	0.42
Basal T (ng/mL)	1.5±1.4	1.6±0.8	0.76
Basal DHT (ng/mL)	0.3±0.2	0.4±0.1	0.59
Basal T/DHT ratio	10.5±7.3	6.9±6.3	0.22
Prepubertal	n=7	n= 38	
Age (years)	4.0±2.9	3.8±2.9	0.67
LH (mIU/mL)	0.7±0.9	0.5±1.6	0.23
FSH (mIU/mL)	1.2±1.0	1.5±2.5	0.86
Basal T (ng/mL)	0.3±0.4	0.2±0.4	0.82
Stimulated T (ng/mL)	5.1±2.9	4.3±2.4	0.38
Stimulated DHT (ng/mL)	1.5±1.6	0.6±0.5	0.12
Stimulated T/DHT	4.7±2.8	9.3±1.3	**0.04***
Pubertal	n=3	n=8	
Age (years)	10.8±0.3	12.7±3.0	0.13
LH (mIU/mL)	2.4±4.2	3.8±5.0	0.69
FSH (mIU/mL)	2±4.9	6.5±9.4	0.56
Basal T (ng/mL)	4.3±3.1	2.6±2.0	0.51
Basal DHT (ng/mL)	0.5±0.2	0.4±0.3	0.91
Basal T/DHT	9.5±4.5	5.8±3.0	0.16

AGA: appropriate for gestational age, AIS: androgenin sensitivity syndrome, BW: birth weight, CAIS: complete androgen insensitivity syndrome, DHT: dihydrotestosterone, EMS: external masculinization score, FSH: follicle-stimulating hormone, IUGR: intrauterine growth retardation, LGA: large for gestational age, LH: luteinizing hormone, PAIS: partial androgen insensitivity syndrome, SGA: small for gestational age, T: testosterone, SDS: standard deviation score

Endocrine Data

Laboratory results of AIS, 5α-RD and the undiagnosed groups according to their T/DHT ratios are detailed in Tables 1, 2, 3 and 4. 22.2% (n=4) of AIS patients and 36.5% (n=35) of undiagnosed patients had T/DHT ratio ≥20, whereas 28.6% (n=4) of 5α-RD patients had T/DHT ratio <20 (Table 1-4). T/DHT ratios were obtained in 57% (n=73) during hCG test, whereas the remaining were obtained by basal hormone levels during puberty or minipuberty. Twenty four patients had more than one T/DHT ratio according to their ages. The mean values of T/DHT ratios used for hormonal diagnosis were significantly different between

Table 2. Comparison of clinical and hormonal features of genetically proven AIS and undiagnosed patients with a high T/DHT value (≥20)

	Mutation (+)	Mutation (-)	p
T/DHT: High	n = 4	n = 35	
Age at presentation (years)	4.0 ± 4.4	1.3 ± 2.8	0.11
Consanguinity, n (%)	0% (n = 0)	25.8% (n = 8)	0.38
Birth weight SDS	-0.7 ± 1.9	-1.2 ± 1.6	0.7
Gestational age (weeks)	35.0 ± 7.0	35.9 ± 4.1	0.93
Gestational age groups (weeks), n (%)			0.84
< 37	25% (n = 1)	40% (n = 14)	
≥37	75% (n = 3)	60% (n = 21)	
BW groups, n (%)			0.88
AGA (-2 to +2 SDS), n (%)	75% (n = 3)	60% (n = 21)	
SGA (< -2 SDS), n (%)	25% (n = 1)	34.3% (n = 12)	
LGA (> +2 SDS), n (%)	0% (n = 0)	5.7% (n = 2)	
Symptom on admission			**< 0.01***
Ambiguous genitalia	75% (n = 3)	94.3% (n = 33)	
Mass in groin	-	-	
Undescended testes	-	2.8% (n = 1)	
Micropenis	-	2.8% (n = 1)	
Finding testis during inguinal hernia surgery	25% (n = 1)	-	
At current			
Age (years)	7.7 ± 8.6	3.9 ± 4.7	0.34
Weight SDS	0.6 ± 1.8	-0.7 ± 1.2	0.20
Height SDS	-0.3 ± 0.6	-0.9 ± 1.3	0.38
Puberty status			0.07
Tanner stage 1	25% (n = 1)	82.9% (n = 29)	
Tanner stage 2	-	-	
Tanner stage 3	-	-	
Tanner stage 4	-	-	
Tanner stage 5	-	11.4% (n = 4)	
Mini puberty	-	5.7% (n = 2)	
Estrogen replacement after gonadectomy	75% (n = 3)	-	
Reared gender, n (%)			**0.04***
Male	25% (n = 1)	94.3% (n = 33)	
Female	75% (n = 3)	2.8% (n = 1)	
Female reared, changed identity after diagnosis	-	2.8% (n = 1)	
Ouigley scale			**< 0.01***
2	-	8.6% (n = 3)	
3	25% (n = 1)	85.7% (n = 30)	
4	-	2.8% (n = 1)	
5	-	2.8% (n = 1)	
6/7	75% (n = 3)	-	
EMS			**0.01***
1	50% (n = 2)	0% (n = 0)	
2	25% (n = 1)	0% (n = 0)	
3	0% (n = 0)	2.9% (n = 1)	
4	0% (n = 0)	17.1% (n = 6)	
5	0% (n = 0)	8.6% (n = 3)	
6	25% (n = 1)	22.9% (n = 8)	
7	0% (n = 0)	40% (n = 14)	
8	0% (n = 0)	8.6% (n = 3)	
AIS groups, n (%)			
PAIS	25% (n = 1)	100% (n = 35)	**< 0.01***
CAIS	75% (n = 3)	-	

Table 2. Continued

	Mutation (+)	Mutation (-)	p
Additional findings			0.95
None	75% (n = 3)	51.4% (n = 18)	
Prematurity	25% (n = 1)	34.3% (n = 12)	
IUGR	-	11.4% (n = 4)	
Anal atresia	-	2.8% (n = 1)	
Target height SDS	-0.8 ± 0.3	-0.8 ± 0.1	0.93
Laboratory results according to the age			
0-6 months	n = 0	n = 20	
Age (months)	-	2.5 ± 2.1	-
LH (mIU/mL)	-	3.9 ± 3.4	-
FSH (mIU/mL)	-	1.8 ± 1.4	-
Basal T (ng/mL)	-	1.8 ± 1.5	-
Basal DHT (ng/mL)	-	0.08 ± 0.05	-
Basal T/DHT	-	40.0 ± 18.5	-
Prepubertal	n = 2	n = 19	
Age (years)	5.2 ± 5.0	2.2 ± 2.2	0.23
LH (mIU/mL)	0.3 ± 0.1	0.3 ± 0.2	0.76
FSH (mIU/mL)	1.8 ± 1.6	0.8 ± 0.5	0.23
Basal T (ng/mL)	0.05 ± 0.07	0.05 ± 0.07	0.12
Stimulated T (ng/mL)	4.6 ± 2.2	4.0 ± 2.9	0.95
Stimulated DHT (ng/mL)	0.05 ± 0.07	0.2 ± 0.4	0.58
Stimulated T/DHT	94.8 ± 28.7	81.6 ± 15.9	0.49
Pubertal	n = 3	n = 3	
Age (years)	12.3 ± 2.7	12.3 ± 2.7	0.22
LH (mIU/mL)	23.9 ± 3.0	6.1 ± 2.9	**0.04***
FSH (mIU/mL)	20.6 ± 10.4	6.6 ± 5.7	0.12
Basal T (ng/mL)	6.5 ± 1.9	4.4 ± 2.2	0.28
Basal DHT (ng/mL)	0.2 ± 0.08	0.09 ± 0.1	0.64
Basal T/DHT ratio	55.8 ± 29.7	57.3 ± 21.2	0.31

AGA: appropriate for gestational age, AIS: androgenin sensitivity syndrome, BW: birth weight, CAIS: complete androgen insensitivity syndrome, DHT: dihydrotestosterone, EMS: external masculinization score, FSH: follicle-stimulating hormone, IUGR: intrauterine growth retardation, LGA: large for gestational age, LH: luteinizing hormone, PAIS: partial androgen insensitivity syndrome, SGA: small for gestational age, T: testosterone, SDS: standard deviation score

AIS, 5α-RD and undiagnosed groups ($p < 0.05$). 5α-RD patients had significant higher T/DHT ratios than both the AIS and undiagnosed groups. Although basal T/DHT ratio during minipuberty or puberty did not differ, stimulated T/DHT ratio in the prepubertal period was significantly lower in the AIS subgroup with T/DHT < 20 than in the undiagnosed subgroup with T/DHT < 20 (Table 1). In contrast, the stimulated T/DHT ratio in the prepubertal period was significantly higher in the 5α-RD subgroup with T/DHT < 20 than in the undiagnosed subgroup with T/DHT < 20 (Table 3). Clinical and laboratory findings of genetically diagnosed AIS and 5α-RD patients arepresented in Supplementary Table 1 and 2. Only pubertal LH was significantly higher in AIS patients than the undiagnosed group when T/DHT≥20.

Molecular Genetics

Cytogeneticaly, 32 patients had definitive diagnosis and four patients had non-46,XY karyotypes (three with 47,XXY, and one with 47,XYY). Fourteen patients were investigated only for AR, 10 only for *SRD5A2* and 104 patients were investigated for both genes. Variants attributed to disease were found in 32 (24%) patients, whereas the others (75%, n = 96) remained undiagnosed for molecular genetic base. In the total cohort, 14% of patients (n = 18) were molecularly diagnosed as AIS, and 10.9% (n = 14) had 5α-RD genetically. Molecular genetic test results showed four novel *AR* variants (c.94G > T, p.Glu32*; c.330G > C, p.Leu110=; c.2084C > T, p.Pro695Leu; c.2585_2592delAGCTCCTG, p.(Lys862Argfs*16). Among those, c.94G > T and c.2585_2592delAGCTCCTG were classified as pathogenic, c.2084C > T as likely pathogenic, while silent change (c.330G > C) as likely benign. In *in silico* analysis for human splicing finder, c.330G > C is not expected to have significant impact on splicing signals (22). This silent change would cause the alteration of leucine encoded by CTG to CTC. In a study of translation-selection model of human genome, it was shown that CTG is the major codon for leucine-tRNA, being more abundant in a translation environment, an important

Table 3. Comparison of clinical and hormonal features of genetically proven 5α-reductase deficiency and undiagnosed patients with a normal T/DHT value (< 20)

	Mutation (+)	Mutation (-)	p
T/DHT: Normal	n = 4	n = 61	
Age at presentation (years)	12.0 ± 4.9	1.3 ± 2.0	**< 0.01***
Consanguinity, n (%)	75% (n = 3)	23% (n = 14)	**0.02***
Birth weight SDS	0.0 ± 1.0	-1.7 ± 2.0	**0.07**
Gestational age (weeks)	39.7 ± 0.5	36.6 ± 3.7	**0.04***
Gestational age groups (weeks), n (%)			0.12
< 37	0% (n = 0)	39.3% (n = 24)	
≥37	100% (n = 4)	60.6% (n = 37)	
BW groups, n (%)			0.19
AGA (-2 to +2 SDS), n (%)	100% (n = 4)	54% (n = 33)	
SGA (< -2 SDS), n (%)	0% (n = 0)	42.6% (n = 26)	
LGA (> +2 SDS), n (%)	0% (n = 0)	3.3% (n = 2)	
Symptom on admission			**< 0.01***
Ambiguous genitalia	0	93.4% (n = 57)	
Same diagnosis siblings	50% (n = 2)	1.6% (n = 1)	
Mass in groin	50% (n = 2)	1.6% (n = 1)	
Undescended testes	0	1.6% (n = 1)	
Micropenis	0	1.6% (n = 1)	
At current evaluation			
Age (years)	15.9 ± 2.8	6.5 ± 5.4	**< 0.01***
Weight SDS	-0.8 ± 0.7	-0.7 ± 1.2	0.82
Height SDS	-0.6 ± 0.6	-1.0 ± 1.1	0.56
Puberty status			**< 0.01***
Tanner Stage 1	-	77% (n = 47)	
Tanner Stage 2	25% (n = 1)	8.2% (n = 5)	
Tanner Stage 3	-	1.6 (n = 1)	
Tanner Stage 4	-	-	
Tanner Stage 5	-	9.8% (n = 6)	
Mini puberty	-	1.6% (n = 1)	
Estrogen replacement after gonadectomy	75% (n = 3)	1.6% (n = 1)	
Reared gender, n (%)			**< 0.01***
Male	-	96.7% (n = 59)	
Female	75% (n = 3)	1.6% (n = 1)	
Female reared, changed identity after diagnosis	25% (n = 1)	1.6 % (n = 1)	
Ouigley scale on admission			**< 0.01***
2	-	18% (n = 11)	
3	25% (n = 1)	57.4% (n = 35)	
4	-	11.5% (n = 7)	
5	50% (n = 2)	4.9% (n = 3)	
6/7	25% (n = 1)	1.6% (n = 1)	
Applied after operation	-	6.5% (n = 4)	
EMS score			**< 0.01***
1	0% (n = 0)	1.6% (n = 1)	
2	25% (n = 1)	0% (n = 0)	
4	0% (n = 0)	16.4% (n = 10)	
5	0% (n = 0)	23% (n = 14)	
6	0% (n = 0)	19.7% (n = 12)	
7	0% (n = 0)	27.9% (n = 17)	
8	75% (n = 3)	4.9 % (n = 3)	
Applied after operation		6.5% (n = 4)	

Table 3. Continued

	Mutation (+)	Mutation (-)	p
AIS groups, n (%)			**< 0.01***
PAIS	75% (n = 3)	98.4% (n = 60)	
CAIS	25% (n = 1)	1.6% (n = 1)	
Additional findings/diagnosis			**0.03***
None	100% (n = 4)	36% (n = 22)	
Prematurity	-	34.4% (n = 21)	
IUGR	-	9.8% (n = 6)	
Multicystic dysplastic kidney	-	3.3% (n = 2)	
Congenital heart disease	-	4.9% (n = 3)	
Anal atresia	-	3.3% (n = 2)	
Nephrolithiasis	-	1.6% (n = 1)	
Autism	-	1.6% (n = 1)	
Gonadoblastoma	-	1.6% (n = 1)	
Infant of diabetic mother	-	1.6% (n = 1)	
Klinefelter syndrome	-	1.6% (n = 1)	
Target height SDS	-1.0 ± 0.2	-0.9 ± 0.09	0.43
Laboratory results according to the age			
0-6 months	n = 0	n = 26	-
Age (months)	-	1.6 ± 1.6	-
LH (mIU/mL)	-	4.1 ± 3.7	-
FSH (mIU/mL)	-	2.2 ± 1.6	-
Basal T (ng/mL)	-	1.6 ± 0.8	-
Basal DHT (ng/mL)	-	0.4 ± 0.1	-
Basal T/DHT	-	6.9 ± 6.3	-
Prepubertal	n = 1	n = 38	
Age (year)	4.8	3.8 ± 2.9	0.23
LH (mIU/mL)	0.1	0.5 ± 1.6	0.48
FSH (mIU/mL)	0.2	1.5 ± 2.5	0.10
Basal T (ng/mL)	0.2	0.2 ± 0.4	0.08
Stimulated T (ng/mL)	4.1	4.3 ± 2.4	0.89
Stimulated DHT (ng/mL)	0.3	0.6 ± 0.5	0.17
Stimulated T/DHT	16	9.3 ± 1.3	**0.04***
Pubertal	n = 4	n = 8	
Age (years)	14.1 ± 1.2	12.7 ± 3.0	0.64
LH (mIU/mL)	6.6 ± 7.2	3.8 ± 5.0	0.23
FSH (mIU/mL)	12.1 ± 14.6	6.5 ± 9.4	0.23
Basal T (ng/mL)	2.6 ± 2.1	2.6 ± 2.0	0.85
DHT (ng/mL)	0.8 ± 0.6	0.4 ± 0.3	0.48
T/DHT	5 ± 2.31	5.8 ± 3.0	0.64

AGA: appropriate for gestational age, AIS: androgenin sensitivity syndrome, BW: birth weight, CAIS: complete androgen insensitivity syndrome, DHT: dihydrotestosterone, EMS: external masculinization score, FSH: follicle-stimulating hormone, IUGR: intrauterine growth retardation, LGA: large for gestational age, LH: luteinizing hormone, PAIS: partial androgen insensitivity syndrome, SGA: small for gestational age, T: testosterone, SDS: standard deviation score

factor determining translational efficiency (23). Presently, there is no sufficient evidence to support a causative status of c.330G>C and segregation analysis for this family was not performed. The most frequent pathogenic *AR* variant in the study was c.1174C>T, (p.Pro392Ser) with a frequency of 33.3% (n = 6) in all AIS patients (Table 5). All of the patients with this mutation presented with PAIS clinically. All of patients with CAIS (n = 7, 38.9%) had different mutations (two of them novel). However two siblings with c.2676T>A (p.Phe892Leu) mutation had different clinical presentations (one PAIS, one CAIS) and one of these siblings with PAIS also had a 47,XXY karyotype (Supplementary Table 1). The most frequent *SRD5A2* mutations were c.164T>A (p.Leu55Gln), c.453delC (p.(Leu152Tyrfs*8) and c.193G>C (p.Ala65Pro). Two patients with c.453delC (p.(Leu152Tyrfs*8)) also had different Quigley scores, assigend as PAIS in one and CAIS in the other. A patient who had heterozygous mutations with c.164T>A, p.Leu55Gln and c.269A>C (p.His90Pro) presented with a CAIS phenotype (Supplementary Table 2).

Table 4. Comparison of clinical and hormonal features of genetically proven 5α-reductase deficiency and undiagnosed patients with a high T/DHT value (≥20)

	Mutation (+)	Mutation (-)	p
T/DHT: High	n = 10	n = 35	
Age at presentation (years)	6.6 ± 7.2	1.3 ± 2.8	**0.04***
Birth weight SDS	0.6 ± 1.4	-1.2 ± 1.6	**0.007***
Gestational age (weeks)	39.2 ± 0.8	35.9 ± 4.1	**0.02***
Gestational age (weeks), n (%)			**0.03***
< 37	0 % (n = 0)	40 % (n = 14)	
≥37	100 % (n = 10)	60 % (n = 21)	
Consanguinity, n (%)	50 % (n = 5)	25.8 % (n = 8)	0.14
BW groups, n (%)			0.10
AGA (-2 to +2 SDS), n (%)	90 % (n = 9)	60 % (n = 21)	
SGA (< -2 SDS), n (%)	0 % (n = 0)	34.3 % (n = 12)	
LGA (> +2 SDS) n (%)	10 % (n = 1)	5.7 % (n = 2)	
Symptom on admission			0.07
Ambiguous genitalia	60 % (n = 6)	94.3 % (n = 33)	
Mass in groin	10 % (n = 1)	-	
Undescended testes	-	2.8 % (n = 1)	
Micropenis	-	2.8 % (n = 1)	
Primary amenorrhea	10 % (n = 1)	-	
Virilization in puberty	10 % (n = 1)	-	
Cliteromegali	10 % (n = 1)	-	
At current evaluation			
Age (years)	6.9 ± 6.0	3.9 ± 4.7	**0.03***
Weight SDS	0.6 ± 1.0	-0.7 ± 1.2	**0.01***
Height SDS	0.3 ± 0.9	-0.9 ± 1.3	**0.02***
Puberty status			0.95
Tanner stage 1	60 % (n = 6)	82.9 % (n = 29)	
Tanner stage 2	-	-	
Tanner stage 3	10 % (n = 1)	-	
Tanner stage 4	10 % (n = 1)	-	
Tanner stage 5	10 % (n = 1)	11.4 % (n = 4)	
Mini puberty	-	5.7 % (n = 2)	
Estrogen replacement after gonadectomy	10 % (n = 1)	-	
Reared gender, n (%)			**0.02***
Male	50 % (n = 5)	94.3 % (n = 33)	
Female	10 % (n = 1)	2.8 % (n = 1)	
Female reared, changed identity after diagnosis	4 % (n = 4)	2.8 % (n = 1)	
Ouigley scale			**0.03***
2	-	8.6 % (n = 3)	
3	50 % (n = 5)	85.7 % (n = 30)	
4	10 % (n = 1)	2.8 % (n = 1)	
5	20 % (n = 2)	2.8 % (n = 1)	
6/7	20 % (n = 2)	-	
EMS score			**< 0.01***
1	10 % (n = 1)	0 % (n = 0)	
2	10 % (n = 1)	0 % (n = 0)	
3	% (n = 0)	2.9 % (n = 1)	
4	30 % (n = 3)	17.1 % (n = 6)	
5	10 % (n = 1)	8.6 % (n = 3)	
6	10 % (n = 1)	22.9 % (n = 8)	
7	% (n = 0)	40 % (n = 14)	
8	30 % (n = 3)	8.6 % (n = 3)	

Table 4. Continued

	Mutation (+)	Mutation (-)	p
AIS groups, n (%)			**0.03***
PAIS	80% (n=8)	100% (n=35)	
CAIS	20% (n=2)	-	
Additional findings/diagnosis			**0.04***
None	90% (n=9)	51.4% (n=18)	
Prematurity	-	34.3% (n=12)	
IUGR	-	11.4% (n=4)	
Klinefelter syndrome	10% (n=1)	2.8% (n=1)	
Anal atresia	-	51.4% (n=18)	
Target height SDS	-0.9±0.1	-0.8±0.1	0.35
Laboratory results according to the age			
0-6 months	n=2	n=20	
Age (months)	1.5±2.2	2.5±2.1	0.33
LH (mIU/mL)	4.7±1.9	3.9±3.4	0.76
FSH (mIU/mL)	2.3±1.0	1.8±1.4	0.31
Basal T (ng/mL)	2.1±2.0	1.8±1.5	0.73
Basal DHT (ng/mL)	0.5±0.02	0.08±0.05	0.13
Basal T/DHT	52.7±19.7	40.0±18.5	0.13
Prepubertal	n=6	n=19	
Age (years)	4.3±2.3	2.2±2.2	**0.01***
LH (mIU/mL)	0.4±0.4	0.3±0.2	0.89
FSH (mIU/mL)	0.9±0.5	0.8±0.5	0.47
Basal T (ng/mL)	0.07±0.07	0.05±0.07	0.43
Stimulated T (ng/mL)	3.4±2.4	4.0±2.9	0.52
Stimulated DHT (ng/mL)	0.08±0.07	0.2±0.4	0.64
Stimulated T/DHT	67.4±27.5	81.6±15.9	0.50
Pubertal	n=3	n=3	
Age (years)	15.6±2.3	12.3±2.7	0.25
LH (mIU/mL)	12.7±10.0	6.1±2.9	0.27
FSH (mIU/mL)	23.7±22.8	6.6±5.7	0.08
Basal T (ng/mL)	4.8±3.8	4.4±2.2	0.72
DHT (ng/mL)	0.1±0.2	0.09±0.1	0.22
T/DHT ratio	49.8±24.9	57.3±21.2	0.65

AGA: appropriate for gestational age, AIS: androgenin sensitivity syndrome, BW: birth weight, CAIS: complete androgen insensitivity syndrome, DHT: dihydrotestosterone, EMS: external masculinization score, FSH: follicle-stimulating hormone, IUGR: intrauterine growth retardation, LGA: large for gestational age, LH: luteinizing hormone, PAIS: partial androgen insensitivity syndrome, SGA: small for gestational age, T: testosterone, SDS: standard deviation score

Discussion

This study documented the clinical, hormonal and genetic features of 46,XY DSD who were considered AIS or 5α-RD according to clinical and hormonal criteria. We summarized clinical, endocrine, and genetic data of 128 Turkish children with 46,XY DSD, collected in only one center with molecular analyses performed in a single laboratory.

In our cohort, 24% of the 46,XY DSD patients had any variants attributed to disease. This finding is consistent with other studies that reportaround 20-40% of cases achieve a molecular diagnosis whereas the others remain without diagnosis (24). On the otherhand, lower rates of molecularly diagnosed cases, 16.3% (11.6% 5α-RD2, 4.7% AIS) (25) and 12% (8% AIS, 4% 5α-RD2 and 88% without gene abnormality) (26) have also been reported in some studies. Different studies from Turkey also report different rates of *AR* or *SRD5A2* gene mutationsin Turkish populations (2,17,27,28). One of these studies report their 51 patients with the mutation rates of *AR* gene 22% and *SRD5A2* gene 12% which are similar to our results (2). Recently, in a large cohort of Turkish DSD patients, 143 patients with 46,XY DSD were evaluated and 45 (31.4%) were genetically proven. In this recent study, the distribution of the molecular diagnosis of 46,XY DSD patients were also presented as 26.6% *SRD5A2,* and 22.2% *AR* (28). In concordance with most of the literature, our study results showed that the frequency of genetically diagnosed AIS patients were higher than 5α-RD patients in the study sample (27,29,30,31).

Table 5. The characteristics of *AR* and *SRD5A2* variants in patients with 46,XY DSD

ENE	Nucleotide	Peptide	Type	Variant ID	Classification*	Karyotype	Zygosity	Status in patients**	Allele/ Patients	References
AR (NM_000044.4, NP_000035.2), n = 18	c.94G > T	p.E32*	Nonsense	Novel	Pathogenic	46,XY	Hem.	c.[94G > T];[0]	1/1	This study
	c.330G > C	p.L110L	Silent	Novel	Likely benign	46,XY	Hem.	c.[330G > C];[0]	1/1	This study
	c.1174C > T	p.P392S	Missense	rs201934623	Pathogenic	46,XY	Hem.	c.[1174C > T];[0]	6/6	(51)
	c.1823G > A	p.R608Q	Missense	rs137852573	Pathogenic	46,XY	Hem.	c.[1823G > A];[0]	1/1	(52)
	c.2084C > T	p.P695L	Missense	Novel	Likely pathogenic	46,XY	Hem.	c.[2084C > T];[0]	1/1	This study
	c.2169G > T	p.L723F	Missense	-	Pathogenic	46,XY	Hem.	c.[2169G > T];[0]	1/1	(53)
	c.2482T > G	p.F828V	Missense	-	Likely pathogenic	47,XYY	Hem.	c.[2482T > G];[0]	1/1	(54)
	c.2521C > A	p.R841S	Missense	-	Pathogenic	46,XY	Hem.	c.[2521C > A];[0]	1/1	(55)
	c.2585_2592delAGCTCCTG	p.(K862Rfs*16)	Frame shift deletion	Novel	Pathogenic	46,XY	Hem.	c.[2585_2592 delAGCTCCTG];[0]	1/1	This study
	c.2668G > C	p.V890L	Missense	rs886041133	Likely pathogenic	46,XY	Hem.	c.[2668G > C];[0]	1/1	(3)
	c.2668G > A	p.V890M	Missense	rs886041133	Pathogenic	46,XY	Hem.	c.[2668G > A];[0]	1/1	(56)
	c.2676T > A	p.F892L	Missense	-	Likely pathogenic	One with 47,XXY	Hem.	c.[2676T > A];[0]	1/1	(2)
SRD5A2 ((NM_000348.3, NP_000339.2), n = 14	c.164T > A	p.L55Q	Missense	rs121434245	Pathogenic	46,XY	Hom. Com. het.	c.[164T > A];[164T > A] c.[164T > A];[269A > C]	2/3 1/3	(57)
	c.193G > C	p.A65P	Missense	-	VUS	46,XY	Hom.	c.[193G > C];[193G > C]	4/2	(58)
	c.269A > C	p.H90P	Missense	-	Likely pathogenic	46,XY	Com. het.	c.[164T > A];[269A > C]	1/3	(59)
	c.453delC	p.(L152Yfs*8)	Frame shift deletion		Pathogenic	46,XY	Hom.	c.[453delC];[453delC]	2/1	(60)
	c.468-470delAAT	p.(Met157del)	In frame deletion	-	Likely pathogenic	46,XY	Hom.	c.[468-470delAAT];[468-470delAAT]	2/1	(61)
	c.513G > C	p.R171S	Missense	rs756405261	VUS	46,XY	Hom.	c.[513G > C];[513G > C]	2/1	(57)
	c.542C > T	p.P181L	Missense	rs1057517829	VUS	46,XY	Hom.	c.[542C > T];[513C > T]	2/1	(62)
	c.586G > A	p.G196S	Missense	rs121434250	VUS	47,XXY	Hom.	c.[586G > A];[586G > A]	2/1	(57)
	c.736C > T	p.R246W	Missense	rs121434244	Likely pathogenic	46,XY	Hom.	c.[736C > T];[736C > T]	2/1	(57)
	c.753delA	p.(Phe252Serfs*27)	Frame shift deletion	rs587776567	Pathogenic	46,XY	Hom.	c.[753delA];[753delA]	2/1	(63)

Hom: homozygous, Het: heterozygous, Freq: frequency of allele, Hem: hemizygosity

One of the remarkable findings of the current study was the comparison of BW SDS and GA between the subgroups of the study sample. The effect of androgens on fetal growth and BW difference between sexes has been reported in previous studies. Although some studies have shown that BW difference is dependent on fetal androgens, other studies reported that it is not generated by the action of androgens (32). Moreover, it is known that 46,XY DSDs due to nonspecific disorders of undermasculinization are more frequently associated with fetal growth restriction, SGA, and concomitant conditions (33,34,35,36). In the current study, none of the 5α-RD patients had SGA. Moreover, BW SDS and GA of the 5α-RD patients were significantly higher than that of AIS and undiagnosed patients, which may demonstrate that fetal androgens can also affectfetal growth. The 19% of cases of nonspecific XY DSD without any clear diagnosis is reported to be SGA (16). In this study, 40.4% of undiagnosed cases was shown to be SGA. The prematurity and intrauterine growth restriction rates were also higher in the undiagnosed group in the current study.Additional conditions are frequent in DSD, with a rate of 27%, which is over 10 times the birth prevalence of congenital anomalies (16). In our study, the rate of other disease comorbidities was higher in the undiagnosed group, in concordance with literature. The presence of one congenital condition may be associated with the presence of further anomalies because disrupting factors, whether environmental or genetic, are likely to affect multiple developmental processes (16).

The different masculinization scores are standardized ways of recording and conveying the degree of virilization on physical examination.These scores are also used to distinguish between individuals with or without any mutation (37,38). Some studies find a correlation between an identifiable genetic cause and masculinization scores whereas some of them do not (37,38). In our study, the CAIS phenotype was more frequent in all ofthe mutation positive groups than genetically undiagnosed patients. Thus, having an identifiable genetic cause may be presented with a lower EMS (11,12) or higher Quigley score (10) compared to patients without an identifiable genetic cause and this trend appears consistent between historical and modern cohorts, despite changes in genetic technology over time.

Different studies use variable cut off levels of T/DHT to differentiate 5α-RD from AIS in the laboratory (1,17,18,27,30-41). Cut-off values ranging from 8.5 to 30 have been suggested for the T/DHT ratio (18). The diagnostic interpretations of mean values of T/DHT ratio based on different age groups is still debated. With different sensitivities from different studies, variable mean values of T/DHT in patients with 5α-RD during infancy (basal 19.1, peak 29.4), prepuberty (basal 8.0, peak 32.5), adolescence (basal 45.6, peak 71.8) and adulthood (basal 46.6) have been reported (18). In the current study, the same cut-off levels of T/DHT were used for all age groups as a result of missing standardized cut-off levels of ratio according to ages. Lack of precisely determined cut-offs still compromise correct diagnosis, and improperly high or low ratios causes confusion for the reliability of T/DHT value in clinical practice. Although, AIS cases with T/DHT≥20 and 5α-RD cases with T/DHT < 20 were detected in our study, the significant difference between the mean values of T/DHT ratios between the AIS, 5α-RD and undiagnosed groups may show that this ratio can still be a valuable determinant in laboratory diagnosis. Moreover, the current study demonstrated that when T/DHT was lower than 20, stimulated T/DHT ratio in prepubertal period was significantly lower in AIS than undiagnosed, and higher in 5α-RD. Thus, we can speculate that when the ratio is lower than 20, the lowestvalues may be related to a higher probability of AIS, whereas the higher values, closer to 20, may indicate the probability of being 5α-RD. On the other hand, our study found no significance between the basal T/DHT ratio between the subgroups during the minipuberty and puberty. Serum T and DHT show fluctuations after birth, before declining to normal prepubertal concentrations and these hormones levels also differ according to the Tanner Stage in puberty (1). The ratio is reported to be typically higher in adolescents than infants and pre-pubertal children and a normal range of T/DHT may be 1.5-17 in normal male infants (1). These normal variations may influence the interpretation of basal T/DHT in mini puberty or puberty (1). From this point of view,our study may also lead to new questions about the reliability of this ratio in minipuberty or puberty to differentiate AIS, 5α-RD or undiagnosed group when T/DHT ratio is in the same cut-off range. Also, it may suggest that hCG testing may be more useful in evaluating this ratio.

Although it is not common, 47,XXY, 47,XYY or different karyotypes with *AR* or *SRD5A2* mutations are reported (42,43). In our study the rate of Klinefelter Syndrome was 2.3% (n = 3, 1 patient with AIS, 1 patient with 5α-RD and 1 with uncertain diagnosis). Moreover, one patient (0.8%) with 47,XYY and *AR* gene mutation was found. Klinefelter patients classically have complete male sex differentiation, and genital anomalies are rarely recognized as associated features of the syndrome (44). The evaluation of *AR* and *SRD5A2* genes in patients with karyotype anomaliesand ambiguous genitalia is also essential to provide accurate genetic counseling for other members of the family.

Mutations in *AR* are found in most subjects with CAIS, but the rate has varied between 28-73%, depending on the case selection (3,45). In our study, *AR* mutations were found in 63.6% of patients with clinically completely female phenotype and this rate was consistent with that of some previous studies from Turkey (2). In contrast, *SRD5A2* mutations and female external genitalia is reported as rare, 3.9-7.3% in 5α-RD cases (41,46). We described three patients with *SRD5A2* mutations who had a clinical diagnosis as CAIS (21.4% of all *SRD5A2* patients and 2.3% of all patients) which was nearly four-fold of the prior reported rate. Interestingly, we also had one patient with clinically CAIS in whom we could not establish a genetic diagnosis and may require whole exome analysis to reveal the diagnosis.

In this research, 12 different variants, four of which were novel, in *AR* and 10 different variants in *SRD5A2* were detected. More than 1000 different mutations in *AR* leading to AIS have been reported (47). Although exon 1 encodes more than half of the AR protein, exon 1 mutations only represent 25% of all of the mutations in AIS patients (47). More than 70% of AIS mutations in exon 1 appear to cause CAIS, and about 18% of exon 1 mutations are related with MAIS which is due to single-base substitution (47). However, in the current study, most of the *AR* mutations (44.4%, n=8) were located in exon 1, and none of them presented with CAIS phenotype. All of CAIS patients had *AR* mutation in the LBD. Also, the most common mutations of the *AR* gene in AIS are single point mutations that result in an amino acid substitution (45). In parallel to this, in our study thehighest number of mutations were identified as missense type. The c.1174C>T (p.Pro392Ser) variant which was the most frequent pathogenic *AR* variant in this study, has previously been reported to be related with CAIS, MAIS, PAIS and testicular cancer phenotype, although all of the patients with this mutation presented with PAIS clinically in this study. Compatible with the literature, the c.2169G>T (p.Leu723Phe) variant caused CIAS rather than PAIS, and c.1823G>A (p.Arg608Gln) caused PAIS rather than CAIS (47). Two siblings in our cohort with c.2676T>A (p.Phe892Leu) variant had different clinical phenotypes (one PAIS, one CAIS). Identical *AR* mutations can lead to variable phenotypic expression because one mutation can produce different phenotypes and appear in different individuals within a family. Despite many studies, it is known that there is no specific correlations between genotypes and phenotypes identified in the AIS patients (6). Forty-five allelic variants that may result in different phenotypes are currently recorded in the McGill AR mutation database. There are no available qualitative data on penetrance at present. The variable phenotypic expression of particular mutations may be due to differences in affected individuals, such as somatic cell embedding (6). On the otherhand, epigenetic repression of AR transcription in mutation-negative AIS (type II) has been studied recently (48). Cofactors can influence AR activity at the transcriptional as well as posttranscriptional level. Methylation-dependent repression of AR mRNA expression can contribute to an incomplete male genital development in a subset of individuals with AIS type II. This epigenetic regulation of AR expression might be established during embryonic development and maintained after differentiation to ensure proper cellular identity (48). The identification of upstream factors responsible for this epigenetic AR mRNA repression will be the next planned step in undiagnosed cases for the future studies.

To date, more than 100 mutations have been described in *SRD5A2* gene (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php) (46). Itisreported that approximately 60% are in thehomozygous and 40% arein compound heterozygousform (46). However, in our study, we have only one compound heterozygous patient (7.1%) and highhomozygous rate may be related to the consanguinity rate in the Turkish population. As a consequence, a wide phenotypic range has been described, attributed to the residual enzymatic activity and probably to the individual genetic background without a strong genotype-phenotype correlation in literature (46). One of our frequently found mutations, p. Leu55Gln, which causes decrease in enzymatic activity has only been described in Turkish patientsto date. In parallel to this, in the current study, we describe three patients with this mutation (one compound heterozygous, two homozygous). It is reported that this mutation causes less severe phenotypes, with EMS values ranging from 3.0 to 8.0, attributable to different residual enzymatic activities caused by different mutations and a genotype-phenotype correlation seems to be the most difficult in this group. In our study, two homozygous mutation-carrying patients presented as PAIS, whereas the compound heterozygous form of this mutation, with a novel p.His90Pro mutation,presented as CAIS clinically. We suggest that more case reports are needed to support our finding based on genotype-phenotype relationship. Pathogenic alterations that interfere with the NADPH binding domain that are within 3-oxo-5-alpha-steroid 4-dehydrogenase domain were also detected in our study (hom, p.Pro181Leu, hom.p.Gly196Ser, hom. p.Arg246Try, hom. p.Arg171Ser). The EMS scores of these mutations is reported to vary between 2.67 to 4.17 (46). In the current study, homozygous p.Arg246Try alteration was associated witha CAIS phenotype. Although it has been reported that only the p.Gly196Ser variant seems to produce a less variable phenotype (46), allelic variants at exon 4

and indels consistently have recently been shown to cause more severe phenotypes (49). Variant p.Gly196Ser, identified in homozygous form, is associated witha PAIS phenotype with Quigley score 3 in this study. Another known mutations affecting NADPH domain, p.Arg171Ser, is frequently found in different populations (Mexican, Turkish, Spanish, Mediterranean), there are very few homozygous reported cases, being found more frequently in compound heterozygotes (49). However, we have one homozygous p.Arg171Ser mutant patient who presented with PAIS. Although female appearance genitalia is infrequent in 5α-RD cases, we had three CAIS phenotype (one withp. [L55Q];[H90P], one hom. p.Arg246Try, and onewith hom. p.(Leu152Tyrfs*8). Although we have two patients with the same hom. p.(Leu152Tyrfs*8) mutation and one heterozygous form of this mutation in the same family, they presented with different degrees of undermasculization. The genotype-phenotype incongruence occurs even in individuals carrying the same variant and also in individuals from the same family, suggesting that other factors beyond the SRD5A2 enzyme play a role in phenotype (49). Thus, and similar to *AR* mutations, allelic variants in the *SRD5A2* gene, lead to a broad spectrum of external genitalia phenotypes with no strong genotype-phenotype relationship (49) and some other factors that affect phenotype are still unclear for both AR and SRD5A2, and constitute a relevant field for future research.

In our study gonadectomy was performed in 16 patient (n = 7 AIS, n = 5 SRD5A2 and 4 patient without any identified mutation). Intratubular germ cell neoplasia was only seen in one patient with no any detected mutation in *AR* or *SRD5A2*. Indeed, dysgenetic and undescended testes are the major risk factors for testicular cancer, the most common malignancy for men between the ages of 15 and 35 years (50). Our patient with gonadoblastoma was 7.3 years old when the gonadectomy was performed. The other causes of gonadoblastoma in 46XY DSD patients, except for dysgenetic gonads are also reported as AIS, ovotesticular DSD, Klinefelter syndrome, 5α-RD, and 17β-HSD deficiency respectively (50). Unfortunately, we only studied *SRD5A2* and *AR* genes in this patient, and further genetic analysis will be essential to obtain a definitive diagnosis.

Study Limitations

Our study has some limitations. First, the nature of the study required us to rely on data from medical records. Second, serum levels of inhibin B and anti-Mullerian hormone were not examined due to missing data. Third, according to the wide age range of sample, T and DHT were measured in different years with a possibility of different methods that may have led to some inaccuracies. These shortcomings can be overcome in future prospective studies by starting to use genetic analysis earlier with more specific methods, such as liquid chromatography linked with tanden mass spectrometry or immunoassays after organic solvent extraction to detect hormones.This study used the Sanger sequencing method for diagnosis of AIS and 5α-RD patients. However, next-generation sequencing-based targeted sequencing is a promising technique to improve the detection rate of DSD, and it will be more useful for future studies.

Conclusion

Four novel AR variants were identified in our study. T/DHT ratio in the diagnosis of AIS and 5α-RD is an important hormonal criteria, but in some cases, T/DHT ratio may be vary beyond the accepted cut-offs that may lead to diagnostic confusion. So genetic analysis for actual diagnosis seems to be essential, especially for determining the treatment pathway and the sex identity of patients.

Acknowledgement

We would like to convey special thanks to the Scientific Research Projects Coordination Unit of İstanbul University (project ID TYL-2017-24211).

Ethics

Ethics Committee Approval: The study was approved by the İstanbul Faculty of Medicine, İstanbul University of Ethics Committee.

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

Financial Disclosure: This research was supported by Scientific Research Projects Coordination Unit of Istanbul University (project ID TYL-2017-24211).

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Supplementary Table 1. Clinical and laboratory features of AIS patients

	Age on admission (yrs)	Age at current (yrs)	BW kg kg/ SDS (GA-wks)	Co	Reared gender F/M (karyotype)	At diagnosis				At current		Laboratory findings at diagnosis					GE age (years)/ pathology	Genotypes
						W SDS/ H SDS	Tanner Stage	QS (EMS)	Clinical diagnosis PAIS-CAIS/	W SDS/ H SDS	Tanner Stage	LH	FSH	Basal T (ng/mL)	Stim T (ng/mL)	Stim T/DHT ratio		
1	0.1	2.2	1.6/-0.8 (32)	-	M (46,XY)	-2.1/-2.2	MP	3 (6)	PAIS	-1.1/-1.3	1	7	2	0.5	2.9	6.4	-	p.L110.L
2	0.01	7.6	3.9/1.3 (40)	-	M (46,XY)	-0.2/0.7	MP	4 (6)	PAIS	3.8/2.9	1	4.3	1.2	2.4	8.4	4.6	-	p.R608Q
3	0.9	1	2.9/-1.3 (39)	-	M (46,XY)	0.1/-0.2	1	3 (4)	PAIS	-1.9/-0.7	1	0.8	1.0	0.8	5.5	9.4	-	p.P392S
4	3.3	9.6	2.5/-2.9 (40)	+	F (47,XYY)	-1.7/-2.1	1	6 (2)	CAIS	-0.6/-0.7	1	0.7	1.5	0.4	9	3.0	4/immature testis	p.F828V
5	0.02	10.5	4.0/1.7 (40)	-	M (46,XY)	1.1/0.7	MP	3 (6)	PAIS	-0.7/-1.2	1	4.3	4.1	0.6	2.0	5.6	-	p.P392S
6*	1.3	6.7	3.1/-1.0 (40)	-	F (46,XY)	-2.1/-1.5	1	6 (1)	CAIS	-2.0/-2.0	1	0.3	0.9	0.4	6.0	7.5	2.8/ immature testis	p.P892L
7*	0.2	5.5	3.0/-1.1 (40)	-	E (47,XXY)	-0.8/0.04	MP	5 (4)	PAIS	0.3/0.7	1	0.3	0.4	0.1	1.6	6.4	-	p.P892L,
8	1.2	5.4	2.5/-2.0 (38)	+	F (46,XY)	-2.7/-1.0	1	6 (2)	CAIS	0.2/-0.07	1	2.7	1.7	0.2	7.9	1.6	2.3/ immature testis	p.R841S
9	17.4	18.1	3.8/1.4 (38)	-	F (46,XY)	2.7/-0.9	GE	6 (1)	CAIS	2,7/-0.9	GE	20.5	25	4.6	-	21.5	16.6/ immature testis	p.V890L
10	17.6	18.2	2.8/-1.9 (40)	-	F (46,XY)	1.1/0.9	5	6 (1)	CAIS	0.9/0.8	GE	26.3	28	6.5	-	30.9	17.9/ immature testis	p.K862Rfs*16
11	2.8	10.5	3/-0.5 (38)	+	M (46,XY)	-0.2/0.2	1	4 (7)	PAIS	0.04/-0.5	2	0.07	0.05	0.02	1.5	1.07	-	p.E32*
12	0.01	0.2	1.2/-2.3 (33)	-	M (46,XY)	-3.5/-4.9	1	2 (8)	PAIS	-0.4/-0.4	1	13.1	1.9	3.9	-	18.6	-	p.P392S
13	1.0	1.7	0.9/-2.8 (30)	-	M (46,XY)	-0.7/-1.15	1	3 (6)	PAIS	-0.2/0.4	1	0.2	0.7		3	74.4	-	p.P392S
14	0.4	5.0	0.6/-4.2 (28)	-	M (46,XY)	-5.0/-6.4	MP	3 (6)	PAIS	-2.5/-2.1	1	4.8	1.2	2.4	6.3	4.3	-	p.P392S
15	7.2	13.9	3.6/0.4 (40)	-	F (46,XY)	-1.2/0.8	1	6 (1)	CAIS	-0.7/-0.3	GE	0.4	2.9	6.1	-	115.0	13.5/ immature testis	p.V890M
16	1.0	2.0	0.7/-3.5 (28)	-	M (46,XY)	-1.9/-0.2	1	3 (7)	PAIS	-4.0/-2.9	1	0.9	1.3	0.09	4.6	4.6	-	pP392S
17	11.0	11.6	4.3/2.0 (40)	-	M (46,XY)	0.4/0.5	3	Postop	PAIS	0.4/0.7	3	2.4	2	1.9	-	9.5	-	p.P695L
18	14.3	15.4	3.6/1.0 (38)	-	F (46,XY)	0.2/1	3	6 (2)	CAIS	-0.2/0.7	4	27.3	14.3	9.2	-	14.6	14.5/ immature testis	p.L723F

*Siblings, yrs: years, wks: weeks, Co: consanguinity, GA: gestational age, BW: birth weight, F: female, GE: gonadectomy, W: weight, H: height, M: male, MP: minipuberty, Postop: postoperated, QS: quickley score, EMS: external masculinization score, Stim: stimulate

Supplementary Table 2. Clinical and laboratory features of 5α-RD patients

	Age on admisiom (yrs)	Age at current (yrs)	BW kg / SDS (GA-wks)	Co	Reared gender F/M (karyotype)	At diagnosis				At current		Laboratory findings at diagnosis					GE age/ pathology	Genotypes
						W SDS/H SDS	Tanner Stage	QS (EMS)	Clinical diagnosis PAIS-CAIS/	W SDS/ H SDS/	Tanner Stage	LH	FSH	Basal T (ng/mL)	Stim T (ng/mL)	Stim T/DHT ratio		
1	15.2	19.1	3.4/0.5 (38)	-	M (46,XY)	0.7/0.9	5	3 (8)	PAIS	0.2/0.8	5	6.5	7.1	5.1	-	24.7	-	Hom p.P252Sfs
2	7.5	13	3.3/0.6 (40)	-	F (47,XXY)	-2.1/-2.2	1	3 (4)	PAIS	-0.7/-0.5	GE	0.2	0.6	0.02	1.1	23.2	8.8/immature testis	Hom p.G196S
3	1.3	5.3	3.0/-0.9 (39)	-	M (46,XY)	-1.0/-0.2	1	6 (2)	CAIS	0.6/-0.7	1	0.9	1.6	0.2	7.9	37.4	-	Comp p.L55Q/p. H90P
4	0.02	1.8	3.9/1.1 (40)	+	M (46,XY)	-0.6/0.0	MP	6 (1)	CAIS	1.6/-1.4	1	3.5	1.1	1.0	2.0	40.0	-	Hom p.R246W
5	1	5.6	4.4/2.4 (39)	-	M (46,XY)	1.3/0.6	1	3 (4)	PAIS	1.5/1.8	1	0.1	1.1	0.03	2.4	20.7	-	Hom. p.R171S
6	0.01	1.9	4.1/1.7 (40)	-	M (46,XY)	-0.2/-0.8	MP	5 (5)	PAIS	1.4/-0.04	1	6.9	3.2	4.4	-	189.7	-	Hom. p.L55Q
7	14.9	15.2	3.8/0.9 (40)	+	F (46,XY)	1.1/-0.2	3	3 (8)	PAIS	0.6/0.5	3	5.9	7.2	5.4	-	3.4	11.3/ immature testis	Hom. p.A65P
8*	4.8	14.3	3.7/0.7 (40)	-	F (46,XY)	0.2/0.0	1	6 (2)	CAIS	0.2/0.2	2	0.1	0.2	0.02	1.8	14.6	15.2, immature testis	Hom. p.P151fx
9*	14	14.0	3.5/0.3 (39)	+	F to M (46,XY)	0.2/-1.2	2	5 (8)	PAIS	-0.9/-1.0	2	1.2	2.3	3.2	-	6.7	-	Hom. p.P151fx
10**	0.9	3,4	4.0/1.5 (40)	+	F to M (46,XY)	0.2/-0.6	1	3 (6)	PAIS	0.01/0.1	1	0.8	0.6	0.6	2.3	90.4	-	Hom p.P151fx
11	5.9	9.13	3.5/0.3 (39)	+	F (46,XY)	-1.4/-1.2	1	5 (4)	PAIS	-1.4/-1.1	1	0.5	0.5	0.03	4.9	195	-	Hom. p.P181L
12	16.5	17.5	3.1/-0.7 (39)	+	F to M (46,XY)	-0.6/-0.2	3	3 (8)	PAIS	1.4/1.0	3	12.2	17.5	9.2	-	74.4	-	Hom. p.A65P
13	14.4	20	3.6/0.3 (40)	+	F (46,XY)	-1.1/-0.9	2	5 (8)	PAIS	-1.2/-0.9	5	2.2	5.2	2.0	7.5	17.4	14.7, immature testis	Hom. p.G156Gfs/
14	17.5	18.1	4/1.9 (38)	+	F to M (46,XY)	-1.5/-1.3	4	4 (8)	PAIS	-1.0/-0.7	5	4.9	13	5.0	-	50.3	-	Hom. p.L55Q

*Siblings, **First-order cousin of siblings, yrs: years, wks: weeks, Co: consanguinity, GA: gestational age, BW: birth weight, F: female, GE: gonadectomy, W: weight, H: height, M: male, MP: minipuberty, Postop: postoperated, QS: quickley score, EMS: external masculinization score, Stim: stimulated, 5α-RD: 5α-reductase deficiency

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021-10-7

J Clin Res Pediatr Endocrinol 2022;14(2):172-178

Revisiting the Annual Incidence of Type 1 Diabetes Mellitus in Children from the Southeastern Anatolian Region of Turkey: A Regional Report

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What is already known on this topic?

The incidence of type 1 diabetes mellitus (T1D) in children has an increasing trend with a variable rate, depending on region and ethnicity.

What this study adds?

This is the first report examining the change in the incidence rate of pediatric T1D and the clinical and presentation characteristics of cases in Diyarbakır over a ten-year period. It is also the first study to report the incidence of T1D in the 0-18 age group in Diyarbakır.

Abstract

Objective: The incidence of type 1 diabetes mellitus (T1D) in children has an increasing trend globally, with a variable rate depending on region and ethnicity. Our group first reported T1D incidence in Diyarbakır in 2011. The aim of this study was to evaluate the current incidence rate of pediatric T1D in Diyarbakır, and compare the incidence, and clinical and presenting characteristics of more recent cases with those reported in our first report.

Methods: Hospital records of patients diagnosed with T1D in Diyarbakır city between 1st January 2020 and 31st December 2020 and aged under 18 years old were retrieved, and their medical data was extracted. Demographic population data were obtained from address-based census records of the Turkish Statistical Institution (TSI).

Results: Fifty-seven children and adolescents were diagnosed with T1D. Of those, 34 were female (59.6%), indicating a male/female ratio of 1.47. The mean age at diagnosis was 9.5±3.9 years (0.8-17.9). TSI data indicated a population count of 709,803 for the 0-18 years age group. Thus the T1D incidence was $8.03/10^5$ in the 0-18 age group and was higher in the 0-14 age group at $9.14/10^5$. The cumulative increase in the incidence of T1D in the 0-14 age group was 26.9% suggesting an increasing rate of 2.7% per year. The frequency of presentation with diabetic ketoacidosis was 64.9%.

Conclusion: The annual incidence of pediatric T1D in Diyarbakır city increased from $7.2/10^5$ to $9.14/10^5$ within the last decade. The rate of annual increase was 2.7% in the 0-14 age group comparing this study with our earlier report, with a predominance in male subjects and a shift of peak incidence from the 5-9 year age group in the first study to the 10-14 year age group in this one.

Keywords: Type 1 diabetes mellitus, annual incidence, Southeastern Anatolian

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Conflict of interest: None declared
Received: 21.10.2021
Accepted: 15.12.2021

Introduction

Type 1 diabetes mellitus (T1D) is one of the most common chronic autoimmune diseases in children which is characterized by damage to insulin-producing pancreatic β cells (1). The incidence of T1D in children is globally heterogenous, with the highest incidence rate in European countries and Middle-East and North Africa region and the lowest incidence rate in Eastern countries (2). For instance, the incidence rate has been reported as $52.2/10^5$ in Finland, $41.7/10^5$ in Kuwait and $1.9/10^5$ in China (2,3,4). The incidence of T1D in the pediatric population has an increasing trend worldwide. The rate of change in T1D incidence varies depending on ethnic origin, geographical region and industrialization status (5-19).

In the first and single study reporting the nationwide incidence of pediatric T1D in Turkey, the incidence rate was reported as $11.3/10^5$ for 0-14 and $10.8/10^5$ for 0-18 age groups (20). In addition, other regional studies evaluating the incidence rate (21,22) and a regional study assessing the trend of the incidence rate have been published in Turkey (23).

Our group published the first regional report on the incidence of T1D in Diyarbakır city, where the incidence rate was found to be $7.2/10^5$ (21). The aim of this study was to determine the current incidence and the change in the incidence rate of pediatric T1D and compare the clinical and demographic characteristics of pediatric T1D in the Diyarbakır city, a city of Southeastern Anatolian region of Turkey.

Methods

There are three tertiary pediatric endocrinology centres in Diyarbakır, a city in the Southeastern Anatolian region of Turkey. In Diyarbakır, all 0-18 years old patients diagnosed with T1D are referred to one of these three centres. Hospital files of all T1D patients diagnosed and referred to these three centres between 1st January 2020 and 31st December 2020 were retrieved. T1D diagnosis was made according to the criteria of the American Diabetes Association and International Society for Pediatric and Adolescent Diabetes guidelines (24,25). Blood glucose level, insulin, c-peptide level, blood pH, HCO_3 level, glycosylated haemoglobin (HbA1c) value, anti-islet cell, anti-insulin and anti-glutamic acid decarboxylase (anti-GAD) antibodies, coeliac serology, and thyroid function tests performed at the time of diagnosis were evaluated. Patients with venous blood pH of <7.3 or HCO_3 level <15 mEq/L with concomitant ketosis (positive blood/urine ketones) were considered to have diabetic ketoacidosis (DKA). Based on the pH and HCO_3 values at presentation, DKA was classified as mild (pH 7.2-7.3 and HCO_3 10-15 mEq/L), moderate (pH 7.1-7.2 and HCO_3 5-10 mEq/L), and severe (pH <7.1 and HCO_3 <5 mEq/L). Season and month at the time of the diagnosis, anthropometric data and pubertal status were recorded from patient files. Demographic data were obtained from address-based census records of the Turkish Statistics Institution (TSI). The study was approved by the Health Sciences University Diyarbakır Gazi Yaşargil Training and Research Hospital Ethical Committee (ethics approval number: 765, date: 29.5.2021). Patients were included in the study after parental consent was obtained.

Statistical Analysis

Statistical analysis was performed with IBM Statistical Package for the Social Sciences Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA). Incidence of T1D was calculated using the numbers of patients reported for each year by gender and in the following age groupings: 0-4, 5-9, 10-14 and 15-18 years at time of diagnosis. Annual numbers for the age groups in Diyarbakır city were used as denominators, and incidence (per 100,000 per year) was calculated with 95% confidence intervals. The population sizes were obtained from the Turkish census data from 2020 from the address-based population registration system of the TSI. In order to assess the significance of the differences between the groups, normality of variables was tested by Kolmogorov-Smirnov test. Mann-Whitney U and chi-square tests were used. Results are reported as means ± standard deviation. A p value <0.05 was considered statistically significant.

Results

Overall, 57 children and adolescents aged between 0-18 years were diagnosed with T1D between the 1st of January 2020-31st of December 2020. According to TSI data, the overall population in Diyarbakır in 2020 was 1,783,431 and the population within the 0-14 age group was 579,460, and within 0-18 age group was 709,803. T1D incidence was calculated as $9.14/10^5$ and $8.03/10^5$ in 0-14 and 0-18 age groups. The age and sex distribution of cases diagnosed with T1D are illustrated in Table 1.

Of 57 children and adolescents diagnosed with T1D in 2020, 34 (59.6%) were female, and the female/male ratio was 1.47. The mean age at diagnosis was 9.5 ± 3.9 years in the whole cohort, and did not differ between girls (9.5 ± 4 years) and boys (9.4 ± 3.8 years) ($p=0.91$). Regarding pubertal status, n = 18 (52.9%) of females and n = 8 (34.8%) of male subjects, and 26 cases (44.6%) in total were in the

pubertal period (Tanner stage ≥2). In the 15-18 age group, the incidence rate was similar between males and females, while a female predominance was observed in the other age groups. At the time of the diagnosis, mean serum glucose, HbA1c, and mean c-peptide levels are shown in Table 2.

The number of cases diagnosed between the ages of 10-14 was 23 (40.3%), while 22 patients (38.5%) were aged between 5-9 years, eight patients (14%) between 0-4 years, and four patients (7%) between 15-18 years (Table 1). The peak incidence rate of T1D was observed in the 10-14 age group, both in females and males. Overall, the diagnosis was 40.4% in the winter season.

In total, 37 (64.9%) of the cases with T1D presented with DKA. The highest frequency for presentation with DKA was in the 5-9 age groups (n = 17/22; 77.3%), followed by the 0-4 age group (n = 5/8; 62.5%), and 10-14 age group (n = 14/23; 60.9%). Of these, 15 of 57 (26.3%) cases presented with severe DKA, which was most prevalent in the 0-4 age group (n = 3/8; 37.5%). Presentation with severe DKA was observed in 27.3% (6/22) of cases in the 5-9 age group and 26.1% (6/23) of cases in the 10-14 age group. There was no severe DKA in the 15-18 age group. Fourteen cases (24.6%) presented with ketosis, and six cases (10.5%) with hyperglycemia. Mean c-peptide levels were significantly lower in cases presenting with DKA compared to those presenting without DKA (p = 0.001) (Table 3).

Anti-GAD antibody was positive in 40 cases and anti-islet cell antibody in 29 cases, including 16 cases positive for both antibodies. Four cases were negative for both antibodies. Of these, two presented with DKA, one with ketosis and one with hyperglycemia. In the antibody-negative patients HbA1c levels ranged from 9% to 17.9%, c-peptide levels from 0.12 to 1 ng/mL and, blood glucose from 365 to 622 mg/dL. Furthermore, insulin requirement continued with persistent low c-peptide (< 0.8 ng/mL) during follow up.

Anti tissue transglutaminase IgA (anti-TTG IgA) serology was investigated for celiac disease and was positive in 10 (17.5%) cases. One case was diagnosed with celiac disease before diabetes. In addition to the case with a first diagnosis of celiac disease, in six cases, anti-TTG IgA levels were 10 fold or more higher than the upper limit of the lab-specific reference range, and all six presented with DKA. The diagnosis of celiac disease was confirmed in these cases using endoscopic biopsy. In the remaining three cases the anti-TTG IgA levels were 1.5-4 fold higher than the upper limit of reference and the patients were asymptomatic.

Table 1. Distribution of the number of cases with T1D, population number and calculated incidence rate according to sex and age groups

	Number of T1D patients; n (%)	Child population, n	Incidence (per 100,000)
Female (years)			
0-4	5 (14.7)	96,708	5.2
5-9	13 (38.2)	96,666	13.4
10-14	14 (41.1)	89,011	15.7
15-18	2 (5.8)	63,775	3.1
0-14	32 (94.1)	282,385	11.3
0-18	34 (100)	346,160	9.8
Male (years)			
0-4	3 (13.0)	102,333	2.9
5-9	9 (39.1)	101,534	8.8
10-14	9 (39.1)	93,208	9.6
15-18	2 (8.6)	66,568	3.0
0-14	21 (91.3)	297,075	7.1
0-18	23(100)	363,643	6.3
Overall (years)			
0-4	8 (14.0)	199,041	4.0
5-9	22 (38.5)	198,200	11.1
10-14	23 (40.3)	182,219	12.6
15-18	4 (7.0)	130,343	3.1
0-14	53 (92.9)	579,460	9.14
0-18	57 (100)	709,803	8.03

T1D: type 1 diabetes mellitus

Table 2. Presentation characteristics of pediatric T1D patients

	Female (n = 34)	Male (n = 23)	Total (n = 57)	p
Mean age (years)	9.54 ± 4.09	9.42 ± 3.8	9.49 ± 3.9	0.910
BMI SDS	-0.79 ± 1.49	-1.4 ± 1.5	-1.02 ± 15	0.162
Pubertal stage ≥2	18 (52.9%)	8 (34.8%)	26 (45.6%)	0.183
DKA	21 (61.8%)	16 (69.6%)	37 (64.9%)	0.553
Severe DKA	11 (32.4%)	4 (17.4%)	15 (26%)	0.198
Glucose (mg/dL)	462.0 ± 143	557.0 ± 169	500.0 ± 159	0.038*
HbA1c %	12.9 ± 2.85	12.6 ± 2.3	12.8 ± 2.6	0.694
C peptide (ng/mL)	0.7 ± 0.54	0.46 ± 0.3	0.61 ± 0.47	0.064

*p value < 0.05 significant
BMI: body mass index, T1D: type 1 diabetes mellitus, DKA: diabetic ketoacidosis

These cases are under clinical observation with no biopsy performed. Thyroid function tests were normal in all cases. Anti-TPO was positive in two cases, and anti-Tg antibody was positive in another two cases.

Discussion

In the present study investigating the incidence of T1D in children between the ages of 0-18 years in Diyarbakır, a city of Southeastern Anatolian in Turkey, the incidence of T1D was found to be 9.14/10^5 in 0-14 age group and 8.03/10^5 in 0-18 age group.

Incidence of T1D in children varies across the world. The lowest incidence rates have been reported from the Asian populations, such as 1-3 per 100000 in China (3,4,5). A gradual increase has been observed from the South European countries where the incidence rate varies between 10-20 per 100000, to the USA and Scandinavian countries where the incidence rate has been reported to vary between 30 and 60 per 100000 (6,14). The incidence studies performed in Turkey have shown that the incidence rate of pediatric T1D (7.2-10.8/10^5) falls between those reported from Asia and South European populations, corresponding to its geographical location (20,21,22).

Globally, within the last three decades, T1D incidence has been reported to increase by 3-4% per year (5,6,19). In various studies, this increase was found to be more marked in countries with a relatively lower incidence at the baseline (5,6).

There are a few studies from Turkey on the incidence of T1D in children (20,21,22,23). In the first nationwide study based on the records of the national Social Security Institution, Yeşilkaya et al (20) reported the incidence of T1D as 11.3/10^5 in 0-14 age group and 10.8/10^5 in 0-18 age group. These rates are higher than those we found in the 0-14 and 0-18 age groups in Diyarbakır. The difference might be due to variations in population density, industrialization, climate and ethnicity between different regions of Turkey. Indeed, in the study of Yeşilkaya et al (20), Turkey was divided into five regions according to incidence rates, and it was observed that the incidence rate of T1D was lower in the East and Southeastern regions of Turkey, including Diyarbakır, than in other regions. The peak incidence was reported in the 10-14 age group (15.4/10^5), consistent with our findings (22).

In the first incidence study we carried out (in 2011) in Diyarbakır, the incidence of T1D was calculated as 7.2/10^5 in 0-14 age children (21). In the present study, we found T1D incidence in the 0-14 age group to be 9.14/10^5 indicating a 26.9% increase within the last decade, suggesting an average annual increase rate of 2.7%. This increase in the incidence was more pronounced in females (from 8.7 to 11.3/10^5) and 10-14 age group (from 8.4 to 12.6/10^5). This is consistent with the reported trend of increase in regions with low incidence. In the ten years that have elapsed since our first study, no population movement, migration, climate changes, or industrial changes have occurred in Diyarbakır (21). Therefore, the increase in the incidence rate can be attributed to the natural increasing trend of T1D in children. However, the Coronavirus disease-2019 (COVID-19) pandemic experienced in 2020 when we carried out the present study might have contributed to the rise in T1D incidence. It has shown that the risk of DKA, especially severe DKA, increases significantly during the COVID-19 pandemic (26).

In the study of Poyrazoglu et al (22), covering the period of 2013-2015 in the Northwest region of Turkey, the incidence of T1D was reported to be 9.82/10^5, in the 0-14 age group and 8.99/10^5 in 0-17 age group. The peak incidence was shown to have a bi-modal distribution according to the age group, with the highest incidence rate occurring in the 5-9 (11.68/10^5) and 10-14 age groups (11.7/10^5). The lowest incidence was reported in the 15-17 age group (5.04/10^5). Their incidence rates are similar to those found in the present study. However, incidence rates in the West and North regions of Turkey are high compared to our region (20). In the study of Esen and Okdemir (23), in which the

Table 3. Evaluation of cases according to presenting symptoms

	No DKA		DKA			p*
	Hyperglycemia	Ketosis	Mild DKA	Moderate DKA	Severe DKA	
Number of cases	6	14	15	7	15	
Age (years)	8.1 ± 2.7	11.1 ± 4.3	9.1 ± 3	9.9 ± 4.3	8.6 ± 4.5	0.328
Pubertal stage ≥2	4/6	8/14	4/15	4/7	6/15	0.113
Glucose (mg/dL)	457.0 ± 161	474.0 ± 194	487.0 ± 201	598.0 ± 81	499 ± 98	0.334
C-peptide (ng/mL)	1.16 ± 0.9	0.77 ± 0.44	0.53 ± 0.3	0.54 ± 0.32	0.34 ± 0.2	0.001*
HbA1c (%)	10.6 ± 3.1	14.4 ± 2.5	13 ± 2.28	14.2 ± 3.2	11.3 ± 2.0	0.418

*p values indicate the comparison of values for patients with and without DKA at presentation.
DKA: diabetic ketoacidosis, HbA1c: glycosylated haemoglobin

T1D incidence trend was evaluated in children under the age of 15, between year 2009 and 2019, the incidence rate was reported to increase from 10.2/10^5 to 24.1/10^5 over the 10 years period. An annual incidence rise of 7.8% was demonstrated, particularly in the 5-9 age group and in boys. These incidence and increase rates are the highest ever reported in Turkey (23).

Regarding the presenting characteristics of the cases, presentation with DKA was highly prevalent (64.9%) and similar to those we detected in the first report (65.9%) (21). Indeed, in the 10 years since our first study, substantial national and regional changes have been made to improve public awareness of diabetes, such as training about diabetes for school staff, public advertisements, and increase in the number of diabetes care professionals in Diyarbakır city, and easier access to health facilities. Delay in referring to the hospital due to COVID-19 may have played a part in the negligible decrease in DKA as a presenting symptom.

There is a bimodal distribution for the age of peak incidence of T1D. The peak incidence of T1D in Diyarbakır has shown a slight shift from the 5-9 years (in the first study) to the 10-14 years group in the current study, while the distribution of the incidence revealed the highest incidence rate in these age groups, which is similar to those reported by Weng et al (Figure 1) (4).

Regarding the sex-specific disease frequency, the regions with high incidence rates have been reported to have a male-predominant incidence, while in the populations with low incidence, a female predominance has been reported (5). Indeed, a female predominance has been observed in our first (female/male ratio 1.4) and current study (female/male ratio 1.47) (22). However, there was a lack of gender discrepancy in the other epidemiological studies reported from our country with a female/male ratio of 0.9 in the study of Poyrazoglu et al (22), 1.02 in the study of Yeşilkaya et al (20) and 0.93 in the study of Esen and Okdemir (23) all of which reported a higher overall incidence rate compared to our results.

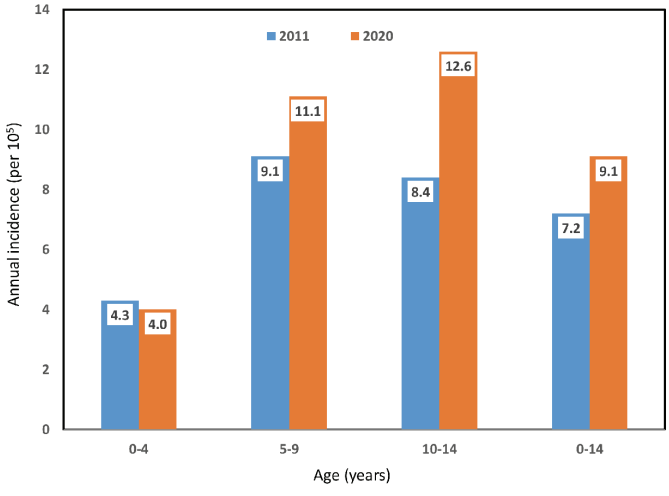

Figure 1. Annual incidence of T1D observed in the first (2011) and current (2020) regional reports conducted in Diyarbakır city revealed a 26.9% increase in the peak incidence and a shift from the 5-9 year age group to the 10-14 years

At the time of diagnosis, in 10 (17.5%) cases, anti-TTG IgA was positive. One of these cases was diagnosed with coeliac disease before diabetes. In 6 patients with an anti-TTG IgA level 10 or more times higher than the upper limit of the reference range, a diagnosis of biopsy-proven celiac disease was considered. All cases with biopsy-proven celiac disease was presented with DKA at a mean age of 9.2 ± 3.9. In the studies of Unal et al, (27) the frequency of biopsy-proven celiac disease was found to be 6.9% and the mean age at diagnosis was 7.58.

Study Limitations

There are limitations of this study. The study period overlapped with the first year of the COVID-19 pandemic, which may have affected both the incidence rate and presenting characteristics, such as the high rate of presentation with DKA. Another limitation is that we calculated the incidence rate crossectional for a one-year period. Therefore, a long-term prospective analysis of the incidence rate consecutively may estimate the most accurate incidence of pediatric T1D in our region.

Conclusion

In conclusion, the annual incidence of pediatric T1D in Diyarbakır city increased from 7.2/10^5 to 9.14/10^5 within the last decade. The rate of annual increase was 2.7% in the 0-14 age group comparing this study with our earlier report, with a predominance in male subjects and a shift of peak incidence from the 5-9 year age group in the first study to the 10-14 year age group in this one. Although consistent with our previous study, the high rate of presentation with DKA, despite several initiatives conducted to increase diabetes awareness of over the last decade, can be attributed to the changes in behaviour due to COVID-19, The pandemic might be associated with a delay in the admission of patients to health centres. Nevertheless, it remains paramount that new strategies are developed to increase awareness of pediatric T1D to alleviate the risk of presentation with DKA.

Ethics

Ethics Committee Approval: The study was approved by the Health Sciences University Diyarbakır Gazi Yaşargil

Training and Research Hospital Ethical Committee (ethics approval number: 765, date: 29.5.2021).

Informed Consent: Patients were included in the study after parental consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Şervan Özalkak, Ruken Yıldırım, Selma Tunç, Edip Ünal, Funda Feryal Taş, Mehmet Nuri Özbek, Concept: Şervan Özalkak, Hüseyin Demirbilek, Mehmet Nuri Özbek, Design: Şervan Özalkak, Hüseyin Demirbilek, Mehmet Nuri Özbek, Data Collection or Processing: Şervan Özalkak, Ruken Yıldırım, Selma Tunç, Edip Ünal, Funda Feryal Taş, Mehmet Nuri Özbek, Analysis or Interpretation: Şervan Özalkak, Hüseyin Demirbilek, Mehmet Nuri Özbek, Literature Search: Şervan Özalkak, Mehmet Nuri Özbek, Writing: Şervan Özalkak, Hüseyin Demirbilek, Mehmet Nuri Özbek.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021-11-1

J Clin Res Pediatr Endocrinol 2022;14(2):179-187

Low Complement C1q/TNF-related Protein-13 Levels are Associated with Childhood Obesity But not Binge Eating Disorder

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What is already known on this topic?

C1q/tumor necrosis factor-related proteins (CTRPs) play an important role in energy metabolism in humans. CTRP-13, a new member of this family, has been shown to increase insulin sensitivity and had an anorexigenic effect.

What this study adds?

We demonstrated significantly lower CTRP-13 levels in children with obesity compared with healthy weight children. A positive correlation between CTRP-13 and high-density lipoprotein-cholesterol levels suggested a possible effect of this adipokine on lipid metabolism.

Abstract

Objective: C1q/tumor necrosis factor-related proteins (CTRPs) are recently described members of the adipokine family. CTRP-13, a new member of this family, has been shown to increase insulin sensitivity and had an anorexigenic effect on food intake in experimental studies. The aim was to investigate serum CTRP-13 levels in children with obesity, and its relationship with other adipokines, metabolic parameters, or binge eating disorder (BED).

Methods: A cross-sectional study was conducted with 105 pubertal children attending a single center. Clinical (metabolic syndrome, BED) and biochemical (glucose, insulin, lipids, leptin, adiponectin, CTRP-13 levels) parameters were assessed.

Results: Sixty children with obesity [24 males (40%); median age 14.7 (13.0-16.4) years] and 45 healthy controls [15 males (33.3%); median age 15.2 (14.1-16.5) years] were included. Serum adiponectin and CTRP-13 levels were significantly lower in children with obesity than controls (7.1 vs 20.1 µg/mL, $p < 0.001$; 64.7 vs 103.8 ng/mL, $p < 0.001$, respectively). CTRP-13 levels correlated negatively with body mass index (Spearman rho = -0.230, $p = 0.018$) and positively with high-density lipoprotein-cholesterol levels (Spearman rho = 0.218, $p = 0.026$). There was no significant difference in serum CTRP-13 concentrations in terms of the presence of metabolic syndrome or BED.

Conclusion: Childhood obesity seems to be causing dysregulation in adipokine production and function, including the down-regulation of CTRP-13. The positive correlation between CTRP-13 and HDL-C levels suggested a possible effect of this adipokine on lipid metabolism. Thus CTRP-13 may be a novel biomarker for dyslipidemia in childhood obesity.

Keywords: C1q/TNF-related proteins, adipocyte, binge eating disorder, metabolic syndrome, pediatrics

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Conflict of interest: None declared
Received: 02.11.2021
Accepted: 20.12.2021

Introduction

Obesity has become an important public health problem for children and adolescents in the last few decades. Obesity is characterized by an increase in body fat mass, developing when energy intake is greater than energy consumption. In other words, obesity is a complex and multifactorial disease that is accompanied by endocrine and metabolic changes as a result of enhanced body fat tissue (1). Since childhood obesity is associated with metabolic and cardiovascular diseases in both children and later adulthood, diagnostic and preventive approaches are essential (2,3).

Appetite regulators play a significant role in ensuring the balance between energy intake and consumption. Among them, insulin and leptin are the major hormones that control dietary intake and the feeling of satiety (4). Adiponectin is one of the main hormones released from adipose tissue and regulates cardiovascular and metabolic functions (5). It has been shown in several studies that decreased adiponectin serum levels were associated with obesity, insulin resistance, metabolic syndrome, and type 2 diabetes mellitus (DM) in pediatric patients (6,7). Binge eating disorder (BED) is characterized by loss of control in food intake and presentes as recurrent episodes of eating large amounts of food until feeling uncomfortably full. BED has an increasing prevalence of 1-5% among children and adolescents, and obesity is one of the main physical results of BED (8).

C1q/tumor necrosis factor-related proteins (CTRPs) are recently described members of the adipokine family, secreted from adipose tissue, like adiponectin. Some types of CTRPs play an important role in energy metabolism in humans (9,10). CTRP-13, a new member of this family, has been shown to increase insulin sensitivity and had an anorexigenic effect on food intake in experimental studies (11,12). Clinical studies have also shown that a decrease in the serum level of CTRP-13 was a risk factor for the development of type 2 DM, coronary artery disease, and non-alcoholic fatty liver disease in adults with obesity (13,14,15).

In this study, we aimed to investigate whether serum CTRP-13 levels were altered by children with a diagnosis of obesity or BED and how CTRP-13 levels were related to other adipokines or metabolic parameters. To our knowledge, this is the first study in children to evaluate CTRP-13 levels in the context of obesity.

Methods

This was a single-center, cross-sectional study and we recruited the study participants from amongst individuals from the same geographic region, aged 12-18 years, who attended our department between May 2020 and April 2021. Before participating in the present study, a comprehensive physical examination was performed for all subjects. Also, laboratory tests, including thyroid function and serum cortisol were performed for children with obesity to diagnose possible endocrine disorders. Children with any acute or chronic systemic diseases, a history of drug use (such as anti-epileptics, anti-psychotics, and steroids), obesity-related syndromes, and endocrine disorders (hypothyroidism and Cushing syndrome) were excluded. In addition, prepubertal subjects or those under 12 years of age were not included in the study. Children without obesity and attending for routine check-up were recruited as a healthy control cohort.

Anthropometric Measurements

Height was measured using a Harpenden stadiometer with an accuracy of 0.1 cm without shoes, and body weight was measured using a scale (SECA, Hamburg, Germany) with a sensitivity of 0.1 kg while wearing light clothing. Body mass index (BMI) was calculated by dividing body weight (kg) by height in metres squared (m^2). Standard deviation (SD) scores for weight, height and BMI were calculated with the online calculator for pediatric endocrinologists (Child Metrics) (16), using the reference created for the Turkish population by Neyzi et al (17). Obesity was defined as BMI >2 SD score, according to the criteria of the World Health Organization (18).

Waist circumference (WC) was measured at the end of a gentle expiration using a non-stretchable tape with a sensitivity of 0.1 cm, at the midpoint between the lowest extent of the rib cage and the iliac crest, without clothing (19). WC percentiles and SD scores were calculated according to the age and gender, using reference data for Turkish children (20). The pubertal stage of each participant was assessed according to Tanner and Whitehouse (21). Breast development of stage 2 and above in girls or testicular volume of ≥4 mL in boys was accepted as pubertal findings. Blood pressure measurements were performed using a calibrated sphygmomanometer by a single investigator using a validated protocol. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice on the right arm after 10 minutes of rest in a seated position. Blood pressure percentile and SD scores were calculated according to age, gender, and height (16).

Metabolic Syndrome

Homeostasis model assessment of insulin resistance (HOMA-IR) was used to evaluate the status of insulin

resistance. A cut-off value of >4.0 for pubertal patients was defined as insulin resistance (22). Metabolic syndrome was described according to the criteria of the International Diabetes Federation Consensus (23):

- For patients between the ages of 10-16, metabolic syndrome was defined as the presence of central obesity (WC ≥90th percentile or adult cut-off if lower) plus any two of the following factors: triglyceride level of ≥150 mg/dL; high-density lipoprotein-cholesterol (HDL-C) level of <40 mg/dL; SBP ≥130 or DBP ≥85 mmHg; fasting serum glucose level of ≥100 mg/dL and/or known type 2 DM.

- For patients ≥16 years old, metabolic syndrome was defined as: Presence of central obesity (WC ≥94 cm for males and ≥80 cm for females) plus any two of the following factors: triglyceride level of ≥150 mg/dL; HDL-C level of <40 mg/dL in males and <50 mg/dL in females; SBP ≥130 or DBP ≥85 mmHg or treatment of previously diagnosed hypertension; fasting serum glucose level of ≥100 mg/dL; or known type 2 DM.

Binge Eating Disorder

A trained psychiatrist determined the diagnosis of BED using a survey, based upon the criteria of the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V). Binge-eating episodes at least one day a week for three months accompanied by marked distress and a lack of inappropriate compensatory behaviors and additional questions regarding the DSM-V criteria were assessed for diagnosis, such as (i) eating much more rapidly than normal, (ii) eating large amounts of food when not feeling physically hungry, (iii) eating until feeling uncomfortably full, (iv) eating alone because of being embarrassed by how much one is eating, and (v) feeling disgusted with oneself, depressed, or very guilty after overeating. An episode was described as follows; eating an amount of food in a discrete period of time that is definitely larger than most people would eat under similar circumstances and with the sense of lack of control over eating during this time (24).

Sample Collection and Storage

Blood samples for analyzing glucose, insulin, lipid profile, leptin, adiponectin, and CTRP-13 levels were obtained via venepuncture from the antecubital vein, after 10-12 hours of overnight fasting. To avoid run-to-run difference, all the samples were analyzed in the same run. Therefore, no correction or calibration factor was required. After allowing 60 minutes for spontaneous blood clotting, the plain tubes were centrifuged at 1200 x g for 10 minutes, and the serum samples were collected into tubes using plastic Pasteur pipettes and stored at -80 °C until analysis. Serum samples were diluted prior to analysis according to the manufacturer's instructions.

Biochemical Analysis

Fasting serum glucose, triglyceride, total cholesterol, and HDL-C levels were measured enzymatically using DP Modular Systems (Roche Diagnostic Corp., Indianapolis, IN, USA). Low-density lipoprotein-cholesterol (LDL-C) levels were calculated using the Friedewald formula when triglyceride levels were <400 mg/dL. Serum insulin level was measured by an electrochemiluminescence immunoassay method using an automated immunoassay analyzer (Immulite 2500, Diagnostic Products Corporation, Los Angeles, CA, USA).

Serum leptin (Catalog number: EK0437, Boster Biological Technology Co. Ltd., Wuhan, China) and adiponectin (Catalog number: EK0595, Boster Biological Technology Co. Ltd., Wuhan, China) levels were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits based on the principle of solid-phase enzyme immunoassay. CTRP-13 (Catalog number: SK00333-06, Aviscera Bioscience Inc., Santa Clara, CA, USA) level was measured by a commercial ELISA kit employing the sandwich phase enzyme immunoassay technique. The ELISA tests for leptin, adiponectin and CTRP-13 had a sensitivity of <10 pg/mL, <60 pg/mL and 2 ng/mL, with a detection range of 62.5-4000 pg/mL, 1.56-100 ng/mL and 3.9-250 ng/mL, respectively. Intra- and inter-assay coefficients of variation were <7.6% and <8.4% for leptin, <7.8% and <9% for adiponectin, 4-6% and 8-12% for CTRP-13, respectively.

This study was approved by the Dokuz Eylül University Local Ethics Committee (ethics approval number: 2020/01-26, date: 06.01.2020) and performed in accordance with the principles of the Declaration of Helsinki. An informed written consent form was obtained from the children and their parents before the study.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences application for Windows, version 24.0 (IBM Co., Armonk, NY, USA). The distribution of data was evaluated using the Kolmogorov-Smirnov test. Clinical data are presented as number (%) for categorical variables and median (25th-75th percentiles) for continuous variables. Comparisons of categorical and continuous variables were performed with the Pearson chi-square test and the Mann-Whitney U test, respectively. The Spearman correlation test was applied for correlations between CTRP-13 and continuous variables. Then, any correlation was investigated among the identified significant variables after

adjusting for age, gender, and BMI. A two-sided p value of < 0.05 was considered statistically significant.

Results

A total of 105 pubertal children [39 males (37.1%); median age 14.9 (13.5-16.5) years] were enrolled in this study. The group with obesity consisted of 60 children [24 males (40%); median age 14.7 (13.0-16.4) years] and the control group included 45 healthy children [15 males (33.3%); median age 15.2 (14.1-16.5) years]. There was no significant difference between the two groups in terms of age and gender (p = 0.154 and p = 0.484, respectively) (Table 1).

Significant differences were observed between children with obesity and children of healthy weight in terms of BMI, BMI SD scores, serum insulin, HOMA-IR, triglyceride, HDL-C, leptin, and adiponectin levels, whereas serum glucose, total cholesterol, and LDL-C concentrations were similar (Table 1). Individuals with obesity had significantly lower CTRP-13 levels than the control group (p = 0.013) (Table 1).

Eighteen of the subjects with obesity (30%) had metabolic syndrome and among children with and without metabolic syndrome, statistically significant differences were observed in terms of hypertension, serum insulin, HOMA-IR, triglyceride, and HDL-C levels (Table 2). Among children with obesity, although adiponectin was found to be significantly lower in those with metabolic syndrome (p = 0.021), no significant difference was observed in leptin or CTRP-13 levels (Table 2). When subgroup analysis was performed regarding gender, there was no significant difference in circulating CTRP-13 levels in boys (p = 0.655) or girls (p = 0.596).

Thirty-two of the patients with obesity (53.3%) had insulin resistance. When individuals with obesity were compared according to the presence of insulin resistance, serum adiponectin concentration was significantly lower in the insulin resistance-positive group [4.2 (1.9-10.9) vs 9.9 (6.1-18.3) µg/mL, p = 0.008]. However, serum leptin and CTRP-13 levels were found to be similar in both groups, with or without insulin resistance [40.1 (30.3-58.9) vs 55.3 (35.4-99.8) ng/mL, p = 0.093 and 64.7 (29.5-109.0) vs 63.5 (36.3-100.6) ng/mL, p = 0.717, respectively].

Twenty-three (38.3%) of the patients with obesity were diagnosed with BED. Among groups with and without BED, significant differences were observed in BMI and BMI SD scores (p = 0.033 and p = 0.029, respectively). However, metabolic and biochemical parameters, including CTRP-13 levels, were similar in both groups (Table 3). Moreover, there was no significant difference found when conducting subgroup analyzes according to gender, in boys (p = 0.976) or girls (p = 0.427).

CTRP-13 correlated negatively with BMI (Spearman rho = -0.230, p = 0.018), BMI SD score (Spearman rho = -0.237, p = 0.015) and positively with HDL-C (Spearman rho = 0.218, p = 0.026) in all participants (Figure 1). After adjustment for age, gender, and BMI, serum CTRP-13 levels showed a positive correlation with HDL-C levels in subjects with obesity (Spearman rho = 0.313, p = 0.018).

Table 1. The clinical and laboratory characteristics of children with obesity and healthy controls

	Children with obesity (n = 60)	Controls (n = 45)	p
Age (year)	14.7 (13.0-16.4)	15.2 (14.1-16.5)	0.154[a]
Gender (male) [n (%)]	24 (37.1)	15 (40%)	0.484[b]
BMI (kg/m²)	32.0 (30.0-35.6)	20.5 (18.2-22.3)	**< 0.001[a]**
BMI SD score	2.7 (2.3-3.1)	-0.5 [(-1.2)-0.6]	**< 0.001[a]**
Glucose (mg/dL)	85.5 (80.3-91)	85 (81-92)	0.766[a]
Insulin (uIU/mL)	19.3 (15.8-25.9)	10.9 (7.7-14.6)	**< 0.001[a]**
HOMA-IR	4.1 (3.3-5.8)	2.3 (1.5-3.3)	**< 0.001[a]**
TG (mg/dL)	90.5 (77.8-140.3)	82 (59.5-101.5)	**0.017[a]**
TC (mg/dL)	171 (155-195)	166 (152.5-192)	0.524[a]
LDL-C (mg/dL)	103.9 (86.1-123.7)	95.4 (83.1-110.3)	0.138[a]
HDL-C (mg/dL)	53 (47.5-64)	48 (41-54.8)	**0.004[a]**
Leptin (ng/mL)	46.4 (31.1-80.5)	7.2 (2.5-13.4)	**< 0.001[a]**
Adiponectin (µg/mL)	7.1 (3.1-13.1)	20.1 (7.3-46.4)	**< 0.001[a]**
CTRP-13 (ng/mL)	64.7 (35.7-103.9)	103.8 (42.9-167.8)	**0.013[a]**

Data are given as mean ± standard deviation or median (25^{th}-75^{th} percentile). [a]Mann-Whitney U test, [b]Pearson chi-square test, $p < 0.05$.
BMI: body mass index, BMI SD score: standard deviation score of BMI, HOMA-IR: homeostasis model assessment-insulin resistance, TG: triglyceride, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, CTRP-13: C1q/tumor necrosis factor-related protein-13

Discussion

CTRPs are new members of the adipokine family, which are secreted from adipose tissue, like adiponectin and leptin. It has been suggested that CTRPs play an important role in energy metabolism. CTRPs also have significant and distinct effects on the immune, endocrine, vascular, skeletal, and sensory systems (9,10). Fifteen types of CTRPs have been identified so far, some of which have metabolic functions

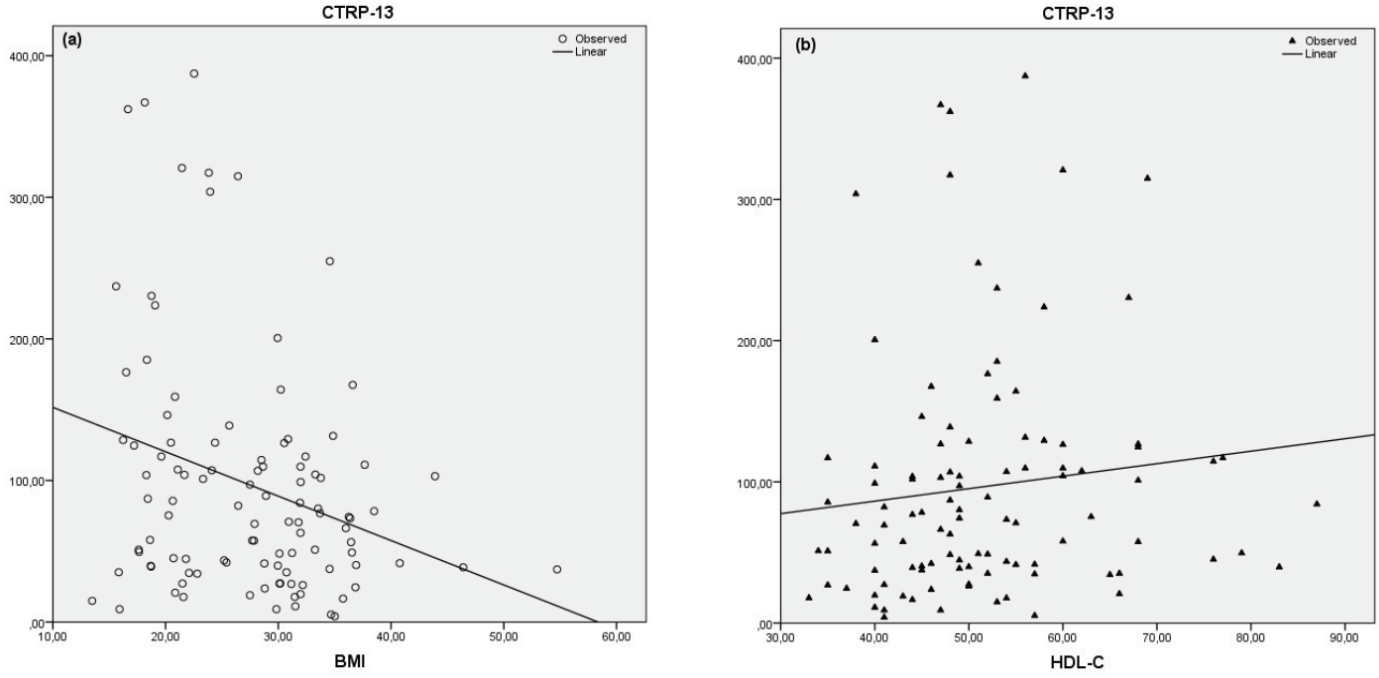

Figure 1. The correlation between serum C1q/tumor necrosis factor-related protein-13 levels and (a) BMI, (b) HDL-C

HDL-C: high-density lipoprotein-cholesterol, BMI: body mass index

Table 2. The clinical and laboratory characteristics of children with obesity according to the presence of metabolic syndrome or not

	MS (+) group (n = 18)	MS (-) group (n = 42)	p
BMI (kg/m²)	32.3 (31.5-34.6)	31.1 (29.6-36.3)	0.233
BMI SD score	2.7 (2.4-3.1)	2.6 (2.3-3.3)	0.411
WC (cm)	3.6 (3.0-4.0)	3.6 (3.1-4.0)	0.974
SBP SD score	2.2 (1.8-2.3)	1.2 (0.6-2.3)	**0.011**
DBP SD score	1.6 (0.7-2.1)	0.7 (0.4-1.6)	0.051
Hypertension, n (%)	15 (83.3)	20 (47.6)	**0.010**
Glucose (mg/dL)	89.5 (80.5-100)	85 (80-90.3)	0.320
Insulin (uIU/mL)	24.7 (16.3-32.6)	17.9 (14.8-24.4)	**0.044**
HOMA-IR	5.7 (3.4-6.9)	3.8 (3.2-5.0)	**0.025**
TG (mg/dL)	156.5 (86.8-181.5)	87 (68.5-107.3)	**0.001**
TC (mg/dL)	188 (143.8-202.8)	168.5 (155-186)	0.266
LDL-C (mg/dL)	114.2 (87.3-127.1)	97.8 (85.7-122.8)	0.287
HDL-C (mg/dL)	44.5 (37.3-50.3)	49 (43.8-55.3)	**0.025**
Leptin (ng/mL)	38.2 (23.8-63.0)	51.2 (22.9-98.0)	0.129
Adiponectin (µg/mL)	4.6 (1.7-7.6)	9.9 (3.3-17.6)	**0.021**
CTRP-13 (ng/mL)	64.7 (34.6-105.9)	63.5 (33.2-104.9)	1.000

Data are given as median (25^{th}-75^{th} percentile). Mann-Whitney U test was used to determine the differences of variables between the two groups, $p < 0.05$.
MS: metabolic syndrome, BMI: body mass index, BMI SD score: standard deviation score of BMI, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment-insulin resistance, TG: triglyceride, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, CTRP-13: C1q/tumor necrosis factor-related protein-13

(9,10). CTRP-13 is one of the members of this family, mostly expressed from adipose tissue and brain in mice and predominantly from adipose tissue in humans (11). The CTRP family has homologous effects on energy homeostasis and may prove to be novel pharmacological targets for diseases secondary to obesity. CTRPs can dynamically adjust the response to changes in short-term nutritional or long-term metabolic status. Excess caloric uptake in obesity and related inflammatory disorders usually disrupt the signaling pathways regulated by CTRPs, with changes in structural configurations and post-translational modifications of these molecules (9). Previous studies demonstrated reduced levels of CTRP-3, CTRP-6, CTRP-7, CTRP-9, CTRP-12, and CTRP-15 in mice or humans with obesity (25,26,27,28). In a childhood study, Chen et al (29) found that CTRP-3 concentrations significantly decreased in children with obesity, demonstrating a negative correlation with BMI. They indicated that the circulating level of CTRP-3 was down-regulated in the pro-inflammatory state of obesity, but the exact mechanism has not been clarified. Adiponectin and CTRPs share similar structures and functions, like organizing anti-inflammatory responses and regulating energy metabolism by improving insulin sensitivity (30). As an example for the relationship between CTRPs and adiponectin, CTRP-9 was shown to associate with adiponectin to form a heterotrimeric structure and to share the same receptor (31). Shanaki et al (32) showed that CTRP-13 and adiponectin inversely correlated with BMI in women, and CTRP-13 positively correlated with adiponectin, too. They also found that decreased serum levels of CTRP-13 may be related to visceral fatness, but no pathophysiological explanation was suggested (14). On the other hand, Omidifar et al (33) found no significant association between mRNA expression levels of CTRP-13 in subcutaneous or visceral adipose tissue in women with obesity and normal weight women. There was no relevant data concerning CTRP-13 in pediatric patients in the existing literature with which to compare our findings. We found that obesity in pubertal children was associated with lower serum CTRP-13 concentrations compared to healthy weight children. As a sign of visceral adiposity, CTRP-13 was negatively correlated with BMI in those individuals. However, in contrast with adult studies, we could not find any correlation between CTRP-13 and adiponectin levels. Although the exact molecular mechanism is not understood, our results suggested that childhood obesity disrupts metabolic homeostasis and causes dysregulation in adipokine production and function, including CTRP-13.

Adipokine dysregulation and inflammatory disorders in obesity result in metabolic problems, such as insulin resistance and type 2 DM (9,34,35). Experimental studies have shown that CTRP-13 has an insulin-sensitizing effect by promoting glucose uptake in adipocytes, myotubes, and hepatocytes, via the adenosine monophosphate-activated protein kinase signalling pathway. It also inhibits the c-Jun

Table 3. The clinical and laboratory characteristics of children with obesity according to the presence of binge eating disorder or not

	BED (+) group (n = 23)	BED (-) group (n = 37)	p
BMI (kg/m²)	33.3 (31.5-36.8)	31.2 (29.4-34.6)	**0.033**
BMI SD score	2.9 (2.6-3.6)	2.5 (2.3-3.0)	**0.029**
WC (cm)	3.6 (3.0-4.3)	3.6 (3.1-3.9)	0.563
SBP SD score	1.9 (0.9-2.3)	1.6 (0.8-2.3)	0.502
DBP SD score	1.4 (0.3-2.1)	0.8 (0.5-1.6)	0.784
Hypertension [n (%)]	15 (65.2)	20 (54.1)	0.394
Glucose (mg/dL)	85 (79-91)	87 (81-92)	0.428
Insulin (uIU/mL)	18.0 (15.8-23.2)	21.0 (15.4-29.4)	0.261
HOMA-IR	3.8 (3.2-5.7)	4.5 (3.4-6.5)	0.334
TG (mg/dL)	92 (81-165)	89 (75.5-126.5)	0.503
TC (mg/dL)	171 (148-195)	170 (156-197.5)	0.773
LDL-C (mg/dL)	104 (84.4-123.8)	103.8 (87.7-124.6)	0.429
HDL-C (mg/dL)	47 (40-56)	48 (41-52)	0.927
Leptin (ng/mL)	49.5 (32.4-98.9)	42.1 (30.7-78.2)	0.538
Adiponectin (µg/mL)	6.6 (3.5-13.2)	7.7 (2.6-14.1)	0.855
CTRP-13 (ng/mL)	73.3 (40.2-103.0)	57.5 (27.1-105.5)	0.447

Data were given as median (25^{th}-75^{th} percentile). Mann-Whitney U test was used to determine the differences of variables between the two groups, $p < 0.05$.
BED: binge eating disorder, BMI: body mass index, BMI SD score: standard deviation score of BMI, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment-insulin resistance, TG: triglyceride, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, CTRP-13: C1q/tumor necrosis factor-related protein-13

N-terminal kinase (JNK) signal pathway, which is activated by fatty acids and ameliorates lipid-induced insulin resistance in hepatocytes (11). In addition, CTRP-13 has significant impact on gluconeogenic enzymes to reduce glucose output in hepatocytes (11). All these literature data, gained from experimental studies, support a significant effect of CTRP-13 on glucose homeostasis. In several clinical studies, a decrease in the serum level of CTRP-13 was found to be a risk factor for the development of type 2 DM or coronary artery disease in adult patients (13,15). Shanaki et al (14) found that CTRP-13 had a negative correlation with insulin, triglyceride levels, and HOMA-IR. They also observed lower CTRP-13 levels in patients with type 2 DM and non-alcoholic fatty liver disease, suggesting CTRP-13 as a potential marker for these clinical conditions (14). An et al (36) demonstrated significant differences in CTRP-13 levels in adults with non-alcoholic fatty liver disease when compared to the control group, and a correlation between CTRP-13 and triglyceride levels in those patients. However, the exact mechanism of CTRP-13 effects on glucose and lipid metabolism in humans has not yet been established. However, Bai et al (37) showed no difference in CTRP-13 levels among age- and BMI-matched adults with or without type 2 DM. They observed similar concentrations at 0 and 2 hours of the oral glucose tolerance test (37). Fadaei et al (15) reported that circulating levels of CTRP-13 did not correlate with lipid profiles in adults. We observed a positive correlation between CTRP-13 and HDL-C levels, which might be a result of the possible effect of this novel adipokine on lipid metabolism, as recently shown with CTRP-3 levels in pediatric patients (38). This alteration was thought to be caused by the modulatory effects of CTRP-13 through signaling pathways, including JNK in hepatocytes, as a defence mechanism against the metabolic complications of obesity. Nevertheless, we did not find any significant difference in CTRP-13 levels according to the presence of metabolic syndrome in children with obesity, as well as insulin resistance. In light of the conflicting literature data and our results, questions still need to be answered about the molecular and functional role of CTRP-13 in inflammatory pathways leading to metabolic complications secondary to obesity. Although CTRP-13 levels were decreased in our patients with obesity, we hypothesized that the metabolic dysregulating consequences of this decrease take some time and hence may present in adulthood. Therefore, a novel therapeutic approach on correcting CTRP-13 levels in childhood may be protective against metabolic complications of obesity seen in later life.

BED is defined by the loss of control of food intake, which results in eating large amounts of food and therefore obesity (8). Insulin and leptin are the main hormones that control food intake and feeling of satiety, which play an important role in maintaining the balance between energy intake and consumption (4). Leptin is an adipokine produced from adipose tissue that controls food intake in the brain through leptin receptors in the arcuate nucleus. It suppresses the release of hypothalamic peptides, such as neuropeptide-Y, resulting in decreased appetite (39). While leptin acts as a "satiety signal" to prevent excess food intake and links adipose tissue to hypothalamic centers regulating energy homeostasis, we could not find any relationship between serum leptin levels and BED. In an experimental study with mice, CTRP-13 was found to be an anorexigenic factor and its secretion from the hypothalamus increased after high-fat feeding and reduced after food restriction. Also in the same study, it was observed that appetite was suppressed and weight loss was achieved in mice given CTRP-13 exogenously (12). Therefore, a hypothalamic feedback loop including orexigenic neuropeptides, such as neuropeptide-Y and agouti-related protein, together with CTRP-13, was thought to be modulating food intake in mice (12). Although the underlying pathophysiology of BED is unclear, we investigated the role of CTRP-13 in this psychiatric disorder based on the data from experimental studies. However, we could not find any evidence of a relationship between a diagnosis of BED and circulating CTRP-13 levels in subjects with obesity. This result may be related to the complex etiopathogenesis of BED.

Study Limitations

To the best of our knowledge, this is the first study evaluating CTRP-13 levels in children with obesity. However, our study has some limitations. First of all, we could not reach the targeted sample size due to the COVID-19 pandemic and the time-limited nature of the study. Therefore, we performed a post-hoc power analysis using G*Power software (version 3.1.9.4, http://www.gpower.hhu.de/en.html) with values of mean and SD gained from the results of this study, and found power at 0.90, with an effect size (d) of 0.60 and type I error at 0.05. Secondly, the limited number of participants in subgroup analyzes might have a negative impact on the accuracy of these results. Therefore, prospective clinical and molecular studies with larger sample sizes are required in this field to elucidate the exact mechanism of the relationship between CTRP-13 and obesity. Finally, BED diagnosis was determined according to a clinical survey, which may be affected by the subjective responses of the individuals with obesity. Nevertheless, a trained psychiatrist performed the surveys and this method was compatible with the daily clinical practice to determine the diagnosis of BED.

Conclusion

We demonstrated significantly lower CTRP-13 levels in children with obesity than in age-matched children with healthy weight. The positive correlation between CTRP-13 and HDL-C levels suggested a possible effect of this adipokine on lipid metabolism and it can be used as a marker for dyslipidemia in childhood obesity. However, unlike adult studies, we could not find any difference in CTRP-13 levels in regard to obese children with or without metabolic syndrome or insulin resistance. A novel therapeutic approach may be to optimize CTRP-13 levels in childhood which may be protective against metabolic complications of obesity seen in later life but much more evidence is required to support this hypothesis.

Ethics

Ethics Committee Approval: This study was approved by the Dokuz Eylül University Local Ethics Committee (ethics approval number: 2020/01-26, date: 06.01.2020) and performed in accordance with the principles of the Declaration of Helsinki.

Informed Consent: An informed written consent form was obtained from the children and their parents before participating the study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: İbrahim Mert Erbaş, Ahu Paketçi, Serkan Turan, Ali Rıza Şişman, Korcan Demir, Ece Böber, Ayhan Abacı, Concept: İbrahim Mert Erbaş, Ayhan Abacı, Design: İbrahim Mert Erbaş, Ayhan Abacı, Data Collection or Processing: İbrahim Mert Erbaş, Ahu Paketçi, Serkan Turan, Ali Rıza Şişman, Korcan Demir, Ece Böber, Ayhan Abacı, Analysis or Interpretation: İbrahim Mert Erbaş, Ahu Paketçi, Serkan Turan, Ali Rıza Şişman, Korcan Demir, Ece Böber, Ayhan Abacı, Literature Search: İbrahim Mert Erbaş, Ahu Paketçi, Serkan Turan, Ali Rıza Şişman, Korcan Demir, Ece Böber, Ayhan Abacı, Writing: İbrahim Mert Erbaş.

Financial Disclosure: This study was supported by a grant from the Dokuz Eylül University Department of Scientific Research Projects (2020.KB.SAG.057).

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2022.2021-10-6

J Clin Res Pediatr Endocrinol 2022;14(2):188-195

How Vitamin D Levels of Children Changed During COVID-19 Pandemic: A Comparison of Pre-pandemic and Pandemic Periods

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What is already known on this topic?

Vitamin D has immunomodulatory effects and this has effects on infections, including Coronavirus disease-2019 (COVID-19). Vitamin D deficiency has been reported to be associated with clinical severity in COVID-19 disease, in both adults and children.

What this study adds?

Vitamin D levels of healthy children (>6 years old) and adolescents decreased in the first year of the pandemic due to the pandemic-related restrictions.

Abstract

Objective: The synthesis of vitamin D is related to sun exposure, thus the restrictions during the Coronavirus disease-2019 (COVID-19) pandemic may have affected the levels of vitamin D in all age groups. The aim of this study was to evaluate vitamin D levels of healthy children and adolescents during the first year of the pandemic.

Methods: The study group included healthy children and adolescents who were admitted for general check-ups and evaluated with 25(OH)D levels. Then, it was divided into two groups: Group 1 "pre-pandemic", and Group 2 "pandemic". Vitamin D levels were recorded from the hospital database and were compared according to age groups, gender, and the season, retrospectively.

Results: The study group [mean age = 90.29 ± 59.45 median age = 79 interquartile range (IQR): 102 months, male/female: 1409/1624] included 3033 children and adolescents (Group 1/Group 2 n = 1864/1169). Although the mean 25(OH)D levels among preschool children did not differ between groups, the vitamin D levels of school-aged children and adolescents were significantly lower in the pandemic period than in the pre-pandemic period [Group 1 median = 16.50 (IQR: 10.5) vs Group 2 median = 15.9 (IQR: 11.3) in 6-12 age group (p = 0.026); Group 1 median = 13.30 (IQR: 10.2) vs Group 2 median = 11.20 (IQR: 9.7) in 12-18 age group (p = 0.003)]. Moreover, the 25(OH)D levels of adolescents showed seasonal variance with lower levels in winter, and unexpectedly, in summer.

Conclusion: Pandemic-related restrictions have caused significant decreases in vitamin D levels of school-aged children and adolescents. We suggest that children and adolescents should be given vitamin D supplementation in order to maintain sufficient levels of vitamin D during the pandemic.

Keywords: Vitamin D, COVID-19, children, pandemic

Introduction

In recent years, the interest in the extraskeletal effects of vitamin D, especially its immunomodulatory effects, has increased. Moreover, this interest has increased during the pandemic as the association of vitamin D and infections has previously been reported. Meta-analysis of randomized controlled trials has indicated that vitamin D has a protective effect against respiratory tract infections (1). Recently, the possible protective and/or preventive effect of vitamin D against Coronavirus disease-2019 (COVID-19) has also been reported (2,3).

A very recent study from Turkey revealed an association between vitamin D deficiency and clinical severity in

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Conflict of interest: None declared
Received: 14.10.2021
Accepted: 06.02.2022

pediatric COVID-19 cases (4). These authors suggested that prophylactic vitamin D supplementation may be considered in order to prevent deficiencies, especially in the adolescent age group. However, this study reported findings from children infected with COVID-19 and there is a lack of data about how the levels of vitamin D in the general population of children and adolescents changed during the pandemic.

In December 2019, after an epidemic of a new viral infectious disease was firstly reported from China, later identified as COVID-19 disease, the World Health Organization declared a pandemic of global health caused by Severe acute respiratory syndrome-Coronavirus-2 (5). After the disease was shown to spread rapidly in many different ways, but primarily through the respiratory tract, many countries implemented a series of social distancing policies, restricting travel and movement to reduce transmission, requested citizens to stay-at-home, and lockdowns. As the synthesis of vitamin D is directly modulated by sunlight, stay-at-home behavior and/or lockdowns to prevent COVID-19 spreads may have affected vitamin D levels in all age groups-adults, children, and adolescents.

The aim of this study was to determine the vitamin D levels of children and adolescents in the first year of the pandemic and compare the results with a one year period pre-pandemic. In addition, a further aim was to identify possible at risk groups for age, gender, and season that were more likely to be affected by lockdowns in terms of vitamin D levels.

Methods

Study Design and Patients

The study population included 1-18 year-old children who attended the University of Health Sciences Turkey, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, İzmir, Turkey between April 1, 2019, and April 1, 2021, and who were evaluated for vitamin D levels for routine health checks. Infants (<1 year of age) were not included in the study as they are eligible to receive prophylactic vitamin D in Turkey through a national vitamin D supplementation program. Children with a medical history of vitamin D-related metabolic disorders, such as skeletal or gastrointestinal system diseases, liver or kidney diseases, genetic syndromes, obesity, malnutrition, or malabsorption disorders were excluded from the study. Data on the date of birth, gender, hospital visit dates, and 25(OH)D levels available in the Hospital Information System (PROBEL) were obtained retrospectively. Then, patients selected for the study were divided into two groups according to the blood sampling date for the 25(OH)D measurement. Repeated measurements in the same subject were not included in the study. Group 1 included patients admitted in the 'pre-pandemic' period (April 1, 2019-March 31, 2020) while Group 2 included patients admitted in the 'pandemic' (April 1, 2020-April 1, 2021) period. Data of the 25(OH)D levels were compared between Group 1 and Group 2 according to age, gender, and also the season. Groups were divided into three age groups: 1-6 years (13-72 months; toddlers and preschool children); 6-12 years (73-144 months; school-age children); and 12-18 years (adolescents). To evaluate seasonal variability, the pre-pandemic/pandemic periods were divided into spring (March, April, May), summer (June, July, August), autumn (September, October, November), and winter (December, January, February).

Serum 25(OH)D levels were measured by an electrochemiluminescence immunoassay method using a standard kit available on the Abbott Architect System and analyzed using an automated biochemical analyzer, Abbott, I 2000, (Abbott Laboratories, Abbott Park, IL, USA). Serum 25(OH)D levels <12 ng/mL, between 12-20 ng/mL, and levels >20 ng/mL were defined as vitamin D deficiency, insufficiency, and sufficiency, respectively (6).

Lockdown Measures for Pediatric Age During the First Year of the Pandemic

After the global pandemic declaration in December 2019, the first patient with COVID-19 disease was reported on 11 March 2020 in Turkey. Then, on 16 March 2020, the Turkish Ministry of National Education declared the cessation of face-to-face education and offered online education for both elementary, secondary and high schools. In addition, the Government of Turkey announced that all children, adolescents, and young adults (<20 years old) were under curfew on weekdays and for the whole day at weekends (7). The restrictions for <20 year-old citizens continued until June 2020 along with >65 year-old citizens. Table 1 summarizes the opening and closure of schools following the lockdown periods during the study period.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University of Health Sciences Turkey, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, İzmir (protocol no: 554, decision no: 2021/09-02, date: 06.05.2021).

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences, version 21. Categorical data are presented as percentages whereas numerical data with Gaussian distribution are presented as mean ± standard deviation (SD)

and abnormally distributed data are presented as median [interquartile range (IQR)]. The chi-square or Fisher's exact test was used to compare proportions between groups, where appropriate. Student's t-test or Mann-Whitney U test, as appropriate, was used to analyze numerical data between different groups. For correlation analysis, the Spearman rank coefficient was calculated for variables without a normal distribution, and Pearson's linear correlation coefficient was used for variables with a normal distribution. A p value < 0.05 was considered statistically significant.

Results

The study group included 3033 children between 1 year and 18 years old [mean ± SD = 90.29 ± 59.45; median age = 79 (IQR: 102) months] The male to female ratio (M/F) was 1409/1624 with 46.5% of the study population male. Group 1 (pre-pandemic group) consisted of 1864 children [mean ± SD = 91.08 ± 59 months; median = 80 (IQR = 102)] months with a M/F of 856/1008 (45.9% male) while Group 2 (pandemic group) consists of 1169 children [mean ± SD = 89.43 ± 60 months; median = 77 (IQR: 103)] months with a M/F of 616/553 47% male). The main characteristics of the participants are presented in Table 2. The mean serum 25(OH)D level in the study group was 18.69 ± 9.98 ng/mL. The mean serum 25(OH)D level of girls participating in the study was 17.78 ± 10.11 ng/mL and lower than the mean serum 25 (OH) vitamin D level of boys 19.76 ± 9.72 ng/mL ($p < 0.05$).

The rate of vitamin D deficiency [25(OH)D level < 12 ng/mL] and insufficiency (12-20 ng/mL) in the study group was 27.5% (833/3033) and 34.1% (1034/3033), respectively; and the rate of children with sufficient vitamin D levels [25(OH)D level > 20 ng/mL] was only 38.4% (1166/3033). When evaluating the groups in terms of sufficient 25(OH)D vitamin levels in different age groups, the rate of children with adequate levels of vitamin D in preschool children (1-6 years), school-aged children (6-12 years) and adolescents (12-18 years) were 23.7% (n = 718), 10% (n = 302) and 4.8% (n = 146), respectively. Moreover, a negative correlation was found between age (months) and 25(OH)D vitamin level (Group 1, $r = -0.396$ $p < 0.01$; Group 2, $r = -0.504$ $p < 0.01$), respectively.

Group 1 (pre-pandemic group) included 1864 children, 835 (44.8%) of whom were 1-6 years, 578 (31%) were 6-12 years and 451 (24.2%) were 12-18 years old. In Group 2 (pandemic group), there were 1268 children, 561 (48%) of them were 1-6 years while 339 (29%) were 6-12 years and 269 (23%) were 12-18 years old. When evaluated according to gender difference, in Group 1, the median 25(OH)D level of girls was 16.1 (IQR: 11.5) ng/mL and significantly lower than the median 25(OH)D level of boys [18.5 (IQR: 13.2) ng/mL] ($p < 0.01$). In Group 2, the median 25(OH)D level was 15.8 (IQR: 14.1) ng/mL in girls and 17.8 (IQR: 12.8) ng/mL in boys, and the difference was again significant ($p < 0.01$). Table 3 presents the mean values of vitamin D levels according to gender in different age groups.

In the study group, the mean 25(OH)D (ng/mL) levels were similar between groups (18.75 ± 9.76 in Group 1 vs 18.60 ± 10.31 in Group 2) when not accounting for age, gender, and seasonal differences ($p > 0.05$). However, to

Table 1. The periods of open and closed schedules of schools according to the Turkish Ministry of National Education during the study period in İzmir

	Spring	Summer	Autumn	Winter
Kindergarten	Open	Open	Open	Open
Elementary school	All classes closed except 02.03.2021-31.05.2021	All classes closed except 01.06.2021-03.07.2021	All classes closed except 28.09.2020-09.11.2020 1st classes open 01.09.2020-28.09.2020	All classes closed
Secondary school	All classes closed except Only 12. Class 05.04.2021-16.04.2021 26.04.2021-29.04.2021	All classes closed except 14.06.2021-02.07.2021	All classes closed	All classes closed

Table 2. The main characteristics of the study group

	Group 1 (pre-pandemic) (n = 1864)	Group 2 (pandemic) (n = 1169)	p
Age (months) mean ± SD median (IQR)	91.08 ± 59 80 (102)	89.43 ± 60 77 (103)	0.063
Gender (male/female)	856/1008	616/553	0.08
25(OH)D mean ± SD*	18.75 ± 10.4	18.60 ± 10.31	0.69

*ng/mL.
SD: standard deviation, IQR: interquartile range

evaluate the effect of lockdowns and closure of schools, the 25(OH)D levels of the children were compared according to age groups. Our results show that the 25(OH)D levels among toddlers and preschool children (1-6 years) did not differ between groups 1 and 2 [Group 1 median = 19.80 (IQR: 13.2) vs Group 2 median = 21.60 (IQR: 14.3); $p > 0.05$]. However, for school-aged children and adolescents, the levels of 25(OH)D were lower in the pandemic period (Group 2) than in the pre-pandemic period (Group 1). Iin school-aged children (6-12 years) for Group 1 the median value was 16.50 (IQR: 10.5) vs Group 2 with a median of 15.90 (IQR: 11.3; $p = 0.026$). In adolescents (12-18 years old) the Group 1 median value was 13.30 (IQR: 10.2) vs Group 2 with a median of 11.20 (IQR: 9.7; $p = 0.003$) (Table 4).

The deficiency, insufficiency and sufficiency proportions during pre-pandemic and pandemic periods in different age groups are presented in Table 5. In this study population the rate of vitamin D deficiency was significantly higher in the pandemic period than in pre-pandemic period in the adolescent age group (12-18 years) [42.6% in Group 1 vs 53.2% in Group 2]. In addition, the rate of vitamin D insufficiency in the pre-pandemic period (35.9%) was significantly higher than in pandemic period (28.6%) while the rate of vitamin D sufficiency was similar between groups in this age group (21.5% in Group 1 vs 18.5% in Group 2). The rates of the deficiency, insufficiency and sufficiency ratios of vitamin D did not differ significantly during pre-pandemic and pandemic periods in other age groups ($p > 0.05$).

Table 6 presents the seasonal variation of 25(OH)D levels in both groups by age. When the mean 25(OH)D levels of both groups were evaluated by season, no significant difference was determined between the groups in the 1-6 years and 6-12 years age groups in all seasons ($p > 0.05$). However, in the 12-18 age group, the mean 25(OH)D levels in Group 2 were significantly lower during winter and summer than in Group 1. In winter in Group 1 the median value was 10.3 (IQR: 8.3) vs Group 2 with a median of 8.7 (IQR: 6.8) ng/mL ($p = 0.016$) while in summer the mean values were Group 1 19.83 ± 7.12 ng/mL vs Group 2 14.67 ± 6.62 ng/mL ($p = 0.03$).

Discussion

This study examined the effect of the restrictions applied in the pandemic on vitamin D levels in children by taking into account seasonal variability, gender, and age groups. We found that the rate of vitamin D insufficiency and deficiency was high among children during both pre-pandemic and pandemic periods. However, children between 6-12 and 12-18 years old had even lower levels of vitamin D in the pandemic period than in the pre-pandemic period. Thus, they were affected negatively, probably because of the closure of schools and stay-at-home restrictions during the COVID-19 pandemic. In addition, this negative effect showed seasonal variance in adolescents, as the mean levels

Table 3. Vitamin D levels according to gender in different age groups during pre-pandemic and pandemic periods

	Pre-pandemic			Pandemic		
	Girls	**Boys**	**p**	**Girls**	**Boys**	**p**
1-6 years, n (%)	440 (53)	395 (47)		267 (47.5)	294 (52.5)	
25(OH)D ng/mL*	18.9 (12.3)	20.5 (13.4)	0.13	21.1 (14.6)	21.7 (13.9)	0.07
6-12 years, n (%)	313 (54)	265 (46)		179 (52)	160 (48)	
25(OH)D ng/mL*	15.7 (9.4)	17.4 (11.2)	0.013	15.2 (11.6)	16.5 (10)	0.03
12-18 years, n (%)	300 (66)	151 (34)		170 (63)	99 (37)	
25(OH)D ng/mL*	13.9 (10.6)	15.3 (9.6)	0.001	10.1 (8.9)	13.9 (10.6)	0.001
Total, n (%)	1008 (54)	856 (46)		616 (53)	553 (47)	
25(OH)D ng/mL**	16.1 (11.5)	18.5 (13.2)	0.001	15.8 (14.1)	17.8 (12.8)	0.001

*Median (IQR), **mean ± SD.
SD: standard deviation, IQR: interquartile range

Table 4. 25(OH)D levels of the children between groups according to age groups

	Pre-pandemic	Pandemic	p
1-6 years (n)	835	561	0.97
25(OH)D ng/mL*	19.80 (13.2)	21.60 (14.3)	
6-12 years (n)	578	339	**0.026**
25(OH)D ng/mL*	16.50 (10.50)	15.90 (11.3)	
12-18 years (n)	451	269	**0.003**
25(OH)D ng/mL*	13.30 (10.2)	11.20 (9.7)	

*Median (IQR).
IQR: interquartile range

of vitamin D were lower especially during the summer and winter months.

Vitamin D deficiency remains a significant global public health problem despite the availability of supplementation and numerous published guidelines for its prevention (8,9,10,11,12,13). The subject continues to be an important problem both in our country, in Europe, and the World (14,15,16,17,18,19). The results of our study show that the rate of vitamin D deficiency remains around a quarter of healthy children. The rate of vitamin D insufficiency (12-20 ng/mL) and deficiency (< 12 ng/mL) in the study group were found to be 34.1 % and 27.5 %, respectively. The Global Consensus Recommendations on Prevention and Management of Nutritional Rickets have recently reported that vitamin D deficiency is preventable and suggests supplementation and food fortification with vitamin D to prevent deficiency (20). In our country, a free vitamin D supplementation program was initiated by the Ministry of Health in 2005 and free vitamin D drops have been distributed to all newborn babies within the framework of this program, following the recommendations of the Endocrinology and Diabetes Association Bone Health Group (21,22). As all infants in this age group in Turkey are eligible to receive prophylactic vitamin D, regardless of the pandemic, infants < 1 year of age were not included in this study.

Table 5. The deficiency, insufficiency and sufficiency rates of 25(OH)D during pre-pandemic and pandemic periods in different age groups

			Pre-pandemic n (%)	Pandemic n (%)	p
25(OH)D*	1-6 years	< 12	149 (17.8)	98 (17.5)	0.115
		12-20	274 (32.8)	157 (28.0)	
		> 20	412 (49.3)	306 (54.5)	
	6-12 years	< 12	144 (24.9)	107 (31.6)	0.074
		12-20	233 (40.3)	131 (38.6)	
		> 20	201 (34.8)	101 (29.8)	
	12-18 years	< 12	192 (42.6)	143 (53.2)	**0.22**
		12-20	162 (35.9)	77 (28.6)	
		> 20	97 (21.5)	49 (18.2)	

*ng/mL.

Table 6. The seasonal variation of 25(OH)D levels according to age group

	Preschool	School-aged	Adolescent
Spring			
n (Group 1/Group 2)	194/147	123/104	81/62
25(OH)D, ng/mL			
Group 1	17.8 (12.8)*	14.4 (7.9)*	12.1 (7.8)*
Group 2	17.7 (11.8)*	13.7 (9.5)*	10.55 (8.4)*
p	0.92	0.61	0.25
Summer			
n (Group 1/Group 2)	112/43	61/24	68/23
25(OH)D, ng/mL			
Group 1	25.1 (9.9)*	22.68 ± 6.22**	19.83 ± 7.12**
Group 2	27.1 (13)*	21.81 ± 8.99**	14.67 ± 6.62**
p	0.20	0.62	**0.03**
Autumn			
n (Group 1/Group 2)	183/139	131/67	111/66
25(OH) D, ng/mL			
Group 1	23.6 (11.4)*	20.9 (10.8)*	17.07 ± 8.08**
Group 2	26.1 (7.7)*	23.3 (10.8)*	18.28 ± 8.19**
p	0.21	0.05	0.33
Winter			
n (Group 1/Group 2)	346/232	263/144	191/118
25(OH)D, ng/mL			
Group 1	16.9 (13.1)*	14.4 (8.8)*	10.3 (8.3)*
Group 2	17.8 (14.3)*	13.4 (9)*	8.7 (6.8)*
p	0.48	0.12	**0.016**

*Median, IQR, **mean ± SD.
SD: standard deviation, IQR: interquartile range

It was previously reported that levels of vitamin D are significantly lower in girls, adolescents, those with low physical activity, during the winter season, and in areas with little or no sun exposure (23-30). Our results were in keeping with these findings; the vitamin D levels of girls were significantly lower than boys especially in 6-12 and 12-18 years old children and adolescents. In addition, in our study population, vitamin D levels were negatively correlated with age. This negative correlation was present in both the pre-pandemic and pandemic periods

As the synthesis of vitamin D is directly modulated by sun exposure, stay-at-home orders and/or lockdowns to prevent COVID-19 spreads may have affected vitamin D levels in both children and adolescents. İzmir, where the current study was conducted, is on the coast of western Anatolia, located on 38.25°N latitude and is temperate and sunny during most of the year (31). However, despite the geographic location with an agreeable and relatively sunny climate for vitamin D synthesis, vitamin D insufficiency and deficiency was a problem before the pandemic and continues to be a problem after the pandemic in this region (32).

Although the vitamin D levels were similar in Group 1 and 2 without taking age, gender, and seasonal differences into consideration, as we hypothesized that limited sun exposure due to lockdowns, closure of schools, and stay-at-home calls may have decreased vitamin D levels among specific age groups, we firstly compared the levels of vitamin D according to age subgroups. Our results showed that vitamin D levels of 6-12 year-old and 12-18 year-old children were significantly lower in the pandemic period (Group 2) than in the pre-pandemic period (Group 1). In a similar study of the relationship between serum levels of vitamin D and pandemic-related restrictions in children in Guangzhou, it was reported that vitamin D deficiency increased in the pandemic period in children under 6 years of age (33). Controversially, in another study conducted among 18-19 year-old male adolescents, the authors reported that they did not identify any difference in vitamin D levels before and during the pandemic in their study population (34). During the pandemic, the timing of the closure of schools, the season during the stay-at-home periods, and the duration of lockdowns vary from country to country (33,34,35). As soon as the first case was reported from our country, schools stopped face-to-face education. Moreover, children under 18 years were restricted in going out which led to limited sun exposure, especially in school-aged children.

In a very recent study, Bayramoğlu et al (4) reported that vitamin D insufficiency (38.4%) and deficiency (41.7%) were evident among adolescent COVID-19 patients and suggested prophylactic use of vitamin D in this age group. Our results add that vitamin D levels are also lower in healthy adolescents in the pandemic period than in the pre-pandemic period, and this decrease has caused an increased rate of vitamin D deficiency in this age group. We also add that the vitamin D levels were lower, not only in adolescents but also in school-aged children during the pandemic. Thus, we suggest that these age groups are affected due to pandemic-related restrictions and should be offered prophylactic vitamin D to prevent vitamin D insufficiency and deficiency. As for toddlers and preschool children (1-6 years), the vitamin D levels were similar between groups. In our opinion, the reason for this result might be that this age group either has no schooling or has been attending kindergarten which were not closed due to pandemic-related restrictions. Thus, we believe, sun exposure in this age group did not change between the pre-pandemic and pandemic periods. However, considering the continuing effect of COVID-19 related restrictions, this age group should also be offered prophylactic use of vitamin D to maintain sufficient levels of vitamin D.

There are many published studies reporting seasonal variations of vitamin D levels due to sun exposure (36). Rustecka et al (35), reported that the vitamin D levels in children decreased during the COVID-19 pandemic and the rate of children with vitamin D deficiency has increased in all seasons, except winter, in Poland. They also stated that although the characteristic seasonal variability was observed before the pandemic, no seasonal variability was observed during the pandemic. In our study, vitamin D levels were significantly lower in the pandemic period than pre-pandemic levels in 12-18 year old adolescents during winter and summer. Unexpectedly, in addition to the characteristic seasonal decrease in winter, the levels of vitamin D were also decreased during summer, indicating that adolescents could not benefit from sunlight because of restrictions.

Study Limitations

The most important limitation of this current study is its retrospective design. As we evaluated hospital records of children, we did not have data about nutritional intake, vitamin D supplementation status, physical activity, and body mass index of the children, all of which are known to affect vitamin D levels directly. Second, vitamin D levels are closely related to geographic properties, specifically the local climate and lockdown restrictions also varied between regions. Thus, the results are not generalizable. A strength of this current study, which goes some way to mitigating the lack of additional data, is the size of the study cohort, all of whom were without chronic disease and who could be correctly identified by hospital records.

Conclusion

We report that school-aged children (6-12 years) and adolescents (12-18 years) are at risk of vitamin D deficiency and insufficiency in both the pre-pandemic and pandemic periods in our country. Moreover, pandemic-related restrictions have caused significant decreases in vitamin D levels in these age groups. Thus, we suggest that children and adolescents should be given vitamin D supplementation in order to maintain sufficient levels of vitamin D. In addition, due to the continuing effects of the pandemic, since the time spent outdoors and, consequently, sun exposure is limited, dietary intake of vitamin D (fish, food fortified with vitamin D) should be supported to prevent vitamin D deficiency in all age groups.

Ethics

Ethics Committee Approval: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, İzmir (protocol no: 554, decision no: 2021/09-02, date: 06.05.2021).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

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Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2022.2021-10-4

J Clin Res Pediatr Endocrinol 2022;14(2):196-206

The Role of American Thyroid Association Pediatric Thyroid Cancer Risk Stratification and *BRAF^{V600E}* Mutation in Predicting the Response to Treatment in Papillary Thyroid Cancer Patients ≤18 Years Old

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What is already known on this topic?

American Thyroid Association (ATA) pediatric initial risk stratification was documented to be useful and effective in predicting recurrence and response to treatment in papillary thyroid cancer (PTC). *BRAF^{V600E}* mutation has been found to be associated with an increased risk of lymph node metastasis, recurrence, and poor prognosis in adult patients. There is limited data about the impact of *BRAF^{V600E}* mutation on prognosis in pediatric PTC.

What this study adds?

This study documented that ATA initial pediatric risk stratification effectively predicted the risk of recurrent and persistent disease and final response to treatment in PTC patients ≤18 years old. The presence of *BRAF^{V600E}* mutation was highly predictive for locoregional recurrence but had no significant effect on the final rate of excellent response to treatment.

Abstract

Objective: This study aimed to evaluate the role of risk stratification by the American Thyroid Association (ATA) pediatric thyroid cancer risk levels and *BRAF^{V600E}* mutation to predict the response to treatment in papillary thyroid cancer (PTC) patients ≤18 years old.

Methods: Clinical outcomes during a median period of 6 (2-21.8) years were assessed in 70 patients, according to ATA pediatric risk stratification, *BRAF^{V600E}* mutation status, and dynamic risk stratification (DRS) at final follow-up.

Results: Of 70 patients, 44 (63%), 14 (20%), and 12 (17%) were classified initially as low-, intermediate-, and high-risk, respectively. *BRAF^{V600E}* mutation analysis data was available in 55 (78.6%) patients, of whom 18 (32.7%) had the *BRAF^{V600E}* mutation. According to the final DRS, 61 (87%), two (3%), six (9%), and one (1%) patients were classified as an excellent, incomplete biochemical, incomplete structural, and indeterminate response, respectively. All ATA low-risk patients showed excellent response to treatment, whereas the rate of excellent response was 65.4% in intermediate- and high-risk levels ($p < 0.001$). The rates of excellent response in *BRAF^{V600E}* positive and negative patients were 83% and 92%, respectively ($p = 0.339$). The rate of locoregional recurrence was significantly higher in *BRAF^{V600E}* positive vs negative patients (33.3% vs 2.7% respectively, $p = 0.001$).

Conclusion: ATA pediatric risk stratification is effective in predicting response to treatment in PTC patients ≤18 years old. The presence of *BRAF^{V600E}* mutation was highly predictive for recurrence but had no significant impact on the rate of excellent response to treatment at final follow-up.

Keywords: *BRAF^{V600E}* mutation, dynamic risk stratification, pediatric thyroid cancer, thyroid cancer

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Conflict of interest: None declared
Received: 26.10.2021
Accepted: 20.01.2022

Introduction

Differentiated thyroid cancer (DTC) in pediatric and adolescent populations is uncommon, and constitutes approximately 2-4% of all pediatric malignancies (1,2). However, global trends in the incidence of thyroid cancer in children and adolescents showed rapid increases between 1998-2002 and 2008-2012 in almost all countries (3). Thyroid cancer is the most common endocrine malignancy in the 0-19 year age group (4). Papillary thyroid cancer (PTC) constitutes almost 90% of all thyroid carcinoma in this age group (5). The initial presentation, clinical course, and mortality of DTC in pediatric patients exhibit differences compared to adult patients. The rates of lymph node involvement, distant metastasis, and recurrence are much higher in pediatric and adolescent patients compared to adults, but the mortality rate at 20 years is less than 1% (6,7). The use of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM staging system is recommended for patients with DTC to predict disease-related mortality (8,9). The role of the AJCC/UICC TNM staging system to predict prognosis in pediatric DTC is limited due to the very low mortality rate (8). Considering the differences between adult and pediatric DTC, the American Thyroid Association (ATA) published Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer in 2015 (1). This guideline recommended initial postoperative stratification of pediatric PTC patients into low-, intermediate-, or high-risk levels to predict the patients at risk of persistent or recurrent cervical disease (1). ATA pediatric initial risk stratification was documented to be useful and effective in predicting recurrence in many studies (10,11,12,13,14). The dynamic risk stratification system (DRS), which was suggested to assess the response to therapy in DTC, has been validated in adult patients and has also been evaluated in pediatric DTC patients in some studies (12-20).

BRAF^{V600E} mutation has been found to be associated with an increased risk of lymph node metastasis, recurrence, and poor prognosis in adult patients (21,22). *BRAF*^{V600E} mutation status was incorporated into the ATA 2015 Modified Risk Stratification System for adult DTC patients and continuous risk scale for the assessment of structural recurrence risk to assist clinicians in proper risk stratification when mutation status data were available (8). There is limited data about the impact of *BRAF*^{V600E} mutation on prognosis in pediatric PTC (23,24,25,26). There are some reasons for the limited data. The rate of *BRAF*^{V600E} mutation is low in pediatric PTC compared to adults; it exhibits great differences according to the age of the patient and is very low in young patients (21,27). There is only one study in the literature that evaluated the correlation between *BRAF*^{V600E} mutation status and ATA pediatric initial risk stratification. The authors detected no significant correlation between the *BRAF*^{V600E} mutation status and ATA pediatric initial risk stratification (24). The impact of *BRAF*^{V600E} mutation on response to treatment by DRS has not been previously investigated in pediatric PTC patients.

The aim of this study was to evaluate the role of ATA pediatric thyroid cancer risk stratification and *BRAF*^{V600E} mutation status to predict the response to treatment in pediatric and adolescent PTC patients.

Methods

A total of 119 patients ≤18 years old underwent thyroid surgery in the Division of Endocrine Surgery of Istanbul Faculty of Medicine, Department of General Surgery between 1995 and 2020. Of these 119 patients, 85 (71.4%) were treated for PTC. This retrospective study included 70 (82%) of the 85 patients in whom all clinicopathological and follow-up data were available. *BRAF*^{V600E} mutation analysis was performed in 55 (78.6%) of 70 patients. Preoperative evaluation included thyroid hormone assay, neck ultrasonography (US), fine-needle aspiration biopsy (FNAB) of suspicious nodules, and FNAB or FNAB-thyroglobulin (Tg) washout of suspicious lymph nodes. The extent of initial thyroidectomy was either lobectomy or total thyroidectomy. Modified radical neck dissection (MRND) and therapeutic central neck dissection (TCND) were performed in patients with proven lateral neck metastasis. TCND was also performed in patients without lateral neck metastasis but with pre- or intra-operative evidence of clinically involved central lymph nodes. Routine prophylactic central neck dissection (PCND) was performed after 2010 in our institution in pediatric DTC patients.

Postoperative management of the patients was accomplished with a multidisciplinary approach, including the departments of pediatric endocrinology, nuclear medicine, and endocrine surgery. Stimulated Tg assay (sTg) and neck US was done 4-6 weeks after surgery in all patients. The patients were initially stratified as low-, intermediate-, and high-risk postoperatively, according to the ATA risk stratification system for pediatric and adolescent DTC patients. Postoperative radioactive iodine (RAI) treatment was performed in all ATA intermediate- and high-risk patients. The decision to use RAI treatment in ATA low-risk patients was individualized according to the clinicopathological features and postoperative sTg and anti-Tg (anti-Tg) values. A whole-body scan (WBS) was obtained 1 week after RAI treatment. Thyroid-stimulating hormone

(TSH) suppression treatment was given in all patients aiming to keep TSH levels lower than 0.1 mIU/L.

Neck US, either stimulated or nonstimulated Tg and anti-Tg assay were repeated every 6-12 months according to the clinical course and initial risk stratification. WBS with 2-5 mCi I^{131} was performed 12 months after RAI treatment with concurrent measurement of sTg and anti-Tg in patients who received RAI treatment. The trend of serum anti-Tg levels was evaluated to manage the follow-up strategy in patients with positive Tg autoantibodies (TgAb). In patients with either detectable/rising levels of nonstimulated Tg or rising/persistently high anti-Tg levels, diagnostic WBS and/or contrast-enhanced computerized tomography of neck and chest were performed when neck US was negative.

BRAF^{V600E} Mutation Analysis

BRAF^{V600E} mutation analysis was performed in formalin-fixed, paraffin-embedded thyroid tissue of thyroid tumor specimens. The QIAamp DNA tissue kit (Qiagen, Hilden, Germany) was used for genomic DNA preparation, following the manufacturer's instructions. *BRAF*^{V600E} mutation was determined by pyrosequencing using the Qiagen PyroMark Q24 pyrosequencer (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions, as has been reported previously (28).

Definitions of Recurrence and Response to Treatment

In patients who underwent total thyroidectomy, with or without RAI treatment, a disease-free state was defined as a nonstimulated Tg level < 0.2 ng/mL or sTg < 1 ng/mL (in the absence of TgAb) concurrent with negative imaging at any time during the follow-up. Recurrence was defined as the detection of biochemical or structural evidence of disease following any disease-free period.

The final response to treatment was evaluated at the time of the final follow-up. Patients were classified as an excellent, incomplete biochemical, incomplete structural, or indeterminate response to treatment according to the previously reported response to therapy definitions and based on initial treatment (15,16,17,18, 29).

In patients who underwent total thyroidectomy and RAI treatment, the excellent response was defined as nonstimulated Tg < 0.2 ng/mL or sTg < 1 ng/mL (in the absence of TgAb) and negative imaging. The incomplete biochemical response was defined as nonstimulated Tg > 1 ng/mL or sTg > 10 ng/mL and negative imaging. The indeterminate response was defined as nonstimulated Tg 0.2-1 ng/mL or sTg 1-10 ng/mL, or stable or declining anti-Tg levels and nonspecific imaging findings.

In patients who underwent thyroidectomy without RAI treatment, the excellent response was defined as nonstimulated Tg < 0.2 ng/mL or sTg < 2 ng/mL (in the absence of TgAb) and negative imaging. An incomplete biochemical response was defined as nonstimulated Tg > 5 ng/mL or sTg > 10 ng/mL, or rising Tg or anti-Tg levels over time and negative imaging. An indeterminate response was defined as nonstimulated Tg 0.2-5 ng/mL or sTg 2-10 ng/mL, or stable or declining anti-Tg levels and nonspecific imaging findings.

In patients who underwent lobectomy, an excellent response was defined as nonstimulated Tg < 30 ng/mL (in the absence of TgAb) and negative imaging. An incomplete biochemical response was defined as nonstimulated Tg > 30 ng/mL or rising Tg or anti-Tg levels over time and negative imaging. An indeterminate response was defined as stable or declining anti-Tg levels or nonspecific imaging findings.

An incomplete structural response was defined as evidence of structural and functional disease with any Tg or anti-Tg level, regardless of the extent of initial treatment.

Evaluation of Outcomes

Demographic data, clinicopathological features [history of irradiation, tumor size, subtypes of PTC, multifocality, lymphovascular invasion and extrathyroidal extension (ETE), and autoimmune thyroiditis], *BRAF*^{V600E} mutation status, the extent of initial surgery, data related to RAI treatment, recurrence, and response to treatment were obtained. The clinicopathological features and clinical outcomes were assessed according to ATA risk levels and *BRAF*^{V600E} mutation. The correlations between the clinicopathological features, ATA initial risk level, *BRAF*^{V600E} mutation, and recurrence and final response to treatment were analyzed.

The study was approved by the Ethics Committee of İstanbul University Faculty of Medicine (approval number: 478485, date: 21.09.2021).

Statistical Analysis

Continuous variables with normal distribution are reported as the mean ± standard deviation (SD), non normal distribution as median (range), and categorical variables as numbers and percentages. The Student's t-test or Mann-Whitney U test was used to compare the differences in continuous variables with normal or non-normal distribution, respectively. The chi-square test or Fisher's exact test was used in comparative analyses of categorical variables. A $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using IBM Statistical Package for the Social Sciences Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA).

Results

Baseline Clinicopathological Characteristics and Treatment in Pediatric Patients with PTC

The median age of the patients was 16 (5-18) years, with a female to male ratio of 55/15 (3.67:1). Eight (11%) patients had a history of head and neck irradiation. The majority (44/70, 63%) of the patients presented with a solitary thyroid nodule. Fifteen (21%) patients had palpable cervical lymph nodes at the time of initial diagnosis. Preoperative neck US revealed metastatic lymph nodes, in both the central and lateral neck in 15 (21%), lateral neck only in five (7%), and central neck only in three (4%) patients. Cytologic examination of FNAB specimens of suspicious thyroid nodules revealed Bethesda 6 cytology in 37 (52%), Bethesda 5 in 11 (16%), Bethesda 4 in 11 (16%), Bethesda 3 in seven (10%), and benign in four (6%) patients.

Total thyroidectomy was performed in 68 (97%) patients, whereas two (3%) patients underwent lobectomy. Lymph node dissection (LND), additional to total thyroidectomy, was done in 42 (60%) patients. The types of LND were TCND with MRND in 21 (50%), PCND in 18 (43%), and TCND only in three (7%) of 42 patients.

The median tumor size was 15 (3-50) mm. Histopathological examination revealed classical, follicular or aggressive variants of PTC in 28 (40%), 29 (41%), and 13 (19%) patients, respectively. Forty-one patients (59%) had multifocality, 17 (24%) ETE, 31 (44%) lymphovascular invasion, and 26 (37%) autoimmune thyroiditis. Lymph node metastasis was found in 28 (40%) patients. RAI treatment was performed in 52 (74%) patients with a median I^{131} dose of 125 (30-300 mCi). Distant metastasis to the lungs was detected in five (7%) patients on WBS after postoperative RAI treatment. The median follow-up was 6 (2-21.8) years.

Twenty locoregional recurrences developed in 12 (17%) patients during the follow-up. No recurrence at distant sites was observed. When the risk of locoregional recurrence according to clinicopathological factors was analyzed, classical variant of PTC ($p = 0.001$), ETE ($p < 0.001$), lymphovascular invasion ($p < 0.001$), and lymph node metastasis ($p = 0.001$) were significant risk factors (Table 1). The total dose of I^{131} administered for RAI treatment in patients who had recurrence was significantly higher than the patients with no recurrence ($p < 0.001$) (Table 1). There was no disease-related mortality.

ATA Initial Pediatric Risk Stratification

According to the ATA initial pediatric risk stratification, 44 (63%), 14 (20%), and 12 (17%) patients were classified as low-, intermediate-, and high-risk, respectively. The comparison of clinicopathological features and clinical outcomes in ATA low-risk patients vs intermediate- and high-risk patients are summarized in Table 2. An ATA low-risk state was significantly associated with smaller tumor size, lower rates of aggressive variant PTC, multifocality, ETE, lymphovascular invasion, lymph node metastasis, LND, RAI treatment and locoregional recurrence, and a higher rate of follicular variant PTC (FVPTC) compared to paptients classified as ATA intermediate- and high-risk (Table 2). None of the ATA low-risk patients had distant metastasis, whereas lung metastasis was observed in 19% of ATA intermediate- and high-risk patients ($p = 0.003$).

All intermediate- and high-risk patients underwent total thyroidectomy, whereas lobectomy was performed in two (4%) of 44 low-risk patients. The median total dose of administered I^{131} was significantly higher in intermediate- and high-risk vs low-risk patients (150 mCi vs 67.5 mCi, respectively; $p < 0.001$) (Table 2).

Final Response to Treatment

Response to treatment according to DRS at the end of follow-up revealed excellent response in 61 (87%), incomplete biochemical response in two (3%), indeterminate response in one (1%), and incomplete structural response (persistent disease) in six (9%) patients. Five of the six patients with persistent disease were ATA high-risk patients and the rate of persistent disease in the high-risk group was 42% (5/12). The rate of excellent response was 100%, 93%, and 33% in ATA low-, intermediate-, and high-risk levels, respectively. The rate of excellent response was significantly lower in ATA intermediate- and high-risk patients when compared to ATA low- risk patients (65.4% vs 100%, $p < 0.001$) (Table 2). When we compared the clinicopathological features in patients with and without excellent response, older age, FVPTC, unifocality, absence of lymphovascular invasion, initial lymph node metastasis or distant metastasis, and ATA low-risk significantly predicted excellent response to treatment (Table 3). The total dose of I^{131} used for RAI treatment was lower in patients with excellent response compared to those without excellent response, but the difference did not achieve statistical significance ($p = 0.055$) (Table 3). The patients who developed locoregional recurrences during the follow-up showed a significantly lower rate of excellent response to treatment at final follow-up compared to those without recurrences (50% vs 95%, $p < 0.001$) (Table 1).

BRAF^{V600E} Mutation Status

BRAF^{V600E} mutation was positive in 18 (33%) of 55 patients with available data. The median age of these 55 patients

was 16 (5-18) years. The median (range) tumor size was 14 (4-50) mm. The correlation between the *BRAF*^{V600E} mutation status and clinicopathological features, ATA initial risk stratification, the extent of surgery and clinical outcomes are summarized in Table 4. Age, gender, history of irradiation and tumor size showed no significant difference between the patients with or without the mutation. Classical variant PTC was significantly associated with the presence of the *BRAF*^{V600E} mutation ($p = 0.01$), whereas the rate of FVPTC was significantly higher in *BRAF*^{V600E} (-) patients compared to *BRAF*^{V600E} (+) patients ($p = 0.01$). The rate of the aggressive variant of PTC showed no significant difference between the *BRAF*^{V600E} (+) and (-) patients. Although the rate of multifocality was higher in *BRAF*^{V600E} (+) patients compared to *BRAF*^{V600E} (-) patients, the difference did not achieve statistical significance (83% vs 57%, respectively; $p = 0.052$). There was no correlation between *BRAF*^{V600E} mutation and lymphovascular invasion, ETE, autoimmune thyroiditis, the extent of thyroidectomy, LND, lymph node metastasis, distant metastasis, and ATA initial risk levels (Table 4). The recurrence rate was significantly higher in *BRAF*^{V600E} (+) patients compared to *BRAF*^{V600E} (-) patients (33% vs 3%, $p = 0.001$). A total of 14 recurrences were observed in six of 18 *BRAF*^{V600E} (+) patients, whereas there was only one recurrence in one of 37 *BRAF*^{V600E} (-) patients ($p < 0.001$). The total dose of I^{131} used for RAI treatment was significantly higher in *BRAF*^{V600E} (+) compared to *BRAF*^{V600E} (-) patients ($p < 0.001$). The rate of excellent response in *BRAF*^{V600E} (+) and (-) patients were 83% and 92%, respectively, and showed no significant difference ($p = 0.3$). The biochemical incomplete, indeterminate and structural incomplete response rates showed no significant difference between the *BRAF*^{V600E} (+) and (-) patients (Table 4).

Discussion

In our study, we found that all ATA low-risk and 93% of intermediate-risk patients had an excellent response to treatment at final follow-up. The presence of *BRAF*^{V600E} mutation was highly predictive for locoregional recurrence but had no significant effect on the final rate of excellent response to treatment.

Thyroid cancer is rare in children, and there is a limited number of studies with a large number of patients. The

Table 1. The clinicopathological features and clinical outcomes in patients with and without locoregional recurrences

	Recurrence (+) (n = 12)	Recurrence (-) (n = 58)	p
Median (range) age, years	14.5 (8-18)	16 (6-18)	0.3
Gender, n (%)			
Female	9 (75)	46 (79)	0.7
Male	3 (25)	12 (21)	
History of irradiation, n (%)	0 (0)	8 (14)	0.1
Tumor size, mm.	20.5 (9-42)	14 (3-50)	0.1
Histologic type, n (%)			
Classical	10 (83)	18 (31)	0.001
Folicular	0 (0)	29 (50)	0.001
Aggresive	2 (17)	11 (19)	0.8
Pathological features, n (%)			
Multifocality	10 (83)	31 (53)	0.056
Extrathyroidial extension	9 (75)	8 (14)	0.0001
Lymphovascular invasion	11 (92)	20 (34)	< 0.001
Autoimmune thyroiditis	4 (33)	22 (38)	0.6
Total thyroidectomy, n (%)	12 (100)	56 (97)	0.5
Total LND, n (%)	10 (83)	32 (55)	0.06
TCND + MRND, n (%)	10 (83)	11 (19)	< 0.001
Lymph node metastasis, n (%)	10 (83)	18 (31)	0.001
Distant metastasis, n (%)	2 (17)	3 (5)	0.1
ATA low risk, n (%)	1 (8)	43 (74)	< 0.001
RAI treatment, n (%)	12 (100)	40 (69)	0.02
Median (range) total I^{131} dose (mCi)	150 (60-300)	100 (30-150)	< 0.001
Excellent response, n (%)	6 (50)	55 (95)	< 0.001

LND: lymph node dissection, TCND: therapeutic central neck dissection, MRND: modified radical neck dissection, RAI: radioactive iodine, ATA: American Thyroid Association

initial presentation, clinical course, and mortality of PTC in children shows major differences compared to adult patients. The rate of multifocal disease ranged between 28% and 57%, ETE between 36% and 59%, central and/or lateral neck metastasis between 60% and 70%, and initial distant metastasis between 4.7% and 14.6% in pediatric DTC (14,24,30,31). Classical variants constitute the majority of PTC in pediatric and adolescent patients (13,14,24,30,31). Although the initial presentation of childhood DTC is more severe compared to adults, the long-term outcome is favorable, with very low mortality rates (3,7). We found a high rate (41%) of FVPTC in our cohort. In a former study from our instution, the rate of FVPTC was reported as 37.2% in adult PTC patients (32). The reported rates of FVPTC in pediatric PTC patients ranged between 10.4% and 36.8%, and was 22.7% in a large database study, which included 1,956 pediatric patients (24,30,31,33,34). The relatively high rate of FVPTC in our pediatric patients might be an incidental finding in a particulary small cohort or might be a reflection of regional and environmental differences in PTC features.

The local or distant recurrence rates are reported to range between 15.9-34% in pediatric and adolescent DTC patients (13,14,30,35). Recurrence was significantly associated with multifocality, large tumors, palpable cervical lymph nodes, lymph node metastasis, ETE or distant metastasis at diagnosis in pediatric and adolescent PTC (30,35). In the study by Welch Dinauer et al (35), the authors showed that focality was the best predictor of recurrence by multivariate analysis. In our study, the increased rate of locoregional recurrence was significantly associated with classical variant PTC, ETE, lymphovascular invasion, lymph node metastasis, and the presence of *BRAF*^{V600E} mutation. However, age, gender, autoimmune thyroiditis,

Table 2. The comparison of the clinicopathological features, extent of surgery and clinical outcomes in ATA pediatric low-and intermediate/high-risk patients

	ATA low risk (n = 44)	ATA intermediate and high risk (n = 26)	p
Median (range) age, years	16 (6-18)	15.5 (5-18)	0.9
Gender, n (%)			
Female	35 (79.5)	20 (77)	0.8
Male	9 (20.5)	6 (23)	
History of irradiation, n (%)	8 (18)	0 (0)	0.02
Median (range) tumor size, mm	10.5 (3-50)	21 (9-45)	0.001
Histologic type, n (%)			
Classical	14 (32)	14 (53.8)	0.07
Folicular	27 (61)	2 (7.7)	< 0.001
Aggresive	3 (7)	10 (38.5)	0.001
Pathological features, n (%)			
Multifocality	21 (48)	20 (80)	0.017
Extrathyroidial extension	0 (0)	17 (65.4)	< 0.001
Lymphovascular invasion	7 (16)	24 (92.3)	< 0.001
Autoimmune thyroiditis	18 (41)	8 (31)	0.4
Total thyroidectomy, n (%)	42 (96)	26 (100)	0.2
Total LND, n (%)	17 (39)	25 (96)	< 0.001
TCND + MRND, n (%)	1 (2.3)	20 (80)	< 0.001
Lymph node metastasis, n (%)	4 (9)	24 (92.3)	< 0.001
Distant metastasis, n (%)	0 (0)	5 (19)	0.003
RAI treatment, n (%)	26 (59)	26 (100)	< 0.001
Median (range) total I^{131} dose (mCi)	67.5 (30-300)	150 (50-300)	< 0.001
Recurrence, n (%)	1 (2.2)	11 (42.3)	< 0.001
Number of recurrences, n (%)	1 (2.3)	19 (73)	0.003
Final DRS, n (%)			
Excellent response	44 (100)	17 (65.4)	< 0.001
Biochemical incomplete/indeterminate	0 (0)	3 (11.5)	0.047
Structural incomplete	0 (0)	6 (23.1)	0.002

LND: lymph node dissection, TCND: therapeutic central neck dissection, MRND: modified radical neck dissection, RAI: radioactive iodine, ATA: American Thyroid Association, DRS: dynamic risk stratification

tumor size, and distant metastasis were not significantly associated with local recurrence in our cohort. Although the rate of multifocality was approximately 1.5-fold higher in patients who developed locoregional recurrence compared to patients with no recurrence, the difference was not statistically significant.

Recently, the ATA recommended that pediatric PTC patients should be initially stratified into ATA pediatric low-, intermediate-, or high-risk levels, based on clinical presentation, tumor size, and evidence of regional invasion and metastasis to identify the patients at risk of persistent or recurrent cervical disease (1). The ATA's initial pediatric risk stratification system has been validated by several studies, and the findings of our study were similar to the results of these other studies (11,12,13,14,20). We observed significantly higher rates of locoregional recurrence and persistent disease in ATA intermediate- and high-risk patients compared to ATA low-risk patients.

The ATA 2015 Modified Risk Stratification System for adult DTC patients does not suggest a routine analysis of *BRAF*^{V600E} status for initial risk stratification (8). However, *BRAF*^{V600E} mutation has been included in the continuous risk scale for the assessment of the risk of structural disease in adults to help clinicians to perform proper risk stratification in cases where mutation information is available (8). The rate of mutation positivity exhibited great difference according to the age of the patient in a pediatric population and was very low in younger children (26,27). In the study by Nikita et al (27), 89% of *BRAF*^{V600E} mutations were detected in patients older than 15 years and 11% in those younger than 15 years old. In our study, we observed that the median age of *BRAF*^{V600E} (+) patients was older compared to *BRAF*^{V600E} (-) patients, but the difference was not significant. The studies that evaluated the correlation between *BRAF*^{V600E} mutation and histopathological features, the extent of disease, and prognosis in pediatric PTC patients showed that *BRAF*^{V600E} mutation was not significantly associated with adverse histopathological features, lymph node metastasis, or distant metastasis and did not predict an aggressive clinical course, as it does in adult PTC (24,25,31,33). *BRAF*^{V600E} mutation was found to be very frequent in classical variant PTC compared to non-classical variants in several studies (25,31,33). Similarly, we observed that the rate of classical variant PTC was approximately 2-fold higher in *BRAF*^{V600E} (+) patients compared to *BRAF*^{V600E} (-) patients, in our

Table 3. The clinicopathological features and treatment in patients with and without excellent response to treatment

	Excellent response (+) (n = 61)	Excellent response (-) (n = 9)	p
Median (range) age, years	16 (6-18)	13 (5-18)	0.045
Gender, n (%)			
Female	49 (80)	6 (67)	0.3
Male	12 (20)	3 (33)	
History of irradiation, n (%)	8	0	0.2
Median (range) tumor size, mm	14 (3-50)	20 (9-45)	0.2
Histologic type, n (%)			
Classical	22 (36)	6 (67)	0.08
Folicular	29 (48)	0 (0)	0.007
Aggresive	10 (16)	3 (33)	0.2
Pathological features, n (%)			
Multifocality	32 (52)	9 (100)	0.007
Extrathyroidial extension	10 (48)	7 (78)	< 0.001
Lymphovascular invasion	23 (38)	8 (89)	0.004
Autoimmune thyroiditis	23 (239	3 (339	0.8
Total thyroidectomy, n (%)	59 (98)	9 (100)	0.5
Total LND, n (%)	33 (54)	9 (100)	0.008
TCND + MRND, n (%)	12 (16)	9 (100)	< 0.001
Lymph node metastasis, n (%)	19 (319	9 (100)	< 0.001
Distant metastasis, n (%)	0 (0)	5 (56)	< 0.001
Recurrence, n (%)	6 (9.8)	6 (67)	< 0.001
ATA low risk, n (%)	44 (72)	0 (0)	< 0.001
RAI treatment, n (%)	43 (70)	9 (100)	0.06
Median (range) total I^{131} dose (mCi)	100 (30-300)	150 (50-300)	0.055

LND: lymph node dissection, TCND: therapeutic central neck dissection, MRND: modified radical neck dissection, RAI: radioactive iodine

study. Geng et al (24) showed that the *BRAF*^{V600E} mutation was significantly associated with both a low AJCC and low AMES tumor stage. The authors reported the rates of *BRAF*^{V600E} mutation as 63.6%, 40%, and 22.2% in ATA low-, intermediate-, and high-risk patients, respectively, with no statistical difference according to the risk level (24). In our study, we also found no significant correlation between the *BRAF*^{V600E} mutation and ATA pediatric initial risk levels. Nor did we observe any significant correlation between the *BRAF*^{V600E} mutation and adverse histopathological features and initial presentation of PTC. Although, *BRAF*^{V600E} mutation was not associated with unfavorable clinicopathological risk factors initially, we observed that it was a significant predictive factor for recurrence in our patients. In our study, the rates of locoregional recurrence in *BRAF*^{V600E} (+) vs (-) patients were 33% vs 3%, respectively.

The DRS has been proposed for re-staging patients according to response to treatment by re-evaluating the clinical, biochemical, imaging, and cytopathologic findings at any time during follow-up (8,15,16,17,18). The DRS has been validated in patients treated with total thyroidectomy and RAI treatment, and a modified DRS system could also be applied to DTC patients who underwent lobectomy or total thyroidectomy without RAI ablation (15,18,29).

Sohn et al (20) showed that the prevalence of structural persistent disease increased as ATA initial risk classification increased in pediatric DTC. Other studies have reported that low-risk patients had the highest probability of an excellent response to initial treatment while high-risk patients had the highest probability of incomplete structural response and the lowest probability of an excellent response (13,14). Our findings were compatible with these studies. We found the

Table 4. The comparison of clinicopathological features, extent of surgery, ATA initial risk stratification, and response to treatment according to *BRAF*^{V600E} mutation status

	BRAF^{V600E} (+) (n = 18)	*BRAF*^{V600E} (-) (n = 37)	p
Median (range) age, years	16 (14-18)	15 (5-18)	0.064
Gender, n (%)			
Female	15 (83)	29 (78.4)	0.6
Male	3 (17)	8 (21.6)	
History of irradiation, n (%)	0 (0)	6 (16)	0.07
Median (range) tumor size, mm	15 (6-50)	14 (4-50)	0.4
Histologic type, n (%)			
Classical	11 (61)	10 (27)	0.01
Folicular	4 (22)	21 (57)	0.01
Aggresive	3 (7)	6 (16)	0.9
Pathological features, n (%)			
Multifocality	15 (83)	21 (57)	0.052
Extrathyroidial extension	6 (33)	8 (22)	0.3
Lymphovascular invasion	10 (55)	12 (32)	0.1
Autoimmune thyroiditis	8 (44)	14 (38)	0.6
Total thyroidectomy, n (%)	18 (100)	36 (97)	0.48
Total LND, n (%)	12 (67)	23 (62)	0.75
TCND + MRND, n (%)	6 (33)	10 (27)	0.6
Lymph node metastasis, n (%)	9 (50)	13 (35)	0.2
Distant metastasis, n (%)	0 (0)	3 (8)	0.2
ATA low risk, n (%)	10 (55)	27 (73)	0.1
RAI† treatment, n (%)	16 (89)	25 (68)	0.09
Median (range) total dose of I^{131} (mCi)	150 (100-300)	75 (30-250)	0.001
Patients with recurrence, n (%)	6 (33)	1 (3)	0.001
Number of recurrences, n (%)	14 (78)	1 (3)	< 0.001
DRS Response to treatment, n (%)			
Excellent	15 (83)	34 (92)	0.3
Biochemical incomplete/indeterminate	2 (11)	1 (2.7)	0.2
Structural incompelete	1 (6)	2 (5.3)	0.9

LND: lymph node dissection, TCND: therapeutic central neck dissection, MRND: modified radical neck dissection, RAI†: radioactive iodine, ATA: American Thyroid Association, DRS: dynamic risk stratification performed at the end of the folllow up

rate of excellent response to be 100 %, 93 % and 33 % in ATA low-, intermediate- and high-risk patients, respectively. In our study, excellent response to treatment was significantly associated with ATA low-risk, older age, FVPTC, unifocal tumors with no invasion and metastasis, and no recurrences during the follow-up.

The impact of *BRAF*^{V600E} mutation status on response to treatment evaluated by DRS has not been previously investigated in pediatric PTC patients. When we analyzed the response to treatment in *BRAF*^{V600E} (+) and (-) patients, no significant difference was found between the two groups in respect of excellent response to treatment at final follow-up. Although the rate of biochemical incomplete/indeterminate response was higher in *BRAF*^{V600E} (+) patients compared to *BRAF*^{V600E} (-) patients, the difference was not statistically significant (11 % vs 2.7 %, p = 0.2). Our findings suggest that *BRAF*^{V600E} mutation might be associated with a higher rate of locoregional recurrence but probably do not increase the long-term risk of incomplete structural response to treatment.

Some studies have reported that younger age was found to be associated with initial high risk and recurrent/persistent disease in pediatric PTC, while others found no correlation with age and prognosis (12,36,37,38,39). We observed that older age was significantly associated with excellent response to treatment. Gender was not associated with either the initial risk stratification or response to treatment in our study and this finding was compatible with the studies in the literature (36,37,38,39).

Study Limitations

This study has some limitations. This is a retrospective study with a relatively small sample size. Pediatric DTC is a rare disease, and studies reporting outcomes of more than 100 children are few. There is the possibility of selection bias as all of the patients in this study were treated in a single tertiary referral center. *BRAF*^{V600E} analysis was performed in 78.5% of the cohort. The small sample size might be insufficient to determine the correlation between *BRAF*^{V600E} mutation and recurrence or response to treatment.

Conclusion

We showed that ATA initial pediatric risk stratification effectively predicted the risk of recurrent and persistent disease and final response to treatment in PTC patients ≤18 years old. All ATA low-risk and 93 % of intermediate-risk patients had an excellent response to treatment at final follow-up. The presence of *BRAF*^{V600E} mutation was highly predictive for locoregional recurrence but had no significant effect on the final rate of excellent response to treatment. During initial risk evaluation of pediatric PTC patients, investigation of *BRAF*^{V600E} mutation status in addition to ATA initial stratification might provide a better estimate of the probability of recurrence in those patients in whom *BRAF*^{V600E} mutation status can be determined. Further studies with a large number of patients are needed to determine the role of the *BRAF*^{V600E} mutation on recurrence and response to treatment in pediatric PTC.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of İstanbul University Faculty of Medicine (approval number: 478485, date: 21.09.2021).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Yasemin Giles Şenyürek, Yalın İşcan, İsmail Cem Sormaz, Şükran Poyrazoğlu, Fatih Tunca, Concept: Yasemin Giles Şenyürek, Yalın İşcan, Fatih Tunca, Design: Yasemin Giles Şenyürek, Fatih Tunca, Data Collection or Processing: Yasemin Giles Şenyürek, Yalın İşcan, İsmail Cem Sormaz, Analysis or Interpretation: Yasemin Giles Şenyürek, Fatih Tunca, Literature Search: Yasemin Giles Şenyürek, Şükran Poyrazoğlu, Writing: Yasemin Giles Şenyürek, Fatih Tunca.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2022.2021-9-10

J Clin Res Pediatr Endocrinol 2022;14(2):207-215

Comparison of National Growth Standards for Turkish Infants and Children with World Health Organization Growth Standards

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What is already known on this topic?

Incorporation of World Health Organization (WHO) standards into pediatric practice has been the subject of debate in many countries, particularly those using national reference data for child growth assessment.

What this study adds?

WHO growth standards do not reflect the growth of Turkish children and may substantially alter the prevalence of short stature and underweight in the 0-5 years age group.

Abstract

Objective: Using World Health Organization (WHO) standards in pediatric practice is still controversial in many countries. It is suggested that national growth charts best reflect the genetic and ethnic characteristics of a population. The aim of this study was to compare length/height, body weight, and body mass index (BMI) in healthy Turkish children of ages 0 to 18 with those proposed by WHO as the international growth standards.

Methods: The data of Turkish children were collected from infant/child population aged 0-5 years (2391 boys, 2102 girls) and children of ages between 6-18 years (1100 boys, 1020 girls). For comparison, the 50th, 3rd, and 97th percentile curves for length/height, weight, and BMI in Turkish children were plotted together with respective WHO data.

Results: Heights were essentially similar in the Turkish and WHO data at ages between 3-10 years. Turkish children were markedly taller compared to the WHO standards after the age of 10 years. Evaluation of the 3rd percentile data revealed that Turkish boys were shorter than the WHO subjects in the first 2 years of life. From 6 months of age, Turkish children showed higher weight for age values in the 3rd, 50th, and 97th percentiles. In all age groups between 6 months and 3 years, and in between 6-18 years of age, Z-score values, as well as the 50th, 15th, 85th, and 95th percentile values were higher in Turkish children. The differences were particularly noteworthy at ages 1-2 years and in the pubertal years.

Conclusion: WHO growth standards do not reflect the growth of Turkish children and may substantially alter the prevalence of short stature and underweight in Turkish children in the 0-5 years age group. When assessing the nutritional and growth status of children, national growth standards may be more appropriate.

Keywords: Growth charts, Turkish children, WHO standards

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Conflict of interest: None declared
Received: 16.10.2021
Accepted: 11.01.2022

Introduction

Monitoring the growth and development of each individual child from birth onwards is an essential part of pediatric care. The monitoring of growth enables the physician to diagnose aberrations in physical growth at an early stage and to initiate treatment when indicated. Anthropometry is not only an important diagnostic method in the evaluation of the growth of individual children, but is also a reliable indicator of the nutritional state of a community. Growth charts are important tools in the assessment of children's development. Local growth charts, if prepared in accordance with standard methodology, best reflect the genetic and ethnic characteristics of a population. Thus, in almost all developed countries, national or local growth charts, based on measurements of healthy infants/children living in those communities, are used in the assessment of the growth of infants and children. The World Health Organization (WHO) standards have been in use, mainly in countries that have not yet developed their national growth charts (1,2).

In an effort to document the hypothesis that provision of optimal nutrition and environmental conditions can eliminate the differences in growth resulting from ethnic, nutritional, socioeconomic, and climatic factors and to advocate that the same growth charts can be used in all countries, WHO has created international standard growth charts for infants and children of ages 0 to 5, based on data obtained from six different countries (1). In contrast, the "international growth standards" proposed by WHO for children aged 5-18 years are based on the 1977 growth data of children from the United States of America (USA) (2).

Despite the statement that these international standards are valid for all countries, there are a great number of publications from European and Asian countries stating that their findings do not conform to the WHO charts (3,4,5,6,7,8,9). On the other hand, WHO charts are recommended for use in the United Kingdom, the USA, and France. Prevention of obesity constitutes the main reasoning lying behind this recommendation (10,11,12,13).

This study was designed to compare body weight, length/height, and body mass index (BMI) values in healthy Turkish children of ages 0 to 18 with those proposed by WHO as international growth standards.

Methods

The data on Turkish children presented in this paper are based on previously reported studies. One of these studies was conducted on an infant/child population aged 0-5 years attending the Well Child Clinic of a University Hospital between the years 1992 and 2006 (14). Preterm infants born before 37 completed gestational weeks were not included in this study. Families attending the Well Child Clinic are relatively homogeneous in socio-economic and cultural levels. The parents of all subjects in this study were literate and the majority of the mothers had at least 5 years of schooling. The majority of the fathers were high school graduates. The routine follow-up schedule of the Clinic for the first year of life started at age 2 weeks and included visits at 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, 9^{th}, 12^{th}, 15^{th}, and 18^{th} months and every 6 months thereafter until 5 years of age. A complete physical examination including anthropometric measurements was performed at each visit. Pediatric residents and nurses provided breastfeeding counseling. All data on the infants/children attending the Clinic were recorded by computer. Length/height, weight, and head circumference measurements were performed by two trained nurses. The naked weights were obtained on an electronic digital scale (Seca, 727; Kimeks Chemical Materials and Anitary Appliances Trade. Inc. Norm İş Merkezi, Şişli-İstanbul; e-mail: eticaret@kimeks.com), accurate to 5 g. A locally manufactured standard measuring board, with increments in millimeters, was used to measure supine length. After the age of 3 years, standing height was measured using a Leicester Height Measure (Child Growth Foundation, manufactured by Invicta Plastics Ltd, Roadby, Leicester, UK). The study sample consisted of 2391 boys and 2102 girls between 15 days and 60 months of age. The data set included a total of 19 523 boys' and 16 807 girls' measurements for length/height, as well as 19 714 boys' and 17 035 girls' measurements for weight. BMI values were calculated from weight and height measurements of 19 433 boys and 16 740 girls. The mean number of measurements per child was 8.2 ± 3.6.

The data on children of ages 6 to 18 years are also based on previous studies (15,16,17). The study sample consisted of 1100 boys and 1020 girls attending primary and secondary schools located in six different districts of Istanbul city. All six schools were located in relatively well-off districts. The data were collected between the years 1989 and 2002 by biannual visits to the schools by a team consisting of one pediatrician, two trained technicians, and two physicians training in pediatrics. Using the school files, all children in one class at a time, whose birthdays were ± 3 months from the prospective date of examination, were selected as subjects to be measured at the next visit. Information on the study and on the importance of height and weight measurements was given to children in groups. Written parental consent was obtained with the help of the school administration. Children who refused to cooperate were excluded. Younger children (6-10 years) constituted the

subjects in the first 3-4 years of the study and over time, measurements were repeated on the same children but other children were also added into the study to provide adequate numbers for the older age groups. Thus, the total sample consists of a mixture of children followed longitudinally over different periods of time. Chronological age was computed from the birth date reported by the child and verified by the school files. If these two sources disagreed, the child was not included in the study. Chronic or debilitating disease, assessed by history and a brief physical examination, was also a reason for exclusion. Heights were measured in a standing position with bare feet, using a portable measuring device (the Leicester height measure, Invicta Plastics Ltd, Roadby, Leicester, UK). A portable scale, sensitive to 0.1 kg, was used for weight measurements, which were conducted with the children in their underclothes. All measurements were performed by the same two trained technicians. Height and head circumference measurements were repeated twice and the mean value was calculated. After all data were collected, the subjects were allocated to socioeconomic classes (SEC), using an arbitrary classification based on the education level of both parents and the occupation of the fathers (15,18). Since no significant differences were noted in height and weight values between SEC classes 1 and 2, data on children falling into both classes (SEC 1 and 2) were included in this presentation. The dates of birth of the children ranged between 1974 and 1989. The data set for children of ages 6 to 18 included a total of 6007 height measurements for boys and 5657 for girls, 6008 weight measurements for boys, and 5647 for girls. The mean number of measurements per child was 5.5 ± 3.3. With the exception of age groups 6, 17.5, and 18 years, each half age group included measurements over 100 subjects.

The study was approved by the Institutional Ethical Review Board of İstanbul University, İstanbul Faculty of Medicine (protocol no: 1272, date: 26.06.2015).

Statistical Analysis

The final data were obtained by the merging and smooth transition of the anthropometric results of younger children with those of children older than six years (17). The LMS method, developed by Cole, was used as the statistical technique for reference construction in all the above studies (19,20). For comparison, the 50th, 3rd, and 97th percentile curves for length/height, weight, and BMI in Turkish boys and girls were plotted together with the respective WHO data. The horizontal line denoted as "0" represents the WHO data.

Results

Figure 1 depicts Z-score values, as well as the 50th, 3rd, and 97th percentile values, for length/height for age in Turkish girls and boys versus the WHO standards. Values pertaining to children aged 0 to 3 years are shown on the left and those for older children on the right panel of the figure. WHO values are expressed as the "0 lines" in the figures. It should be noted that the Z-score, as well as the 50th percentile values, were comparable to WHO standards in the first 3 months and between 3-10 years of life. Higher values for length/height existed in the age groups between 3 months and 3 years. The differences ranged between 0.1 standard deviation (SD) and 0.5 SD (roughly between 0.3 cm and 1.2 cm). Heights were essentially similar in the Turkish and WHO data at ages between 3 and 10 years. However, Turkish children were notably taller compared to the WHO curves after age 10 years, showing differences as great as 1.7 cm. The 97th percentile values also showed differences similar to those noted in the 50th percentile curves and the Z-score values. On the other hand, an evaluation of the 3rd percentile data revealed that Turkish boys were shorter than the WHO subjects in the first 2 years of life, a difference in the range of 0.5 cm and 1.4 cm. Third percentile values in Turkish boys were comparable to the WHO standards between ages 2 years and 10 years. As to girls, the 3rd percentile values for length/height were comparable to WHO standards between ages 0 to 10 years. In both sexes, 3rd percentile values for height were greater in Turkish children. Weight for age values in Turkish children as compared with WHO values are shown in Figure 2. It is notable that, starting at age 6 months, Turkish children showed higher weight for age values in the 3rd, 50th, and 97th percentiles as well as in the Z-score charts.

Percentile curves and Z-score curves for BMI in Turkish children as compared to the WHO standards are depicted in Figure 3. In all age groups between 6 months and 3 years, and in age groups between 6 and 18 years, Z-score values, as well as the 50th, 15th, 85th, and 95th percentile values were greater in Turkish children. The differences were particularly noteworthy (as high as 1.7 cm) at ages 1-2 years and in the pubertal years. BMI values in children aged between 3 to 6 years were comparable to WHO standards.

Prevalence estimates of short stature in the children's sample according to the Turkish and WHO standards and prevalence estimates of obesity in the children's sample according to the Turkish and WHO standards are illustrated in Figure 4 and Figure 5.

In the age groups from 0 to 3 years, absolute differences between Turkish children and WHO standards, which were

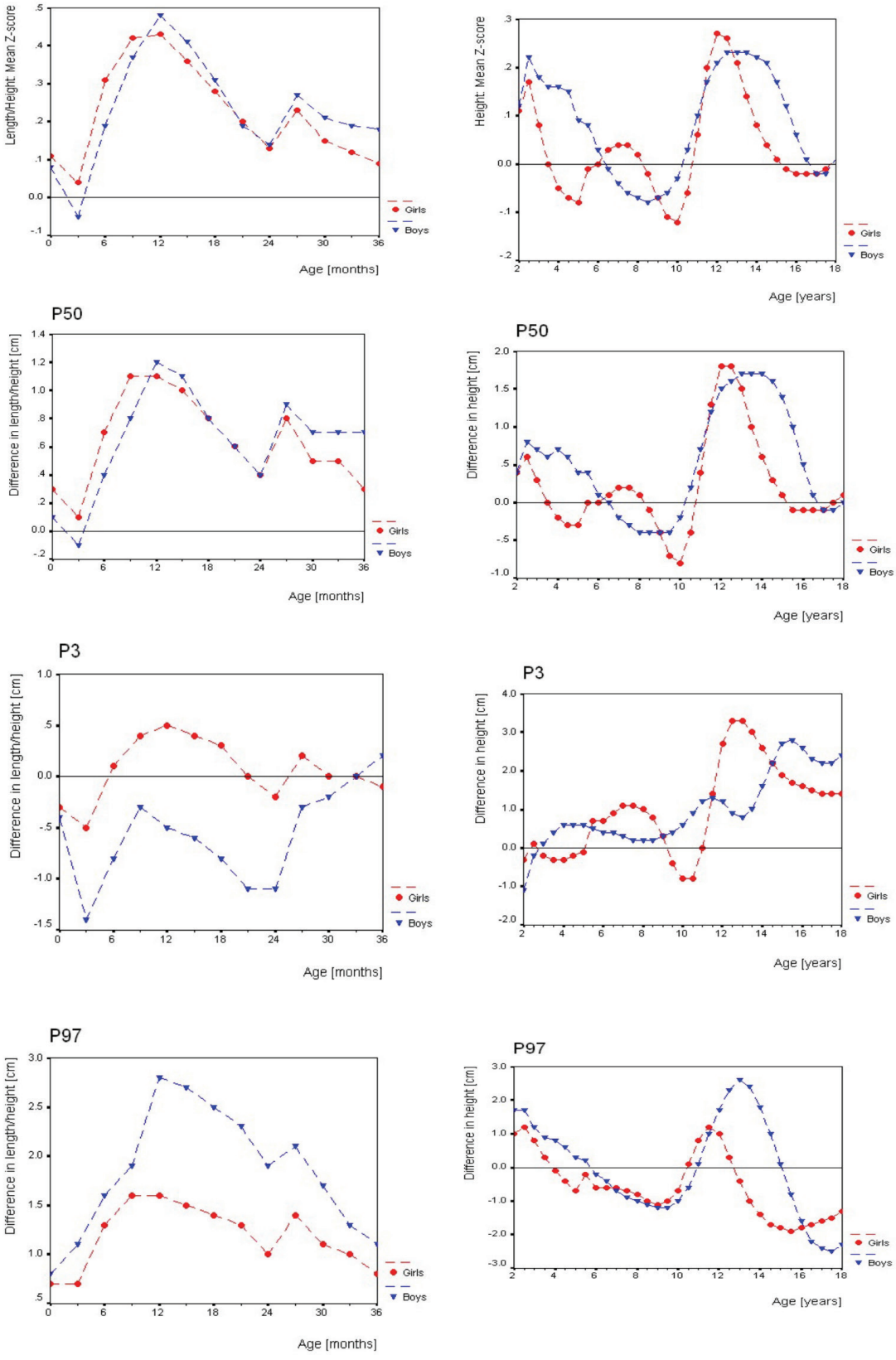

Figure 1. Z-score and percentile values for length/height for age in Turkish children versus the World Health Organization (WHO) standards (values pertaining to children aged 0 to 3 years are shown on the left and those for older children on the right panel of the figure. WHO values are expressed as the "0 line" in the figures)

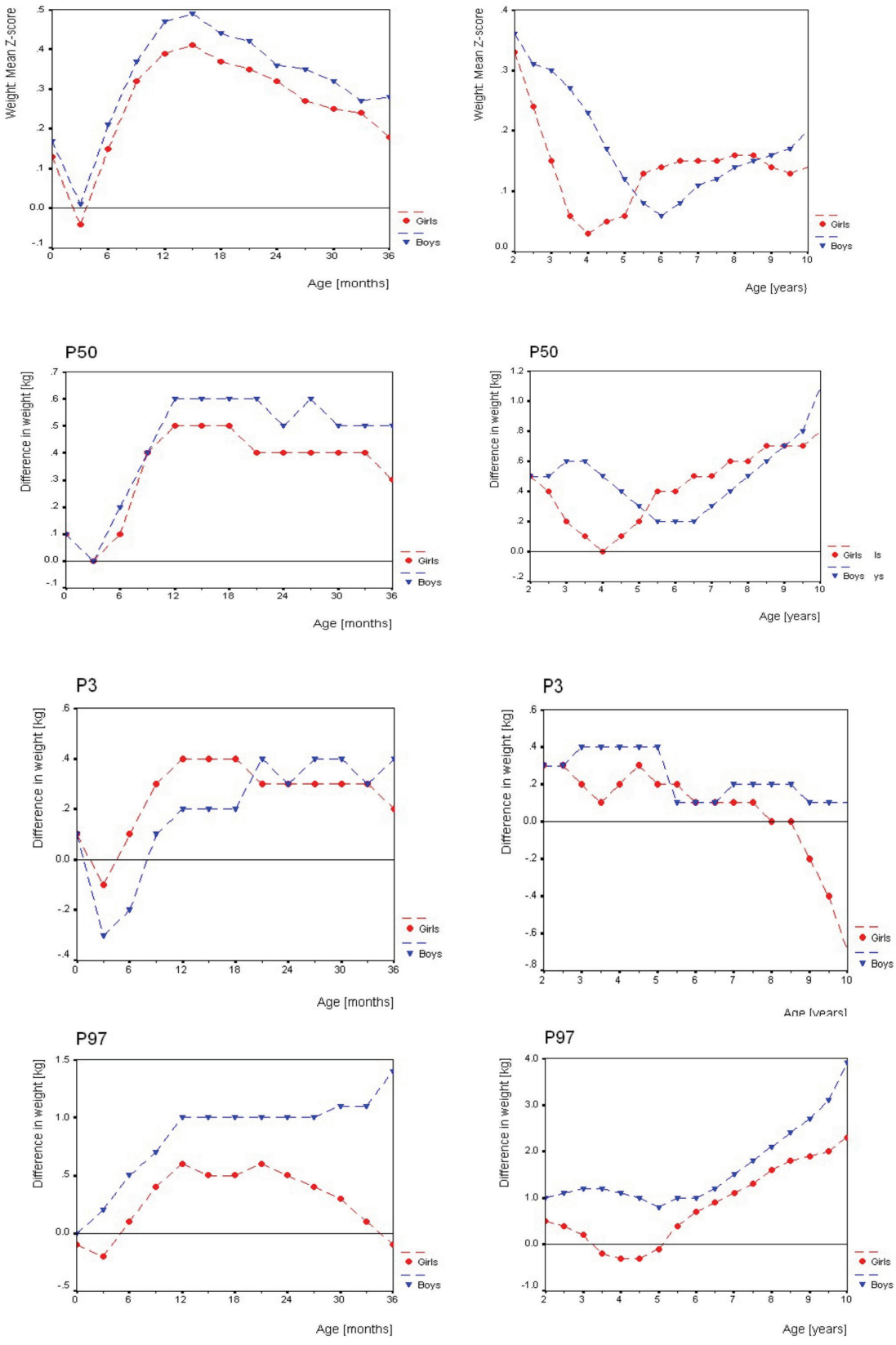

Figure 2. Z-score and percentile values for weight for age in Turkish children versus the World Health Organization (WHO) standards (values pertaining to children aged 0 to 3 years are shown on the left and those for older children on the right panel of the figure. WHO values are expressed as the "0 line" in the figures)

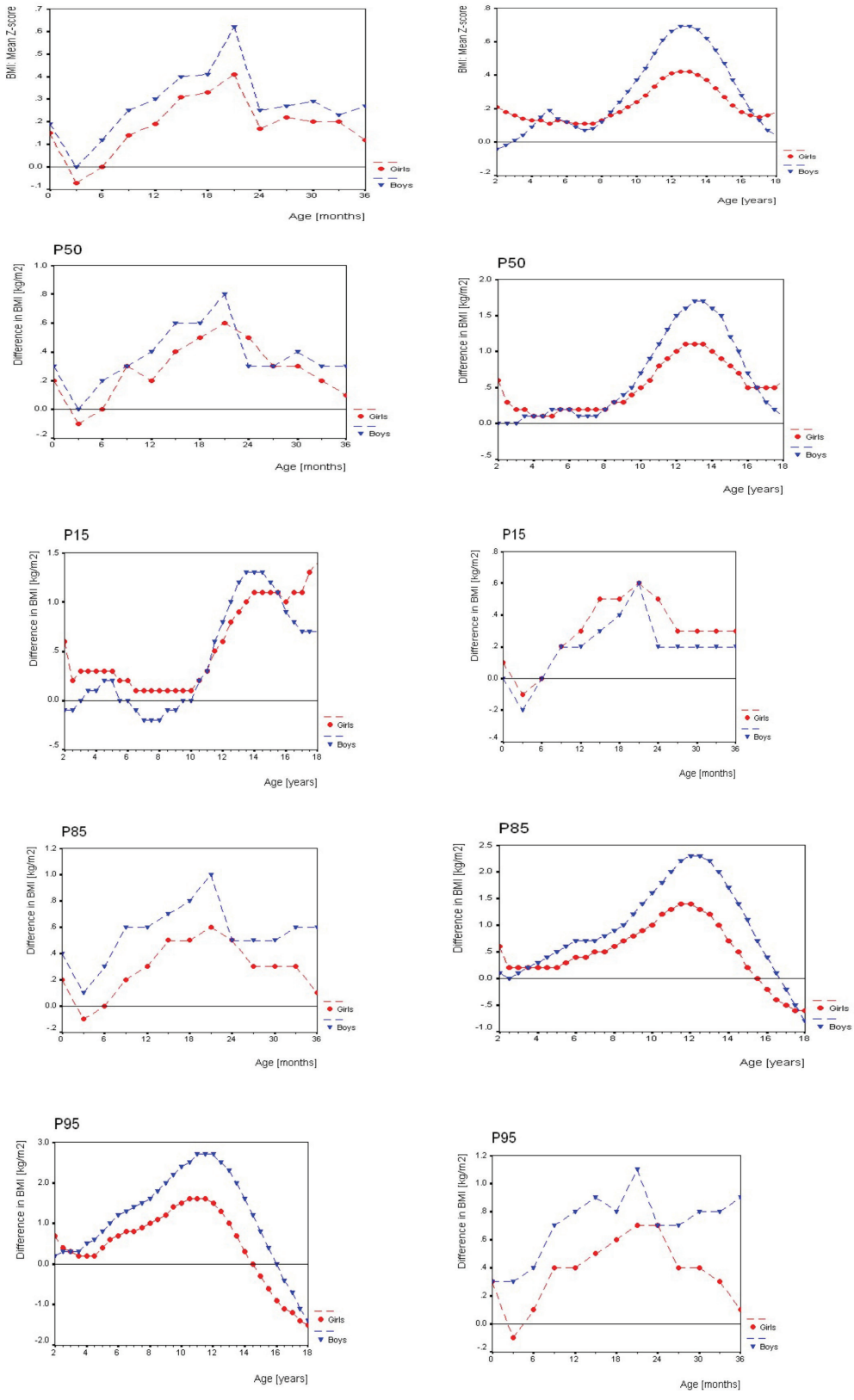

Figure 3. Z-score and percentile values for body mass index for age in Turkish children versus the World Health Organization (WHO) standards (values pertaining to children aged 0 to 3 years are shown on the left and those for older children on the right panel of the figure. WHO values are expressed as the "0 line" in the figures)

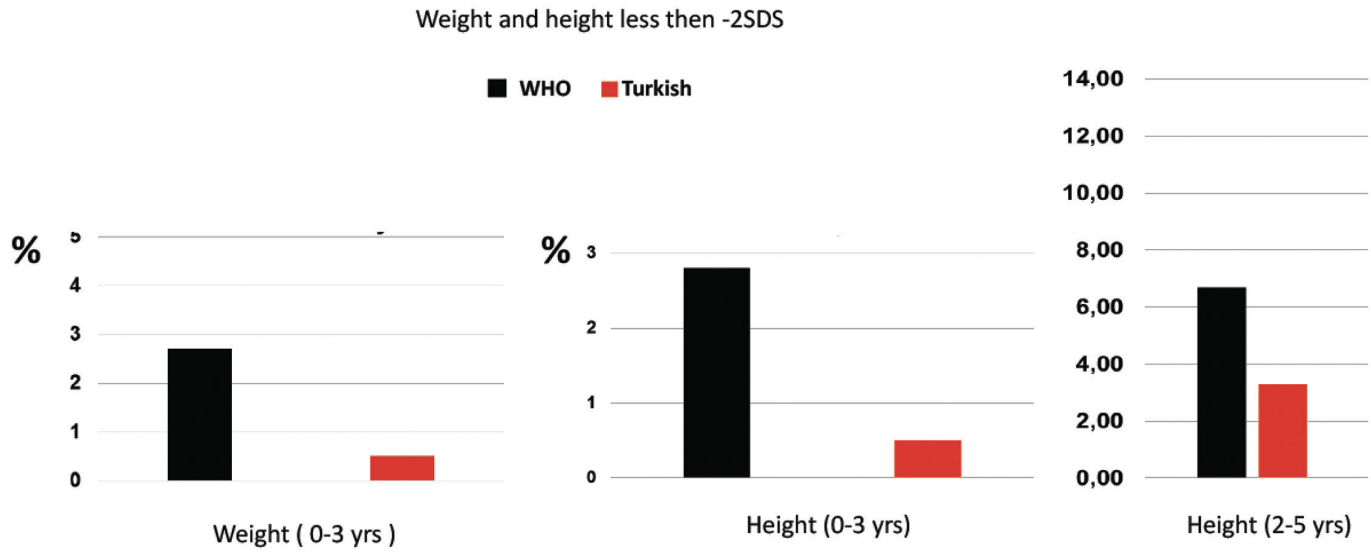

Figure 4. Prevalence estimates of short stature in the children's sample according to the Turkish and World Health Organization standards

SDS: standard deviation score

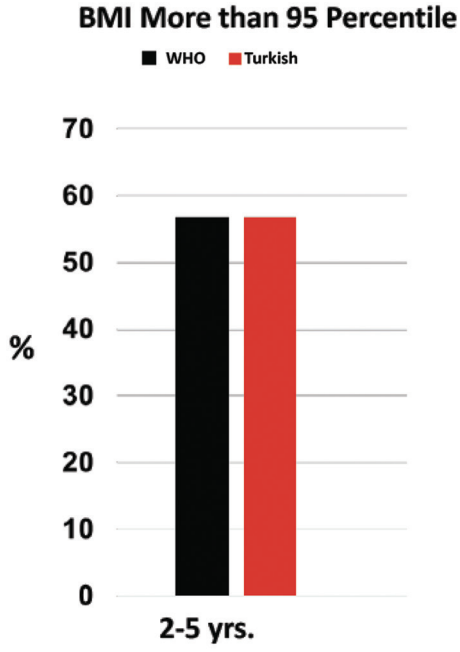

Figure 5. Prevalence estimates of obesity in the children's sample according to the Turkish and World Health Organization standards

BMI: body mass index

larger than 0.3 cm (height/length), 0.15 kg (weight), and 0.1 kg/m^2 (BMI) were significant at the 0.05 levels.

In the age groups from 3.5 to 18 years, absolute differences between Turkish children and WHO standards, which were larger than 1 cm (height), 1 kg (weight), and 0.5 kg/m^2 (BMI) were significant at the 0.05 levels.

Discussion

The implementation of incorporating WHO standards into pediatric practice has been the subject of debate in many countries, especially in those using national reference data for child growth evaluation. A number of studies have been published indicating that the new WHO Growth Standards do not fit with national references, including for Dutch, Belgian, Norwegian, Danish, and German populations (3,6,21,22). Populations in these countries demonstrate tall stature and a large number of children have length/height above + 2 SD according to the WHO 2006 Growth Standards. The study on the growth of Belgian and Norwegian children showed that the proportion of children below -2 SD of the WHO Growth Standards was lower and that above 2 SD of the WHO Growth Standards was higher in length/height, weight, BMI, and head circumferences (6). The applicability of the new WHO Child Growth Standards to the East Asian populations was studied by Hui et al (4) who found that Hong Kong Chinese children were generally shorter and fatter than the WHO standards. Due to higher birth weight in the UK90 reference compared to the WHO standards, growth curves for pre-term children and for the age 0-14 days were preserved, but for the other ages, the WHO standards are used (23,24). Also, in the US, using the WHO standards was recommended by the CDC expert panel for children from birth to 24 months only (12).

Except for the first 6 months and 3 to 6 years of age, BMI values were higher in Turkish children than WHO standards. After 3 months of age, the weight and length/height values of the infants and children in our study were well above the WHO Growth Standards. Heights were essentially similar in the Turkish and WHO data at ages between 3 and 10 years but Turkish children were notably taller compared to the WHO curves after age 10 years. Although not well documented, population differences in growth become more striking in older children and during puberty. These differences are probably associated with earlier development of puberty in Turkish children (25,26). To compare national and WHO growth references and explore their differences in assessing growth, a group of Turkish children between 0-5 years of age who attended outpatient clinic was measured. Differences between the two references in the evaluation of weight and height status in a group of Turkish children are highlighted in Figures 4 and 5. The highest prevalence of underweight and short stature was recorded using the WHO reference. With regards to obesity, estimations using the Turkish and WHO references were identical.

Hui et al (4) reported that Hong Kong Chinese toddlers at 3 years of age were, on average, shorter when compared with the WHO growth standards. The researchers speculated that this difference may be due to the result of epigenetic constraints on growth rather than failure to thrive or stunting due to less optimal living conditions. It is known that there are marked differences in final height among countries, even among those that are equally well off and also geographically close to one another (27,28,29). Eveleth and Tanner, reviewing reports from different populations, stated that growth and body proportions are determined by the genetic constitution of the population. Comparing western European populations to those in the eastern part of the world, the authors stated that western populations are longer limbed and taller (30). Indeed, we also found in one of our studies that Turkish children have a higher sitting height/height ratio than Dutch children and lower than Chinese children, supporting the statement of the authors on the influence of genetic differences (31). Since the WHO study is a mixture of populations from different parts of the world with different backgrounds and different growth potential, the WHO study appears to have underestimated the genetic influence. Yet, it seems that genetic influence is very important. Most studies comparing the use of the WHO standards as country-specific growth references suggest that the latter may describe the growth of children more faithfully than the WHO standards (21,22).

Study Limitations

The limitation of our study is that data were analysed cross-sectionally. Because the majority of the children were followed for different periods of time, so longitudinal data covering ages 6 to 18 were not available for all children, although a relatively large sample size was attained for this national study. The other limitation of the study is the sample of the child population aged 0-5 years was based on a relatively well-off urban population.

Conclusion

In conclusion, as has been reported in many other countries, WHO growth standards do not precisely reflect the growth of Turkish children and may substantially alter the prevalence of short stature and underweight in Turkish children 0-5 years of age. We suggest that the WHO's growth standards can be used to compare the growth and development of children from different countries. However, when assessing the nutritional and growth status of children, national growth standards, where available, may be more appropriate.

Ethics

Ethics Committee Approval: The study was approved by the Institutional Ethical Review Board of İstanbul University, İstanbul Faculty of Medicine (protocol no: 1272, date: 26.06.2015).

Informed Consent: Written parental consent was obtained with the help of the school administration.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Rüveyde Bundak, Olcay Neyzi, Gülbin Gökçay, Zehra Yavaş Abalı, Hülya Günöz, Feyza Darendeliler, Design: Rüveyde Bundak, Gülbin Gökçay, Hülya Günöz, Firdevs Baş, Data Collection or Processing: Rüveyde Bundak, Gülbin Gökçay, Firdevs Baş, Zehra Yavaş Abalı, Hülya Günöz, Feyza Darendeliler, Analysis or Interpretation: Andrzej Furman, Rüveyde Bundak, Zehra Yavaş Abalı, Literature Search: Rüveyde Bundak, Olcay Neyzi, Zehra Yavaş Abalı, Writing: Olcay Neyzi, Rüveyde Bundak, Zehra Yavaş Abalı.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2022.2021-11-16

J Clin Res Pediatr Endocrinol 2022;14(2):216-220

The Impact of the CEDD-NET on the Evaluation of Rare Disorders: A Multicenter Scientific Research Platform in the Field of Pediatric Endocrinology

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What is already known on this topic?

It is known that in some rare and special diseases, studies cannot be performed due to the insufficient number of patients, and thus experiences are published only in the form of case reports.

What this study adds?

We present pediatric endocrinologists with an example of a platform where they can produce large studies with low cost and a high number of patients.

Abstract

Objective: The database http://cedd.saglik-network.org (CEDD-NET) has been operating since 2013 in Turkey. All pediatric endocrinologists can propose projects to this network. The aim of our study was to determine the impact of CEDD-NET on the transformation of multicenter studies into scientific publications and assess the academic characteristics of the studies that have been transcribed into publication.

Methods: All the studies that were opened to patient admission on the website between August 26, 2013 and March 1, 2021 were reviewed.

Results: A total of 30 studies were accepted and opened for data entry. The median data collection period was 12 (1.5-24) months, while the median number of researchers participated was 23 (3-180), the median number of cases was 120 (26-192). The average cost was $2113 (1370-3118). Out of 30 studies, data entry was completed for 27. Sixteen publications were produced from 14 studies, 13 ot them have not published yet. The median time from the end of data entry to publication of the study was 686 (168-1608) days. While the median impact factor of the journals in which the studies were published was 1.803 (1.278-5.399), the median number of citations was 6.5 (0-49), and cited by 99 times in Web of Science indexed journals in total.

Conclusion: CEDD-NET appears to be productive and effective as all the publications are of high quality that have been published in the Q1-Q2 categories. This study demonstrated the benefits and necessity of establishing nationwide databases, even covering more than one country, in specialized branches, such as pediatric endocrinology where rare diseases are of concern.

Keywords: Nationwide studies, pediatric endocrinology, rare diseases, CEDD-NET

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Conflict of interest: None declared
Received: 15.12.2021
Accepted: 07.01.2022

Introduction

Pediatric endocrinology is a specialized discipline that deals with common endocrine disorders, such as obesity and hypothyroidism, in addition to several rare disorders. Disorders of sex development, hypophosphatemic rickets, hypoparathyroidism, and childhood thyroid cancers are among these rare diseases. The rarity of these diseases makes it almost impossible to conduct new studies from a single center, highlighting the need for large multicenter studies.

The Pediatric Endocrinology and Diabetes Society (PEDS) in Turkey is an association with a total of 357 members; that includes only pediatric endocrinologists. Thanks to the activities of this association, the database http://cedd.saglik-network.org (CEDD-NET) was established and has been operating since 2013. All pediatric endocrinologists can propose projects to this network. The project is evaluated by the members of the advisory board, consisting of 15 members who are the chairs of various Working Groups, in terms of ethical compliance and scientific content. Appropriate studies are finalized for data entry by the must approve of at least 8 out of 15 members of the advisory board. Software engineers create the data set and announce the project to all pediatric endocrinology and diabetes centers in the country via email. Those who want to participate in the research project, log-in to the website and report their intent. Later, the application is evaluated by the CEDD-NET coordinator. Centers whose participation is approved are then included in the study. The aim of this network is to conduct high quality, multicenter studies in the field of pediatric endocrinology, to investigate approaches and treatment responses in rare diseases, and to shed light on treatment approaches and guide future studies for physicians.

The aim of our study was to determine the impact of CEDD-NET on the transformation of multicenter studies into scientific publications and assess the academic characteristics of the studies that have been transcribed into publication.

Methods

In our study, all the studies that were opened to patient admission on the website http://cedd.saglik-network.org between August 26, 2013 and March 1, 2021 were reviewed. The number of participants in the studies, the number of cases, the number of parameters investigated, and the cost (at the dollar exchange rate on the date the study was opened for case recruitment) were documented. In the completed studies, the publication status of the study was investigated through Pubmed and Google Academic databases. The date of the publication, the impact factor of the published journal, the Q category, the number of citations by any publication, and by Web of Science journals were searched.

Statistical Analysis

Categorical data were described using observed frequencies and percentages, and continuous variables were summarized by their medians and minimum maximum values in case of serious deviation from normality with Statistical Package for the Social Sciences (SPSS), version 21.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered to be statistically significant.

Results

A total of 30 studies were accepted and opened for data entry in the 7.5-year period from August 26, 2013, when the first study started, to March 1, 2021. While data entry was still ongoing in three of these studies, it had been closed in 27 studies and had been terminated. The median data collection period of the studies was 12 (1.5-24) months. While the median number of researchers who participated was 23 (3-180), the median number of cases was 120 (26-192). The median number of investigated parameters was 154 (41-564). The average cost was $2113 (1370-3118). Out of 30 studies, data entry was completed for 27. Sixteen publications were produced from 14 studies, 13 of them were not yet published. From the data of one study, three different publications were produced (Table 1). The median time from the end of data entry to publication of the study was 686 (168-1608) days. While the median impact factor of the journals in which the studies were published was 1.803 (1.278-5.399), the median number of citations was 6.5 (0-49), and the median number of citations by Web of Science journals was 4 (0-22).

Discussion

The specialized field of endocrinology and metabolism is a field that deals with health problems, such as type 2 diabetes and obesity (1), which affects up to 30% of the population, as well as diseases with a frequency of $< 1/1000000$ such as congenital lipodystrophy (2). Based on the bibliographic review of the studies conducted in the field of endocrinology and metabolism in the world, Turkey is in a good place (ranked 16th) among the countries producing the most publications (3). Thanks to the developments in the field of medicine, many new treatments are produced and the way for many new studies is paved.

Table 1. Publication outputs and features of the studies conducted with the CEDD-NET system

Publication title	Participating center	Researcher (n)	Case (n)	Parameter (n)	Journal	Publication year	Impact factor	Q category	Cost (American dollars)	Cite (n)	Citation in web of science journals (n)
1. Clinical Characteristics and Growth Hormone Treatment in Patients with Prader-Willi Syndrome*	13	15	67	190	JCRPE	2021	1.803	Q2	3005	0	0
2. Molecular Diagnosis of Monogenic Diabetes and their clinical/laboratory features in Turkish Children*	29	36	205	80	JCRPE	2021	1.803	Q2	2113	0	0
3. Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: Outcomes of Extended Pilot Study in 241,083 Infants*	17	19	66	426	JCRPE	2020	1.803	Q2	1637	5	3
4. Clinical Characteristics of 46,XX Males with Congenital Adrenal Hyperplasia*	9	10	26	145	JCRPE	2020	1.803	Q2	1370	0	0
5. Nationwide Turkish Cohort of Hypophosphatemic rickets*	75	151	155	120	JCRPE	2020	1.803	Q2	1882	3	3
6. Characteristics of Turkish children with Type 2 diabetes at onset: a multicentre, cross-sectional study (7)	40	46	366	440	Diabetic Medicine	2019	3.083	Q1	2531	7	1
7. Comparison of Treatment Regimens in Management of Severe Hypercalcemia Due to Vitamin D Intoxication in Children*	21	25	88	538	JCRPE	2019	1.803	Q2	2737	4	2
8. Clinical and Laboratory Characteristics of Hyperprolactinemia in Children and Adolescents: National Survey*	32	41	238	154	JCRPE	2018	1.803	Q2	2457	4	2
9. Genotype–phenotype correlation, gonadal malignancy risk, gender preference, and testosterone/dihydrotestosterone ratio in steroid 5-alpha-reductase type 2 deficiency: a multicenter study from Turkey (8)	24	25	121	76	J Endocrinol Invest	2019	4.256	Q2	1834	7	7
10. Response to growth hormone treatment in very young patients with growth hormone deficiencies and mini-puberty (9)	20	24	69	41	JPEM	2018	1.278	Q2	1834	6	5
11.Clinical, biochemical and genetic features with nonclassical 21-hydroxylase deficiency and final height (10)	23	29	266	384	JPEM	2017	1.278	Q2	3118	10	7
12. The Growth Characteristics of Patients with Noonan Syndrome: Results of Three Years of Growth Hormone Treatment: A Nationwide Multicenter Study (11)	21	23	124	319	JCRPE	2016	1.803	Q2	2282	15	8
13. The Etiology and Clinical Features of Non-CAH Gonadotropin-Independent Precocious Puberty: A Multicenter Study*	30	33	136	361	JCEM	2016	5.399	Q1	2282	22	12
14. **A. Turner Syndrome and Associated Problems in Turkish Children: A Multicenter Study*	36	47	848	127	JCRPE	2015	1.803	Q2	1941	48	23
B. Anthropometric findings from birth to adulthood and their relation with karyotpye distribution in Turkish girls with Turner syndrome*					AJMG-PART A	2016	2.125	Q2		13	6
C. Growth curves for Turkish Girls with Turner Syndrome: Results of the Turkish Turner Syndrome Study Group*					JCRPE	2015	1.803	Q2		49	22

*They can be accesible on the link https://jcrpe.org

**After termination of the study, 3 publications were produced from this data.

JCRPE: Journal of Clinical Research in Pediatric Endocrinology, JPEM: Journal of Pediatric Endocrinology and Metabolism, AJMG: American Journal of Medical Genetics, CEDD-NET: http://cedd.saglik-network.org

When citations of publications are examined, among the sub-fields of pediatrics, pediatric endocrinology is the third most cited field (4). Some endocrine disorders in this field are rare and do not allow clinical research from a single center (5). Examples of these diseases are Prader-Willi syndrome, and 46 XX congenital adrenal hyperplasia reared as males. In addition, due to the insufficient number of cases followed by clinicians, their clinical experience is limited in these diseases (6). Through the data network established by PEDS, physicians both share their experiences with each other and get ideas about treatment approaches. When the publications of the PEDS are examined, the publication "Response to growth hormone treatment in very young patients with growth hormone deficiencies and mini-puberty" was reported as the largest series evaluating patients who were diagnosed with growth hormone deficiency and started treatment under the age of three years. Similarly, the publication "Genotype-phenotype correlation, gonadal malignancy risk, gender preference, and testosterone/dihydrotestosterone ratio in steroid 5-alpha-reductase type 2 deficiency: a multicenter study from Turkey", and "Clinical and laboratory characteristics of hyperprolactinemia in children and adolescents: national survey" are the studies with the largest patient series in their specific field. Likewise, the study "Comparison of treatment regimens in management of severe hypercalcemia due to vitamin D intoxication in children", which evaluated vitamin D intoxication, made a great contribution to the literature with the largest patient series in an area that is very difficult to study. All these papers can be accessible on the link https://jcrpe.org.

When the cost calculation of the studies was made, the highest amount was found to be $3,118. Despite the fact that this amount was below the cost of many scientific research projects, publications of the high quality were produced. This suggests that studies with a data network can also be cost-effective.

One of the issues to be discussed is that only 51.8% of the studies have been published. The reasons that studies have not been published may include the low number of cases, insufficient data collection or difficulties experienced by corresponding authors to bring them to the publication stage. The fact that four publications have not been published, even though it has been more than 4 years since data entry was closed, reveals the necessity of reviewing researchers at the publication stage by the evaluation board.

Study Limitations

One of the limitations of the study is that the corresponding authors were not contacted individually. This would have allowed assessment of the negative and positive aspects of the data evaluation using the CEDD-NET system but we were unable to assess this. Furthermore, the problem of unpublished studies and if this was due to the data source, was also not investigated. The productivity and effectiveness of CEDD-NET would better have been assessed by canvassing the anonymous opinions of the researchers who provided data to the various studies.

Conclusion

In conclusion, the CEDD-NET database is cost-effective and useful, as it creates a data network that can easily reach many physicians across the country. It is productive and effective, as all the publications are of Q1-Q2 quality to date and have been cited by 99 Web of Science indexed journals in total. This study shows the benefits and necessity of establishing nationwide databases, even extending to more than one country, in specialized branches of medicine, such as pediatric endocrinology, where rare diseases are of concern and good quality evidence is scarce.

Ethics

Ethics Committee Approval and Informed Consent: Ethical approval and was not required for this research, as this study did not directly or indirectly involve human participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Feyza Darendeliler, Concept: Aysun Ata, Feyza Darendeliler, Design: Samim Özen, Feyza Darendeliler, Data Collection or Processing: Aysun Ata, Analysis or Interpretation: Samim Özen, Literature Search: Samim Özen, Feyza Darendeliler, Writing: Samim Özen, Aysun Ata.

Financial Disclosure: The authors declared that this study received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2021.2020.0278

J Clin Res Pediatr Endocrinol 2022;14(2):221-226

A Novel Mutation in the Thyroglobulin Gene Resulting in Neonatal Goiter and Congenital Hypothyroidism in an Eritrean Infant

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What is already known on this topic?

Congenital hypothyroidism (CH) may be caused by thyroid dysgenesis or thyroid dyshormonogenesis. Thyroid dyshormonogenesis is cause by mutations in genes involved in hormonogenesis and is usually inherited in an autosomal recessive manner. Mutations in the thyroglobulin gene (*TG*) are a recognized cause of dyshormonogenic CH, which may cause fetal or neonatal goiter

What this study adds?

The *TG* c.5686+1delG pathogenic variant has not been previously described as causing congenital goitrous hypothyroidism.

Abstract

Congenital hypothyroidism (CH) due to dyshormonogenesis may occur due to mutations in any of the key genes involved in thyroid hormone biosynthesis (*TG, TPO, DUOX2, DUOXA2, SLC5A5, IYD, SLC26A4* and *SLC26A7*). Mutations in the thyroglobulin gene (*TG*) are frequently associated with goiter, which may present fetally or neonatally, although a spectrum of phenotypes is reported. We present the case of a woman of Eritrean origin who presented in the third trimester of pregnancy in the early stages of labor. Ultrasound at presentation revealed a fetal neck swelling consistent with a goiter. Following delivery by Caesarian section with minimal respiratory support, the infant was found to be hypothyroid with undetectable serum levels of thyroglobulin. Sequencing of the *TG* revealed a homozygous donor splice site pathogenic variant (c.5686+1delG) not previously described in the literature. Levothyroxine treatment resulted in normal growth and psychomotor development. Goitrous CH with inappropriately low thyroglobulin has previously been reported in patients harbouring homozygous single nucleotide substitutions at the same *TG* donor splice site, which result in exon skipping and retention of malformed thyroglobulin by the endoplasmic reticulum. We conclude that the *TG* c.5686+1delG pathogenic variant is the likely basis for our patient's fetal goiter and CH, and that the clinical phenotype associated with *TG* c.5686+1delG is comparable to that seen with single nucleotide substitutions at the same site.

Keywords: Congenital goiter, hypothyroidism, thyroglobulin, novel mutation, case report

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Conflict of interest: None declared
Received: 01.12.2020
Accepted: 06.03.2021

Presented in: This work was presented at the European Society of Pediatric Endocrinology annual meeting, Vienna, Austria, September 2019.

Introduction

Congenital hypothyroidism (CH) is the commonest neonatal endocrine condition with recent reports demonstrating an incidence of around 1 in 1500 live births (1). Untreated CH can result in profound neurodevelopmental delay but since the introduction of newborn screening for CH in most Western countries, there has been a dramatic improvement in neurodevelopmental outcomes associated with the condition (2). CH is traditionally subdivided into thyroid dysgenesis (TD) and dyshormonogenesis, where TD refers to structural abnormalities of the thyroid including thyroid agenesis or an ectopic/sublingual thyroid gland and dyshormonogenesis describes inadequate thyroid hormone biosynthesis by a normally-located, often goitrous gland, due to molecular defects in the thyroid hormone biosynthetic machinery (3). Dyshormonogenesis usually has an identifiable monogenic basis, involving mutations in thyroglobulin (*TG*) or genes involved in iodine transport, organification and recycling (*SLC5A5, SLC26A4, DUOX2, DUOXA2, TPO, SLC26A7, IYD*), although oligogenic mutations may also contribute (3). Inheritance of dyshormonogenesis is usually autosomal recessive although CH due to *DUOX2, DUOXA2* and *IYD* mutations may also be dominantly inherited.

Dyshormonogenesis may present with goiter in the neonatal period or later, and may rarely be associated with fetal goiter. The mechanical consequences of fetal dyshormonogenic goiter may be associated with significant morbidity. Polyhydramnios may occur due to esophageal compression, and at delivery, neck hyperextension may result in malpresentation. Additionally, neonatal tracheal compression may cause fatal respiratory compromise. Complications can also arise from the underlying foetal thyroid dysfunction that can negatively affect development *in utero* and subsequent neurodevelopmental outcomes (4,5,6,7).

The management of hypothyroid fetal goiter remains controversial. Since fetal goiter is a marker of serious thyroid dysfunction, many recommend the determination of thyroid function and the consideration of *in utero* treatment. Although fetal ultrasound, magnetic resonance imaging and amniotic fluid thyroid stimulating hormone (TSH) levels have all been methods suggested for determining fetal thyroid status, these have been shown to have variable levels of accuracy and cordocentesis has been recommended as the gold standard for determining fetal thyroid status. However, it must be taken into account that this may be associated with complications, including cord bleeding, chorioamnionitis and preterm delivery, among others (8).

When considering the decision to treat fetal goiter with associated hypothyroidism, one needs to take into account the size of the goiter, the effect on surrounding structures and associated features such as polyhydramnios. Treatment with both intra-amniotic levothyroxine and administration via the umbilical vein have been described with varying degrees of success, both with regards to reduction in goiter size, reduction in perinatal complications and improvement of fetal thyroid hormone levels (5,6,7). However, conservative management, with radiological surveillance and elective delivery with respiratory support where necessary have also achieved favorable outcomes in some cases (4).

Genetic evaluation has been infrequently undertaken in cases with dyshormonogenic fetal goiter although underlying mutations in *TG, TPO*, and *DUOXA2* have been reported in this context (5,6,7).

Case Report

A 35-year-old woman of Eritrean origin was referred to a tertiary obstetric centre due to the finding of a neck mass on fetal ultrasound on presentation at term. Exact gestation was unknown as she had had no previous antenatal follow up or ultrasound scans.

Maternal history was notable for multiparity, with four previous healthy live births with no history of any congenital anomalies or CH. She had no significant past medical history. Her non-consanguineous partner was also Eritrean with no significant past medical history.

On fetal ultrasound exam, a large mass was visualized in the neck and upper chest consisting of two lobes consistent with an enlarged thyroid gland (Figure 1). The mass including both lobes measured 55x63 mm. The trachea was noted to pass through the two lobes with no narrowing noted. Both carotids were displaced laterally by the mass. In addition, the superior vena cava was significantly enlarged, the heart was enlarged and significant tricuspid regurgitation was visualized.

In anticipation of difficulties in airway management following delivery, a Caesarean section was planned with pediatric ear nose and throat and anesthetic staff present. A live female infant was delivered in good condition with Apgar scores of 8 and 9 at one and five minutes respectively. Oxygen saturation was low and the infant was treated with high flow nasal cannula oxygen. Initial examination was notable for a large diffuse neck swelling (Figure 2) and mild respiratory distress with no other abnormal examination findings. The infant was transferred to the neonatal unit for further investigation and management.

In light of prenatal ultrasound findings and clinical examination, initial studies were carried out to investigate thyroid structure and function. Initial thyroid function in the first 24 hours of life, showed primary hypothyroidism although free tri-iodothyronine (fT3) levels were preserved: TSH 272.4 mIU/l (0.4-20), free thyroxine (fT4) 6.3 pmol/L (10-30), and fT3 5.5 pmol/L (2.5-9.8). Thyroglobulin was inappropriately low at 0.7 mcg/L (0-55), without detectable levels of thyroglobulin antibodies (< 20 U/mL).

An ultrasound showed enlargement of both lobes of the thyroid gland, including the isthmus. The gland was reportedly of normal texture and was hyperemic. A thyroid technetium scan was also performed (Tc-99m) which demonstrated a diffusely enlarged thyroid gland with diffusely increased uptake.

In view of the laboratory findings of primary hypothyroidism, treatment was commenced on day 1 of life with high dose levothyroxine (18 mcg/kg) with rapid normalization of thyroid function tests. The dose was gradually tapered down accordingly. The child continues endocrine follow up and at the age of 3 years is currently well managed with medical therapy, with a very small goiter which increases in size in accordance with increasing TSH levels. She has no additional medical problems and shows normal growth and psychomotor development.

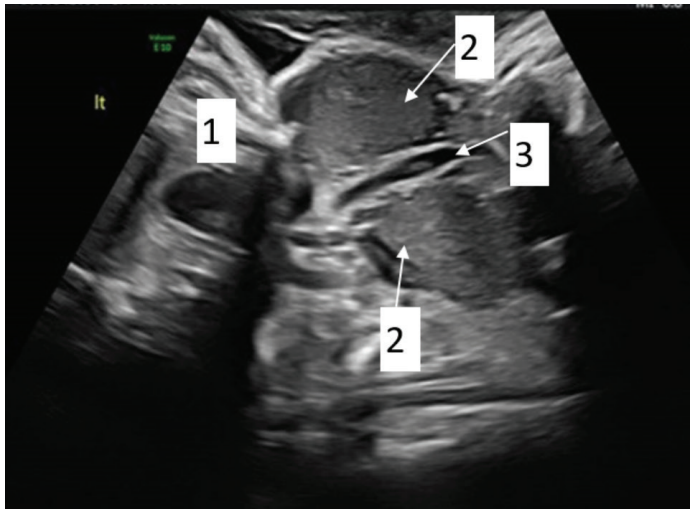

Figure 1. Foetal goiter as visualised on antenatal ultrasound. 1. Head, 2. Thyroid, 3. Trachea

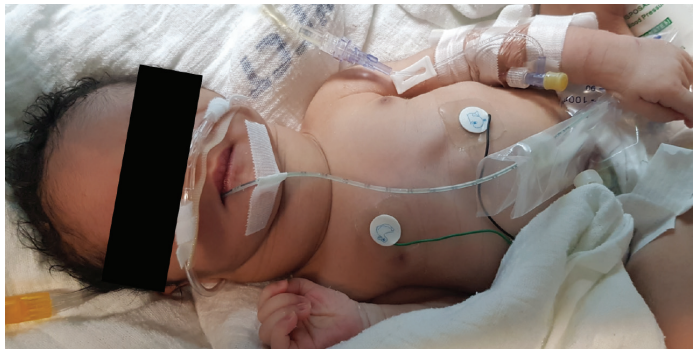

Figure 2. Neonatal goiter

Due to the association of neonatal goiter with laboratory evidence of hypothyroidism, low serum concentration of thyroglobulin and diffuse uptake of technetium on nuclear scanning, a genetic defect in the thyroglobulin gene was suspected and genetic studies were undertaken with written informed parental consent.

Genetic Studies

Sanger sequencing of the thyroglobulin gene (*TG*, ENST00000220616.9) revealed a novel donor splice site pathogenic variant at the exon 30-intron 30-31 boundary; c.5686 + 1delG which is absent from the gnomAD database (Figure 3) (9). The patient was homozygous for this pathogenic variant. Due to parental reluctance to pursue further genetic testing, DNA was not available from her parents or four siblings for genotyping.

Discussion

Thyroglobulin is a large secretory protein which is crucial for thyroid hormone biosynthesis and storage in the thyroid follicular lumen. The *TG* gene encodes a protein of 2768 amino acids in length including a 19 amino acid N-terminal signal peptide. The recently-solved protein structure of TG has defined five regions (N-terminal domain, core, flap, arm and C-terminal domain) containing domains of type-1 to type-3 cyteine rich TG repeats and a C-terminal choline-esterase-like domain (ChEL) as well as a probable four hormonogenic acceptor tyrosines and five donor tyrosines (10). TG is synthesized in the endoplasmic reticulum (ER) and folds with the assistance of molecular chaperones before trafficking to the apical membrane. The complex protein folding and intracellular trafficking of TG are essential for its normal follicular secretion and require both ER chaperones

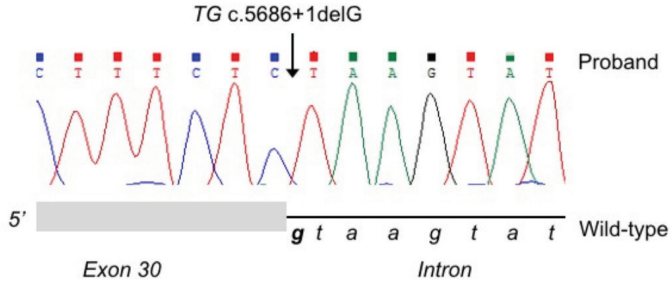

Figure 3. Sanger sequencing chromatogram for the *TG* exon 30-intron boundary, demonstrating a single nucleotide deletion et the donor splice site (ENST00000220616.9, c.5686 + 1delG)

TG: triglyceride gene

and oxidoreductases, as well as specific intramolecular interactions (11). The ChEL domain has an important role in permitting TG intracellular trafficking and secretion and intradomain disulfide bonds between the many cysteine residues in TG are essential for the correct folding of newly synthesized TG (11,12,13).

TG mutations are a common cause of dyshormonogenesis, with an estimated frequency of at least 1:100,000 and are usually inherited in an autosomal recessive manner, although CH has rarely been associated with monoallelic mutations (13,14,15). To the best of our knowledge, *TG* c.5686+1 delG has not previously been reported. However, three different point mutations resulting in single nucleotide substitutions at the same site have been reported in association with congenital goitrous hypothyroidism; *TG* c.5686+1G>A, 5686+1G>C and 5686+1G>T (16,17,18,19,20,21). Analysis of patient-derived thyroidal tissue has confirmed that *TG* c.5686+1G>T and c.5686+1G>C mutations cause skipping of exon 30 with a resultant in-frame deletion of 46 amino acids in the TG type III repeat domain, causing the loss of 1- putative N-linked glycosylation site and modifying the TG protein structure (17,20). In common with most pathogenic *TG* mutations, *TG* c.5686+1G>T results in TG misfolding and retention within the ER with decreased export to the colloid (16). The fact that *TG* c.5686+1 delG disrupts the same canonical donor splice site guanine residue, suggests that it is highly likely to be pathogenic, although functional studies to confirm this were not undertaken in our study. Due to parental reluctance to pursue further genetic testing, we were unable to obtain DNA to confirm segregation of homozygosity for the mutation with CH phenotype.

Individuals harbouring *TG* mutations exhibit a spectrum of thyroid dysfunction, ranging from biochemically severe CH to euthyroid goiter (15). Goiter occurs frequently, commonly manifesting in the neonatal period, although onset may be delayed, and a small minority of cases exhibit fetal goiter (6,18). The biochemical hallmark of CH due to a *TG* mutation comprises an inappropriately low or undetectable circulating thyroglobulin level despite elevated circulating TSH concentration or goiter, and failure of exogenous TSH to stimulate a rise in serum TG. Thyroidal iodide uptake is enhanced and organification of iodide is usually preserved (17,21). In some cases, the fT3/fT4 ratio is elevated (20).

Previously reported cases with homozygous disruption of the same donor splice site have shown variable biochemical phenotypes, although goiter is a consistent feature. Two Brazilian siblings who were homozygous for the TG c.5686+1G>T mutation exhibited fetal or neonatal goiter and severe CH (18). An additional two Brazilian siblings with the same mutation initially presented with congenital goiter. Interestingly, although the eldest sibling exhibited severe hypothyroidism, his sister had a milder biochemical phenotype with low serum total T4 but normal total T3. It was hypothesized that the variable expressivity seen in this family may be partly explained by iodine status, since the elder sibling was raised predominantly in an iodine deficient area whereas the family's relocation to an iodine replete region when his sister was aged 2 years may have ameliorated her thyroid dysfunction (17). A homozyous *TG* c.5686+1G>C mutation was also identified in an iodine replete Pakistani girl, presenting aged 10 years with a massive goiter but normal TSH, subnormal fT4 and raised fT3 levels (20). It is likely that in this case and in the mildly hypothyroid Brazilian sibling, small quantities of mutated thyroglobulin molecules reached the follicular lumen, permitting iodination and synthesis of thyroid hormones, which is facilitated by adequate iodine intake. The elevated fT3/fT4 ratio may be at least in part due to increased thyroidal type 2 deiodinase activity (20). *TG* c 5686+1 G>A was detected in compound heterozygosity with *TG* p.Q310P in two Japanese cases for whom detailed individual data was not presented although both were on treatment for screening-detected CH and had goiter in early childhood (21).

Here, we report a female patient of Eritrean origin with a large fetal goiter detected whilst *in utero* prior to delivery. Lack of ultrasound data from earlier in the pregnancy preclude definitive comments regarding the onset of goitrogenesis, however its large size and local compressive effects suggest it may have originated some weeks earlier. She had significant primary hypothyroidism at birth with preserved fT3 levels despite subnormal fT4 levels, and her thyroglobulin level was inappropriately low. These clinical and biochemical features are all recapitulated in previously reported patients harbouring mutations at the *TG* c.5686+1 donor splice site and we believe her homozygous TG c.5686+1 delG pathogenic variant to be the likely cause of her thyroid dysfunction, although future functional studies will be required to confirm this.

Urinary iodine was not measured contemporaneously with presentation in this patient but since there is evidence to suggest that iodine intake during pregnancy may be inadequate in some areas of Israel, suboptimal maternal iodine status may have contributed to the fetal goitrogenesis although the patient's normal fT3 suggests some iodination of mutant TG was occurring in the follicular lumen. Although conservative management of her fetal goiter resulted in a relatively uncomplicated delivery, radiological monitoring for fetal goiter would be advisable during future pregnancies,

given the presumed 25% risk of having another affected child if both parents are heterozygotes for the *TG* c.5686 + 1 delG pathogenic variant. Additionally, maternal iodine status should be optimized.

Conclusion

We report a case of an infant presenting in late pregnancy with a large fetal goiter, CH determined by laboratory studies following delivery and an undetectable serum thyroglobulin. Sequencing of the thyroglobulin gene revealed an as yet unreported homozygous donor splice site pathogenic variant at the exon 30-intron 30-31 boundary; c.5686 + 1delG. Previous single nucleotide substitutions and this site have been described with variable phenotypes including both neonatal goiter, goitrous hypothyroidism, and goitrous euthyroidism. Genetic evaluation, when carried out, revealed skipping of exon 30 in the mRNA and subsequent generation of a shortened protein. Functional evaluation of single nucleotide substitutions at this site demonstrated retention of the TG protein within the ER and resulting decreased export to the follicular lumen. Due to parental refusal further genetic testing on unaffected family members was not carried out. However, based on previous studies as described and the patient's clinical, biochemical and imaging phenotype, we concluded that this pathogenic variant was the likely cause for the patient's clinical condition.

Acknowledgements

The authors wish to thank Mrs. Carmel Byers for her assistance in acquiring genetic testing.

Ethics

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Adeline K. Nicholas, Concept: Eve Stern, Eran Kassif, Orit Pinhas Hamiel, Design: Eve Stern, Eran Kassif, Orit Pinhas Hamiel, Nadia Schoenmakers Data Collection or Processing: Nadia Schoenmakers, Analysis or Interpretation: Eve Stern, Nadia Schoenmakers, Adeline K. Nicholas, Eran Kassif, Orit Pinhas Hamiel, Yonatan Yeshayahu, Literature Search: Eve Stern, Nadia Schoenmakers, Adeline K. Nicholas, Eran Kassif, Orit Pinhas Hamiel, Yonatan Yeshayahu, Writing: Eve Stern, Nadia Schoenmakers, Adeline K. Nicholas, Eran Kassif, Orit Pinhas Hamiel, Yonatan Yeshayahu.

Financial Disclosure: The authors declared that this study received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0265

J Clin Res Pediatr Endocrinol 2022;14(2):227-232

TRMT10A Mutation in a Child with Diabetes, Short Stature, Microcephaly and Hypoplastic Kidneys

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What is already known on this topic?

Over 40 different genetic subtypes of monogenic diabetes have been identified to date. *TRMT10A* mutations cause a distinct syndrome that includes abnormal glucose homeostasis, intellectual disability, short stature and microcephaly.

What this study adds?

Our report expands the phenotypic description of this syndrome. We report for the first time hypoplastic kidneys and inadequate response to growth hormone stimulation tests in a girl with this syndrome. Making a specific molecular diagnosis helps to predict the clinical course and enable genetic counseling, as well as personalized treatment.

Abstract

A new syndrome of diabetes, short stature, microcephaly and intellectual disability has been described in association with mutations in the tRNA methyltransferase 10 homologue A *(TRMT10A)* gene. We report a patient who presented with fasting hyperglycemia, a raised hemoglobin A1c and positive islet cell autoantibodies. Additional clinical features included intellectual disability, hypoplastic kidneys and short stature. In view of the syndromic features coexistant with diabetes, genetic evaluation was carried out, revealing a homozygous mutation in the *TRMT10A* gene (c.616G > A, p.G206R). The case highlights the importance of genetic evaluation of patients with diabetes with atypical features that can further progress our understanding of the pathophysiology of the rarer subtypes of diabetes.

Keywords: Monogenic diabetes, short stature, microcephaly, hypoplastic kidneys

Introduction

Monogenic diabetes is uncommon, accounting for approximately 1 % to 6 % of pediatric diabetes patients (1,2). The disease may be inherited within families as a dominant or recessive trait, and rarely through mitochondrial inheritance. It may also present as a spontaneous case due to a *de novo* mutation. Over 40 different genetic subtypes of monogenic diabetes have been identified to date, each having a typical phenotype and a specific pattern of inheritance. Maturity onset diabetes of the young (MODY) is by far the commonest type of monogenic diabetes. All currently known subtypes of MODY are caused by dominant heterozygous mutations in genes important for the development or function of β-cells (3). Over the last few years, a number of forms of monogenic diabetes clinically and genetically distinct from MODY, have been identified.

In 2013, a new autosomal recessive syndrome, including short stature, microcephaly, intellectual disability and diabetes mellitus, was described in association with mutations in the tRNA methyltransferase 10 homologue A (*TRMT10A*) gene (4). Since the first description, only a few case reports of this syndrome have been published (5,6,7,8,9).

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Conflict of interest: None declared
Received: 25.11.2020
Accepted: 28.12.2020

The aim of this case report is to describe a girl that presented with hyperglycemia and positive autoantibodies with a presumed diagnosis of type 1 diabetes. Identification of the genetic etiology of a *TRMT10A* mutation improved the understanding of the disease course and enabled personalized clinical care.

Case Report

An 11-year-old girl was referred to the emergency room in light of high fasting glucose of 153 mg/dL (normal values 70-100 mg/dL) and a hemoglobin A1c (HbA1c) of 9.9% (normal values 3.8-6.4%). The patient was born to Jewish parents of Uzbekistan descent; parents were distantly related. Two older siblings were healthy and there was no family history of diabetes or any other autoimmune condition. Pregnancy was notable for intrauterine growth retardation (IUGR) demonstrated from week 17 of pregnancy. Birth weight was 2190 grams at term with no perinatal complications. Developmental milestones were delayed and the patient had been diagnosed with attention deficit disorder in early childhood.

At the age of two years old following repeated urinary tract infections, an ultrasound scan revealed bilateral mildly hypoplastic kidneys. Consequently, she was under nephrology follow-up that showed stable renal growth and normal renal function.

At the age of 11 years, her height was 127.3 cm [-2.3 standard deviation (SD)], weight 33.5 kg (-0.5 SD), body mass index (BMI) 20.7 kg/m² (1.0 SD) and head circumference 49 cm (-2.0 SD) (Figure 1). Growth velocity was 4.2 cm/year. Due to the short stature the patient was referred for endocrinological assessment. Laboratory investigations revealed fasting blood glucose of 110 mg/dL (normal values 70-100 mg/dL) and insulin-like growth factor 1 168 ng/mL (normal range 118-448 ng/mL). Her bone age determined using the Greulich-Pyle method was eight years and ten months at chronological age of ten years and nine months. On repeat laboratory investigations, fasting blood glucose was 153 mg/dL and HbA1c 9.9% (normal values 3.8-6.4%). Medical history was negative for polydipsia, polyuria or significant weight loss. Subsequent laboratory examinations showed positive anti-islet cell antibodies (76.9 IU/mL, normal range 0-30) with weakly positive anti-GAD antibodies (7.4 IU/mL, normal range 0-5). She was started on a low dose of long acting insulin with a working diagnosis of type 1 diabetes.

In view of the combination of IUGR, developmental delay, hypoplastic kidneys, short stature and diabetes, a genetic etiology was suspected. The presence of hypoplastic kidneys raised the suspicion for a diagnosis of MODY type 5, which can include congenital renal malformations as a feature. Therefore, she was referred to the nephro-genetic clinic in our institute. On examination, dysmorphic features including microcephaly, narrow nasal bridge, retrognathia and beaked nose were observed. In addition, a large hyperpigmented skin lesion on her left thigh and bilateral fifth finger clinodactyly were noted. Microarray was reported as normal. Whole exome sequencing was carried out which revealed a homozygous missense mutation in the *TRMT10A* gene (NM_001134665, c.616G>A, p.G206R). This mutation has previously been reported (5). Both parents were found to be heterozygous for the mutation.

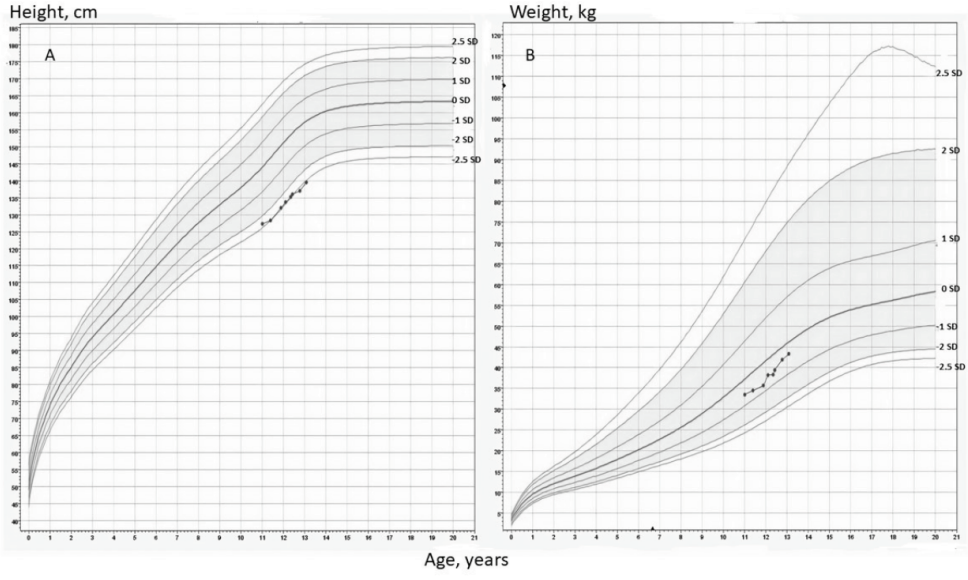

Figure 1. Height (A) and weight (B) curves of the patient

Growth hormone stimulation tests were performed due to short stature, low growth velocity and delayed bone age. Peak growth hormone was 7.3 mcg/L after administration of clonidine and 1.8 mcg/L after administration of arginine, establishing a diagnosis of growth hormone deficiency. Magnetic resonance imaging (MRI) of the pituitary was reported as normal. Growth hormone replacement therapy was discussed with the family who decided against treatment.

In the subsequent months following diagnosis, glucose levels were well controlled with low doses of long acting insulin with no need for boluses of short acting insulin with meals. Subsequently short acting insulin was started with meals.

Two years following diagnosis, she was clinically well. Her height was 139.5 cm (-2.5 SD), weight 43.3 kg (-0.3 SD) and BMI 22.4 kg/m² (1.0 SD) (Figure 1). Pubertal status was Tanner stage 3. The insulin requirement was 0.4 units/kg/day. Glycemic control was good with fasting glucose 147 mg/dL and HbA1c of 7%. C-peptide level at the same time was still detectable at 2.47 ng/mL (normal range 0.9-7.1 ng/mL). Monitoring glucose levels using the FreeStyle Libre flash glucose monitoring system demonstrated relatively stable glucose levels with no hypoglycemic events (Figure 2). Ophthalmic examination was normal with no evidence of retinopathy.

Informed consent from the parents of the patients was obtained for publication of the case.

Discussion

Here we present a case of monogenic diabetes due to *TRMT10A* mutation. The elevated fasting glucose and HbA1c of the patient met the criteria for a diagnosis of diabetes mellitus. Diabetes mellitus is a heterogeneous group of disorders with different genetic patterns and pathophysiological mechanisms. Type 1 diabetes is the most common type in the pediatric population and the patient had positive anti-islet cell antibodies. However, the low insulin requirements, detectable C-peptide levels and presence of extra pancreatic features (IUGR, developmental delay, hypoplastic kidneys, short stature) raised the suspicion of a genetic syndrome. The presence of hypoplastic kidneys was suspicious for a diagnosis of MODY type 5. However, no mutation in *HNF1*β was detected on whole exome sequencing. Type 2 diabetes is becoming an increasing problem in obese adolescents but the patient's BMI was in the normal range for her age.

TRMT10A mutation was first described in three siblings born to consanguineous parents of Moroccan descent, each with short stature and intellectual disability. They were diagnosed with diabetes between the ages of 14 and 22 years (4). All were negative for anti-GAD, islet cell and anti-insulin antibodies, in addition to having an HLA phenotype that did not confer risk of developing type 1 diabetes. Whole exome sequencing was carried out on one of the probands and one candidate mutation in chromosome 4, a homozygous c.379G>A in exon 4 of *TRMT10A* was identified. This was predicted to replace an arginine residue with a premature stop codon at position 127 of the polypeptide chain. Both affected siblings were found to be homozygous for the

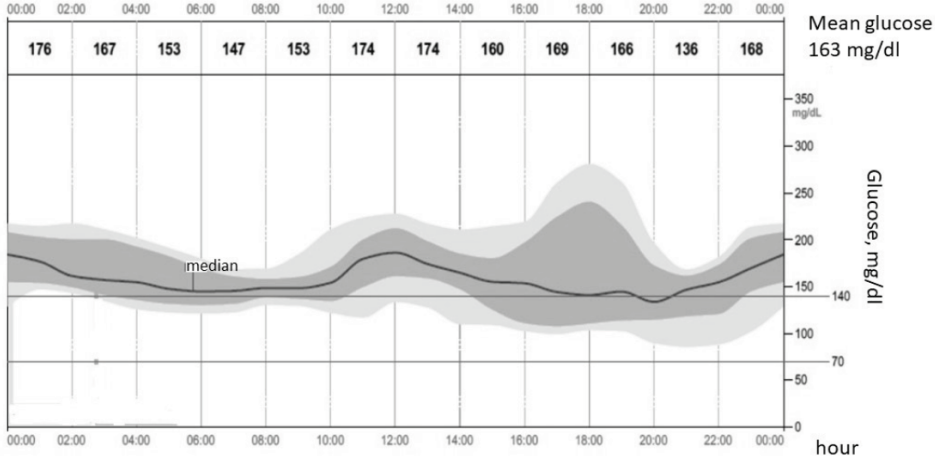

Figure 2. Averaged diurnal glucose levels within two weeks as measured by continuous glucose monitoring (Libre) two years after the diagnosis. At that time the daily insulin dose was 0.4 units/kg/day. The dark gray area represents the inter quantile range (IQR 25-75) of glucose levels and the black line the median

IQR: interquartile range

mutation while the parents and an unaffected sibling were found to be heterozygous for the mutation.

Following this report, nine additional individuals from five different families were reported as having homozygous mutations, compound heterozygous or deletion in the same gene (5,6,7,8,9). Similar to our patient, all reported patients exhibited intellectual disability and microcephaly (Table 1). In some patients the microcephaly presented at birth and resolved later and others had persistent microcephaly. Some patients had epilepsy and two patients had abnormal brain MRI findings (6).

Another main feature of the syndrome is abnormal glucose homeostasis. A variety of clinical presentations have been reported, including diabetes with or without ketosis, hyper insulinemic hypoglycemia, insulin resistance and postprandial hyperglycemia. The age at diabetes diagnosis ranged from nine to 28 years. Our patient was diagnosed with diabetes at the age of 11 years without ketoacidosis. Interestingly, she had positive islet cell antibodies, as was previously described in another patient (8). She had good glycemic control with relatively low insulin doses (0.4 units/kg/day) and detectable c-peptide two years after the diagnosis. This feature of well-preserved insulin secretion was previously reported in this syndrome (4,7,8). Of note,

Table 1. Clinical characteristics of 13 patients from seven families with *TRMT10A* mutations

Reference	No.	Gender	*TRMT10A* mutation	Impaired glucose metabolism	Treatment	Microcephaly	Intellectual disability	Epilepsy	Short stature	Other features
Igoillo-Esteve et al (4) 2013	1	F	c.379 G > A; p. Arg127Stop	Diabetes	Insulin	Yes	Yes	Yes	Yes	Dysmorphic features, osteoporosis
	2	F	c.379 G > A; p. Arg127Stop	Diabetes	Insulin	Yes	Yes	NR	Yes	
	3	M	c.379 G > A; p. Arg127Stop	diabetes	Insulin	Yes	Yes	NR	Yes	
Gillis et al (5) 2014	4	F	c.616G > A, p. Gly206Arg	Hyperinsulinaemic hypoglycemia and postprandial hyperglycemia	Diet	Yes	Yes	Yes	Yes	Delayed puberty
	5	M	c.616G > A, p. Gly206Arg	As his sister	Diet	Yes	Yes	Yes	Yes	
	6	M	c.616G > A, p. Gly206Arg	As his sister	Diet	Yes	Yes	Yes	Yes	
Zung et al (8) 2015	7	F	4q23 deletion	Ketotic diabetes. Positive islet cell antibodies	Insulin	Yes	Yes	Nr	Yes	Delayed puberty
Yew et al (7) 2016	8	F	c.79G > T; p. Glu27Ter	Diabetes, insulin resistance	Insulin, metformin	Yes	Yes	Yes	No	Buffalo hump
	9	M	c.79G > T; p. Glu27Ter	Diabetes, insulin resistance	Metformin	Yes	Yes	Yes	No	Delayed puberty
Narayanan et al (6) 2015	10	F	c.277C > T, p. Arg93* and c.397C > T, p. Arg133*	No	-	Yes	Yes	NR	NR	Dysmorphic features, abnormal brain MRI
	11	M	c.277C > T, p. Arg93* and c.397C > T, p. Arg133*	No	-	Yes	Yes	Yes	NR	Pulmonary infections, abnormal brain MRI
Lin et al (9) 2020	12	M	c.496–1G > A	Diabetes	Metformin	Yes	Yes	Yes	Yes	
Present report	13	F	c.616G > A, p. Gly206Arg	Diabetes	Insulin	Yes	Yes	No	Yes	IUGR, dysmorphic features, growth hormone deficiency, hypoplastic kidneys

F: female, M: male, NR: not reported, IUGR: intrauterine growth retardation, MRI: magnetic resonance imaging

our patient had the same mutation as the family described by Gillis et al (5). Both families originated from the same small and isolated Jewish community in Uzbekistan, yet the clinical phenotype differed between the two families. The three siblings had mainly hyperinsulinamic hypoglycemia (5), while our patient had diabetes with no documented hypoglycemic events.

Our novel finding is the hypoplastic kidneys and abnormal growth hormone stimulation tests, that were not previously described. Although short stature was previously described as part of this syndrome, this is the first time that growth hormone deficiency was diagnosed by stimulation tests. Additional features of the syndrome that were described in some of the patients included dysmorphic features and delayed puberty.

Transfer RNAs (tRNAs) are non-coding RNA molecules essential for protein synthesis (10). Across many species, tRNAs undergo complex post translational modifications including modification of tRNA nucleotide bases and sugars crucial for cellular function. Multiple enzymes have been identified that catalyze these posttranscriptional tRNA modification reactions. *TRMT10A* encodes a protein that has tRNA m^1G_9 methyltransferase activity (7). The protein was first discovered in yeast and its homologues are widely conserved across eukarya and archaea (11). The functional characterization of human *TRMT10A* was recently studied (12). This nuclear protein is expressed in several tissues including the liver, kidney, spleen, lung and adipose tissue. The expression is enriched in the brain and pancreatic islet cells. This is consistent with the main features of the syndrome described: microcephaly, intellectual disability and abnormal glucose homeostasis. *TRMT10A* silencing has been shown to induce apoptosis, which suggests that mutations may negatively affect beta cell mass and the number of neurons in the developing brain (4).

Conclusion

In summary, our report expands the phenotypic description of this syndrome. This case demonstrates that genetic testing should be performed in those diabetic patients with preserved β-cell function over an extended period or with extra-pancreatic features. Further studies are needed to shed light on the pathogenesis resulting from *TRMT10A* inactivation.

Ethics

Informed Consent: Informed consent from the parents of the patients was obtained for publication of the case.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Eve Stern, Asaf Vivante, Yael Levy-Shraga, Concept: Eve Stern, Asaf Vivante, Yael Levy-Shraga, Design: Eve Stern, Yael Levy-Shraga, Data Collection or Processing: Eve Stern, Ortal Barel, Yael Levy-Shraga, Analysis or Interpretation: Eve Stern, Asaf Vivante, Ortal Barel, Yael Levy-Shraga, Literature Search: Eve Stern, Asaf Vivante, Ortal Barel, Yael Levy-Shraga, Writing: Eve Stern, Asaf Vivante, Ortal Barel, Yael Levy-Shraga.

Financial Disclosure: The authors declared that this study received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0249

J Clin Res Pediatr Endocrinol 2022;14(2):233-238

46,XY Sex Development Defect due to a Novel Homozygous (Splice Site) c.673_1G>C Variation in the *HSD17B3* Gene: Case Report

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What is already known on this topic?

The 17-beta hydroxysteroid dehydrogenase type 3 (17β-HSD3) enzyme is primarily found in the testes and is involved in transforming Δ4-androstenedione, a weak androgen, to the more biologically active form, testosterone. Defects in the *HSD17B3* gene, which encodes this enzyme, cause 17β-HSD3 deficiency.

What this study adds?

In this article, we describe a previously unreported c.673_1G>C homozygous variation that was identified in the *HSD17B3* gene of a 46,XY patient.

Abstract

The enzyme 17-β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) catalyzes the biosynthesis of testosterone (T) from Δ4-androstenedione, and plays an important role in the final steps of androgen synthesis. 17β-HSD3 deficiency originates from mutations in the *HSD17B* gene, causing an autosomal recessive 46,XY sex developmental disorder (DSD). Patients with 46,XY karyotype can exhibit a wide phenotypic spectrum, varying from complete external female genitalia to male genitalia with hypospadias. Here we report a case of 17β-HSD3 deficiency diagnosed in the infantile period who was later found to have a novel *HSD17B3* gene variation. The 14-month old patient, who exhibited a female phenotype, presented with a bilateral lump in the inguinal area. Imaging revealed bilateral testicular gonads in the inguinal area. Hormonal evaluation showed low levels of basal and stimulated serum T, a high level of androstenedione (A), and a low T/A ratio. Chromosomal analysis showed 46,XY karyotype. Sequence analysis of the *HSD17B3* gene revealed a c.673_1G>C homozygous class 2 (splice site) variation in intron 9. The consanguineous parents were sequenced, and both were heterozygous for the same mutation. This variation has not been previously reported in the literature. In conclusion, a 46,XY DSD should be considered in patients with a female phenotype who exhibit gonad(s) in the inguinal area at an early age. Furthermore, in patients with insufficient T synthesis and high levels of androstenedione, 17β-HSD3 should be considered, and molecular analysis should be done for a definitive diagnosis and subsequent genetic counseling.

Keywords: 17 beta-hydroxysteroid dehydrogenase type 3, 46,XY disorders of sex development, *HSD17B3* gene

Introduction

The 17-beta hydroxysteroid dehydrogenase (17β-HSD) enzyme family includes at least 14 isoenzymes identified to date. These contribute to the development of reproductive organs by taking part in the final steps of androgen and estrogen synthesis. The 17-beta hydroxysteroid dehydrogenase type 3 (17β-HSD3) enzyme is primarily found in the testes and is involved in transforming Δ4-androstenedione (A), a weak androgen, to the more biologically active form testosterone (T) (1). Defects in the *HSD17B3* gene, which encodes this enzyme, causes 17β-HSD3 deficiency.

17β-HSD3 enzyme deficiency was initially described in 1971 and shows an autosomal recessive inheritance

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Conflict of interest: None declared
Received: 27.10.2020
Accepted: 28.12.2020

(2,3). Although not known exactly, the incidence rate has been reported to be 1/147,000 live births and the rate of heterozygosity to be 1/135 (4). Furthermore, in populations with a high rate of consanguineous marriage, such as the Gaza Strip Arabs, the incidence has been reported to be as high as 1/100-300 (5,6). Problems in T synthesis during fetal development result in insufficient development of male external genital organs. Although T synthesis is insufficient, the production of anti-Müllerian hormone (AMH) continues normally and prevents the development of Mullerian structures (7).

These patients with a 46,XY karyotype can exhibit a wide phenotypic spectrum varying from female external genitalia to male external genitalia with hypospadias, or ambiguous genitalia with microphallus (8). During puberty, the increase in gonadotropin levels lead to an increase in A levels and extra-testicular conversion of A to T, which ultimately leads to evident virilization. The degree of virilization can vary depending on 17β-HSD3 isoenzyme residue in the testes and the activity of other isoenzymes, such as 17β-HSD5 (9,10,11). Individuals with a 46,XX karyotype generally have normal female genitalia and are asymptomatic, making the condition difficult to diagnose (5,12).

In the laboratory analysis of 17β-HSD3 deficiency, low serum T levels and high serum A levels are observed. A human chorionic gonadotropin (hCG) stimulation test generally results in a serum T/A ratio of below 0.8 (13). A final diagnosis is made through molecular genetic testing.

In this article, we describe a case who presented with a history of bilateral lumps in the inguinal area during the infantile period, the patient's physical examination revealed external genitalia of female phenotype and palpable gonads. The evaluations were consistent with 17β-HSD3 deficiency, and a previously unreported c.673_1G>C homozygous variation was identified in the *HSD17B3* gene of the 46,XY patient.

Case Report

A 14-month old female patient presented with a history of a bilateral lump in the inguinal area. Her mother and father were first-degree cousins. Physical examination showed female-appearing external genitalia with the absence of clitoromegaly. Gonads were palpable in both inguinal regions. Ultrasound imaging revealed gonads that were compatible with testes in both inguinal areas, and no Mullerian structures were observed in the pelvis. It was speculated that the patient may have 46,XY disorder of sex development (DSD). Laboratory testing was used to assess gonad functions, showing that the serum follicle stimulating hormone level was 2.29 IU/L (0.26-3.0), luteinizing hormone (LH) was 1.19 IU/L (0.02-0.3), and T level was <0.693 nmol/L. Her serum AMH level was >73 ng/ML (maximum 3.9 for female, 9.9-444.1 for male) and inhibin B level was 388 ng/L (91-400). Evaluation of basal hormone levels revealed that T synthesis was insufficient. In order to precisely evaluate T synthesis ability, an hCG stimulation test was conducted. Both basal and stimulated serum T levels were found to be low and the serum T level did not increase following stimulation. The patient's T synthesis defect was confirmed. Serum T/A ratio was 0.14 (Table 1). A standard dose (250 μg) synacthen test was done to exclude disorders in which T synthesis defect and adrenal insufficiency may be seen together (such as 17-alpha hydroxylase deficiency). The test showed an adequate level of stimulated cortisol, and normal basal and stimulated serum progesterone and dehydroepiandrosterone sulphate levels. 17-alpha hydroxylase deficiency was ruled out. The karyotype was 46,XY, and fluorescence *in situ* hybridization analysis showed the absence of *SRY* gene variations. Pelvic magnetic resonance imaging (MRI) was used to comprehensively examine internal gonadal structures. The MRI revealed structures proximal to both inguinal canals that were compatible with testes, and a 30x4 mm structure that was compatible with vaginal tissue between the bladder and rectum. Diagnostic cystoscopy and laparoscopy showed the presence of gonads identical to testes in both inguinal canals. The vagina was 2 cm long. Since AMH levels were high and imaging showed the absence of Mullerian structures, gonadal dysgenesis was excluded.

A gonad biopsy was made for pathological evaluation. The pathology report stated "Bilateral testes containing seminiferous tubules and surrounded by the tunica albuginea were observed. Spermatogonia were not observed in the seminiferous tubules. Leydig cells were not present with hematoxylin and eosin staining. Of the immune markers used, Inhibin led to a strong positive immunoreaction in Sertoli cells and a mild positive immunoreaction in a small number of Leydig cells; calretinin led to a mild, positive reaction in Leydig cells; mild staining with PLAP; CD138

Table 1. Serum androgen concentrations before and after human chorionic gonadotropin stimulation

Serum hormone levels	Pre-hCG	Post-hCG
T (nmol/L)	<0.693	<0.693
DHT (nmol/L)	0.11	0.3
Androstenedione (Δ4) (nmol/L)	<0.83	3.83
T/DHT	5.0	1.81
T/Δ4	0.66	0.14

T: testosterone, DHT: dihydrotestosterone, hCG: human chorionic gonadotropin

negative; OCT3/4 negative; LH receptor showed a negative reaction." As the pathology report stated that the Leydig cells were insufficient and LH receptors were absent with specific staining, Leydig cell hypoplasia was considered but LH/choriogonadotropin receptor *(LHCGR)* gene analysis revealed no mutation. The patient, who had a T synthesis defect as well as a low serum T/A ratio, and did not have adrenal insufficiency or gonadal dysgenesis, was considered to have 17β-HSD3 deficiency. A sequence analysis of the patient's *HSD17B3* gene revealed a c.673_1G>C homozygous class 2 (splice site) variation on intron 9 (Figure 1). Both parents exhibited an identical heterozygous variation. This variation has not been reported in the literature previously, and was most likely pathological according to *in silico* analyses (Table 2). It was reported that in 46,XY patients with 17β-HSD3 deficiency who exhibit a total female phenotype, it is possible to achieve a penis size within normal limits through

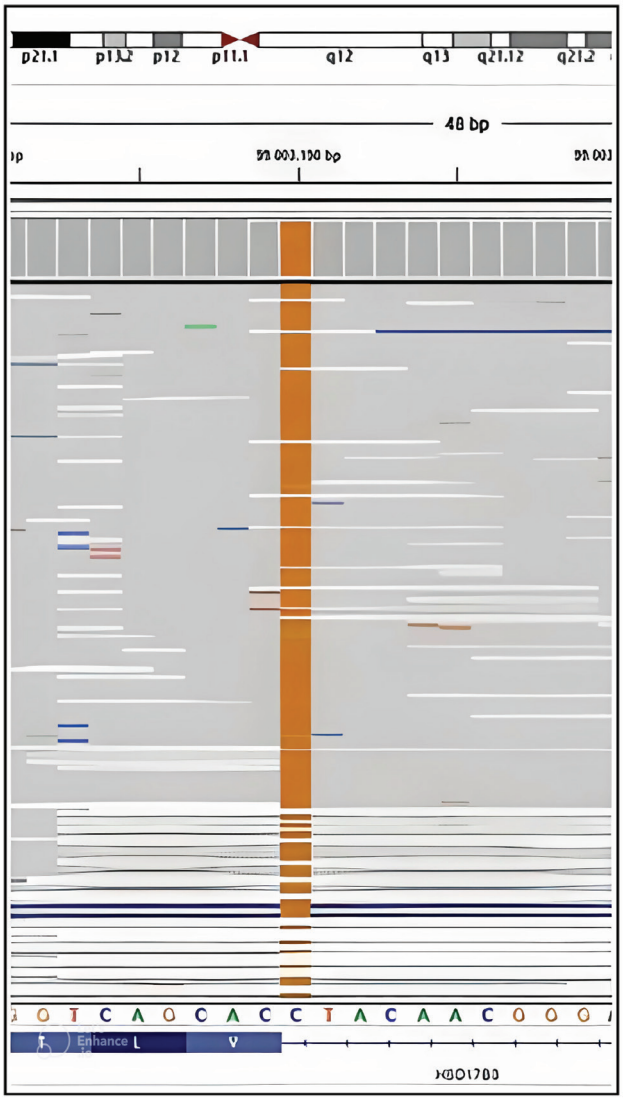

Figure 1. *HSD17B3* gene, c.673_1G>C homozygous class 2 (splice site) variation on intron 9

treatment with 25-50 mg/dose of intramuscular T for 3-9 months during the infantile period (14). Accordingly, our patient was treated with 50 mg/month of intramuscular T and gender determination was made based on response to treatment.

The parents gave their written consent for sharing the patient's examination, laboratory, imaging, and genetic results in scientific publications, on the condition that the child remains anonymous.

Discussion

The clinical signs of 17β-HSD3 deficiency may vary due to its wide phenotypic spectrum. These 46,XY patients may have differing external genital appearances depending on the residual activity of enzymes. Patients most frequently have a complete external female genital structure, usually with separate urethral and vaginal openings. However, some patients have been reported to only have a short urogenital sinus (3,11,15). Patients with complete external female genitalia are usually diagnosed late, and are often raised as female individuals. These patients usually present during puberty with primary amenorrhea and varying degrees of virilization. In patients with evident lumps in the inguinal canals or labioscrotal folds, the palpation of gonads may lead to an early diagnosis, similar to our patient (4,11,16). 46,XY patients may less frequently present with micropenis and hypospadias, in which case the patient is generally raised as a male individual (5).

Table 2. The *in silico* analysis, revealing the genetic variation in the *HSD17B3* gene of our patient

Gene *HSD17B3*	
Genbank transcript ID	NM-000197.2
Chromosomal Locus	9q22.32
DbSNP	Novel
Variant	c.673-1G>C
Variant Location	Intron9
Variant Type	Splice-site
Mutation Taster	Disease causing
Polyphen-2	Damaging
Varscak Splice-site Prediction	Class5 (Splicing Effect)
Eigen score	Pathogenic
ExAC (allele frequency)	Not found
GnomAD exomes	No entry
ClinVAR	-
Conservation	Conserved
DANN score	0.9952
ACMG Classification	Likely pathogenic
ACMG Pathogenity Criteria	PVS1, PM2, PP3

Due to their female phenotype and evident virilization during puberty, 46,XY patients with 17β-HSD3 deficiency are clinically similar to other conditions, such as androgen insensitivity syndrome (AIS), partial 5-α-reductase type 2 deficiency, or steroidogenic factor 1 deficiency (17). Boehmer and colleagues reported that 19 patients who were initially believed to have AIS were diagnosed with 17β-HSD3 deficiency after further investigation (4). Leydig cell aplasia is also included in the differential diagnosis of 46,XY female patients who have been diagnosed at an early age.

The typical hormonal findings for 17β-HSD3 deficiency includes reduced T and increased A levels. While it is possible to diagnose patients through basal hormone levels during adulthood, puberty or minipuberty, an hCG stimulation test must be performed in the other age periods, or the diagnosis may be missed (11,13). In our patient, T levels did not increase with the hCG stimulation test and the T/A ratio was found to be low, suggesting 17β-HSD3 deficiency. Through imaging techniques, the observation of Wolffian structures and the absence of Mullerian structures are supportive in diagnosing 17β-HSD3 deficiency. However, since these findings are also present in both 5α-reductase deficiency and androgen receptor mutations, they are inadequate for a definitive diagnosis. In individuals with 17β-HSD3 deficiency, while histological examination can reveal near normal testicular structure at early ages, patients who reach adulthood with undescended testes usually display characteristics of testicular atrophy (exaggerated thickening of the basement membrane, evident decrease in the seminiferous tubule germinative epithelium, interstitial fibrosis, increased Leydig cells) (18). According to the literature, in the pathological examination of gonads that were removed for prophylactic measures, 2-3% of cases had germ cell tumors (19). In 40 patients diagnosed with 17β-HSD3 deficiency histological examination of testicular tissue stained with hematoxylin and eosin revealed that 5% of cases had germ cell tumors (18). On medical imaging, our patient had gonads in both inguinal canals that were compatible with testes and Müllerian structures were absent; moreover on the pathology report, the gonad biopsy was described as testicular tissue.

17β-HSD3 deficiency arises from the compound heterozygous or homozygous mutation of the *HSD17B3* gene. 17β-HSD3 deficiency shows an autosomal recessive inheritance pattern and is a frequent cause of 46,XY DSD among populations with high rates of consanguineous marriage. The *HSD17B3* gene consists of 11 exons and is located on chromosome 9q22. To date, more than 30 mutations have been identified in this gene, including insertion, exonic deletion, missense, and nonsense mutations (8,20,21,22,23). Most of these mutations have been identified in the Arab population of the Gaza strip. The most widespread mutation in the Arab population is the p.Arg80Gln mutation on exon 3 (4). In the Turkish population, c655-1;G-A, p.Ala188Val, and c.777-783del_GATAACC mutations have previously been identified (24). In a study by Özen et al (25), 20 patients being followed-up for 46,XY DSD, who did not have mutations in genes *SRD5A2* and *AR*, were analyzed using targeted next generation sequence (TNGS) analysis for 56 potential genes which may be involved in the etiology of 46,XY DSD. Mutations were identified in the *HSD17B3* gene in 30% of patients. It was reported that two patients had a homozygous p.Y287X variation, one patient had combined heterozygous p.R80Q and p.E93K variations, and three patients each had one homozygous p.T54A, p.R175T, or p.R80Q variation (25). The literature has reported no genotype-phenotype correlation in 17β-HSD3 deficiency (26). Our patient exhibited a c.673_1G>C homozygous class 2 (splice site) variation in intron 9 of the *HSD17B3* gene. This variant has not been previously reported in the Human Gene Mutation Database. *In silico* analyses (Human Splicing Finder; VarSome; Mutation- Taster; https://varsome.com) predicted this variant as likely to be pathogenic.Similar to other DSDs, gender selection proves to be a difficult decision in individuals with 17β-HSD3 deficiency, especially in cases diagnosed at an early age. The 2006 report of the Chicago Consensus Meeting recommends discussing both the fertility potential (unclear) and the development of sexual identity (mostly male) while determining sex in patients with 17β-HSD3 deficiency diagnosed during infancy (27). Male individuals with cryptorchidism and 17β-HSD3 deficiency show regression in spermatogenesis over time. These patients have an uncertain fertility potential, and a fertile 46,XY patient has not been previously identified. Gonads that are preserved should be lowered into the scrotum and routinely checked for malignancy (18,27). However, prepubescent gonadectomy is recommended for patients that are raised female because of the potential risks of germ cell tumors and virilization caused by a pubertal increase in androgens.

A significant proportion of female individuals (39-64%) who did not undergo gonadectomy and experienced virilization during their adolescence, later transitioned to the male sex (28). However, females who did undergo gonadectomy during their childhood were usually satisfied and very few individuals exhibited a desire for future sex change (4,8,29). It was reported that no individual with male-dominant phenotype who was raised as a male desired a change in sex (28).

It is crucial that every case is evaluated individually while trying to determine sex. Since our patient was diagnosed early and evaluations showed that the patient and family embraced the individual's male identity, T-based treatments were given and the patient awaited the response of the external genital structures to androgen therapy. We plan to determine the sex based on the patient's response to the treatment.

Conclusion

17β-HSD3 deficiency is an autosomal recessive form of 46,XY DSD. Although the diagnosis can be made with the appropriate endocrinological evaluations, it is confirmed by molecular genetic analysis. Our case showed a novel variation (c.673_1G > C homozygous) in the *HSD17B3* gene. 46,XY DSD should be considered in females who present with inguinal lumps and/or mild clitoromegaly during infancy or childhood, and in adolescent females who experience virilization during puberty. For a definitive diagnosis and subsequent genetic counseling, molecular analysis should be performed in cases with insufficient T synthesis and high androstenedione levels who are suspected of 17β-HSD3 deficiency. An early and accurate diagnosis is important for determining sex, patient management, and genetic counseling.

Ethics

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nurdan Çiftci, Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Concept: Nurdan Çiftci, Emine Çamtosun, Ayşehan Akıncı, Design: Nurdan Çiftci, Emine Çamtosun, Ayşehan Akıncı, Data Collection or Processing: Nurdan Çiftci, Analysis or Interpretation: Nurdan Çiftci, Emine Çamtosun, Ayşehan Akıncı, Literature Search: Nurdan Çiftci, Emine Çamtosun, Writing: Nurdan Çiftci, Emine Çamtosun, Ayşehan Akıncı.

Financial Disclosure: The authors declared that this study received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2021.2020.0269

J Clin Res Pediatr Endocrinol 2022;14(2):239-243

The Successful Treatment of Deep Soft-tissue Calcifications with Topical Sodium Thiosulphate and Acetazolamide in a Boy with Hyperphosphatemic Familial Tumoral Calcinosis due to a Novel Mutation in *FGF23*

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What is already known on this topic?

The central point of treatment in hyperphosphatemic familial tumoral calcinosis (HFTC) is to control the serum phosphorus level. In addition to low phosphate diet, medical therapies including phosphate binders, calcitonin, bisphosphonates, calcium channel blockers, corticosteroids, acetazolamide, probenecid and colchicine, have been used in different combinations. However, their success is variable and the main concern is the limited resolution of tumoral calcifications. Finally, topical sodium thiosulfate (STS) has been found to be effective only in superficial soft-tissue calcifications.

What this study adds?

We found a novel homozygous mutation in *FGF23* in a patient with HFTC and also used a new combined therapy with topical STS and acetazolamide. Deep soft-tissue calcifications resolved completely with this treatment, with no relapse for three years. The findings in our case suggest that the combination of topical STS and acetazolamide added to phosphate-lowering agents may be effective in resolving deep soft-tissue calcifications in HFTC.

Abstract

Hyperphosphatemic familial tumoral calcinosis (HFTC) is a rare autosomal recessive disorder. Topical sodium thiosulfate (STS) and acetazolamide can be a safe and effective treatment for patients who do not respond to conventional therapy for ectopic calcifications. We report the successful treatment of deep soft-tissue calcifications with topical STS and acetazolamide in a boy diagnosed with HFTC due to a novel homozygous mutation of *FGF23*.

Keywords: Hyperphosphatemic familial tumoral calcinosis, sodium thiosulphate, acetazolamide, tumoral calcinosis, children

Introduction

Hyperphosphatemic familial tumoral calcinosis (HFTC) (OMIM 211900) is a rare autosomal recessive disease characterized by decreased renal phosphate excretion, hyperphosphatemia, and tumor-like subcutaneous soft-tissue calcifications around large joints. This disorder is caused by inactivating autosomal recessive mutations in three genes, including fibroblast growth factor 23 (*FGF23*), polypeptide N-acetylgalactosaminotransferase 3 (*GALNT3*), and klotho (*KL*). Serum FGF23, encoded by *FGF23*, is responsible for the inhibition of both sodium phosphate cotransporter in proximal renal tubules and 1α- hydroxylase enzyme expression. So, FGF23 increases urinary phosphate excretion while decreasing serum 1.25-dihydroxy vitamin D [$1.25(OH)_2D$] level.

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Conflict of interest: None declared
Received: 12.11.2020
Accepted: 03.02.2021

Klotho, a co-receptor protein encoded by *KL*, together with FGF23 receptor should be intact to elicit these effects of FGF23. *GALNT* codes the enzyme named UDP-N-acetyl-alpha-D galactosamine or polypeptide N-acetylgalactosaminyltransferase-3. This enzyme protects intact FGF23 from enzymatic degradation. Therefore, any mutations in *GALNT3* lead to a decrease in serum FGF23 level. The net effects of these three intact genes are to decrease serum phosphorus and $1.25(OH)_2D$ levels. However, any inactivating mutation in these genes causes hyperphosphatemia and elevated serum $1.25(OH)_2D$ level due to decreased renal tubular phosphate excretion and increased 1α-hydroxylase activity, respectively (1,2,3,4). Serum calcium, alkaline phosphatase, and parathyroid hormone levels in the patients with HFTC are typically normal. Therefore, HFTC is considered to be the biochemical mirror of disorders that lead to excessive serum FGF23 levels, such as tumor-induced osteomalacia, X-linked hypophosphatemic rickets, and autosomal dominant hypophosphatemic rickets (5).

The treatment of HFTC is not standardised. The main components of treatment are low phosphate diet and drugs that bind phosphate or promote phosphate excretion from the kidneys. However, clinical response to these treatments is quite variable, and the medical treatment of ectopic calcifications can be difficult with conventional treatment (5,6). Surgical intervention can be performed in subjects with functional impairment or severe pain, but it is not routinely undertaken because calcinosis often recurs (7).

Here, we report the successful treatment of deep soft-tissue calcifications with topical sodium thiosulfate (STS) and acetazolamide in a boy diagnosed with HFTC due to a novel homozygous mutation of *FGF23*. The findings in this case suggest that the combination of topical STS and acetazolamide added to phosphate-lowering agents may be effective in resolving deep soft-tissue calcifications in HFTC.

Case Report

A 15-year-old boy who was followed up by the orthopedic clinic presented with a new, painful and progressive swelling in his right hip for a year. Surgical resections were performed four times in the last five years due to similar swelling in his left hip and both elbows. Histopathologic examination of the tissues was reported as tumoral calcinosis. He was not receiving any medical treatment. There was no history of fever, polyuria, kidney stones or fractures. His parents were third degree relatives. The rest of his family background was unremarkable. At admission, body weight and height were 46.0 kg [-1.8 standard deviation (SD)] and 157.5 cm (-1.8 SD), respectively. Physical examination revealed a 4x5 cm firm swelling in the right hip, which caused pain and limited hip range of motion with passive movements. Except for the surgical scars on the relevant joints, other examination findings were unremarkable. Laboratory tests, including complete blood count, urogram, blood gases, serum glucose, blood urea nitrogen, creatinine, sodium, potassium, magnesium, calcium, alkaline phosphatase, parathormone, 25-hydroxy vitamin D, liver and thyroid function tests, and spot urine calcium/creatinine ratio were within normal limits. Serum phosphorus level was 7.8 mg/dL (N = 2.5-5.0 mg/dL) (Table 1). X-ray and magnetic resonance imaging examination confirmed the extra-osseous calcification (Figures 1A, 2A, and 3A). Genetic analysis of *GALNT3* gene was normal. However, *FGF23* exon 1 analysis revealed a novel homozygous guanine-to-cytosine transversion at position 162 (c.162G > C), resulting in a novel glutamine (Q)-to-histidine (H) amino acid substitution at position 54 (p.Q54H) (Figure 4). The parents were heterozygous for the same variant and their clinic and laboratory findings were normal. A low-phosphate diet and an oral phosphate binding agent, sevelamer (40 mg/kg/d, three doses), were given. At the end of one year of this treatment, although the patient had good compliance with the conventional therapy, the size of the swelling did not regress or worsen, new lesions did not develop, and serum phosphorus level was 6.1 mg/dL (Table 1). At that time, oral acetazolamide (20 mg/kg/d) and

Table 1. Biochemical markers on admission and under therapy

Follow up	Ca (mg/dL)	P (mg/dL)	ALP (IU/L)	PTH (pg/mL)	25(OH)D (ng/mL)	Spot urine Ca/Cr
On admission (low phosphate diet and sevelamer was started)	10	7.8	186	18.9	20.2	0.06
12 months later (acetazolamide and STS cream were added to the therapy)	9.8	6.1	165	28.2	19.5	0.06
15 months later (continued with low phosphate diet + sevelamer, and acetazolamide + STS cream were stopped)	9.3	4.2	93	32	20.5	0.04
51 months later (low phosphate diet + sevelamer)	9.2	3.9	102	35	21	0.02

Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, PTH: parathyroid hormone, 25(OH)D: 25-hydroxy vitamin D, Cr: creatinine, STS: sodium thiosulfate

a topical cream consisting of STS ($Na_2S_2O_3$) dispersed into a Galen's cerate (cold cream, from 4/96 to 10/90 wt/wt) were added to the therapy. A thin layer of cream was applied over the swelling twice a day. The mass disappeared dramatically both clinically and radiologically after 3 months (Figures 1B, 2B, and 3B). The patient tolerated this treatment well and no side effects were detected. At that time, acetazolamide and topical STS treatments were discontinued while low phosphate diet and sevelamer treatments were continued. Three years later, there was still no calcification or newly developed lesion on the radiograph of the right hip (Figure 1C). The laboratory tests performed at first admission were repeated at each visit and serum phosphorus levels were within normal limits (Table 1).

Discussion

In most cases with deep soft-tissue calcifications due to HFTC, the therapeutic effects of drugs are either negligible or short-lived. Given these disappointing results, the combination of topical STS and acetazolamide added to phosphate-lowering agents, as in our case, appears to provide a promising contribution in resolving deep soft-tissue calcifications. Additionally, this case report broadens the spectrum of *FGF23* mutations.

FGF23, encoded by *FGF23*, is the primary regulator of extracellular phosphate concentration. FGF23 synthesized in bone is released into the circulation and causes urinary phosphate excretion by acting on the proximal renal tubule. In addition, FGF23 decreases renal production of $1.25(OH)_2D$ by inhibiting 1α-hydroxylase, thereby reducing intestinal phosphate absorption. Activating mutations in *FGF23* are

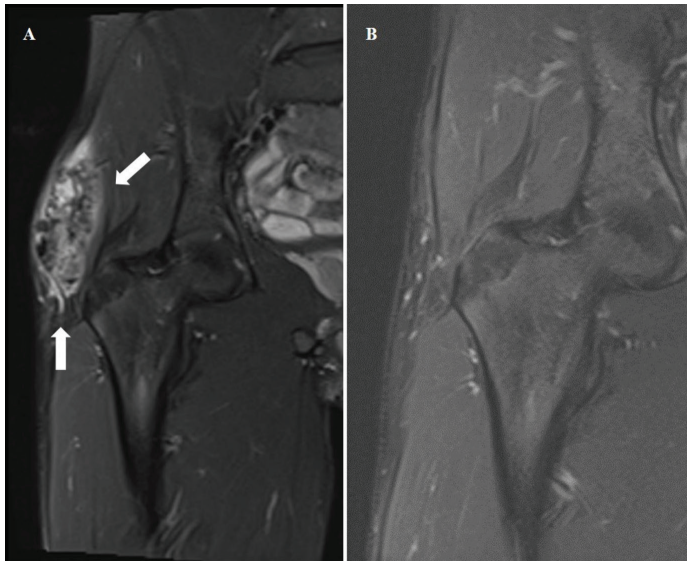

Figure 2. (A) Frontal plan magnetic resonance imaging showing soft tissue calcification around the right hip before the treatment (white arrows). (B) 3 months after topical STS and acetazolamide

STS: sodium thiosulfate

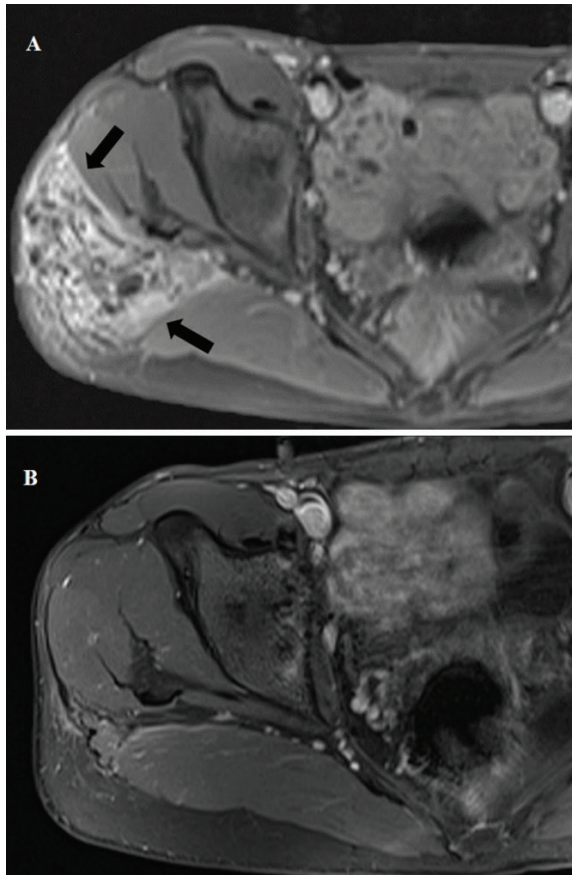

Figure 3. (A) Horizontal plan magnetic resonance imaging showing soft tissue calcification around the right hip before the treatment (black arrows). (B) 3 months after topical STS and acetazolamide

STS: sodium thiosulfate

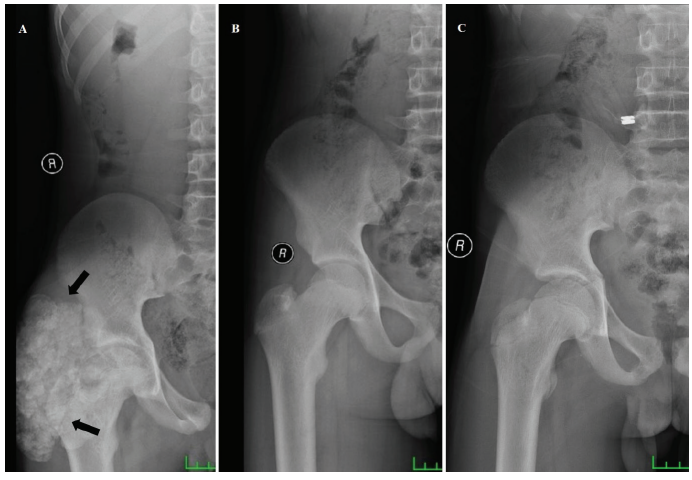

Figure 1. (A) Soft tissue calcification on the anteroposterior radiograph of the right hip before the treatment (black arrows). (B) 3 months after topical STS and acetazolamide. (C) 36 months after acetazolamide and topical STS treatments were stopped

STS: sodium thiosulfate

inherited in an autosomal dominant manner and lead to configurational changes that prevent inactivation of intact *FGF23*, resulting in autosomal dominant hypophosphatemic rickets. On the other hand, the mode of inheritance of inactivating mutations in *FGF23* is autosomal recessive and they cause inadequate FGF23 production, resulting in HFTC (1,8). A cytosine substitution for guanine at position 162 (c.162G > C) in *FGF23* exon 1 has been reported as a likely pathogenic heterozygous variant, coded rs193922701 in ClinVar and Mutation Taster databases for autosomal dominant hypophosphatemic rickets. However, our patient was homozygous for the same variant and this resulted in glutamine (Q)-to-histidine (H) amino acid substitution at position 54 (p.Q54H) and clinical and laboratory findings consistent with HFTC. In addition to these results, Garringer et al (9) reported the transversion of cytosine to adenine at position 160 (c.160C > A) in *FGF23* exon 1 of a patient with HFTC, resulting in glutamine (Q)-to-lysine (K) amino acid substitution at position 54 (p.Q54K). All these findings suggest that this codon encoding glutamine amino acid in *FGF23* exon 1 is sensitive to base changes. In order to evaluate the pathogenicity of the novel variant, we used *in silico* prediction tools, mutation databases (Human Gene Mutation Database and Clinvar), allele frequency in population studies [1000 Genome, Genome Aggregation Database (gnomAD)], segregation analysis and American College of Medical Genetics and Genomics criteria (10). We identified that this variant had not been found in genomAD and that its site had been a highly preserved region across species. We performed segregation analysis to establish the risk of disease for this variant and found that p.Q54H could segregate with the disease phenotype by causing the neutral-polar acidic amino acid to be replaced with a basic one. To the best our knowledge, our patient is the first

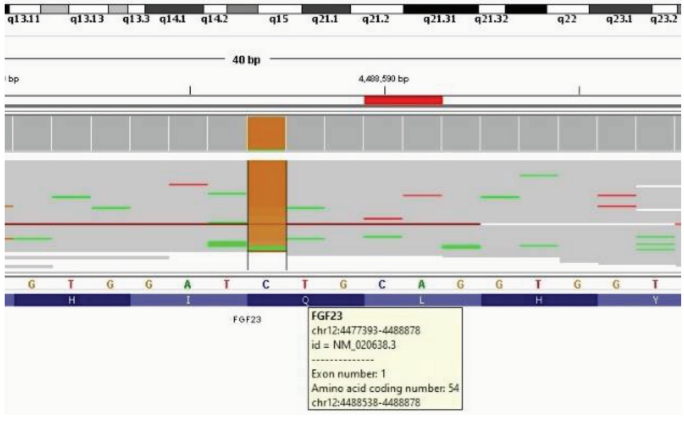

Figure 4. Sequence analysis of *FGF23*. A novel homozygous guanine-to-cytosine transversion at position 162 (c.162G > C), resulting in a novel glutamine (Q)-to-histidine (H) amino acid substitution at position 54 (p.Q54H)

case with HFTC resulting from a novel mutation (p.Q54H; c.162G > C) of *FGF23*.

Data on the optimal treatment of HFTC are very limited. This is because HFTC is a very rare disease and there are no randomized clinical trials. All the treatments described are derived from case reports or small case series with varying success rates, possibly due to heterogeneous patient population and non-standardized methods. In addition, the criteria for success in treatment are highly variable. Some studies focus on the treatment effect on tumoral calcification size and symptomatic improvement while others focus on laboratory measurements such as changes in serum phosphate or urinary phosphate excretion (11). The central point of treatment in HFTC is to control the serum phosphorus level and reduce pain. Unless calcinosis causes the restriction of joint motion, surgery is not recommended because of frequent relapses. In addition to low phosphate diet, medical therapies including phosphate binders, calcitonin, bisphosphonates, calcium channel blockers, corticosteroids, acetazolamide, probenecid and colchicine have been used in different combinations. They have variable and limited success in completely resolving tumoral calcifications due to not adhering to the difficult dose regimen (4,5,12). At the beginning of the treatment, we used a low phosphate diet and sevelamer as a phosphate binder. This approach prevented the progression of the swelling size and the formation of new tumors for a year. However, it failed to reduce the tumor size. Therefore, we added oral acetazolamide and topical STS to the treatment. Acetazolamide, a carbonic anhydrase inhibitor, causes phosphaturia by inducing proximal renal tubular acidosis (5). The exact mechanism of topical STS is unknown. However, it is suggested that STS induces calcium removal through chelation by creating metabolic acidosis and inhibits crystal formation and vascular calcification (6,13). The first study in which topical STS was used for the local treatment of ectopic calcifications in HFTC patients was published in 2016 (6). In that study the ratio of STS dispersed into a Galen's cerate was from 10/90 to 25/75 wt/wt and no acetazolamide was used. The authors realized that topical STS alone might be effective for superficial soft tissue calcifications, but not deep ones. We used acetazolamide to take advantage of its synergistic effect in combination with lower doses of topical STS (4/96 to 10/90 wt/wt) and found that this combined therapy was effective to completely eliminate deep soft-tissue tumoral calcifications in three months. This combination was also effective in keeping serum phosphorus levels within normal limits. These findings suggest that topical STS dosage should be determined individually, based on response to previous treatments, and topical STS may be more effective for deep soft-tissue calcifications when combined with

acetazolamide. The optimal duration of topical STS therapy has not been established and there are no data on possible relapse after cessation of therapy. However, our case shows that a 3-month treatment is sufficient to remove the lesions. In addition, despite the discontinuation of acetazolamide and topical STS treatments, no new calcification occurred for three years under low phosphate diet and sevelamer treatment. It seems reasonable to continue low phosphate diet and sevelamer therapy and to preserve acetazolamide and topical STS treatments for possible new or recurrent calcifications. However, it should be kept in mind that our findings are observational and the clinical course of the patient cannot be fully explained with this treatment. Therefore, prospective, controlled, multicenter studies are required to verify treatment efficacy and optimize the treatment procedure in children with HFTC.

Conclusion

In conclusion, this report broadens the spectrum of *FGF23* mutations. The soft-tissue calcifications in patients with HFTC may be difficult to treat with conventional drugs. At this point, topical STS and acetazolamide may be a safe and effective treatment. The dose of topical STS should be adjusted individually and its use with acetazolamide may be more effective in resolving deep soft-tissue calcifications.

Acknowledgments

We thank "Intergen Genetic Disorders and Diagnose Center" for genetic analysis in this patient.

Ethics

Informed Consent: Informed consent was obtained from the parents of the patient for publication of this case.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: Hakan Döneray, Ayşe Özden, Kadri Gürbüz, Design: Hakan Döneray, Data Collection or Processing: Hakan Döneray, Ayşe Özden, Kadri Gürbüz, Analysis or Interpretation: Hakan Döneray, Ayşe Özden, Literature Search: Hakan Döneray, Ayşe Özden, Writing: Hakan Döneray.

Financial Disclosure: The authors declare that this study has received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2021.2020.0175

J Clin Res Pediatr Endocrinol 2022;14(2):244-250

A Novel *SCNN1A* Variation in a Patient with Autosomal-recessive Pseudohypoaldosteronism Type 1

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What is already known on this topic?

Autosomal-recessive pseudohypoaldosteronism type 1 (PHA1) is a rare genetic disorder caused by different variations in the epithelial sodium channel (ENaC) subunit genes. Most of these variations appear in *SCNN1A*, mainly in exon eight, which encodes for the alpha subunit of the ENaC. Variations are nonsense, single-base deletions or insertions, or splice site variations, leading to mRNA and proteins of abnormal length. In addition, a few new missense variations have been reported.

What this study adds?

We report a novel mutation [c.729_730delAG (p.Val245Glyfs*65)] in exon 4 of the *SCNN1A* gene in a case of autosomal recessive PHA1. A patient with PHA1 requires early recognition, proper treatment, and close follow-up. Parents are advised to seek genetic counseling and plan future pregnancies.

Abstract

Pseudohypoaldosteronism type 1 (PHA1) is an autosomal-recessive disorder characterized by defective regulation of body sodium (Na) levels. The abnormality results from mutations in the genes encoding subunits of the epithelial Na channel. Patients with PHA1 present in infancy as being in adrenal crisis. A 41-day-old female who presented with recurrent adrenal crisis did not adequately respond to hydrocortisone and required mineralocorticoid therapy. The patient's demographic data and clinical features were recorded. Blood samples were collected and tested for endocrine and metabolic characteristics and for use in genetic studies. Bidirectional Sanger sequencing of *SCNN1A* was conducted. The entire coding region of 12 exons and 20 bp of flanking intron were sequenced. Genetic analyses revealed a new mutation - c.729_730delAG (p.Val245Glyfs*65) - in *SCNN1A* exon four. Adrenal crisis during the neonatal period highlights the importance of early screening for PHA1. Genetic testing could help to anticipate the prognosis, severity, onset of the disease, and the mode of inheritance, especially given its extensive phenotype.

Keywords: Pseudohypoaldosteronism, hyperkalemia, hyponatremia, adrenal crisis, congenital adrenal hyperplasia

Introduction

Pseudohypoaldosteronism type 1 (PHA1) is a rare disorder of mineralocorticoid resistance that is characterized by defective regulation of body sodium (Na) levels because of the inability of aldosterone to exert its effect on target tissues, aldosterone resistance. PHA1 is a life-threatening disease that presents during the neonatal period with dehydration, severe salt wasting, and failure to thrive, accompanied by hyponatremia, hyperkalemia, metabolic acidosis, and increased plasma renin, with an elevated aldosterone level that is consistent with aldosterone resistance despite normal renal and adrenal functions (1,2). PHA1 can be divided into renal (PHA1A, OMIM

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Conflict of interest: None declared
Received: 14.10.2020
Accepted: 03.02.2021

#600983) and systemic (PHA1B, OMIM #264350) types based on its physiologic and genetic characteristics. Renal type PHA1A is an autosomal-dominant disorder with heterogeneous inactivating mutations in the *NR3C2* gene that codes for the aldosterone receptor (3,4). The renal autosomal-dominant type is characterized by Na loss that is restricted to the kidneys. It is usually less severe with gradual clinical improvement during the first several years of life, thus allowing Na supplementation to be terminated at some point. In contrast, the systemic PHA1 type has an autosomal-recessive inheritance pattern caused by inactivating mutations in any subunits of the epithelial Na channel (ENaC) encoded by SCNN1A, -1B, -1G (5). The systemic autosomal-recessive type typically presents shortly after birth. It is characterized by Na loss from the kidneys and other mineralocorticoid target tissues, such as the colon, lungs, salivary glands, and sweat glands, which increases the frequency of lower respiratory tract infections. The disorder is more common and severe in infancy, persists into adulthood, and requires lifelong therapy using Na supplementation (6,7).

There are no reports of the frequency of PHA1 in Arab populations or the Middle East. However, Al-Shaikh reported two Omani children who presented with PHA1 during their first week of life, both of whom came from a consanguineous family. The first child (a male) had a severe clinical course with skin manifestations, recurrent episodes of severe chest infections, and electrolyte imbalances. Feeding difficulty and treating the electrolyte imbalances were challenges for the treating physician. A genetic analysis was not conducted in this case. The second case (a female) had a milder clinical course with fewer episodes of respiratory infections. Her genetic analysis revealed a new mutation in the *SCNN1A* ENaC subunit (8).

Case Report

A 41-day-old female was referred to our hospital as a case of possible congenital adrenal hyperplasia. She was a product of a consanguineous marriage and an uneventful pregnancy. The patient was born at a gestational age of 37 weeks by normal spontaneous vaginal delivery with a birth weight of 3 kg without complications. At the age of six days, she began to have difficulty feeding, lethargy, and frequent vomiting. She was taken to the emergency room with severe dehydration and acidosis (arterial pH = 7.20; serum bicarbonate = 11 mmol/L), high potassium (K) of 10 mmol/L, and low Na of 119 mmol/L. Cardiopulmonary resuscitation was initiated and the patient was intubated. The treating team considered the possibility of an adrenal crisis and conducted initial hormonal studies (Table 1). The patient was treated with a stress dose of hydrocortisone, along with a fluid bolus and Na bicarbonate ($NaHCO_3$). Hyperkalemia was managed with calcium gluconate, insulin, and glucose. The patient was admitted to the neonatal intensive care unit and tested for sepsis and metabolic issues, both of which were negative. She was later discharged from the hospital and treated with hydrocortisone and fludrocortisone with no Na chloride (NaCl).

This patient had an elder sibling and a cousin who died with similar presentations. At the age of 20 days, the newborn was readmitted to the hospital with episodes of vomiting, poor feeding, and lethargy and was found to have high K and low Na levels. The conditions were managed using a stress dose of hydrocortisone, a fluid bolus, and $NaHCO_3$. Hyperkalemia was managed using calcium gluconate, insulin, glucose, and emergency peritoneal dialysis to lower her K levels. After the patient was stabilized, she was referred to our hospital for further investigation and management. On examination, the patient was conscious, well hydrated and was not dysmorphic. She had normal female genitalia and showed no systemic abnormalities.

Our initial lab results revealed Na: 138 mmol/L, K: 5 mmol/L, and bicarbonate (HCO^-_3) 23 mmol/L. The urinalysis and urine cultures were normal. A fluorescence *in situ* hybridization study was sent from the primary hospital, which was negative for the sex-determining region gene, and chromosomal analysis revealed an XX female karyotype. The results of abdominal and pelvic ultrasound examinations were normal. The results of hormonal estimations are provided in Table 1.

Initially, the patient's symptoms were managed with hydrocortisone and fludrocortisone treatment for possible congenital adrenal hyperplasia. However, because the initial lab results for hormone levels (Table 1) were not consistent with congenital adrenal hyperplasia, and the patient had presented with recurrent adrenal crisis since the neonatal period, she did not adequately respond to hydrocortisone and required mineralocorticoid therapy. In addition, follow-up examinations revealed papular skin rashes and

Table 1. Results of initial hormonal studies at presentation

Investigation	Results	Normal range
17-hydroxyprogesterone, ng/dL	72	5-115
Serum renin activity, nmol/L/hr	23	4-12
Serum aldosterone, pmol/L	4244.2	1000-3800
Serum ACTH, pmol/L	5	1.6-13.9
Serum cortisol, µg/dL	94.1	2.3-11.9
DHEA-s, µg/dL	13	5-35

DHEA-s: dehydroepiandrosterone sulfate, ACTH: adrenocorticotropic hormone

recurrent chest findings, which suggested a diagnosis of pseudohypoaldosteronism. A sweat-chloride test showed elevated levels (128 mmol/L; normal range, 0-39 mmol/L for patients >4 months).

The present study was approved by the research and ethics committee of King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia (IRB log number: 18-609).

The patient's demographic data and clinical symptoms were recorded. Blood samples were collected and tested for endocrine and metabolic characteristics and for use in genetic studies. Molecular genetic testing was conducted initially for *NR3C2* sequencing for autosomal-dominant pseudohypoaldosteronism. Bidirectional Sanger sequencing of *SCNN1A* was then conducted. The entire coding region of 12 exons and 20 bp of flanking intron were sequenced. Informed consent for all tests was obtained from the patient's parents.

The results of the hormone tests on the patient are presented in Table 1. Molecular genetic testing for *NR3C2* sequencing for autosomal-dominant pseudohypoaldosteronism wereperformed and did not show any sequence variant that may have caused the disease. Based on the majority of reported mutations, Bidirectional Sanger sequencing of *SCNN1A* was then conducted. The results revealed that the patient was homozygous for a novel mutation at c.729_730delAG in exon 4 of *SCNN1A* (Figure 1),which was predicted to result in a frameshift and premature protein termination (p. Val245Glyfs*65).

Using *in silico* prediction tools, such as SIFT-indel and Mutation Taster, this variant is predicted to be deleterious (Table 2). Moreover, the amino acid valine in position 245 is highly conserved among different species (Figure 2). This variant is not found in gnomAD (PM2).

Targeted sequencing for this variant was conducted in both parents, who were found to be carriers for the sequence variant c.729_730delAG (p.Val245Glyfs*65) in *SCNN1A* in heterozygous form.

Discussion

"Adrenal crisis" was the initial impression, which included adrenal hypoplasia and congenital adrenal hyperplasia, and was based on the patient's clinical presentation and a history of poor feeding, lethargy, frequent vomiting, consanguinity, family history of sudden infant death, hyperkalemia, hyponatremia, and metabolic acidosis. The patient was

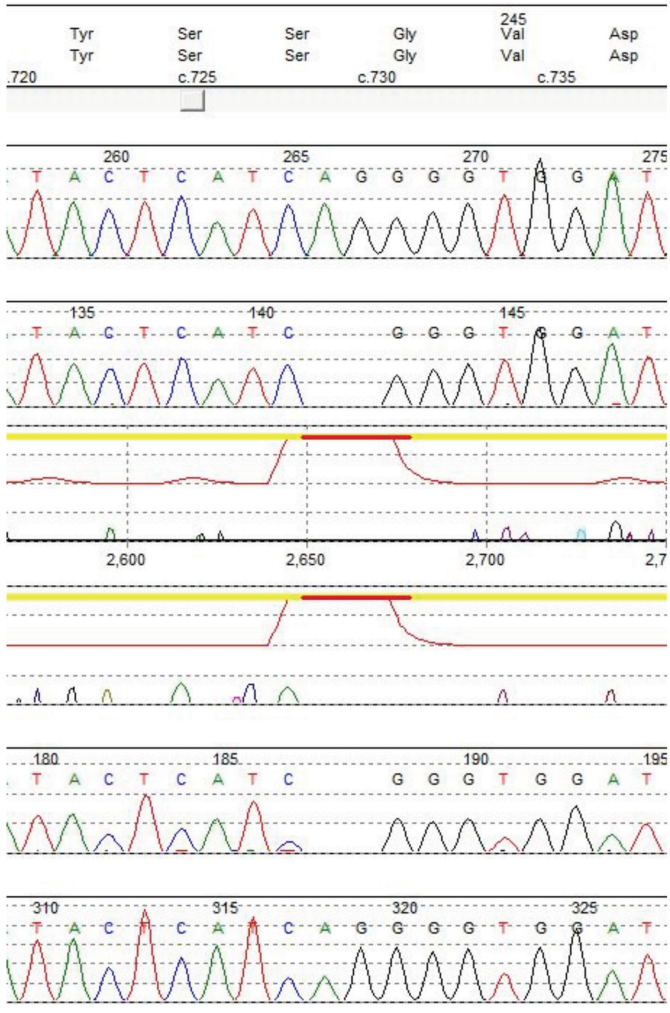

Figure 1. Electropherogram of the novel variant in *SCNN1A*: c.729_730delAG

Table 2. *In silico* analysis of the *SCNN1A* gene mutation founded in our patient and ACMG classification

Mutation (nucleotide)	Protein change	Mutation Taster	SIFT-indel	gnomAD Exomes MAF	ACMG criteria	Classification
c.729_730delAG	p.Val245Glyfs*65	Disease causing	Damaging	-	PVS1 PM2 PP3	Pathogenic

PVS1 (very strong): Null variant (frame-shift), in gene *SCNN1A*, for which loss-of-function is a known mechanism of disease (gene has 8 pathogenic LOF variants and LOF Z-score = 1.97 is greater than 0.7), associated with pseudohypoaldosteronism, type 1, Liddle syndrome 3 and bronchiectasis with or without elevated sweat chloride 2.
PM2 (moderate): Variant not found in gnomAD exomes (unable to check gnomAD exomes coverage).
Variant not found in gnomAD genomes (good gnomAD genomes coverage = 30.5).
Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium.
PP3 (supporting): Pathogenic computational verdict based on 1 pathogenic prediction from GERP vs no benign predictions.

accordingly treated with hydrocortisone. She had normal female genitalia, hyponatremia, and hyperkalemia with normal androgen precursor levels but a high level of plasma aldosterone, renin activity, and cortisol, all of which were more consistent with PHA1 than congenital adrenal hyperplasia.

Other differential diagnoses, based on the results of the initial electrolyte levels, included other causes of hyperkalemia and hyponatremia, including secondary PHA, which is a transient form of aldosterone resistance secondary to a urinary tract infection. A urinary tract malformation was also a possibility, but was unlikely because the results of the urine tests and ultrasound were normal. Renal tubular acidosis type 4 is characterized by low levels of ammonia in the urine and is associated with hyperkalemia, mild hyponatremia, and metabolic acidosis with hyperaldosteronism or aldosterone insensitivity.

A poor response of the patient to corticosteroids indicated aldosterone resistance and either renal or systemic PHA1. Patients with PHA1 usually present in the neonatal period with hyponatremia, hyperkalemia, and metabolic acidosis, and the condition can be diagnosed by significantly elevated plasma aldosterone levels and renin activity (9,10,11).

The clinical manifestations of renal PHA1 may vary in asymptomatic patients and can only be diagnosed using the presentation of hyperaldosteronism and elevated renin levels compared with patients presenting with a salt-wasting crisis. The clinical characteristics and severity of this condition can vary widely. Severe phenotype has been reported due to mutations in both alleles in the *NR3C2* gene (12). Overall, PHA1 has a mild clinical course followed by remission over time (13,14,15,16,17). The systemic form of PHA1 leads to salt loss from organs that express ENaC, such as the kidneys, salivary and sweat glands, and the colon. Children typically present shortly after birth with electrolyte disturbances mimicking an adrenal crisis (4). Acute initial presentation, frequency of respiratory symptoms, and a positive sweat-chloride test led to the diagnosis of systemic PHA1 in our patient.

In vitro screening of several mutant genes has led to advances in understanding the physiology of the mineralocorticoid receptors and ENaC. Therefore, the underlying molecular pathology of renal and systemic PHA1 has been associated with mutations in the mineralocorticoid receptor and ENaC subunit genes, *SCNN1A*, *SCNN1B*, and *SCNN1G*, respectively (11). Aldosterone plays a central role in electrolyte homeostasis and maintenance of fluid in the distal nephron. Loss-of-function mutations in two key components of the aldosterone response-the mineralocorticoid receptor and ENaC-lead to PHA1, a rare genetic disease of aldosterone resistance characterized by salt loss, dehydration, failure to thrive, hyperkalemia, and metabolic acidosis (14).

The prickly heat-like skin rash in our patient, which was typically aggravated by salt deprivation, is a characteristic feature of systemic PHA1. As a consequence of ENaC expression, this manifestation is consistent with recent studies that have reported that skin manifestations are common in systemic PHA1 patients, as are other phenotypic features, such as cholelithiasis, polyhydramnios, and hypercalciuria leading to hypocalcemia (18,19,20,21,22,23,24).

A sweat-chloride test is positive in both systemic PHA1 and cystic fibrosis because both disorders lead to salt loss from the sweat glands. The gold standard for a diagnosis of cystic fibrosis is based on a clinical diagnosis plus an abnormal sweat-chloride test and the determination of a mutation in the cystic fibrosis transmembrane conductance regulator gene *CFTR*. Therefore, based on our patient's initial clinical presentation and her symptom improvement after salt supplementation and K exchange resins, cystic fibrosis was a less-likely diagnosis and a

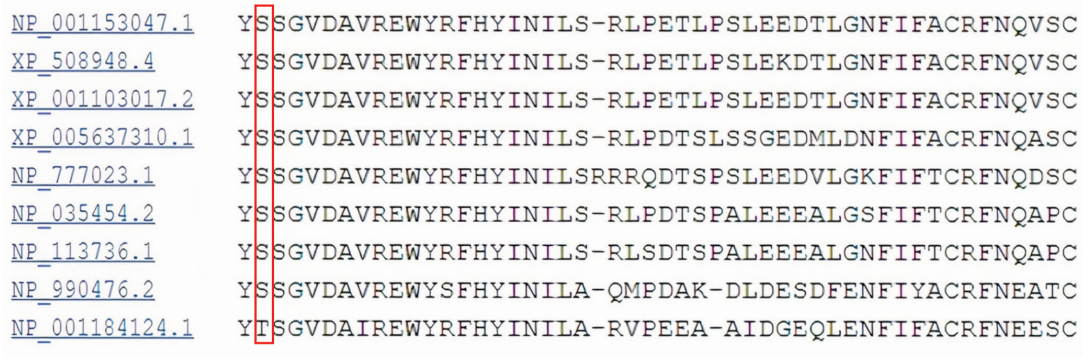

Figure 2. Conservation of the amino acid (valine) at position 245 of the amino acid sequence among different species

NP_001153047.1: H. sapiens, XP_508948.4: P. troglodytes, XP_001103017.2: M. mulatta, XP_0ç05637310.1: C. lupus, NP_777023.1: B. taurus, NP_035454.2: M. musculus, NP_113736.1: R. norvegicus, NP_990476.2 :G. gallus, NP_001184124.1: X. tropicalis

CFTR mutation analysis was not necessary in this case (25). *CFTR* encodes for a membrane protein and chloride channel, which play important roles in transepithelial salt absorption and secretion in some epithelial tissues, such as the intestine, airways and sweat glands, and reproductive system. Studies have shown that *CFTR* modulates the function of ENaC in salt absorption, but the mechanism is still controversial. *CFTR* mutations lead to an inadequacy in the *CFTR*-ENaC relationship, which may lead to increased Na + absorption in the airways, resulting in hypersecretion of mucus, decreased mucociliary clearance, and bacterial colonization (26,27,28).

PHA1 was first reported by Cheek and Perry in 1958 as a sporadic occurrence with a severe salt-wasting syndrome. Since 1958, the literature has mainly consisted of case reports or case series but the number of patients with PHA1 has increased over the past decade.. Based on HGMD, more than 71 *NR3C2*, 42 *SCNN1A*, 47 *SCNN1B*, and 20 *SCNN1G* mutations have been reported. Most of the reported mutations were single-base deletions/insertions, or splice-site mutations with severe courses while missense mutations usually follow a mild course. Several new mutations have been reported with phenotypic variations (23,25). In 2017, Nur et al (24) reported one case and reviewed 27 others from clinical presentation to follow-up and outcome. In 2018, Guran (26) summarized the diagnosis and current management of PHA1.

To the best of our knowledge, this is the first recognized case of PHA1 with a new mutation that results in a frameshift and premature protein termination (p. Val245Glyfs*65), which leads to a sequence variant designated as c729_730delAG in *SCNN1A* exon four. This finding confirms the hypothesis that an autosomal-recessive or a sporadic PHA1 systemic type is a genetically heterogeneous disease involving other unidentified genes (15). However, null variant (frame-shift), in *SCNN1A* is a known mechanism of disease, single nucleotide deletion in the same coding region has been classified as pathogenic SCNN1A:c.729delA which leads to different frameshift (p.Val304TrpfsTer4). This reported mutation is associated with lung symptoms (recurrent lower respiratory tract infections) without development of chronic lung disease, which is a similar finding to our case (29).

Renal type 1 PHA1, an autosomal-dominant disease resulting from heterogeneous mutations in the *NR3C2* gene that encodes for the mineralocorticoid receptor, results in a condition of aldosterone not being able to bind to its receptor. Severe phenotype has been reported due to mutations in both alleles in the *NR3C2* gene. Approximately 50 distinct mutations have been reported, but there have been no reports on genotype/phenotype correlation (11). In contrast, systemic type 1 PHA1 is an autosomal-recessive disorder that typically presents during the neonatal period with severe symptoms, such as hyponatremia, hyperkalemia, and metabolic acidosis. This condition can be diagnosed by significantly elevated plasma aldosterone levels and renin activity with the symptoms persisting into adulthood (15,17,18).

Treatment of PHA1 should be considered a challenging emergency, and life-saving measures are needed to rectify dehydration, replace Na loss, and correct hyperkalemia and acidosis during the acute phase (4).

Renal PHA1 can be managed with salt supplementation, which is often decreased over the lifetime of the patient. Although systemic PHA1 requires aggressive management, including salt supplementation, intensive fluid administration, K exchange resins, and dietary manipulation to reduce hyperkalemia, the use of indomethacin may be beneficial in some patients and when treating systemic complications of the disease. The course of the disease is often interrupted by recurrent episodes of salt-loss crisis, with hyperkalemia associated with nausea, vomiting, and difficulty feeding, which might require gastrostomy/jejunostomy feeding, as well as a chronic pulmonary syndrome, with or without associated chest infections (18,19).

Na supplementation may be given in the form of $NaHCO_3$, NaCl (1 g contains ~17 mEq Na), or Na citrate. Up to 110 mEq/kg/d can be given. Generally, it is difficult for patients to tolerate a high dose of salt orally so the dose intake can be divided and mixed with baby formula and food. It may need to be administered by nasogastric tube/jejunostomy in poor tolerance situations (18,26).

Severe hyperkalemia and acidosis can be managed by the intravenous slow infusion of $NaHCO_3$ and then oral replacement when the condition stabilizes. A combination of glucose and insulin infused slowly at the usual ratio of 1 unit of insulin to every 5 g dextrose is an effective measure by which to reduce serum K. Salbutamol infusion or continuous nebulization has been added as adjunctive therapy. In severe persistent hyperkalemia, peritoneal dialysis is used, after which calcium chloride is infused to protect the heart from arrhythmias until the serum K levels are normalized.

K exchange resins, which act as K-binding agents, are widely used in hyperkalemia management. The most commonly used K-binding agents are Na polystyrene sulfonate and calcium polystyrene sulfonate. Usually the Na-containing resin is preferred to the calcium-containing resin because it simultaneously corrects both hyponatremia and

hyperkalemia. It is used orally or rectally at approximately 1-2 g/kg/dose every six hours when high doses administered orally cannot be tolerated. In these cases, rectal administration of the drug is a good option (20,26).

Genomic testing for a carrier state in the asymptomatic siblings and the parents is highly recommended to detect homozygosity or compound heterozygosity (8). One limitation to the present study was that we did not measure testosterone levels during the patient's hospitalization.

Conclusion

Adrenal crisis during the neonatal period highlights the importance of early screening for PHA1 to facilitate early recognition, proper treatment, close follow-up, and genetic counseling for the family. Parents should be advised to seek genetic counseling and testing when planning future pregnancies.

Ethics

Informed Consent: Informed consent for all tests was obtained from the patient's parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

Financial Disclosure: The authors declared that this study received no financial support.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2021.2021.0151

J Clin Res Pediatr Endocrinol 2022;14(2):251-257

Bilateral Ovarian Germ Cell Tumor in a 46,XX Female with Nijmegen Breakage Syndrome and Hypergonadotropic Hypogonadism

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**Contributed equally to the study*
***Contributed equally to the study as mentors*

What is already known on this topic?

Nijmegen breakage syndrome (NBS) is an autosomal recessive disease characterized by chromosomal instability and increased risk of various malignancies, including solid tumors. Pure gonadal dysgenesis (PGD) resulting in hypergonadotropic hypogonadism is frequently encountered in females with NBS. Disorders of sex development, including PGD, are associated with an increased risk of germ cell tumors. The risk of ovarian germ cell tumors seems not to be significantly increased in NBS females with PGD therefore a routine prophylactic oophorectomy is not recommended in these patients.

What this study adds?

In 46,XX NBS females diagnosed with ovarian germ cell tumor, an early prophylactic oophorectomy of the remaining nonfunctional streak ovary should be considered to exclude or avoid second germ cell tumor. Early detection and complete resection of ovarian malignancy in female NBS patients may enable successful treatment without the need for adjuvant chemotherapy, which should be avoided in patients with NBS as it significantly increases the risk of development of subsequent cancers.

Abstract

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disease, affecting mainly patients of Slavic origin. It is caused by a defect in the *NBN* gene, resulting in defective nibrin protein formation. This leads to chromosomal instability, which predisposes to cancer, with lymphoid malignancies predominating. Nibrin is also involved in gonadal development and its disfunction in females with *NBS* frequently results in a pure gonadal dysgenesis (PGD) causing hypergonadotropic hypogonadism. However, only a few ovarian tumors in NBS patients have been reported to date. We describe the first case of a girl with NBS with PGD, who developed metachronous bilateral ovarian germ cell tumors (dysgerminoma and gonadoblastoma). Pathogenesis of PGD, neoplastic transformation and therapeutic approach in females with NBS are discussed.

Keywords: Pure gonadal dysgenesis, solid tumor, gonadoblastoma, dysgerminoma, children, cancer predisposition

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Conflict of interest: None declared
Received: 01.06.2021
Accepted: 25.08.2021

Introduction

Nijmegen breakage syndrome (NBS; OMIM#251260, ORPHA:647) is a rare autosomal recessive disease characterized by chromosomal instability, similar to that of ataxia-telangiectasia, with preferential involvement of chromosomes 7 and/or 14 in a variable proportion of analyzed metaphases. The majority of patients are of Slavic origin with founder effect for the most common pathogenic variant, namely NM_002485.5(*NBN*):c.657_661delACAAA (p.Lys219Asnfs*16; rs587776650) (previously referred to as c.657_661del5) in exon 6 of the *NBN* gene. The *NBN* gene encodes nibrin, the crucial component of the of the Mre11/Rad50/*NBN* (MRN) complex, responsible for repair of DNA-double-strand breaks. A genetic defect in this gene causes defective nibrin protein formation, which in turn impairs G2/M checkpoint control during the cell cycle. This results in spontaneous chromosomal instability and hypersensitivity to ionizing radiation (1,2).

The hallmark features of NBS patients include microcephaly, distinct dysmorphic facial features (sloping forehead, prominent midface and receding mandible), growth retardation in the first years of life, *café au lait* spots and some minor developmental defects (3). While microcephaly is a dominating feature, mental capabilities of NBS patients may not be affected in infancy and early childhood, but cognitive functions usually worsen with age (1). Combined immunodeficiency of both the cellular and humoral arms of the immune system is an essential feature of NBS (4) and increased susceptibility to severe infections, particularly of the respiratory tract, is commonly observed (5).

Malignancies are the most frequent cause of mortality in NBS patients (6). The risk of malignancy is approximately 50-fold higher, with risk for lymphomas 250-fold higher than in general population (5,7,8). In NBS, lymphoid malignancies also occur significantly more frequently than in other chromosome-breakage disorders, such as ataxia-telangiectasia, Bloom syndrome, and Fanconi Anemia (5,6,7). Malignant solid tumors are uncommon, constituting less than 10% of cases. Among them, medulloblastoma and perianal rhabdomyosarcoma predominate (6,9,10), while breast cancer, prostate cancer, thyroid cancer, neuroblastoma and melanoma have been reported occasionally (6,11).

Evidence of hypergonadotropic hypogonadism affecting females with NBS has been reported, based on a longitudinal observation of a large cohort of Polish patients and anecdotal case-reports (12,13). Interestingly, homozygous and compound heterozygous pathogenic variants in *NBN* gene have also been found in patients with isolated infertility, lacking other typical NBS features. The infertility was caused either by premature ovarian insufficiency (14,15,16) or by oligo-terato-asthenozoospermia (15). These reports suggest that nibrin may play a role in normal development and function of gonads, and explain the cause and pathomechanism of gonadal insufficiency in NBS patients lacking function of this protein (12).

Various specific forms of disorders of sex development, in which pure gonadal dysgenesis (PGD) is one of examples, are prone to development of germ-cell tumors. In NBS, only one case of ovarian gonadoblastoma and one of dysgerminoma have been previously reported (6,11). However, heterozygous carriers of c.657_661del5 variant have been shown to be at increased risk of developing rare types of ovarian cancers and other malignancies (17,18,19).

In this report, we document a girl with NBS who developed bilateral ovarian germ cell tumor. The first clinically appeared as a dysgerminoma and, two years later, gonadoblastoma was detected contralaterally in the prophylactic ovariectomy.

Case Report

A 13-year-old girl with NBS was admitted to the Department of Pediatrics, Hematology and Oncology of the University Clinical Center in Gdańsk, Poland, in 2007 due to an asymptomatic tumor of the left ovary, detected by screening ultrasonography (US) imaging. At the age of eight years, the patient had been diagnosed with NBS, based on typical clinical features, including: microcephaly; typical facial features; growth retardation; immunodeficiency; and recurrent respiratory tract infections. The diagnosis of NBS was confirmed by molecular testing, detecting a homozygous five base-pair deletion NM_002485.5(*NBN*):c.657_661delACAAA in exon 6. The family history of the patient was remarkable. Among five siblings she had an older sister with very similar clinical features, suggestive of NBS, but never confirmed molecularly. In 1999, at the age of 17 years, her sister died of rapidly progressing, abdominal, diffuse large B-cell lymphoma. Parents are both Polish and not related.

On admission, our patient was in a good condition, assessed as Tanner stage I. Her height was 146 cm, weight 35 kg, head circumference 49 cm and chest circumference 60 cm - all measurements were below the 3rd percentiles. US examination showed a solid and well demarcated tumor of the left ovary, measuring 35x30x26 mm. There was an abundant pathological vasculature invading from the periphery of the lesion and a very low resistivity index of 0.51. The presence of a localized ovarian tumor was confirmed on magnetic resonance imaging (MRI) (Figure 1a). No lymph nodes and distant metastases were detected. The serum levels of lactate dehydrogenase, uric acid, C-reactive

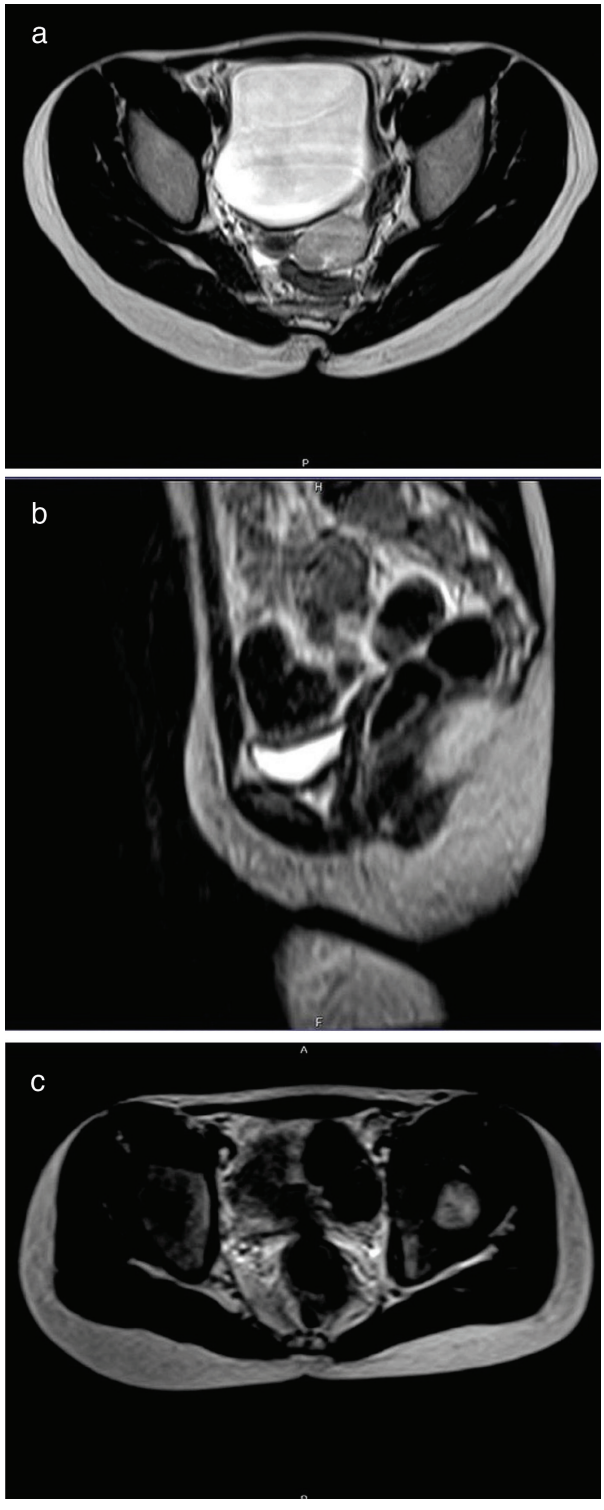

Figure 1. a) Axial T2-weighted magnetic resonance (MR) scan shows well demarcated hyperintense pathological mass in the left ovary, which does not infiltrate the ovarian capsule. The tumor is oval and homogeneous. b) Sagittal T2-weighted MR image shows a small hypoplastic uterine remnant in the anatomic location of the uterus. c) Axial T2-weighted MR image shows a hypoplastic ovary on the right side (the longest diameter of the ovary is 13 mm). The morphology of the right ovary is normal without any visible pathological structure inside

protein, beta-chorionic gonadotropin alpha-fetoprotein and carcinoembryonic antigen were within normal ranges for age. The suspicion of ovarian non-Hodgkin lymphoma was raised, and a laparoscopic left oophorectomy was performed at the Department of Pediatric Surgery, Gdańsk. Histopathology revealed total ovarian involvement by dysgerminoma with a typical immunophenotype. The ovarian capsule was not infiltrated and no angioinvasion was detected (Figure 2a). The right ovary was found to be normal on intraoperative macroscopic evaluation. According to the International Federation of Gynecologists and Obstetricians the tumor was classified as stage 1A, which did not require adjuvant oncologic therapy.

After the surgery, the patient remained under regular follow-up by a pediatric oncologist, with screening US of the abdomen and pelvis performed every 3 months and MRI every 6 months. No recurrence of the left ovarian tumor was found. However, both a small uterus and a streaked hypoplastic right ovary were recorded in subsequent examinations (Figure 1b, c). Finally, at the age of 15 years, primary amenorrhea with no secondary signs of puberty was diagnosed in the girl. Based on serum hormone levels, including follicle-stimulating hormone (FSH) - 131.32 mIU/L, luteinizing hormone - 35.86 U/L, and estradiol < 37 pmol/L, hypergonadotropic hypogonadism was diagnosed and right ovary dysplasia was suspected. Cytogenetic studies revealed female karyotype with a number of distinct, nonclonal aberrations involving chromosome 7 and 14 in a significant proportion (13%) of the cells analyzed. A paracentric inversion of chromosome 14 was also present in all metaphases studied. No *SRY* signal was detected by fluorescence *in situ* hybridization.

After multidisciplinary consultation, it was decided to remove the remaining non-functioning ovary and to start the patient on hormone replacement therapy. The small right (1.5x1.0 cm) gonad was resected by laparoscopy, histologically showing gonadal dysgenesis with gonadoblastoma. The gonad contained fibrous, focally evident ovarian type stroma with scattered granulosa cell groups and dispersed microcalcifications. Moreover, Sertoli cell nodules, as well as germ cells accompanied by Sertoli/granulosa cells in cord-like structures were present. Neither primordial follicles nor seminiferous tubules were present. Coexisting gonadoblastoma nests presented typical immunophenotype with Oct 3/4, placental alkaline phosphatase, CD117, and inhibin positivity (Figure 2b, c, d, e, f).

The patient was started on hormone replacement therapy, and in three months she developed secondary sexual characteristics (breast Tanner stage 3 and growth gain of 5

cm). On follow-up US examinationsa few months later, an enlargement of the uterus from 11x5.7x22 mm to 31x14x55 mm and prominent endometrium were detected. Currently, this 27-year-old patient is alive and free of any symptoms of cancer recurrence. She is under the care of a hematologist and on continuous immunoglobulin supplementation. However, after she turned 18 and was discharged from pediatric care, she discontinued hormone replacement therapy. In a recently performed US examination, her uterus size was 24.5x11.6x30.5 mm, with no visible endometrium. An informed consent from the patient and her parents to publish this case-report has been obtained.

Discussion

NBS is a rare and possibly still underdiagnosed disease. It has been estimated that approximately 20-30% of patients are diagnosed with neoplasms prior to the diagnosis of this syndrome. Our patient was diagnosed with NBS soon after her sister's death from cancer, based on their specific phenotypic similarity. This led to discontinuation of repeated diagnostic X-rays of chest and paranasal sinuses during her recurrent infections. These procedures are now highly contraindicated in NBS patients, due to their hypersensitivity to ionizing radiation and the high risk of malignancy induction (20). Since then, our patient underwent regular prophylactic US examinations, leading to the discovery of an asymptomatic left ovarian dysgerminoma at an early stage.

Gonadal dysgenesis is defined as an incomplete or defective formation of gonads, resulting from a disturbed process of migration of the germ cells and the organization of the primitive gonads. It is caused by aberrations of sex chromosomes or defects in genes involved in the formation of the urogenital ridge and sex determination of the primarily bipotential gonad (21). Gonadal dysgenesis predisposes to

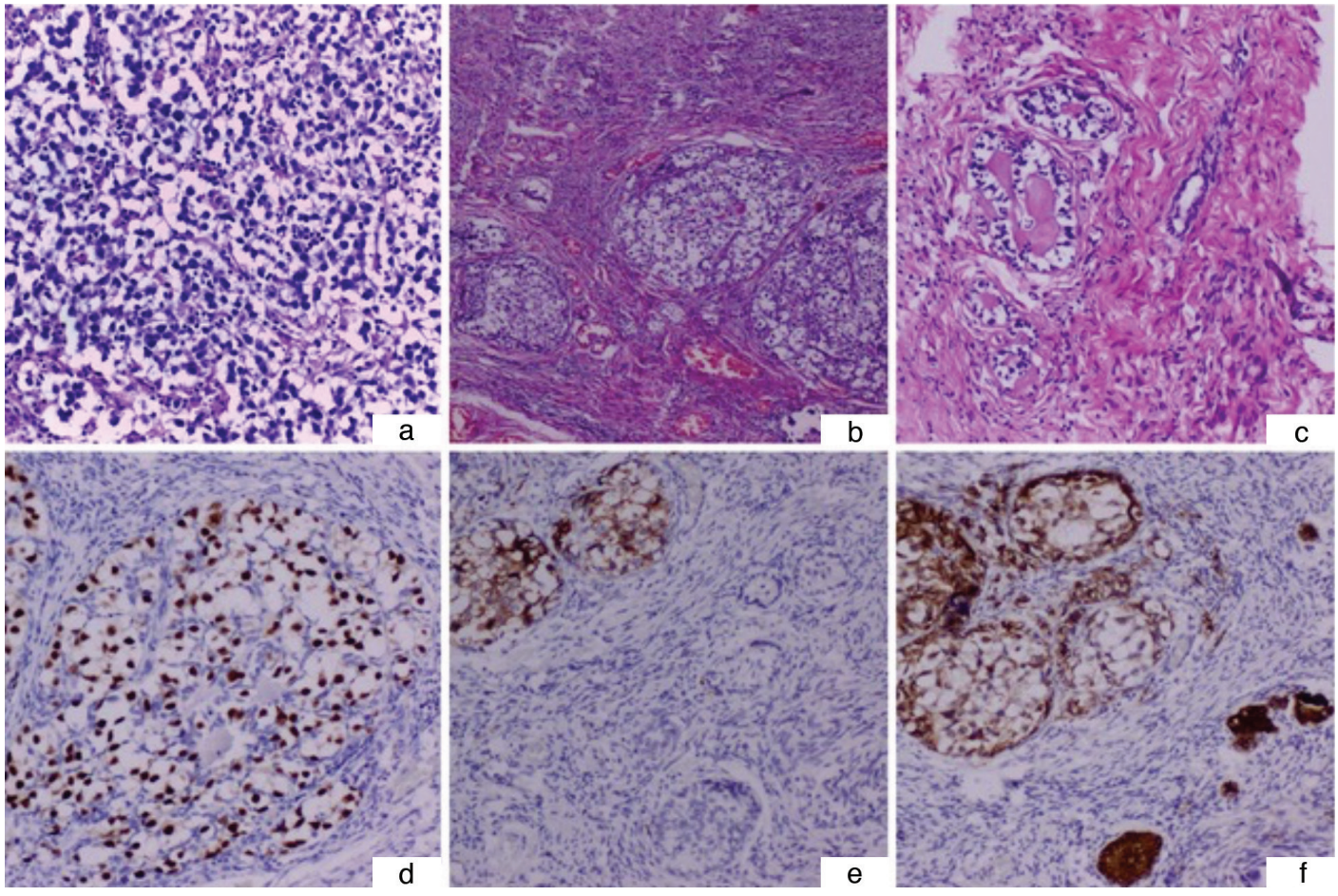

Figure 2. a) Histology of the dysgerminoma from the left ovary [hematoxylin-eosin (HE) 200x]. b) Dysgenetic gonad containing gonadoblastoma foci within the ovarian type stroma (HE 100x). c) A small group of tubular structures of sex cord cells arranged around the hyaline cores, scattered in the stroma of the dysgenetic gonad (HE 400x). d) Oct 3-positive germ cells within gonadoblastoma structures (Oct 3, 400x). e) CD117 expression within the germ cell component of gonadoblastoma, and immunonegative sex cord elements within the surrounding gonad (CD117, 200x). f) Inhibin alpha staining present within the sex cord element of gonadoblastoma and stromal sex cord structures (inhibin, 200x)

the development of gonadoblastoma and dysgerminoma, particularly in patients with 46,XY karyotype (22). However, rare cases of these tumors in patients with female 46,XX karyotypes have been reported (23,24). Capito et al (25) suggested that a highly elevated serum FSH level might be an indirect marker of suspected ovarian tumor in patients with PGD. Gonadal dysgenesis resulting from defective nibrin function is a typical feature of females with NBS.

Dysgerminomas can be associated with many genetic syndromes, such as: Frasier; ataxia-telangiectasia; WAGR (Wilms tumor, aniridia, genitourinary anomalies and intellectual disability); and Apert syndromes (26,27,28,29). However, to the best of our knowledge, only one case of dysgerminoma in a patient with NBS has been reported to date (6). In our patient, the 46,XX karyotype was confirmed; however, numerous abnormalities involving chromosomes 7 and 14 were detected in a significant percentage of cells. These aberrations are characteristic for NBS patients, reflecting the flag mark of the disorder - chromosomal instability. In our patient, along with the typical abnormalities of chromosomes 7 and 14 in 13% of cells, all metaphases revealed the presence of pericentric inversion of chromosome 14. It is generally acknowledged that aberrations involving chromosomes 7 and 14 are present in 10-50% of the cells (30). To some extent, the rate observed in our patient might be age-related, resulting from simple accumulation of individual aberrations over time. This patient was cytogenetically evaluated at the age of 20 years, while most of the published NBS patients had karyotype performed in early childhood. We cannot exclude, however, the parental origin of chromosome 14 inversion, because the parents were unavailable for genetic testing at that time.

The prognosis and treatment of dysgerminomas depend on their pathological and clinical stage. Patients with disease limited to one ovary may be treated by unilateral oophorectomy alone, especially when fertility is to be maintained. However, in patients with PGD, early bilateral gonadectomy is recommended to avoid the risk of subsequent neoplasm development. In our patient, no secondary signs of puberty with primary amenorrhea were observed at the age of 15. On US and MRI, performed repeatedly after left ovariectomy, the hypoplastic uterus and right ovary were described. With very low estradiol levels and significantly elevated FSH concentrations, hypergonadotropic hypogonadism was diagnosed and right ovarian dysplasia was suspected. Due to the history of dysgerminoma in the left ovary, a decision to perform prophylactic right oophorectomy was made, following the rules established for PGD XY females (Swyer syndrome) (25). Histopathological examination of the resected right ovary confirmed its dysgenetic features and revealed areas of gonadoblastoma. The surgery was complete so the patient did not require adjuvant oncological therapy and remains cancer-free. Hormone replacement therapy produced a satisfactory clinical effect with induction of secondary sexual characteristics.

Our patient is the first case with metachronous ovarian gonadoblastoma and dysgerminoma developing within the dysgenetic ovaries, occurring in a patient with NBS and 46,XX karyotype. Although it seems that nibrin may play a role in normal development and function of the ovary, only one case of gonadoblastoma and one of dysgerminoma in NBS patients have been previously reported to date (6,11).

The optimal management of NBS females with hypergonadotropic hypogonadism suggesting PGD is controversial. In 2010 Chrzanowska et al (12) published results of a longitudinal study showing that primary ovarian insufficiency with associated hypergonadotropic hypogonadism are common and typical features in females with NBS. The authors suggested that careful, but minimally invasive diagnostics, including auxiological follow-up, US examinations and basic hormonal assays are sufficient in these patients. Accordingly, Huang et al (24) demonstrated within a group of 33 patients with XX PGD, that the risk of malignant transformation in case of absence of the Y chromosome is low. Therefore, in XX PGD patients prophylactic gonadectomy is not recommended and only careful follow-up should be provided. However, in patients in whom ovarian germ cell tumor has already been diagnosed within one gonad, we advocate for early prophylactic oophorectomy of the remaining nonfunctional streak ovary to exclude or avoid a second germ cell tumor. This recommendation is particularly meaningful for XX, PGD females with NBS because early detection and complete resection of malignancy in these patients gives a chance for successful outcome without need of adjuvant chemotherapy, which is extremely risky in terms of development of subsequent cancers. Such a strategy for clinical management was adopted in our patient, resulting in a long-lasting clinical remission of metachronous bilateral ovarian germ cell tumors.

Acknowledgements

We would like to express our appreciation to professor Krystyna H. Chrzanowska from the Department of Medical Genetics, the Children's Memorial Health Institute in Warsaw, Poland for providing valuable meritorical support during the analysis of this patient. We would like to thank the students of The English Division Pediatric Oncology Scientific Circle,

Medical University of Gdańsk: Marta Kedrzycki and Sylwia Mackiewicz for their help in preparing the case report.

Ethics

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Malgorzata A. Krawczyk, Dorota Birkholz-Walerzak, Mariola Iliszko, Beata S. Lipska-Zietkiewicz, Wojciech Kosiak, Ninela Irga-Jaworska, Ewa Bien, Concept: Malgorzata A. Krawczyk, Malgorzata Styczewska, Ewa Izycka-Swieszewska, Ewa Bien, Design: Malgorzata A. Krawczyk, Malgorzata Styczewska, Data Collection or Processing: Malgorzata A. Krawczyk, Malgorzata Styczewska, Wojciech Kosiak, Analysis or Interpretation: Malgorzata A. Krawczyk, Malgorzata Styczewska, Dorota Birkholz-Walerzak, Mariola Iliszko, Beata S. Lipska-Zietkiewicz, Wojciech Kosiak, Ninela Irga-Jaworska, Ewa Izycka-Swieszewska, Ewa Bien, Literature Search: Malgorzata A. Krawczyk, Malgorzata Styczewska, Dorota Birkholz-Walerzak, Mariola Iliszko, Beata S. Lipska-Zietkiewicz, Writing: Malgorzata A. Krawczyk, Malgorzata Styczewska, Dorota Birkholz-Walerzak, Mariola Iliszko, Beata S. Lipska-Zietkiewicz, Wojciech Kosiak, Ninela Irga-Jaworska, Ewa Izycka-Swieszewska, Ewa Bien.

Financial Disclosure: The authors declared that this study received no financial support.

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