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ECE 2017 (19th European Congress of Endocrinology)
20-23 May 2017, Lisbon, Portugal

ECO 2017 (24th European Congress on Obesity)
17-20 May 2017, Porto, Portugal

ESPE 2017 (10th International Meeting of Pediatric Endocrinology)
14-17 September 2017, Washington, DC, USA

ISPAD 2017 (43rd Annual Conference, International Society for Pediatric and Adolescent Diabetes) October 18-21, 2017, Innsbruck, Austria

REVIEW

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Non-Classical Congenital Adrenal Hyperplasia in Childhood

Selim Kurtoğlu, Nihal Hatipoğlu

Erciyes University Faculty of Medicine, Department of Pediatric Endocrinology, Kayseri, Turkey

Abstract

Congenital adrenal hyperplasia (CAH) is classified as classical CAH and non-classical CAH (NCCAH). In the classical type, the most severe form comprises both salt-wasting and simple virilizing forms. In the non-classical form, diagnosis can be more confusing because the patient may remain asymptomatic or the condition may be associated with signs of androgen excess in the postnatal period or in the later stages of life. This review paper will include information on clinical findings, symptoms, diagnostic approaches, and treatment modules of NCCAH.

Keywords: Non-classical congenital adrenal hyperplasia, congenital adrenal hyperplasia, virilization, hirsutism

Introduction

Congenital adrenal hyperplasia (CAH) is a group of diseases which develop as a result of deficiency of enzymes or cofactor proteins required for cortisol biosynthesis (1,2,3,4,5). Due to cortisol deficiency, feedback control mechanism at hypothalamic and hypophyseal levels remains unsatisfactory, a defect which leads to an increase in adrenocorticotropic hormone (ACTH) production and consequently to adrenal hyperplasia (3). CAH is classified as the classical and non-classical types. The classical type constitutes the majority of the cases and results from 21-hydroxylase deficiency (21-OHD), which can present as the simple virilizing or as the salt-wasting types. While there is almost no enzyme activity in the cases presenting with salt wasting, the ratio of enzyme activity corresponds to 1-2% in simple virilizing types and to 20-50% in non-classical types (4).

Non-classical CAH (NCCAH) includes a series of diseases occurring due to gene mutations or disorders in the steroid synthesis steps of steroidogenic acute regulatory protein (StAR) providing the transfer of cholesterol from mitochondrial membrane to the cell. Although NCCAH occurs in the deficiencies of 21-OHD, 11β-hydroxysteroid dehydrogenase (11β-*HSD*) and 3-beta hydroxysteroid dehydrogenase (3β-*HSD),* and StAR mutations, it is most commonly observed in 21 and 11-β OHSD deficiencies. Its prevalence is reported as 1/1000 (6). However, the disease is observed in higher rates among Jewish, Mediterranean, Middle Eastern, and Indian societies (7).

Findings of genital virilization are not observed at birth in NCCAH patients. Although premature pubarche was detected in a 6-month-old infant as the earliest example, clinical findings and symptoms in NCCAH cases usually start from the age of 5 and usually emerge in late childhood, adolescence, and adulthood (8,9). Results of an analysis of 220 female patients with NCCAH showed that the clinical presentation started before the age of 10 in 11% and between the ages of 10-40 in 80% of the cases and that premature pubarche was the first symptom in 92% of the cases under 10 years of age (10). Increased androgen levels constitute the main basis of the clinical symptoms, but mild cortisol deficiency can also occur in some cases. Clinical findings include labial adhesion, perianal hair, clitoromegaly, increased penile length with prepubertal testicular volume, increased bone age, premature pubarche (development of pubic and axillary hair before the age of 8 and 9 for girls and boys, respectively), axillary apocrine odor, precocious puberty, greasy hair, acne, prepubertal gynecomastia, diffuse hair loss in centro-parietal and/or fronto-temporal regions, hoarseness of voice, menstrual irregularities (oligomenorrhea, anovulation, dysfunction), hirsutism, abortions, infertility in both genders, adrenal rest tumors, and short height according to midparental

Address for Correspondence: Nihal Hatipoğlu MD,
Erciyes University Faculty of Medicine, Department of Pediatric Endocrinology, Kayseri, Turkey
Phone: +90 505 578 05 37 **E-mail:** nihalhatipoglu@yahoo.com

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target height (11,12,13,14,15). In a prospective longitudinal study of Swedish children from birth to 18 years, it was demonstrated that permanent teeth eruption occurred at a mean age of 5.7±0.52 in these patients (16). Dental development may also occur at early ages (at ages 4-5 years) in these patients (17).

In a multicenter study including adolescents and adult females, hirsutism, oligomenorrhea, and acne were found in 59%, 54%, and 33% of the subjects, respectively (18). Tallness, accelerated bone maturation, development of premature pubic and axillary hair, and adult apocrine odor are findings which may be noted in both girls and boys in the prepubertal period. Heterozygote non-classical *21-hydroxylase* gene mutation was detected in 8.3% of the girls with premature pubarche (19). *CYP21A2* mutation was detected in 4.7% of the 126 subjects (122 girls, 4 boys) with premature pubarche, hirsutism, or polycystic ovary syndrome (PCOS) presentation (20). Increased penile length can be observed among boys. Prepubertal gynecomastia and adrenocortical incidentaloma are very rare findings detected in male cases (21,22). However, some NCCAH cases may be asymptomatic. In a study (23) which analyzed 330 family members of cases with mutation in terms of phenotype/genotype, a homozygote or compound heterozygote mutation was found in 51 relatives, and 42 of these relatives were clinically asymptomatic. A heterozygote mutation was found in 242 cases and it was observed that 37 cases were unaffected. In this study population, the most common genotypes for homozygote and compound heterozygote mutations were the V281L and V281L/IVS2-13A/C > G, respectively. The parents of the children whose diagnosis was certain were detected as undiagnosed symptomatic individuals.

Pubertal girls affected with NCCAH typically present with hirsutism (24,25). It is speculated that functional changes occur in the hypothalamic-pituitary-ovarian axis of these patients and that these changes lead to increased progesterone and/or 17-hydroxyprogesterone (17-OHP), androgens, 5-α reductase expression in the ovaries and/or directly lead to an increase in corticoid production. Excessive androgen impairs the progesterone sensitivity of the hypothalamus and increases luteinizing hormone (LH) secretion with rapid gonadotropin-releasing hormone (GnRH) pulses (26). Production of androgen from ovarian theca cells increases due to LH hypersecretion and contributes to the hyperandrogenemia. NCCAH due to 21-OHD was detected in 4.9% of 123 adult females who presented with severe heavy acne (27). Two studies on NCCAH from Turkey are worth mentioning. In one study, it was reported that among 285 females with a presentation of premenopausal hyperandrogenemia in Central Anatolia, the frequency of NCCAH due to 21-OHD was 2.1% (28). In another study performed in a similar area, 9.52% of 63 hirsutism cases (43 and 20 of these cases were diagnosed as PCOS and idiopathic hirsutism, respectively) were found to have non-classical 21-OHD (29). Akinci et al (30) have reported that among adolescent patients (age range 13 to 19 years) with hirsutism, 21-OHD NCCAH was detected in only one case (3%). It is known that *in utero* androgen exposure occurs in classical CAH cases, but this is not observed in NCCAH cases (21).

Diagnostic Studies in Non-classical Congenital Adrenal Hyperplasia

It may be difficult to distinguish the clinical symptoms and findings of premature adrenarche from those of PCOS in girls (20). Although a high 17-OHP level is diagnostic in classical CAH cases, this finding may be insufficient for a diagnosis of NCCAH. Therefore, the ACTH test is accepted as the gold standard for a diagnosis of NCCAH.

Non-classical 21-OHD: It should be noted that basal 17-OHP should be measured in the morning hours (06:00-08:00 a.m.) on an empty stomach and at the follicular phase in menstruating females (between the 3rd and 5th postmenstruation days) (21). This is because the 17-OHP value exceeds 2 ng/mL in the luteal phase in half of healthy females (31). Boys suspected of NCCAH should be tested immediately (32). A basal value between 1.7 and 3.0 ng/mL is sufficient for diagnosis (31,33,34). In a study in which late-onset 21-OHD was detected in a rate of 3.2% in 186 children diagnosed with premature pubarche, a basal 17-OHP level of 1.55 ng/mL was suggested as the cut-off value (35). The consensus regarding basal 17-OHP concentration for ACTH test indication is reported as 2 ng/mL. A basal 17-OHP level over 5 ng/mL is regarded as a quite high value (14). NCCAH was found in 4.2% of 238 French children with premature adrenarche and it was understood that a basal 17-OHP level over 2 ng/mL demonstrates 100% sensitivity and 99% specificity (36). After estimation of basal 17-OHP levels, ACTH is applied intramuscularly or intravenously in a dose of 250 mg/1.73 m^2 and a second sample is taken after 60 minutes. The majority of researchers agree that a 17-OHP level over 10 ng/mL at the 60th minute of ACTH application is a criterion for diagnosis of late-onset 21-OH deficiency and this conclusion is in agreement with results of genetic studies (37). However, some authors suggest that 12 ng/mL should be the cut-off limit (5). A 21-deoxycortisol value in addition to 60th minute 17-OHP level with ACTH test for late-onset 21-OHD being exceeding 400 pg/mL is also taken as criterion (38). ACTH and corticotropin-releasing hormone (CRH) are not high in these cases. Furthermore, total and free

testosterone levels and the levels of sex hormone-binding globulin (SHBG), cortisol, and 11-deoxycortisol should be measured at baseline and at the 60th minute. Generally, the value of 60th minute cortisol with ACTH test is expected to be >18 mg/dL. However, it is also important to detect the cases below this limit. Stoupa et al (39) reported that cortisol values measured in the ACTH test were below 18 mg/dL in 60% of 47 children with late-onset 21-OHD. If the cortisol level remains below 18 mg/dL, it should be noted that these cases may be under risk of adrenal deficiency in stress situations.

Non-classical 11β-HSD deficiency: Basal 11-deoxycortisol level is over 10 ng/mL in late-onset 11-OH deficiency (40). According to Reisch et al (40), cut-off values for basal deoxycortisol level are 6.95 ng/mL and 7.23 ng/mL for prepubertal and pubertal cases, respectively. When 60th minute 11-deoxycortisol level is higher than 18 ng/mL in the ACTH test, the diagnosis becomes definitive (5).

Non-classical 3β-HSD deficiency: In 3β-HSD cases, the criteria for diagnosis consist of a basal 17-OH pregnenolone level above 30 ng/mL and a 17-OH pregnenolone/cortisol ratio above 10 SD (5). In girls with oligomenorrhea, anti-Müllerian hormone (AMH) increase may be detected before hyperandrogenemia (41).

Another point to take into account during basal tests is presence of a secondary biosynthetic defect. Eldar-Geva et al (42) detected 3β-HSD, 21-OH, and 11β hydroxylase mutations in 12.3%, 10%, and 8% of 170 females presenting with hirsutism, respectively. They observed partial 11β-OH deficiency in 21-OH cases and partial 3β-HSD deficiency in 11β-HSD cases. These authors suggested that this incident which they named as secondary biosynthetic defect may be associated with intra-adrenal accumulating androgens.

Another point to discuss about diagnostic criteria is the measurement methods. Around the world in general, 17-OHP and 11-deoxycortisol levels are usually measured by immunoassay [radioimmunoassay (RIA), immunochemiluminometric assay (ICMA), electrochemiluminescence immunoassay (ECLIA)] methods. Interference is a common problem in RIA methodology, so there is a need for improvement in purification and extraction methodology. Recently, more reliable and accurate results were reported with liquid chromatography coupled with mass spectrometry (LC-MS/MS) devices (41,43). Ambroziak et al (44) reported a study in which hormone levels were measured by immunoassay and LC-MS/MS methods in 39 females with hyperandrogenism presentation and 29 females in a control group. Total testosterone, dehydroepiandrosterone sulfate (DHEA-S), androstenedione, and 17-OHP were measured with immunoassay and LC-MS/MS methods and it was understood that the values measured with immunoassay methods were higher. It was reported that 85% of the patients were subjected to unnecessary tests and investigations due to high 17-OHP. Moreover, analyzing the urinary steroid metabolites of the cases with capillary gas chromatography/mass spectrometry in selective ion monitoring mode (GC/MS-SIM) device is important. The diagnosis can be definitive by measuring a 17-OHP metabolites in the urine, namely 17-hydroxy pregnenolone (17-OHP, normal value is 63-279 mg/24 hours), pregnanetriol (PT, normal value is 179-992 mg/24 hours), 21-deoxycortisol metabolite pregnanetriolone (PTN, normal value is 3.5-50 mg /24 hours) (44). In addition to these tests, these same authors conducted urinary steroid metabolite studies and genetic tests on 40 adult female patients (age range of 18-39 years) with hyperandrogenism presentation. An ACTH test was conducted in patients with a basal 17-OHP level of 1.7-10 ng/mL. The cases detected to have a basal and post-ACTH stimulation 17-OHP value over 10 ng/mL were subjected to genetic testing for urinary metabolites in 24-hour urine and late-onset 21-OH deficiency type. 17-OHP levels of 21 cases were determined to be over 10 ng/mL after ACTH, but only five of the cases (24%) were diagnosed definitely with NCCAH by using urinary steroid profile and genetic data. The diagnoses based on basal and post-ACTH 17-OHP levels over 10 ng/mL at the end of the study are not conclusive, and since 75% of the results are false-positive, it is suggested that definitive diagnosis should be obtained by using urinary steroid profile and genetic studies (45). Another point to be noted is that 11-deoxycortisol and cortisol demonstrate a cross-reactivity with a rate of 23.3% among measurement methods. Thus, Xu et al (46) reported that the level of Immulite 2000 and cortisol can be false-low, whereas 11-deoxycortisol level can be false-high.

Genetic Studies

Genetic studies in classical and non-classical CAH cases are needed. Siblings and parents should also subjected to genetic analyses. Since the most common type is 21-OH, *CYP21A2–CYP21A1P* mutations are generally investigated. Null 12 G splice, I172N, P3OL, V28IL, P453S.Int2 mutations are observed most commonly and they are positive in 73-87% of the cases (14,22,34,47). NCCAH phenotype is detected in 98 % of the cases with mutation V28IL. 11-β hydroxylase (*CYP11β1*) and 3-β hydroxysteroid dehydrogenase (*HSD3β2*) and StAR mutations observed more rarely should be studied (48).

Differential Diagnosis

Tumors producing androgen leading to premature pubarche presentation, androgen exposure, premature

adrenarche, cortisone reductase deficiency, and DHEA sulphosphotransferase deficiency should be considered in the differential diagnosis (9,21). Indeed, a case diagnosed with NCCAH in early years was reported to have an ovarian steroid cell tumor (49).

The cases observed to have StAR mutation (lipoid adrenal hyperplasia) known as NCCAH can be considered to be cases of familial glucocorticoid deficiency (50). Also, if enzyme P450SCC separating the side chain of cholesterol undergoes a partial defect, it can be mistaken for lipoid adrenal hyperplasia which is a kind of NCCAH (51).

Screening

It is known that NCCAH cases usually cannot be detected during CAH screenings made in the neonatal period. Held et al (52) have reported that detection rates of non-classical 21-OHD at the first and second screenings during neonatal screening for CAH were 1/217 573 and 1/32 465, respectively.

Treatment Planning

If bone age is found to be advanced in a prepubertal girl or boy with NCCAH, final height loss can be prevented by stopping the progress with hydrocortisone. At this decision, diagnosis, bone age, and time of start of treatment need to be considered. Cases in whom treatment was initiated one year prior to onset of puberty and who had a bone age below 9, final height remained within the genetic potential (53). The generally accepted approach is to initiate hydrocortisone treatment in cases observed to have prepubertal growth acceleration or apparent advancement of bone age. A second indication for starting treatment with hydrocortisone depends on the finding that the cortisol level measured at the 60th minute of the ACTH test does not exceed 18 mg/dL (54).

Another issue is to administer growth hormone (GH) and GnRH agonist treatment for their additive effect on final height. In a study where GH and GH + GnRH agonists were given to prepubertal 3 cases and pubertal 3 cases, improvement in predictive heights were observed when compared to an untreated group (55). However, it is generally accepted that routine GH and GnRH should not be given to these patients before the emergence of the signs and symptoms of central precocious puberty or unless predictive adult height is below 2 SD value of the average population value (54).

Hydrocortisone is administered in a dose of 6-15 mg/m^2/day, divided into 3 doses (21). In treatment of adolescent patients with hydrocortisone, several points need to be considered. First, in adolescents, compliance tends to decrease with time. Secondly, while the half-life of hydrocortisone corresponds to 80 minutes during pre/post-pubertal period, it falls to 40 minutes in puberty (2). This is because the increasing IGF-1 level decreases 11-β OHSD type-1 activity and also increases cortisol clearance due to increase in glomerular filtration rate. Adrenal would be suppressed with the administration of hydrocortisone, so in cases of inflammatory disease, surgical operation, and trauma, corticoid should be administered in stress doses (21). When the patients receiving hydrocortisone treatment reach adolescence, the treatment can be ended if there are no findings of hyperandrogenism such as hirsutism, acne, and oligomenorrhea. At this time, an ACTH test is also done to control hypothalamic-pituitary-adrenal axis. If hyperandrogenism findings are clear, administering 0.25 mg of dexamethasone at night is recommended (54). Some clinicians suggest that hydrocortisone treatment should be continued for another 2-3 years in the post-menarcheal period, since girls initially have anovulatory cycle after menarche (2). When boys reach Tanner stage 3 (testis volume 8-10 mL), hydrocortisone is discontinued and normal development of pubertal height is ensured (2). If peak cortisol level of pubertal and adult females measured after ACTH is below 18 mg/dL, steroid treatment is administered only in cases of stress. Insufficiency of adrenomedullary functions does not occur in these cases (56). However, if an increase in levothyroxine or hyperthyroidism occurs, adrenal crisis may arise as the result of increased clearance of cortisol (57). In cases with hirsutism, treatment with oral contraceptive agents leads to an increase in SHBG production in the liver, to a decrease in androgen release from the ovary, and consequently to improvement in menstrual irregularity (14). If necessary, anti-androgens (spironolactone, flutamide, cyproterone acetate, or finasteride) may be added to the treatment. Cosmetic approaches such as laser application and depilatories can also be suggested (2,15,21). In some cases, hydrocortisone treatment can be continued if oral contraceptives and anti-androgens cannot be tolerated or when hyperandrogenemia is quite severe (2).

Transfer to Adult Endocrine Units

The issue of transferring the patients who have completed their adolescent years to adult endocrine units is usually neglected. In this transfer process, the medical and social problems of each patient needs to be well investigated and the information needs to be transferred in detail. The "Kieler Modell" created by Kruse et al (58) can be taken as an example on this subject. The process of transferring to adulthood takes place at ages 17-18 years. It is suggested that contact informing meetings attended by the pediatric and adult teams should be held a year before this process and that the cases should be monitored together. Moreover, the specialists of endocrinology, gynecology, urology, and psychiatry should be encouraged to participate in the meetings organized to introduce the cases and share the information (54,58).

Long-term Problems

In NCCAH cases, a series of problems such as acne, oligomenorrhea, hirsutism, abortion-stillbirth, deep voice in women, infertility, impaired life quality, psychiatric problems (psychosis, suicide, alcohol use, drug use), decrease in bone density, fractures, obesity, dyslipidemia, insulin resistance, diabetes, increase in the thickness of intima media, hypertension, cardiovascular problems, early mortality risk, and tumorigenesis risk may arise (14,59). Although testicular adrenal rest tumors are observed in classical CAH cases, it is reported that they may also occur in NCCAH cases (60). It is known that rest tissue originates from adrenal stem cells. In some cases, when the treatment is insufficient, testicular adrenal rest tumors develop with ACTH stimulation. These tumors are hypoechoic and smooth marginated. Infertility occurs as a result of testosterone production deficiency and oligospermia as a result of mass compression. Therefore, periodic testis ultrasonograhic follow-up is suggested for NCCAH cases (60). Development of rest tissue and tumor in ovaries is less frequent (61). NCCAH may also be the cause of adrenal incidentaloma cases, but the frequency of this complication is not known (15). Androgenic or feminizing adrenal tumors can rarely be seen in NCCAH cases (62,63).

Fetal Problems

The overall rate of miscarriages in NCCAH patients is 20%. NCCAH also increases the risk of classical and non-classical CAH for the offspring babies of affected mothers (18). For these reasons, having more information about this genetics-based condition is very important, particularly in communities where kin marriage is frequent.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Selim Kurtoğlu, Nihal Hatipoğlu, Design: Selim Kurtoğlu, Nihal Hatipoğlu, Data Collection and Processing: Selim Kurtoğlu, Nihal Hatipoğlu, Analysis and Interpretation: Selim Kurtoğlu, Nihal Hatipoğlu, Literature Research: Selim Kurtoğlu, Nihal Hatipoğlu, Writing: Selim Kurtoğlu, Nihal Hatipoğlu.

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Subclinical Hypothyroidism in Danish Lean and Obese Children and Adolescents

Maria Dahl[1], Johanne Dam Ohrt[1], Cilius Esmann Fonvig[1,2], Julie Tonsgaard Kloppenborg[1,3], Oluf Pedersen[2], Torben Hansen[2,4], Jens-Christian Holm[1,2]

[1]Copenhagen University Hospital Holbæk, The Children's Obesity Clinic, Department of Pediatrics, Holbæk, Denmark
[2]University of Copenhagen Faculty of Health and Medical Science, The Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Metabolic Genetics, Copenhagen, Denmark
[3]University of Copenhagen, Herlev Hospital, Department of Pediatrics, Herlev, Denmark
[4]University of Southern Denmark, The Faculty of Health Sciences, Odense, Denmark

What is already known on this topic?

Subclinical hypothyroidism (SH) is a frequent finding in children and adolescents with obesity. Furthermore, SH has been associated with increased risk of exhibiting cardio-metabolic disorders. The mechanisms responsible for the disturbances in thyroid hormone concentrations during obesity are still unclear.

What this study adds?

This study underlines that SH is more prevalent among overweight/obese children compared to their lean peers. We found a positive correlation between waist-height ratio (used as a proxy of central fat accumulation) and fasting serum concentrations of thyroid-stimulating hormone, indicating that central obesity is of importance when evaluating the risk of children with obesity to exhibit thyroid abnormalities.

Abstract

Objective: Thyroid abnormalities are common in obese children. The aim of the present study was to examine the prevalence of subclinical hypothyroidism (SH) and to determine how circulating thyroid hormone concentrations correlate with anthropometrics in Danish lean and obese children and adolescents.

Methods: In this cross-sectional study, we included 3006 children and adolescents, aged 6-18 years, from the Registry of the Danish Childhood Obesity Biobank. The overweight/obese group (n = 1796) consisted of study participants with a body mass index (BMI) standard deviation score (SDS) ≥1.28. The control group (n = 1210) comprised lean children with a BMI SDS < 1.28. All participants were characterized by anthropometrics (weight, height, and waist circumference) and fasting serum concentrations of thyroid-stimulating hormone (TSH), free triiodothyronine, and free thyroxine (fT_4) at baseline.

Results: The prevalence of SH was higher among overweight/obese compared to lean study participants (10.4% vs. 6.4%, p = 0.0001). In the overweight/obese group, fasting serum TSH concentrations were associated positively with BMI SDS (p < 0.0001) and waist-height ratio (WHtR) (p < 0.0001) independent of age, sex, and pubertal developmental stage, whereas fasting serum fT_4 concentrations were associated positively only with WHtR. The odds ratio of exhibiting SH was 1.8 when being overweight/obese compared with lean (p = 0.0007) and 1.8 when presenting with a WHtR > 0.5 (p = 0.0003).

Conclusion: The prevalence of SH was higher among overweight/obese study participants. The positive correlations of circulating TSH and fT_4 with WHtR suggest that central obesity, independent of the overall degree of obesity, augments the risk of concurrent thyroid abnormalities in children and adolescents with obesity.

Keywords: Childhood obesity, thyroid hormones, waist-height ratio

Address for Correspondence: Jens-Christian Holm MD, Copenhagen University Hospital Holbæk, The Children's Obesity Clinic, Department of Pediatrics, Holbæk, Denmark **Phone:** +45 59484200 **E-mail:** jhom@regionsjaelland.dk

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Introduction

Thyroid hormones are known to play an important role in the regulation of thermogenesis (1,2) and are also involved in glucose and lipid metabolism (2,3), making them essential determinants of energy expenditure.

It is well known that overt hypothyroidism can lead to obesity. Nevertheless, modest alterations in thyroid function are more common among obese compared to lean children, including elevated serum concentrations of thyroid-stimulating hormone (TSH) combined with normal serum concentrations of free thyroxine (fT_4)-often termed subclinical hypothyroidism (SH) (4,5,6,7,8,9). SH is a diagnosis of exclusion which does not cause any clinical symptoms of hypothyroidism. Whether SH in obesity is caused by a reduced thyroid function or reflects an adaptive mechanism against obesity in order to increase energy expenditure remains to be clarified. Longitudinal studies in obese children have reported improvements in thyroid status by reductions in TSH concentrations during weight loss (10,11) supporting the latter notion; however, results have been conflicting (6,12).

SH has been suggested a risk factor for cardiovascular and metabolic disorders such as hypertension and dyslipidemia (3,13,14). Likewise, central fat accumulation in obese children is associated with a range of metabolic comorbidities and development of cardiovascular complications (15). Although there exist various studies evaluating thyroid status in children with obesity, most studies have focused on body mass index (BMI) standard deviation score (SDS) as the anthropometric measure to describe the degree of obesity (5,6,7,14). Few studies have investigated associations with other anthropometric measures such as waist circumference (WC) (16,17) and waist-hip ratio (17). Waist-height ratio (WHtR) has been advocated as an effective and convenient measure of central adiposity in children that could potentially be superior to BMI SDS in estimating cardiovascular and metabolic risk (15,18,19).

The aim of the present study was to determine the prevalence of SH and investigate the fasting serum concentrations of thyroid hormones in children and adolescents with overweight/obesity and in their lean peers. Furthermore, we evaluated how anthropometric measures, including WHtR as a proxy of central adiposity, were associated to fasting serum concentrations of thyroid hormones, as we hypothesized that SH in children could be associated to the degree of central adiposity.

Methods

Study Sample and Design

For this cross-sectional study, we recruited children and adolescents from the Registry of the Danish Childhood Obesity Biobank. From August 2007 to May 2014, 1854 overweight/obese children and adolescents were enrolled in the chronic care multidisciplinary treatment program at The Children's Obesity Clinic, Department of Pediatrics, Copenhagen University Hospital Holbæk, Denmark. The chronic care multidisciplinary treatment program is furthermore established in eight municipal clinics throughout Denmark wherefrom 946 overweight/obese children and adolescents were registered from June 2012 to May 2014. From June 2011 to May 2014, a control group was recruited comprising a population sample of 1979 Danish school children from schools in the same geographical regions (Capital Region and Region Zealand, Denmark). None of the recruitment areas in Denmark are known to suffer from iodine deficiency. Inclusion criteria for both groups were: i) Age 6-18 years and ii) a fasting blood sample drawn at the time of inclusion into the Biobank or at initiation of obesity treatment. Overall, the overweight/obese group was defined by a BMI SDS ≥1.28, corresponding to the 90th percentile according to a Danish age- and sex-adjusted reference (20,21). For sub-group analyses, overweight was defined as BMI SDS ≥1.28, while obesity was defined as BMI SDS ≥2.33 (20,21). The lean control group consisted of children and adolescents with a BMI SDS <1.28. The exclusion criteria were: i) An intake of medications known to affect serum concentrations of thyroid hormones (22), ii) a time period of more than 60 days between the anthropometric measures and the fasting blood sampling, and iii) a serum TSH concentration above 10.0 mIU/L or below 0.45 mIU/L, both suggestive cut-offs of overt thyroid disease (23,24).

Methods

Study participants were assessed with concomitant clinical and biochemical characteristics. The children and adolescents recruited from The Children's Obesity Clinic were assessed at treatment enrollment. Clinical characteristics included age, sex, height, weight, BMI SDS, WC, and pubertal developmental stage. Biochemical characteristics included serum concentrations of TSH, fT_4, and free triiodothyronine (fT_3).

Anthropometry

Anthropometric measures were obtained with the study participants wearing light indoor clothing with empty

pockets and without shoes. Height was measured to the nearest mm using a stadiometer. Weight was measured to the nearest 100 grams using a Tanita Digital Medical Scale (WB-100 MA, Tanita Corp., Tokyo, Japan). BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). BMI SDS was calculated using the LMS method (25) by transforming BMI into a normal distribution based on a Danish population with the same sex and age (20). WC was measured to the nearest mm at the end of a normal expiration with a non-elastic measuring tape placed at the level of the umbilicus while the participant was standing with arms down. WHtR was calculated as WC divided by height.

Pubertal Developmental Stage

Determination of pubertal developmental stage was rated according to the classification of Tanner (26,27). A trained pediatrician examined the children and adolescents with overweight/obesity, while pubertal staging of the lean control group was obtained via a questionnaire with picture pattern recognition of the five different Tanner stages accompanied by a text describing each category.

Biochemical Measurements

Venous blood samples were drawn from an antecubital vein after an overnight fast. If required, a local anesthetic cream (lidocaine/prilocaine mixture, Emla, AstraZeneca, Stockholm, Sweden) was applied one hour prior to venipuncture. The blood samples were analyzed immediately after venipuncture. A Cobas 6000® analyzer (F. Hoffmann-La Roche Ltd, Basel, Switzerland) was used to analyze serum concentrations of TSH and fT_4 from August 2007 to May 2013. A Dimension Vista/Centaur analyzer (Siemens, Munich, Germany) was used to measure serum concentrations of TSH, fT_4, and fT_3 on blood samples collected after May 2013, making measures of fT_3 concentrations available in a sub-group of study participants. Both analyzers used immunologic chemiluminescent assays. The analyzers yielded concordant results and were evaluated to be comparable in the analyses of TSH and fT_4. This was based on a linear relationship ($r^2 = 0.985$ for fT_4 and $r^2 = 0.997$ for TSH) between the analyzers and a bias of 6% for TSH and 13.7% for fT_4. Intra-assay coefficients of variation were < 0.10 for both analyzers.

Intervals for normal values were set as follows: TSH 0.45-4.5 mIU/L (23), fT_4 11.1-23.4 pmol/L, and fT_3 3.62-8.71 pmol/L based on analyses of a German pediatric population comprising 722 children and adolescents (28).

SH was defined as an elevated serum concentration of TSH in the range 4.5-10.0 mIU/L combined with a normal serum concentration of fT_4 (24). Euthyroidism was defined as normal serum concentrations of TSH and fT_4 alongside no clinical signs of hypothyroidism.

Statistical Analysis

Data were analyzed as continuous variables between groups by Wilcoxon rank-sum test, and two by two comparisons were analyzed by Fisher's exact test. Multiple linear regression models where used to investigate associations, and logistic regression models of the binomial family were used in the assessment of odds ratios (ORs). Levels of significance were set at $p < 0.05$. All statistical analyses were performed using "R" statistical software version 3.1.2 (http://www.r-project.org).

Ethical Aspects

Informed written consent was obtained from study participants aged 18 years and from the parents if the participants were younger. Informed assent was obtained from all children and adolescents. The study received ethical approval by the Ethics Committee of Region Zealand, Denmark (ID no. SJ-104) and by the Danish Data Protection Agency and was performed in accordance with the ethical standards of the Declaration of Helsinki 2013. The present study has been reported in line with the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) guidelines.

Results

In total, 3114 recruited children and adolescents fulfilled the inclusion criteria. Of these, 59 subjects were excluded due to intake of medications potentially affecting the thyroid hormones, 47 were excluded due to a period of more than 60 days between anthropometric measures and blood sampling, and two volunteers were excluded due to a serum TSH concentration above 10 mIU/L. The remaining 3006 eligible study participants consisted of 1796 (985 girls) overweight/obese and 1210 (729 girls) lean children and adolescents. Serum fT_3 concentrations were available in 440 overweight/obese (237 girls) and 534 lean children and youths (318 girls). Overall, the group of overweight/obese children and adolescents had a median BMI SDS of 2.75 [interquartile range (iqr) 2.22-3.22] and a median age of 11.6 (iqr 9.5-13.9), while the median BMI SDS in the lean group was 0.06 (iqr -0.53-0.61) combined with a median age of 12.1 (iqr 9.6-14.9), with the group of lean girls showing a higher age than the other groups. Sex-stratified clinical and biochemical baseline characteristics are shown in Table 1.

Table 1. Baseline characteristics of 1210 lean and 1796 overweight/obese Danish children and adolescents

	Girls			Boys			OW/OB girls vs. boys	Lean girls vs. boys
	OW/OB	Lean	p	OW/OB	Lean	p	p	p
n	985	729		811	481			
Age, years	11.5 (9.2; 14.1)	12.3 (9.9; 15.3)	**< 0.0001**	11.7 (9.7; 13.6)	11.6 (9.1; 14.3)	0.86	0.62	**0.001**
BMI SDS	2.61 (2.14; 3.01)	0.06 (-0.55; 0.62)	**< 0.0001**	2.94 (2.35; 3.46)	0.07 (-0.50; 0.58)	**< 0.0001**	**< 0.0001**	0.51
WHtR	0.58 (0.54; 0.63)	0.43 (0.41; 0.45)	**< 0.0001**	0.59 (0.54; 0.63)	0.43 (0.41; 0.45)	**< 0.0001**	0.91	0.64
TSH, mIU/L	2.5 (1.9; 3.5)	2.3 (1.7; 3.1)	**< 0.0001**	2.7 (2.0; 3.6)	2.4 (1.8; 3.2)	**< 0.0001**	**0.002**	0.24
fT_4, pmol/L	14.4 (12.9; 15.9)	14.0 (12.5; 15.7)	**0.0005**	14.7 (13.2; 16.2)	14.1 (12.5; 15.8)	**0.0001**	**0.01**	0.36
fT_3, pmol/L	5.7 (5.2; 6.4)	5.5 (4.9; 6.1)	**0.02**	5.9 (5.6; 6.4)	5.9 (5.5; 6.3)	0.54	**0.008**	**< 0.0001**
SH, n (%)	91/985 (9.2%)	51/729 (7.0%)	0.11	95/811 (11.7%)	26/481 (5.4%)	**0.0002**	0.09	0.28

Data are given as medians with interquartile range.

BMI: body mass index, fT_3: free triiodothyronine, fT_4: free thyroxine, OW/OB: overweight/obese, SDS: standard deviation score, TSH: thyroid-stimulating hormone, WHtR: waist-height ratio, SH: subclinical hypothyroidism

The prevalence of SH was higher among overweight/obese compared to lean children and adolescents (10.4% vs. 6.4%, p = 0.0001). Stratifying for sex, the SH prevalence was higher in overweight/obese boys than in lean boys, whereas no significant difference in the prevalence rates was observed when comparing overweight/obese and lean girls (Table 1).

Overall, the overweight/obese children and youths exhibited higher concentrations of serum TSH (p < 0.0001) and serum fT_4 (p < 0.0001) when compared with lean children and adolescents. Overweight/obese girls had higher serum concentrations of TSH, fT_4, and fT_3 compared to lean girls (Table 1). Furthermore, serum TSH concentrations were higher in obese as compared with overweight girls (Figure 1). Serum concentrations of TSH in overweight/obese boys were higher than in lean boys (Table 1 and Figure 1). Also serum fT_4 concentrations were higher in overweight/obese boys compared to their lean peers, while no significant difference in serum fT_3 concentrations was observed among boys (Table 1). In the overweight/obese group, boys exhibited higher concentrations of serum TSH, fT_4, and fT_3 compared to girls (Table 1). The serum fT_3 concentration in boys with SH was higher than in euthyroid boys and higher than in their female peers exhibiting SH (Table 2).

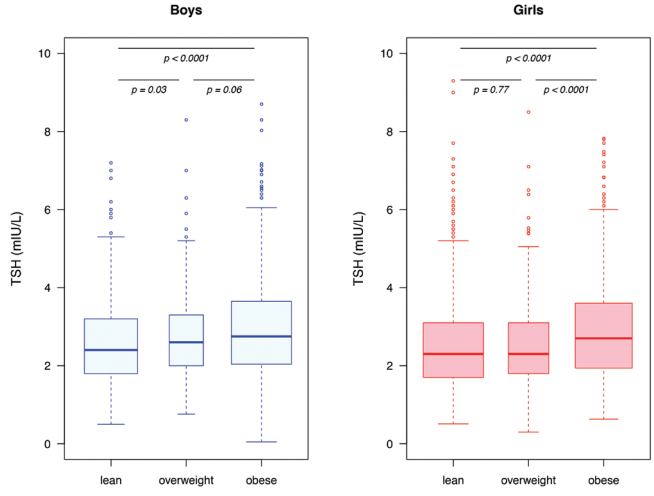

Figure 1. Box-plots showing serum concentrations of thyroid-stimulating hormone in the 3006 Danish lean, overweight, and obese boys and girls. TSH: thyroid-stimulating hormone

Overweight/obese children and adolescents had higher WHtR (p < 0.0001) compared with their lean peers. In the overweight/obese group, boys had a significantly higher BMI SDS than girls, while there was no sex difference in WHtR among the overweight/obese children. Children and adolescents having SH had higher BMI SDS (p < 0.0001)

Table 2. Baseline characteristics of Danish girls and boys exhibiting subclinical hypothyroidism compared with euthyroid girls and boys

	Girls			Boys			SH girls vs. boys	Euthyroid girls vs. boys
	SH	Euthyroid	p	SH	Euthyroid	p	p	p
n	142	1467		121	1116			
Age, years	11.8 (9.3; 14.6)	11.6 (9.4; 14.4)	0.97	11.1 (8.4; 13.6)	11.6 (9.6; 13.8)	**0.03**	**0.049**	0.39
BMI SDS	2.39 (0.52; 2.99)	1.81 (0.25; 2.71)	**0.002**	2.82 (1.45; 3.57)	2.11 (0.39; 3.11)	**< 0.0001**	**0.0006**	< 0.0001
WHtR	0.55 (0.45; 0.62)	0.52 (0.44; 0.59)	**0.004**	0.57 (0.48; 0.62)	0.52 (0.44; 0.60)	**0.0003**	0.25	0.14
TSH, mIU/L	5.2 (4.7; 6.1)	2.3 (1.7; 3.1)	**< 0.0001**	5.2 (4.8; 5.9)	2.5 (1.9; 3.1)	**< 0.0001**	0.82	0.003
fT_4, pmol/L	14.5 (13.2; 16.0)	14.4 (13.0; 15.9)	0.49	14.7 (13.3; 16.2)	14.6 (13.0; 16.2)	0.97	0.89	0.05
fT_3, pmol/L	5.6 (5.4; 6.0)	5.8 (5.2; 6.3)	0.63	6.3 (6.0; 6.8)	5.9 (5.5; 6.3)	**0.0009**	**0.0006**	**0.001**

Data are given as medians with interquartile range.

BMI: body mass index, fT_3: free triiodothyronine, fT_4: free thyroxine, SDS: standard deviation score, SH: subclinical hypothyroidism, TSH: thyroid-stimulating hormone, WHtR: waist-height ratio

Table 3. Multiple linear regressions showing the relationship between fasting serum thyroid-stimulating hormone concentrations and anthropometrics (n = 1796) in the overweight/obese group

	Model A			Model B		
	β (SE)	r^2	p	β (SE)	r^2	p
Basic model					0.01	
BMI SDS	0.24 (0.01)	0.02	**< 0.0001**	0.23 (0.05)	0.02	**< 0.0001**
WHtR	2.18 (0.42)	0.02	**< 0.0001**	2.35 (0.46)	0.02	**< 0.0001**

Estimates (β), standard errors (SE), and correlation coefficients (r^2) of the relationship between thyroid-stimulating hormone and the anthropometrics body mass index standard deviation score and waist-height ratio. The "basic model" compromises age, sex, and pubertal developmental stage. Model A is an unadjusted univariate analysis. Model B is a univariate analysis adjusted for age, sex, and pubertal developmental stage.

BMI: body mass index, SDS: standard deviation score, WHtR: waist-height ratio

and WHtR ($p < 0.0001$) compared with the euthyroid study participants (Table 2). In a multivariate regression model including all 3006 lean and overweight/obese children and youths, serum TSH concentrations were associated positively with WHtR ($p = 0.0009$) but not with BMI SDS ($p = 0.83$), age ($p = 0.18$), sex ($p = 0.23$), or pubertal developmental stage ($p = 0.82$). In the overweight/obese group, serum TSH concentrations were associated positively with BMI SDS and WHtR separately before and after adjustment for age, sex, and pubertal developmental stage in univariate regression models (Table 3). A positive correlation was observed in the overweight/obese group between serum fT_4 concentrations and both BMI SDS and WHtR, but only the association between serum fT_4 and WHtR remained significant after adjusting for age, sex, and pubertal developmental stage (Table 4). No associations between serum fT_3 and anthropometrics were found among the overweight/obese study participants (Table 5).

When separately comparing BMI SDS and WHtR with the presence of SH in all the 3006 study participants in a multiple logistic regression model adjusted for age, sex, and pubertal development stage, the OR of exhibiting SH

Table 4. Multiple linear regressions showing the relationship between fasting serum free thyroxine concentrations and anthropometrics (n = 1796) in the overweight/obese group

	Model A			Model B		
	β (SE)	r^2	p	β (SE)	r^2	p
Basic model					0.06	
BMI SDS	0.20 (0.07)	0.00	**0.006**	0.09 (0.08)	0.06	0.25
WHtR	1.93 (0.73)	0.00	**0.008**	2.91 (0.79)	0.07	**0.0002**

Estimates (β), standard errors (SE), and correlation coefficients (r^2) of the relationship between free thyroxine and the anthropometrics body mass index standard deviation score and waist-height ratio. The "basic model" compromises age, sex, and pubertal developmental stage. Model A is an unadjusted univariate analysis. Model B is a univariate analysis adjusted for age, sex, and pubertal developmental stage.

BMI: body mass index, SDS: standard deviation score, WHtR: waist-height ratio

Table 5. Multiple linear regressions showing the relationship between fasting serum free triiodothyronine concentrations and anthropometrics (n = 440) in the overweight/obese group

	Model A			Model B		
	β (SE)	r^2	p	β (SE)	r^2	p
Basic model					0.25	
BMI SDS	0.03 (0.05)	0.00	0.47	-0.07 (0.05)	0.25	0.14
WHtR	-0.19 (0.54)	0.00	0.72	-0.18 (0.52)	0.25	0.73

Estimates (β), standard errors (SE), and correlation coefficients (r^2) of the relationship between free triiodothyronine and the anthropometrics body mass index standard deviation score and waist-height ratio. The "basic model" compromises age, sex, and pubertal developmental stage. Model A is an unadjusted univariate analysis. Model B is a univariate analysis adjusted for age, sex, and pubertal developmental stage.

BMI: body mass index, SDS: standard deviation score, WHtR: waist-height ratio

was 1.8 when being either overweight/obese compared to lean [95% confidence interval (CI): (1.3; 2.6), p = 0.0007] or when presenting with a WHtR > 0.5 compared to the study participants having a WHtR < 0.5 [95% CI: (1.3; 2.6), p = 0.0003].

Discussion

In this cross-sectional study on Danish children and adolescents, we observed a higher prevalence of SH among overweight/obese compared to lean children and adolescents. Concentrations of fasting serum TSH were associated positively with both BMI SDS and WHtR in the overweight/obese group. Independent of the degree of obesity, WHtR appeared to have a higher impact on the concentration of serum TSH compared with BMI SDS. Furthermore, serum fT_4 concentrations correlated positively with WHtR but not with BMI SDS, while no significant associations were found between serum fT_3 and either of the anthropometric measures. The 10.4% prevalence of SH in study participants with overweight/obesity is in accordance with reports from previous comparable studies, where prevalence rates of 7 to 23% have been shown (29). The lower prevalence of SH in the lean group was likewise comparable to results of other studies (7,9).

The higher concentrations of serum TSH observed among overweight/obese individuals compared with lean ones have similarly been observed in other studies conducted on overweight/obese children and adolescents (5,6,7,8,9,10,14). The literature is less univocal regarding data on the concentrations of peripheral thyroid hormones. Mostly, elevated concentrations of TSH have been reported in combination with elevated concentrations of fT_3 (7,10,11,14) and slightly decreased (7) or normal fT_4 concentrations (10,11,14). This is partly in contrast to our results where elevated serum fT_4 concentrations were found among overweight/obese compared to lean children and adolescents, whereas serum fT_3 concentrations did not differ between the two groups. fT_3 is known to be the physiologically active peripheral thyroid hormone with approximately 80% of circulating T_3 derived by extrathyroidal monodeiodination of T_4 (30). A change in the monodeiodination pathway leading to augmented concentrations of active fT_3, possibly resulting in an increased energy expenditure, has been suggested as an explanation of the thyroid hormone derangements in obesity (7,14,30). A disturbance in the negative feedback mechanism regulating the hypothalamus-pituitary-thyroid axis has been proposed as another explanation (30). With the observed elevated serum concentrations of TSH and fT_4 among overweight/obese compared with lean children in

the present study, our results support the latter notion, as people with a normal feedback mechanism and elevated concentrations of serum TSH would be expected to have decreased concentrations of peripheral thyroid hormones. The lacking consistency when evaluating peripheral thyroid hormones in obesity could be due to differences in study populations, as it has been proposed that thyroid function differs between lower grades of overweight and morbid obesity (31). The inconsistency could also point towards the elevated serum TSH concentrations to be the most pertinent thyroid disturbance in obesity. This possibility has previously been considered in a study of 226 obese and 39 lean adults, where serum TSH was associated with BMI and WC (32). TSH has been shown to induce adipogenesis and adipokine production (e.g. leptin) directly through TSH receptors in adipose tissue, independent of the mediating influence of thyroid hormones on energy balance (33). Likewise, signalling via leptin receptors has been shown to affect the TSH secretion by stimulating pro-thyrotropin-releasing hormone receptors in hypothalamus (34). The bidirectional signalling indicates a direct interaction between TSH and adipose tissue regulated differently from the hypothalamus-pituitary-thyroid axis and underlines the possibility that an elevated serum TSH concentration in obesity is more crucial than the varying derangements in peripheral thyroid hormone concentrations.

In line with the elevated serum concentrations of TSH in children with obesity, a positive association between TSH and BMI SDS has almost consistently been reported in previous pediatric studies (6,7,9,11,14,35). We observed a positive association between serum TSH and BMI SDS as well as between BMI SDS and serum fT_4, whereas serum fT_3 did not correlate with BMI SDS. Previously, a positive association between BMI SDS and serum total T_4 has been reported in 190 obese children as compared to 133 age- and sex-matched controls, while no significant associations between BMI SDS and serum fT_4 or serum fT_3 (9) were observed, the latter being compatible with our results.

Few studies have focused on the correlation between thyroid variables and anthropometrics other than BMI. A positive association between serum TSH and WC has been reported in 201 adult euthyroid women (16), whereas another study failed to show any association in 703 multiethnic obese children and adolescents (3). In a study of 240 pre-pubertal children, serum fT_3 concentrations were strongly influenced by augmented central fat accumulation, measured by dual-energy x-ray absorptiometry (DXA), independent of total body fat mass and percentage of body fat. Augmented visceral fat accumulation is associated with the development of several cardio-metabolic risk factors (15,18). BMI SDS is of limited use in the evaluation of body composition due to its inability to differentiate muscle mass from bone and fat mass (36). WHtR takes body composition into account by evaluating WC in accordance to variability in height, and therefore WHtR is considered to be a more appropriate proxy of central adiposity compared with BMI SDS (15,18,19). Conceivably, in the present study, we included WHtR as a surrogate measure of central adiposity. We observed that serum TSH associated positively with WHtR and BMI SDS among overweight/obese individuals, but when all study participants were included in the analysis, serum TSH only showed a significant association with WHtR. Additionally, the stronger determinant of serum fT_4 concentrations appeared to be the WHtR among the overweight/obese children and adolescents. This finding suggests that an augmented WHtR, and thus central obesity, is associated with the risk of thyroid dysfunctions.

A sex difference was demonstrated in the overweight/obese group, as overweight/obese boys showed higher concentrations of thyroid hormones compared with overweight/obese girls. Even though we did not see a sex-specific difference in WHtR, it is possible that the higher concentrations of thyroid hormones in boys can be explained by a correlation between thyroid hormones and the degree of central obesity, as it has been suggested that men accumulate more visceral fat than women, independent of total body fat mass (37). The proposed difference between men and women regarding accumulation of visceral fat is biologically plausible for boys and girls as well. We also observed serum TSH concentrations to be higher in obese compared to overweight and lean girls, while serum TSH concentrations in obese and overweight boys were higher than in lean boys. This indicates that girls need to become relatively more overweight than boys before they show corresponding elevated concentrations of TSH. The theory of visceral fat to accumulate faster among boys compared to girls (37) could contribute to this observed sex difference in thyroid-pituitary gland function. Nevertheless, the mechanisms explaining thyroid abnormalities in obesity are still unclear. Due to the cross-sectional nature of this study, we are unable to clarify whether the higher prevalence of SH among children and adolescents with overweight/obesity plays a pathogenic part in obesity or if rather it reflects an adaptive effort to raise energy expenditure.

One limitation of this study is the lack of thyroid antibody measurements and the possibility that children with autoimmune thyroiditis may have been included in the study sample. However, this is not very likely, since all patients in the overweight/obese group were evaluated clinically by a trained pediatrician regarding their clinical thyroid status. Furthermore, other studies conducted on

obese children and adolescents have reported positive results for thyroid antibodies in 5.7% to 19.5% of obese children with SH, concluding that autoimmune thyroiditis is rarely the cause of elevated thyroid hormone concentrations in obesity (4,5,9). The relative limitation of blood samples to be analyzed for serum fT_3 concentrations is another shortcoming of the present study. Also, the change in method used to analyze fT_4 and TSH concentrations is a limitation. We relied on the quality control performed by our laboratory at the time of method change which determined reliable correlation coefficients and comparable coefficients of variations between the analyzers. Therefore, we believe that the fT_4 and TSH concentrations measured by the two different analyzers are comparable. The maximum interval of 60 days between the obtaining of anthropometric measurements and the blood sampling allowed for the variability caused by the possibility that overweight/obese children enrolled in obesity treatment lost or gained weight before the blood sample was taken. This could mean that the presented result is a conservative estimate of the SH prevalence among the overweight/obese group. Yet, an additional limitation of the study is the self-reported staging of pubertal development of the controls. Tanner staging is by its nature an arbitrary measure as it attempts to characterize a continuous developmental progress into one of five distinct stages and therefore both objectively measured and self-reported staging needs to be carefully interpreted. The primary strength of this study is that it was conducted on a relatively large study population consisting of well-characterized overweight/obese and lean children and adolescents.

In conclusion, this study shows that SH is more prevalent among children and adolescents with obesity compared to their lean peers. Central fat accumulation is related to the thyroid abnormality independent of the degree of overall obesity, indicating central obesity as an aggravated risk for development of SH. Nevertheless, the causality of the association remains to be clarified and longitudinal studies are needed to understand possible links between SH and central fat accumulation.

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Ethics

Ethics Committee Approval: The study received ethical approval by the Ethics Committee of Region Zealand, Denmark (ID no. SJ-104) and by the Danish Data Protection Agency. Informed Consent: Informed written consent was obtained from study participants aged 18 years and from the parents if the participants were younger. Informed assent was obtained from all children and adolescents.

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Authorship Contributions

Concept: Maria Dahl, Johanne Dam Ohrt, Cilius Esmann Fonvig, Julie Tonsgaard Kloppenborg, Jens-Christian Holm, Design: Maria Dahl, Johanne Dam Ohrt, Cilius Esmann Fonvig, Julie Tonsgaard Kloppenborg, Jens-Christian Holm, Data Collection or Processing: Maria Dahl, Johanne Dam Ohrt, Cilius Esmann Fonvig, Julie Tonsgaard Kloppenborg, Jens-Christian Holm, Analysis or Interpretation: Maria Dahl, Johanne Dam Ohrt, Cilius Esmann Fonvig, Julie Tonsgaard Kloppenborg, Oluf Pedersen, Torben Hansen, Jens-Christian Holm, Literature Search: Maria Dahl, Johanne Dam Ohrt, Julie Tonsgaard Kloppenborg, Writing: Maria Dahl, Johanne Dam Ohrt, Cilius Esmann Fonvig, Julie Tonsgaard Kloppenborg, Jens-Christian Holm.

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Neck Circumference to Assess Obesity in Preschool Children

Meda Kondolot[1], Duygu Horoz[2], Serpil Poyrazoğlu[2], Arda Borlu[3], Ahmet Öztürk[4], Selim Kurtoğlu[5], Mümtaz M. Mazıcıoğlu[6]

[1]Erciyes University Faculty of Medicine, Department of Pediatrics, Social Pediatrics Unit, Kayseri, Turkey
[2]The Head of Local Health Authority, Kayseri, Turkey
[3]Erciyes University Faculty of Medicine, Department of Public Health, Kayseri, Turkey
[4]Erciyes University Faculty of Medicine, Department of Biostatistics, Kayseri, Turkey
[5]Erciyes University Faculty of Medicine, Department of Pediatric Endocrinology, Kayseri, Turkey
[6]Erciyes University Faculty of Medicine, Department of Family Medicine, Kayseri, Turkey

What is already known on this topic?

Neck circumference (NC) has been shown to be one of the reliable and practical additional measurements to define obesity in school children and adolescents. Limited information is available about the use of NC in preschool children.

What this study adds?

NC may be useful to define obesity as an additional measurement in preschool children as well.

Abstract

Objective: Limited information is available about the use of neck circumference (NC) to assess obesity in preschool children. This study aims to provide NC percentiles and determine the cut-off levels of NC as a measure to assess obesity in preschool children.
Methods: The data were obtained from the Anthropometry of Turkish Children aged 0-6 years (ATCA-06) study database. A total of 21 family health centers were chosen and children aged 2-6 years old from all socioeconomic levels were randomly selected from the lists of district midwives; 1766 children (874 male and 892 female; 88.3% of sample size) were included in the study. The smoothed centile curves of NC were constructed by the LMS method. Receiver operating characteristic (ROC) analysis was performed to calculate cut-off points for NC using body mass index ≥95th percentile.
Results: Mean NC was greater in males than females. Cut-off values for obesity were found to be statistically significant in both genders other than 3 years old boys. The NC percentiles of Turkish preschool children were slightly greater than those of other European preschool children in both genders. This difference disappeared around the adiposity rebound period. The 97th percentile values for Turkish preschool children continue to be greater in both genders.
Conclusion: NC may be useful to define obesity in preschool children. Since ethnic and various other factors may have a role in incidence of obesity, local reference data are important in assessment of obesity.
Keywords: Preschool children, obesity, neck circumference, percentiles, cut-offs

Introduction

Obesity in early childhood is on the increase and this is of great concern because of the relationship between childhood obesity and metabolic complications and other clinical comorbidities encountered in adult life (1). In the United States, obesity prevalence was reported to be 12.4% for boys and 10% for girls in a recent study in children aged 3 to 5 years (2). In our data set, obesity prevalence was identical in both genders as 5% in Turkish children aged 0-84 months (3). It was reported that children at the 50th percentile of body-mass index (BMI) at the age of 5 years had a 6% probability of being obese at the age of 14 years and that this probability increased to 25% among 5-year-olds at the 85th percentile and to 47% among those at the 95th percentile (1). Since early childhood adiposity may lead to cardiometabolic health effects in later years, it should be closely monitored to prevent these complications (4,5,6,7,8).

Address for Correspondence: Meda Kondolot MD,
Erciyes University Faculty of Medicine, Department of Pediatrics, Social Pediatrics Unit, Kayseri, Turkey
E-mail: medakondolot@gmail.com

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Children have a rapid increase in adiposity during the first year of life. After infancy, adiposity declines and reaches a minimum and rebound at around 5 to 6 years. Subsequently, adiposity shows a steady increase throughout childhood and adolescence (6). In monitoring for obesity, it is important to follow the course of adiposity and identify the age at which excess weight gain occurs. Although BMI is a frequently used measurement to asses total body fat content, it fails to provide sufficient information regarding body fat distribution. Since upper body adiposity is considered to be a significant determinant of cardiometabolic risk factors, it should be monitored rather than assessing total body fat content. Recent studies have shown that neck circumference (NC) is one of the reliable and practical anthropometric measurements to assess upper body adiposity and consequently to assess cardiometabolic risk as a result of irregular fat distribution (9,10,11,12,13,14,15,16,17,18,19).

There are only a few studies that provide reference data on NC measurements for school children and adolescents (20,21). NC reference values for preschool children are especially limited (21).

This present study aims to 1) provide NC percentiles and determine cut-off levels of NC for obesity in preschool children; 2) to explore the importance of NC measurements as an additional tool for assessment of obesity in preschool children.

Methods

The data were obtained from the Anthropometry of Turkish Children aged 0-6 years (ATCA-06) study database. The data were collected between September 2009 to May 2010 in a large city in Central Turkey with a population of over 1,200,000. A two-stage probability sampling was used to select children from each socioeconomic level. In the first stage, Family Medicine Centers (FMCs) located in the city center and suburbs were selected randomly. These Centers address a population of all socioeconomic levels (low, medium, high). In determining socioeconomic levels, we used the evaluation of local health authorities. A total of 21 FMCs were chosen, and children of ages 2-6 years from these centers were randomly selected from the district midwives' lists according to their families' income. Parents along with their children were invited to the FMC. Children whose parents did not consent to participate were excluded from the study.

A total of 2000 children (975 male and 1025 female) were recruited for the study. Children with chronic medical disorders, cervical lymphadenomegaly, neck deformity, and those whose NC measurements were out of range for each gender and age (3^{rd}-97^{th} percentiles) were excluded to obtain normally distributed data. Upon exclusion of those participants who did not match the selection criteria, a total number of 1766 children (874 male and 892 female; 88.3% of sample size) were included in the study.

Chronological age which was calculated by subtracting date of birth from date of observation was used to determine cut-offs. Each quarter year elapsed from their birthday was used to obtain percentiles in short periods. The Ethics Committee of Erciyes University approved this study (2008/28). Written parental consent was obtained prior to the study.

All measurements were performed by well-trained health technicians. NC was measured using a plastic tape measure while the child's head was held erect with eyes facing forward and the neck in a horizontal plane at the level of the most prominent portion, the thyroid cartilage. All measurements were taken with the subjects standing upright, with the face directed forward and shoulders relaxed. BMI was calculated according to the formula: weight (kg)/height $(m)^2$. Children whose BMI was $\geq 95^{th}$ percentile curve according to local references were identified as obese (3).

Construction of the percentile curves was performed with the LMS Chart Maker Pro version 2.3 software program (The Institute of Child Health, London) which fits smooth percentile curves to reference data. The smoothed percentile curves of NC were constructed by the LMS method. This method summarizes percentiles at each age based on the power of age-specific Box-Cox power transformations that are used to normalize data (22). These three quantities depend on age. The final curves of percentiles are produced by three smooth curves representing L (Lambda; skewness), M (M; median), and S (Sigma; coefficient of variation). With estimates of L, M, and S, values of X are connected to the values of z through the above equation. The percentile is obtained from a normal distribution table, where the z-score corresponds to the percentile of interest. In boys, the effective degrees of freedom (edf) for NC were equal to 3 (M curve), 4 (S curve), and 3 (L curve). In girls, edf for NC were equal to 3, 4, and 2, respectively. The median curves of boys and girls were compared to show gender-specific trends through 3-6 years.

Statistical Analysis

Construction of the centile curves was performed with the LMS Chart Maker Pro version 2.3 software program (The Institute of Child Health, London) which fits smooth centile curves to reference data (22). Gender difference in NC was compared with student's t-test. NC cutt-off values were calculated for 3-6-year-old children with receiver operating

characteristics (ROC) analysis according to dependent variable obesity defined by BMI ≥95th percentile (23). The ROC curves demonstrated the overall discriminatory power of a diagnostic test: NC. Sensitivity and specificity were calculated to identify the optimal cut-off values. Calculated cut-offs were checked with Youden index as (J) ≥0.6 good and (J) ≥0.4 moderate. Descriptive statistics for each quarter year (e.g., 3-8 m, etc.) within sex were calculated by SPSS version 15.0 (Chicago, Illinois, USA).

Results

Mean (standard deviation) and median (minimum-maximum) values for NC in Turkish children age of 2-6 years in both genders and comparisons of the means are shown in Table 1. Mean NC values were greater in males than females, and the difference was significant at age groups 30-32, 42-44, 48-50, 57-68, and 72-83 months. The mean increment from 24 to 83 months period was 1.4 cm for males and 0.5 cm for females (Table 1).

The calculated age-specific (at 3-month intervals) 3rd, 5th, 10th, 15th, 25th, 50th, 75th, 85th, 90th, 95th, and 97th percntiles for NC in each gender are given in Tables 2 and 3. The increase in NC for 50th percentile values through the 24 to 83 months period was 1.2 cm in boys and 0.6 in girls. The increase in NC through the 3rd to 97th percentiles for 24-26 months was 5.48 cm in boys. This value was 5.05 cm for 81-83 months (Table 2). The increases in NC through 3rd to 97th percentiles for 24-26 months and for 81-83 months in girls were 5.69 cm and 4.80 cm (Table 3).

ROC analysis was performed to calculate cut-off points for NC using BMI ≥95th percentile (for obesity) as a dependent variables. The results for boys and girls are shown in Tables 4 and 5. Youden index was also calculated for cut-off values and it was found to be statistically significant in both genders except 3 years old group of boys (Tables 4, 5). In case of obesity (BMI ≥95th), we found that cut-off points for NC were 25.9 and 27.0 cm for males who were 4 and 5 years old, respectively (a cut-off point calculated by ROC analysis and confirmed by Youden index ≥0.4) and that these values were

Table 1. Mean (standard deviation), median (minimum-maximum) neck circumference values for Turkish children aged 2-6 years and comparisons of the means

Age groups#	Boys			Girls			t	p
	n*	Mean (SD)	Median (min-max)	n*	Mean (SD)	Median (min-max)		
24-26 months	37	25.2 (1.5)	25.0 (22.5-28.0)	54	24.7 (1.6)	24.1 (22.0-28.0)	1.352	0.180
27-29 months	55	25.3 (1.6)	25.1 (22.5-29.0)	48	25.1 (1.3)	25.0 (23.0-28.0)	0.590	0.556
30-32 months	55	25.4 (1.4)	25.0 (22.9-29.0)	46	24.6 (1.3)	24.9 (22.0-28.0)	2.914	**0.004**
33-35 months	55	25.1 (1.3)	25.0 (22.5-28.1)	47	24.6 (1.4)	24.7 (22.0-27.5)	1.783	0.078
36-38 months	49	25.0 (1.2)	25.0 (23.0-28.3)	51	24.6 (1.5)	24.2 (22.5-28.1)	1.507	0.135
39-41 months	35	25.2 (1.3)	25.0 (23.0-28.0)	53	24.6 (1.6)	24.0 (22.3-29.0)	1.820	0.072
42-44 months	42	25.3 (1.2)	25.5 (22.6-28.0)	41	24.8 (1.2)	24.5 (23.0-27.1)	2.243	**0.028**
45-47 months	53	25.4 (1.2)	25.0 (22.9-28.0)	42	25.1 (1.5)	25.0 (22.8-29.0)	1.218	0.226
48-50 months	40	25.8 (1.5)	25.5 (23.0-29.0)	48	25.0 (1.3)	25.0 (23.0-27.5)	2.836	**0.006**
51-53 months	41	25.6 (1.4)	25.0 (23.5-29.10)	49	25.4 (1.3)	25.5 (23.0-27.7)	0.901	0.370
54-56 months	46	25.8 (1.5)	26.0 (23.0-29.0)	57	25.1 (1.3)	25.0 (22.6-28.0)	2.411	0.180
57-59 months	43	25.8 (1.4)	25.5 (23.0-29.0)	38	24.9 (1.3)	25.0 (22.7-28.0)	2.903	**0.005**
60-62 months	52	26.0 (1.3)	26.0 (24.0-29.0)	41	25.1 (1.4)	25.0 (23.0-29.0)	3.282	**0.001**
63-65 months	50	26.2 (1.3)	26.0 (24.0-29.0)	46	25.5 (1.4)	25.0 (23.2-28.0)	2.308	**0.023**
66-68 months	48	26.4 (1.5)	26.0 (24.0-29.0)	56	25.5 (1.3)	25.5 (23.0-29.0)	2.929	**0.004**
69-71 months	46	26.0 (1.3)	26.0 (24.0-29.0)	57	25.4 (1.6)	25.0 (23.0-29.0)	1.985	0.050
72-74 months	42	26.2 (1.2)	26.0 (24.0-29.0)	44	25.1 (1.2)	25.0 (23.0-28.0)	3.964	**< 0.001**
75-77 months	32	26.2 (1.3)	26.0 (24.0-29.5)	33	25.4 (1.1)	25.5 (24.0-28.2)	2.797	**0.007**
78-80 months	30	26.4 (1.4)	26.1 (24.0-30.0)	22	25.4 (1.3)	25.0 (23.0-28.2)	2.706	**0.009**
81-83 months	23	26.6 (1.6)	26.5 (24.9-30.0)	19	25.2 (1.3)	25.5 (23.0-28.0)	3.053	**0.004**

#Age groups represent completed months.

*The total group includes 1766 children (874 boys, 892 girls)

SD: standard deviation, min-max: minimum-maximum

Table 2. The neck circumference percentile values (3rd to 97th percentiles) for 2-6 years old boys

Age groups	Percentiles*										
	3rd	5th	10th	15th	25th	50th	75th	85th	90th	95th	97th
24-26 months	22.75	23.01	23.44	23.73	24.19	25.10	26.12	26.70	27.12	27.78	28.23
27-29 months	22.81	23.07	23.48	23.77	24.22	25.12	26.11	26.68	27.09	27.74	28.17
30-32 months	22.87	23.12	23.53	23.81	24.25	25.13	26.11	26.67	27.08	27.71	28.14
33-35 months	22.93	23.18	23.57	23.86	24.29	25.15	26.11	26.67	27.07	27.69	28.12
36-38 months	22.99	23.24	23.63	23.91	24.33	25.19	26.14	26.69	27.09	27.70	28.13
39-41 months	23.07	23.31	23.70	23.97	24.39	25.24	26.18	26.73	27.12	27.74	28.16
42-44 months	23.15	23.39	23.78	24.05	24.47	25.31	26.24	26.79	27.18	27.79	28.21
45-47 months	23.24	23.48	23.86	24.13	24.55	25.38	26.31	26.86	27.25	27.86	28.29
48-50 months	23.34	23.57	23.95	24.22	24.63	25.46	26.39	26.94	27.33	27.95	28.37
51-53 months	23.44	23.67	24.04	24.31	24.72	25.55	26.48	27.02	27.42	28.04	28.46
54-56 months	23.53	23.77	24.14	24.40	24.81	25.63	26.56	27.11	27.50	28.13	28.56
57-59 months	23.63	23.86	24.23	24.49	24.90	25.72	26.65	27.20	27.59	28.22	28.66
60-62 months	23.73	23.96	24.33	24.58	24.99	25.81	26.73	27.29	27.68	28.32	28.76
63-65 months	23.83	24.05	24.41	24.67	25.07	25.89	26.82	27.37	27.77	28.41	28.85
66-68 months	23.92	24.14	24.50	24.76	25.15	25.97	26.89	27.45	27.85	28.50	28.95
69-71 months	24.00	24.22	24.58	24.84	25.23	26.04	26.97	27.53	27.93	28.58	29.04
72-74 months	24.09	24.31	24.66	24.91	25.31	26.12	27.04	27.60	28.01	28.66	29.12
75-77 months	24.17	24.39	24.74	24.99	25.38	26.19	27.12	27.68	28.09	28.75	29.21
78-80 months	24.25	24.47	24.82	25.07	25.46	26.26	27.19	27.75	28.16	28.83	29.30
81-83 months	24.33	24.55	24.90	25.14	25.53	26.33	27.26	27.82	28.24	28.91	29.38

*Percentiles are given for each 3-month period. The age groups represent completed months.

27.5 cm for males who were 6 years old (Youden index ≥0.6) (Table 4). Cut-off points for NC were 25.8 (Youden index ≥0.6), 25.8 (Youden index ≥0.6), 25.7 (Youden index ≥0.4), and 25.5 cm (Youden index ≥0.4) for females who were 3, 4, 5, and 6 years old, respectively (Table 5).

Figures 1 and 2 show comparisons of the 3rd, 50th, 97th NC percentiles of our data with the IDEFICS (Identification and prevention of Dietary- and lifestyle-induced health EFfects in Children and infantS) study (conducted on European children) in boys and girls, respectively. The data indicate that NC values of Turkish preschool children were slightly greater than those of other European preschool children in both genders. This difference disappears around the adiposity rebound period, and NC values of Turkish preschool children become slightly lower after this period except for the 97th percentile. This value continues to be greater in the Turkish preschool children in both genders as compared to the European data.

Discussion

BMI is obviously the most commonly used anthropometric measure to assess obesity. However, BMI may fail to describe body fat distribution. Since upper body fat deposition is associated with an increased metabolic and cardiovascular risk (24,25,26), it is absolutely essential to determine fat accumulation in the upper body. Various anthropometric measures and indices may be used to determine upper body fat content and distribution such as waist circumference (WC), mid-upper arm circumference, skinfold thickness, waist/hip ratio, and waist/height ratio. WC is the most frequently used measure, which may have some measuring limitations such as its relationship with mid expiratory movement and consumption of excess food (9,15,27,28,29). Recently, NC has been shown as a reliable and easy alternative tool to WC for determining upper body fat accumulation and distribution (11,12,13,17,18,19). The advantages of measuring NC compared with WC are better inter- and intra-observer reliability, relative stability which is less affected by respiration and clothing (11,30).

In addition, it has been reported that NC may be used to assess both obesity and metabolic disorders (12,13). NC percentiles were also produced for school children and adolescents (20,21). However, information on preschool children is limited. Cunningham et al (1) have emphasized the importance of screening predominantly in preschool children. These authors have shown that the incidence of

Table 3. The neck circumference percentile values (3rd to 97th percentiles) for 2-6 years old girls

Age groups	Percentiles*										
	3rd	5th	10th	15th	25th	50th	75th	85th	90th	95th	97th
24-26 months	22.26	22.53	22.96	23.26	23.73	24.67	25.72	26.34	26.78	27.47	27.95
27-29 months	22.34	22.60	23.02	23.32	23.77	24.70	25.73	26.34	26.78	27.46	27.93
30-32 months	22.40	22.66	23.07	23.36	23.81	24.72	25.74	26.34	26.77	27.45	27.92
33-35 months	22.46	22.71	23.12	23.40	23.85	24.74	25.75	26.34	26.77	27.44	27.90
36-38 months	22.51	22.76	23.16	23.45	23.88	24.77	25.76	26.35	26.77	27.44	27.90
39-41 months	22.57	22.82	23.21	23.49	23.92	24.80	25.78	26.37	26.78	27.44	27.90
42-44 months	22.64	22.88	23.27	23.54	23.97	24.84	25.81	26.39	26.80	27.46	27.91
45-47 months	22.70	22.94	23.32	23.60	24.02	24.87	25.84	26.41	26.82	27.47	27.93
48-50 months	22.77	23.00	23.38	23.65	24.07	24.91	25.87	26.44	26.85	27.50	27.95
51-53 months	22.83	23.06	23.44	23.70	24.12	24.96	25.91	26.47	26.88	27.52	27.97
54-56 months	22.89	23.12	23.49	23.76	24.16	25.00	25.94	26.50	26.91	27.55	28.00
57-59 months	22.95	23.18	23.55	23.81	24.21	25.04	25.97	26.53	26.93	27.57	28.02
60-62 months	23.01	23.23	23.60	23.86	24.26	25.07	26.00	26.56	26.96	27.60	28.05
63-65 months	23.06	23.29	23.65	23.90	24.30	25.11	26.04	26.59	26.99	27.62	28.07
66-68 months	23.12	23.34	23.70	23.95	24.34	25.15	26.07	26.61	27.01	27.65	28.09
69-71 months	23.17	23.39	23.74	23.99	24.38	25.18	26.09	26.64	27.03	27.67	28.11
72-74 months	23.22	23.43	23.79	24.03	24.42	25.21	26.12	26.66	27.05	27.68	28.12
75-77 months	23.27	23.48	23.83	24.07	24.46	25.24	26.14	26.68	27.07	27.70	28.14
78-80 months	23.31	23.52	23.87	24.11	24.49	25.27	26.17	26.70	27.09	27.71	28.15
81-83 months	23.36	23.57	23.91	24.15	24.53	25.30	26.19	26.72	27.1	27.73	28.16

*Percentiles are given for each 3-month period. The age groups represent completed months.

Table 4. Receiver operating curve analysis for neck circumference to determine cut-off points for obesity for 3-6-year-old boys

Age group (years)&	n (%)#	ROC (95% CI)	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	+LR	-LR	+PV	-PV
3.0	179 (5.1)	0.667 (0.593-0.736)	25.3	77.8 (40.1-96.5)	57.4 (49.6-65.0)	1.83	0.39	8.9	98.0
4.0	170 (4.1)	0.743 (0.670-0.807)	25.9**	85.7 (42.2-97.6)	56.8 (48.8-64.5)	1.98	0.25	7.9	98.9
5.0	196 (4.1)	0.745 (0.677-0.804)	27.0**	75.0 (35.0-96.1)	81.2 (74.8-86.5)	3.99	0.31	14.6	98.7
6.0	127 (3.9)	0.917 (0.855-0.959)	**27.5***	80.0 (28.8-96.7)	88.5 (81.5-93.6)	6.97	0.23	22.2	99.1

&Completed ages

#n (%): Total number of children in that age group (prevalence of obesity)

*Youden index (J) ≥0.6

**Youden index (J) ≥0.4

ROC: receiver operating curve, CI: confidence interval, LR: likelihood ratio, PV: predictive value

obesity between the ages of 5 and 14 years was 4 times higher in children who had been overweight at the age of 5 years compared with those of normal weight at that age. Therefore, it is essential to assess adiposity and unstable fat distribution before and during the adiposity rebound period (4,6,7,8). Since it was shown that early childhood adiposity has certain cardiologic and metabolic health consequences in later life, we need a reliable and easy-to-use measure for preschool children.

The available data about NC percentiles of preschool children was from a multicenter study (Sweden, Germany, Hungary, Italy, Cyprus, Spain, Belgium, Estonia) that included normal-weight 2.0-10.9-year-old European children (21). Formisano et al (18) later worked on these data and showed that cardiometabolic risk was associated with increased NC. We believe that this current study will be contributory to the importance of the role of NC measurements in young children by providing local reference data as well as by comparing these references with the most recent and reliable studies in Europe.

In our study, similar to data reported by Nagy et al (21) for children in the multicenter study, we found that NC values

Table 5. Receiver operating curve analysis for neck circumference to determine cut-off points for obesity for 3-6-year-old girls

Age group (years)[&]	n (%)[#]	ROC (95% CI)	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	+LR	-LR	+PV	-PV
3.0	187 (3.3)	0.773 (0.706-0.832)	25.8*	83.3 (36.1-97.2)	78.1 (71.3-83.9)	3.80	0.21	11.4	99.3
4.0	192 (4.7)	0.771 (0.705-0.828)	25.8*	77.8 (40.1-96.5)	72.7 (65.6-79.0)	2.85	0.31	12.3	98.5
5.0	200 (4.0)	0.805 (0.743-0.858)	25.7**	87.5 (47.4-97.9)	62.3 (55.0-69.2)	2.32	0.20	8.9	99.2
6.0	118 (5.1)	0.715 (0.625-0.794)	25.5**	83.3 (36.1-97.2)	65.2 (55.6-73.9)	2.39	0.26	11.4	98.6

[&]Completed ages

#n (%): Total number of children in that age group (prevalence of obesity)

*Youden index (J) ≥0.6

**Youden index (J) ≥0.4

J = maxc [Se(c) + Sp(c) – 1]

ROC: receiver operating curve, CI: confidence interval, LR: likelihood ratio, PV: predictive value

increase with age and that they are slightly higher in males. This gender difference becomes significant in children older than 5 years. However, the increase in NC through 24 to 83 months is relatively smaller than that reported by Nagy et al (21). The increase in 50th percentile NC values was 1.2 cm and 0.6 cm in our study, while the above authors reported an increase of 2.4 cm and 2.1 cm for boys and girls, respectively. Furthermore, NC percentile values in Turkish preschool children were slightly greater than those of the European preschool children in both genders (Figures 1, 2). This difference disappears around the rebound adiposity period and then NC values of Turkish preschool children decrease slightly. However, the 97th percentile NC values of Turkish preschool children continue to be greater than those of their European counterparts in both genders. These findings may be related to several factors such as sample selection, ethnic or geographical differences. The study cited above (21) included data from eight European countries. However, we do not know whether similar differences would exist if our data could have been compared with findings from each individual country separately. We must also note that the similarity of NC values during the rebound adiposity period found in our study slightly differs from those cited in the above study (Figures 1, 2).

We consider that this difference may be explained by environmental and other unknown factors that could have more predominant effects than genetic factors at the adiposity rebound period and later. This should be investigated in further studies.

This study provides NC cut-off values for obesity in Turkish preschool children. Additionally, it is the first study that reports NC percentiles and cut-offs in Turkish preschool children. The power of this study may be the stratification for socioeconomic level in a relatively big sample size which

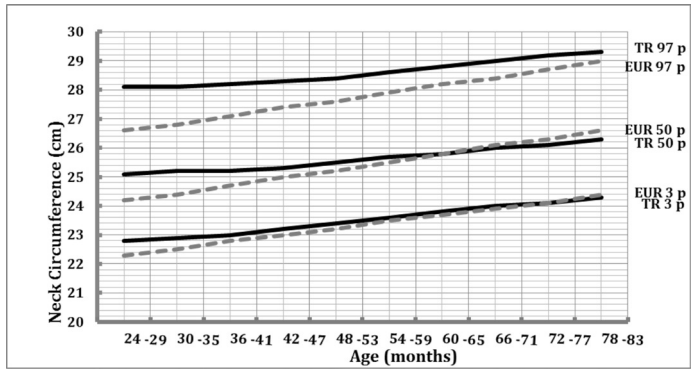

Figure 1. The comparison of 3rd, 50th, and 97th percentiles of neck circumference in boys with European children (Identification and prevention of Dietary- and lifestyle-induced health EFfects in Children and infantS study)

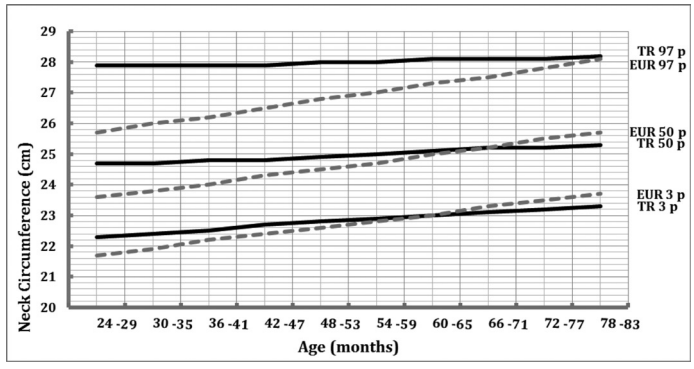

Figure 2. Comparison of 3rd, 50th, and 97th percentiles of neck circumference in the girls with European children (Identification and prevention of Dietary- and lifestyle-induced health EFfects in Children and infantS study)

may represent Turkish population. Our findings show that NC may also be a useful tool to assess obesity in preschool children in addition to BMI, since it represents upper body fat distribution. However, the cut-off values of NC established in our study should be confirmed in subsequent studies.

In conclusion, this study provides reference and cut-off values for preschool Turkish children. Since NC findings may indicate future cardiometabolic risk, both our reference and cut-off values for NC may be useful for screening and follow-up.

Ethics

Ethics Committee Approval: The Ethics Committee in Erciyes University approved this study in 2008 Number: 2008/28. Informed Consent: Written parental consent was obtained prior to the study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Selim Kurtoğlu, Mümtaz M. Mazıcıoğlu, Meda Kondolot, Design: Selim Kurtoğlu, Mümtaz M. Mazıcıoğlu, Ahmet Öztürk, Data Collection or Processing: Duygu Horoz, Serpil Poyrazoğlu, Arda Borlu, Analysis or Interpretation: Mümtaz M. Mazıcıoğlu, Ahmet Öztürk, Literature Search: Meda Kondolot, Mümtaz M. Mazıcıoğlu, Writing: Meda Kondolot, Mümtaz M. Mazıcıoğlu.

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Role of Versican and ADAMTS-1 in Polycystic Ovary Syndrome

Sibel Özler[1], Efser Öztaş[1], Aytekin Tokmak[2], Merve Ergin[3], Meryem Kuru Pekcan[2], Başak Gümüş Güler[4], Halil İbrahim Yakut[5], Nafiye Yılmaz[2]

[1]Zekai Tahir Burak Women's Health Training and Research Hospital, Clinic of Perinatology, Ankara, Turkey
[2]Zekai Tahir Burak Women's Health Training and Research Hospital, Clinic of Obstetrics and Gynecology, Ankara, Turkey
[3]25 Aralık State Hospital, Clinic of Clinical Biochemistry, Gaziantep, Turkey
[4]Liv Hospital, Clinic of Obstetrics and Gynecology, Ankara, Turkey
[5]Zekai Tahir Burak Women's Health Training and Research Hospital, Clinic of Pediatrics, Ankara, Turkey

What is already known on this topic?

Patients having polycystic ovary syndrome (PCOS), show the symptoms of oligo/anovulation, and hyperandrogenism; and their ultrasonographic examination demonstrates the polycystic view of ovaries. The degradation of cumulus oophorus complex (COC) during ovulation, depends on excretion of various cytokines, prostoglandins, extracellular matrix enzymes, and proteases from the neighboring cells. Versican is one of the proteoglycans forming the COC; and it is degraded by the protease, ADAMTS-1, during the ovulation process.

What this study adds?

Our aim in this study was to investigate if serum versican and ADAMTS-1 levels could be used as a serum marker in anovulation patients. We consider these markers to be used in the treatment strategies of infertile patients having PCOS; with the help of upcoming studies, containing more sample sizes.

Abstract

Objective: ADAMTS-1 is a matrix metalloproteinase which cleaves versican in the cumulus oocyte complex under the effect of luteinizing hormone surge in the periovulatory period. Altered levels may have a role in the pathogenesis of polycystic ovary syndrome (PCOS). We aimed to determine the serum versican and ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motif-1) levels in PCOS patients and compare the results with healthy controls.

Methods: Thirty-eight patients with PCOS and forty healthy controls aged between 15 and 22 years were included in the study. They were sampled according to their basal hormone, serum versican, and ADAMTS-1 levels. Serum versican and ADAMTS-1 levels were measured by enzyme-linked immunosorbent assay. A multivariate logistic regression model was used to identify the independent risk factors of PCOS.

Results: Serum versican levels were significantly decreased in the PCOS group when compared with the controls. The best versican cut-off value for PCOS was calculated to be 33.65 with 76.74% sensitivity and 52.94% specificity. Serum versican levels, homeostasis model assessment of insulin resistance index, a Ferriman-Gallwey score higher than 8, and oligomenorrhea were the strongest predictors of PCOS. Serum versican levels were significantly decreased in PCOS patients. Besides, serum ADAMTS-1 and versican levels were significantly and positively correlated with each other.

Conclusion: Serum versican levels were significantly decreased in patients with PCOS. This suggests a possible role of versican in ovulatory dysfunction and in the pathogenesis of PCOS.

Keywords: Versican, ADAMTS-1, polycystic ovary syndrome

Address for Correspondence: Sibel Özler MD,
Zekai Tahir Burak Women's Health Training and Research Hospital, Clinic of Perinatology, Ankara, Turkey
Phone: +90 312 306 50 00 **E-mail:** sibel2ozler@gmail.com

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most frequent endocrinopathies among women of reproductive age, with a prevalence of 6-26%, depending on the diagnostic criteria and ethnicity (1,2). Women with PCOS have increased risk of developing type 2 diabetes. PCOS is also associated with multiple cardiovascular risk factors (3,4). In many women with PCOS, the signs and symptoms start at adolescence (5). Considering the adverse long-term consequences of PCOS, especially when the symptoms begin in the early period of life, early diagnosis and treatment are of great importance for prevention of its progression. It is important to know the underlying factors causing PCOS to provide guidance for early intervention. Although genetic and environmental factors are thought to have a role in the etiopathogenesis of the disease, the exact mechanism is still unclear (5).

PCOS is defined as a syndrome of ovarian dysfunction and its clinical manifestations are mainly attributed to this (4). During both fetal and adult life, continuous remodeling of the ovarian tissue is taking place to maintain normal ovarian function, and this process requires changes also in the extracellular matrix (ECM) (6). Successful ovulation is a complex process consisting of follicular development, ovulation, luteal formation and subsequent regression, and these are all dependent on the cyclical remodeling of ECM (7). Most critical ovulatory mediators were shown to be the ones exerting their effects through the cumulus cell complex surrounding the oocyte (8). Besides, it has been suggested that extensive tissue remodeling of the ovary is mediated by heparan sulfate proteoglycans and matrix metalloproteinases (MMP) (7,9,10).

A disintegrin-like metalloproteinase with thrombospondin motif (ADAMTS) proteases are a distinct group of zinc metalloproteases, comprising 20 members and known to function in the cleavage and degradation of various ECM components (11). Increasing evidence suggests that ADAMTSs have a role in various physiopathological processes such as morphogenesis during embryonic development, follicular development and ovulation, cyclic endometrial remodeling, angiogenesis as well as in development of cancer, of thrombotic and inflammatory conditions (11,12,13,14,15). ADAMTS-1 is a MMP that has been implicated in the inhibition of angiogenesis induced by luteinizing hormone (LH) in the periovulatory follicles of the mouse and rat and is also thought to be a mediator of proteolytic cleavage of the hyaluronan binding proteoglycans, aggrecan, and versican (16,17).

Cumulus oocyte complex (COC) is rich of hyaluronan which is synthesized by cumulus cells, via expression of hyaluronan synthase-2 (18). Versican, a large hyaluronic acid (HA) binding proteoglycan, is expressed by periovulatory granulosa cells and localized within the expanding matrix of COC (18). The secreted active form of ADAMTS-1 has been shown to be localized selectively in COC, and with the effect of LH surge in the periovulatory period, to lead to cleavage of versican (19). Thus, it has been suggested that cleavage of versican in the expanded COC matrix is an important function of ADAMTS-1 in the ovulation process (18,19). Furthermore, previous studies suggested a relationship between versican and atherosclerosis, diabetes, insulin resistance (IR) and endothelial dysfunction, which are well-known long-term consequences of PCOS (3,20,21).

Based on the results of the above-mentioned studies, we hypothesized that altered levels of ADAMTS-1 and versican in the ECM of COC, by causing ovulatory dysfunction, might be causative factors for the clinical manifestations of PCOS. In the present study, we aimed to compare the serum ADAMTS-1 and versican levels in adolescent and young females with PCOS with those of a control group.

Methods

Thirty-eight adolescent and young female subjects with PCOS, aged between 15-22 years, were recruited consecutively from the outpatient clinic of Zekai Tahir Burak Women's Health Training and Research Hospital. The diagnosis of PCOS was based on presence of two of the following three criteria: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries, as proposed by the Rotterdam consensus in 2003 (4). Venous blood from the patients was sampled on the third day of their menstrual cycles. Forty age-matched healthy controls were also recruited.

Exclusion criteria were use of medications known to alter insulin secretion or action and lipoprotein metabolism; hypertension; smoking; family history of cardiovascular diseases; and endocrinopathies including diabetes, Cushing syndrome, androgen-secreting tumors, late-onset 21-hydroxylase deficiency, thyroid dysfunction; current use of oral contraceptives; presence of autoimmune diseases; and hyperprolactinemia. All participants provided a written informed consent and the study protocol was approved by the local ethics committee of our hospital (Approval date/number: 16.10.2014/15).

Clinical examinations were performed and anthropometric measurements were recorded for all participants included in the study. Body mass index (BMI) was calculated by using the formula: weight (kg)/height (m^2). Waist circumference

(WC) was measured as the circumference of the abdomen at its narrowest point between the lower costal (10th rib) border and the top of the iliac crest. Hip circumference was measured at the level of greatest posterior protuberance of the buttocks. Blood samples were obtained by venipuncture after an overnight fasting for at least 12 hours for biochemical evaluation and were processed within 1 hour after withdrawal. The serum samples were stored at -80 °C until the day of analysis.

All analyses were performed with the use of a Beckman Coulter (High Wycombe, United Kingdom) Gen-S automated analyzer. Plasma glucose levels were determined with the glucose hexokinase method. Serum levels of follicle-stimulating hormone (FSH), and LH were determined by immunochemiluminometric assay. Inter-/intra-assay coefficients of variability (CV) for FSH and LH were 2.3%/1.4% and 2.1%/3.1%, respectively. Estradiol, prolactin, dehydroepiandrosterone sulfate (DHEAS), total testosterone (total-T), insulin, and thyroid-stimulating hormone (TSH) were measured using the UniCel DxI 800 radioimmunoassay system (Beckman Coulter, Fullerton, CA, USA). The inter- and intra-assay CVs were 0.1% and 3.2% for estradiol, 1.7% and 3.2% for prolactin, 1.7% and 2.8% for DHEAS, 0.5% and 1.7% for total-T, 8.5% and 2.4% for TSH. Serum levels of 17-hydroxy progesterone (17OH-P) and free testosterone (free-T) were measured by radioimmunoassay (Siemens, Erlangen, Germany). The inter- and intra-assay CVs were 4.6% and 10.7% for 17OH-P and 5.7% and11.4% for free-T, respectively. Homeostasis model assessment of IR (HOMA-IR) (insulin × glycemia in (µmol/L)/ 22.5) was estimated. HOMA-IR >2.5 was considered to indicate the presence of IR (22). The serum levels of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) were determined with enzymatic colorimetric assays via the use of an AU680 Chemistry System (Beckman Coulter, Fullerton, CA, USA). The lipid accumulation product (LAP) index was calculated using the formula [WC (cm)-58] × [TG concentration (mmol/L)] (23).

Serum ADAMTS-1 concentrations were determined by human ADAMTS-1 enzyme-linked immunosorbent assay (ELISA) (Eastbiopharm Co., Ltd., Hangzhou, China) and the results were expressed as ng/mL. Serum versican concentrations were determined by using human versican ELISA kit (Boster Biological Technology Co., California, USA) and the results were expressed as pg/mL.

Statistical Analysis

Data analysis was performed by using Statistical Package for the Social Sciences for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). The data were shown as mean [95% confidence interval (CI)] or number of cases and (percentage), where applicable. The Kolmogorov-Smirnov test was used to determine whether continuous variables were normally distributed or not. Homogeneity of variances was evaluated by the Levene test. Continuous variables were shown as mean ± standard deviation (SD) or median (minimum-maximum), where applicable. Mean differences between case and control groups were compared by student's t-test. Mann-Whitney U-test was used to compare the median values. Nominal data were analyzed by Pearson's chi-square test. Whether the mean differences between groups were statistically significant or not, were evaluated by analysis of covariance (ANCOVA). Degrees of association between continuous variables were evaluated by partial correlation analyses. The optimal cut-off points of laboratory parameters discriminating groups were evaluated by receiver operating characteristic (ROC) analyses, calculating area under the curve (AUC) as giving the maximum sum of sensitivity and specificity for the significant test. Multiple logistic regression analyses were applied for calculating odds ratios (OR) and 95% CIs for each clinical condition. Linear regression model was used to evaluate the relation of independent variables as clinical and laboratory parameters in the group having PCOS. A p-value less than 0.05 was considered statistically significant.

Results

A total of seventy-eight participants (38 adolescent and young females with PCOS and 40 age-matched healthy controls) were enrolled in this case-control study. The baseline clinical, endocrinological, and laboratory characteristics are given in Table 1.

Among the laboratory parameters, fasting plasma glucose, insulin, HOMA-IR, estradiol, and LH levels were significantly higher in PCOS group. LAP index (calculated as 29.86 and 14.17 in PCOS and control groups, respectively) was also significantly higher in PCOS patients ($p < 0.001$). The mean value for WC in PCOS group was 77.23 ± 11.87 cm, which was significantly higher than that of the control group, 72.89 ± 7.78 cm ($p = 0.041$). ADAMTS-1 levels were not significantly lower in PCOS group ($p = 0.959$). The serum versican levels of the PCOS and control groups were detected as 54.69 ng/mL and 95.6 ng/mL, respectively, showing that serum versican levels were significantly decreased in PCOS group when compared with the controls ($p = 0.009$).

Versican levels were re-evaluated using ROC analysis; cut-off levels were determined and AUC was calculated. According to

Table 1. Baseline characteristics, clinical and laboratory parameters of patients with polycystic ovary syndrome and controls

	PCOS (n = 38)	Control (n = 40)	p-value
Clinical findings			
Age (years)	18.38 ± 2.07	18.89 ± 2.59	0.320
Age at menarche (years)	12.67 ± 1.10	13.02 ± 1.17	0.160
BMI (kg/m^2)	23.52 ± 2.91	22.95 ± 3.70	0.439
WHR	0.79 ± 0.07	0.76 ± 0.06	0.068
WC (cm)	77.23 ± 11.87	72.89 ± 7.78	0.041
Laboratory findings			
Fasting blood glucose (mg/dL)	88.5 (76-105)	86 (64-127)	**0.034**
Insulin (µIU/mL)	10.14 (4.72-46.7)	7.05 (2.43-18.79)	**0.004**
HOMA-IR	2.13 (0.98-9.69)	1.63 (0.49-3.94)	**0.004**
Estradiol (pg/mL)	36.82 ± 17.90	25.73 ± 17.38	**0.008**
LH (mIU/mL)	8.19 (3.56-21.13)	4.66 (0.65-31.77)	**< 0.001**
FSH (mIU/mL)	6.25 (2.32-12.60)	6.27 (2.74-21.18)	0.897
DHEA-S (µg/dL)	386 (137.1-953.7)	313.7 (179.7-466.3)	0.075
17-OH progesterone (ng/dL)	1.23 (0.71-8.01)	0.92 (0.27-2.83)	0.073
Total-C (mg/dL)	156.03 ± 27.06	151.92 ± 31.08	0.555
LDL-C (mg/dL)	72.70 ± 23.54	70.32 ± 25.33	0.677
HDL-C (mg/dL)	63.80 ± 8.23	64.63 ± 9.41	0.728
TG-C (mg/dL)	85 (45-298)	71.5 (39-284)	0.081
Total cholesterol/HDL-C ratio	2.31 (1.68-3.70)	2.2 (1.68-4.82)	0.357
LAP index	29.86 (1.66-191.94)	14.17 (-2.28-65.45)	**< 0.001**
Total testosterone	18.78 ± 11.52	20.61 ± 9.98	0.549
Free testosterone	1.46 (0.83-5.77)	1.36 (0.60-3.57)	0.075
ADAMTS-1 (pg/mL)	4.63 (0.14-15.8)	5.03 (1.63-17.02)	0.959
Versican (ng/mL)	54.69 (21.56-158.9)	95.6 (25.1-283.0)	0.009

*Student's t-test; mean ± standard deviation. Mann-Whitney U-test; median (minimum–maximum). p-value < 0.05 is considered as statistically significant; BMI: body mass index, WC: waist circumference, WHR: waist to hip ratio, HOMA-IR: homeostasis model assessment of insulin resistance, LH: luteinizing hormone, FSH: follicle-stimulating hormone, TSH: thyroid-stimulating hormone, DHEA-S: dehydroepiandrosterone sulfate, Total-C: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TG-C: triglycerides-cholesterol, LAP index: lipid accumulation product index, ADAMTS-1: a disintegrin and metalloproteinase with thrombospondin motif-1, 17-OH: 17-hydroxy

Table 2. Regression analysis in the prediction of polycystic ovary syndrome

	Univariate		Multivariate	
	OR (95% CI)	p-value	OR (95% CI)	p-value
HOMA-IR > 2.5	0.212 (0.053-0.852)	**0.029**	1.452 (0.476-4.429)	0.512
Estradiol (pg/mL)	1.037 (1.008-1.067)	**0.011**	1.034 (0.987-1.084)	0.158
LH (mIU/mL)	1.153 (1.026-1.296)	**0.017**	1.248 (1.029-1.513)	**0.024**
LAP index	1.062 (1.019-1.107)	**0.004**	1.038 (0.982-1.098)	0.187
Versican (ng/mL)	0.990 (0.981-0.998)	**0.018**	0.971(0.949-0.994)	**0.015**

p-value < 0.05 is considered as statistically significant; HOMA-IR: homeostasis model assessment of insulin resistance, LH: luteinizing hormone, LAP index: lipid accumulation product index, OR: odds ratio, CI: confidence interval

the ROC analysis performed for the diagnostic performance of serum versican levels for PCOS, the AUC was 0.675 (95% CI: 0.55-0.795; p = 0.009) (Figure 1). The best versican cut-off value for PCOS was 33.65 with 76.74% sensitivity, 52.94% specificity, 97.35% positive and 64.29% negative predictive values. According to the ROC analysis performed to assess the differences of LAP index between groups, the AUC was 0.714 (95% CI: 0.601-0.827; p < 0.001) (Figure 2).

Table 3. Linear regression model for the assessment of polycystic ovary syndrome risk

	Unstandardized coefficients				
	β	Standard error	Beta	t	p-value
Versican	-0.002	0.001	-0.333	-3.348	**0.001**
HOMA-IR	0.085	0.029	0.293	2.984	**0.004**
FGS	0.034	0.010	0.341	3.438	**0.001**
Oligomenorrhea	0.305	0.107	0.286	2.846	**0.006**

HOMA-IR: homeostasis model assessment of insulin resistance, FGS: Ferriman Galloway score

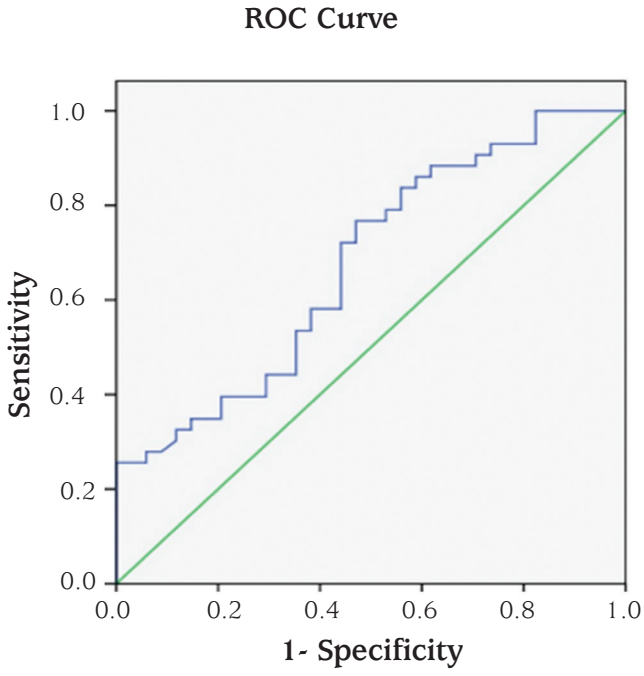

Figure 1. Versican receiver operating characteristic curve.

ROC: receiver operating characteristic

The best cut-off value of versican for distinguishing PCOS was 16.49 with 63.64% sensitivity, 40.73% specificity, 70.0% positive and 64.44% negative predictive values. All statistically significant parameters according to univariate analysis were further evaluated with multivariate logistic regression analysis (Table 2). Considering the laboratory parameters, versican levels less than 33.65 ng/mL were found to be significantly associated with PCOS (OR: 0.971, 95% CI: 0.949-0.994, p = 0.015). Besides, increased levels of LH were also found to be significantly associated with PCOS (OR: 1.247, 95% CI: 1.029-1.513, p = 0.024).

We used multiple linear regression analysis with stepwise method to evaluate the predictive effects of

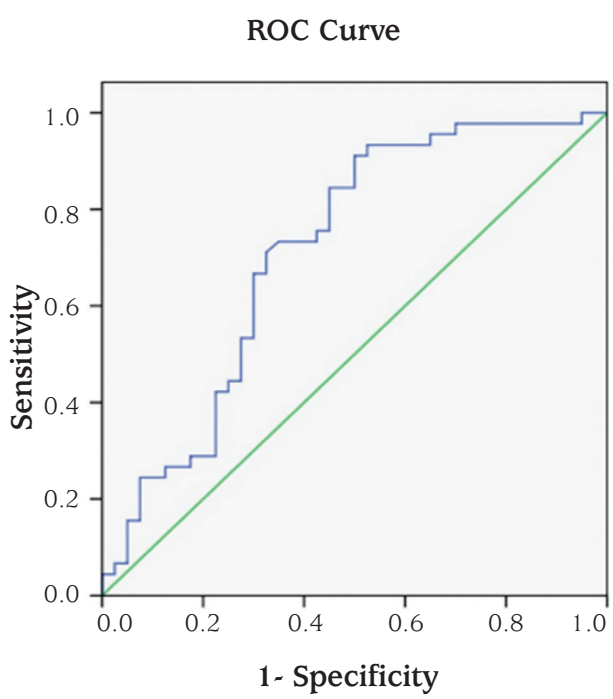

Figure 2. Lipid accumulation product index receiver operating characteristic curve.

ROC: receiver operating characteristic

independent variables [like serum versican level, HOMA-IR index, Ferriman-Gallwey score (FGS) higher than 8, oligomenorrhea] on PCOS which is a dependent variable. The analysis demonstrated that these parameters were the strongest predictors of PCOS, since they explained 25% of variance in PCOS (Table 3).

In the PCOS group, a statistically significant positive correlation was determined only between ADAMTS-1 and versican levels (r = 0.615, p < 0.001). No significant correlations were determined between versican levels and HOMA-IR, LAP index, hyperandrogenism, age at menarche, FGS, and oligomenorrhea.

Discussion

In the present case-control study, significantly decreased levels of serum versican were detected in adolescents and young adults with PCOS when compared with healthy age-matched controls. In addition, a statistically significant positive correlation was determined between serum ADAMTS-1 and versican levels in the PCOS group.

Ovulation requires remodelling of some essential ECM components to permit the release of the COC from the surface of the ovary. This process involves proteolytic

events, as well as the proper formation of the expanded COC matrix (24). ADAMTS-1 is shown to be secreted mainly by mural granulosa cells, localized to the ECM of expanded COCs and induced markedly by LH in ovulating follicles (18,25). Besides, versican was also shown to be present in the expanded COC matrix and was induced by LH surge, consistent with the activity and localization of ADAMTS-1 (18,19). Xiao et al (26) investigated the expression of ADAMTS-1 in granulosa cells of the PCOS patients, both by immunocytochemistry and reverse transcription polymerase chain reaction and demonstrated the decreased expression of ADAMTS-1 in PCOS patients when compared with normally ovulating women. On the contrary, we found no statistically significant difference in ADAMTS-1 levels between PCOS and the control groups. Although not significant, ADAMTS-1 levels were lower in the PCOS group and, in the present study, we also demonstrated a significant positive correlation between ADAMTS-1 and versican levels. A possible explanation for this discrepancy may be the limited number of participants of our study. However, Russell et al (18) showed that versican was cleaved in the COCs of the progesterone receptor knocked out mice, even when ADAMTS-1 levels were markedly decreased, a finding which is consistent with the results of the present study.

We observed significantly decreased serum versican levels in PCOS patients when compared with the controls, in our study. Consistent with our result, Richards et al (27) demonstrated that versican was the primary substrate in COCs not only for ADAMTS-1 but also for ADAMTS-4 and 5, and they were all expressed in spatiotemporal patterns suggesting evident and probable overlapping functions with each other. So in the present study, as the versican levels were significantly decreased in the PCOS group, we suggested that it was the primary component of the COCs cleaved during ovulation by the induction of LH surge. However, the insignificantly lower levels of ADAMTS-1 which were observed in our study suggested its important but not the only role in the degradation of ECM components.

LAP index is a cheap and easily available marker of risk for cardiovascular disease. Previous studies have already shown that it has good and reliable diagnostic accuracy for the detection of IR, metabolic syndrome, and risk for cardiovascular disease, even stronger than BMI, WC, and waist-hip ratio (28,29).

We attributed the lack of differences in levels of DHEAS, free-T, total-T, and TG between the groups, to the smallness of our sample. The small number of patients was the main limitation of our study. However, we consider this study important as one of the first in the field and believe that it will encourage further clinical research in this area which include larger samples yielding more significant results.

In conclusion, serum versican levels were found to be significantly decreased in adolescent girls and young women with PCOS. The results of this study support a possible role of versican in ovulatory dysfunction and in the pathogenesis of PCOS.

Ethics

Ethics Committee Approval: The study protocol was approved by the local ethics committee of our hospital (Approval date/number: 16.10.2014/15). Informed Consent: All participants provided a written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Sibel Özler, Efser Öztaş, Aytekin Tokmak, Halil İbrahim Yakut, Nafiye Yılmaz, Design: Sibel Özler, Efser Öztaş, Aytekin Tokmak, Halil İbrahim Yakut, Nafiye Yılmaz, Data Collection or Processing: Aytekin Tokmak, Meryem Kuru Pekcan, Başak Gümüş Güler, Analysis or Interpretation: Sibel Özler, Aytekin Tokmak, Literature Search: Sibel Özler, Efser Öztaş, Aytekin Tokmak, Merve Ergin, Meryem Kuru Pekcan, Başak Gümüş Güler, Halil İbrahim Yakut, Nafiye Yılmaz, Writing: Sibel Özler, Efser Öztaş, Merve Ergin, Başak Gümüş Güler, Halil İbrahim Yakut, Nafiye Yılmaz.

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ORIGINAL ARTICLE

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The Relationship between Serum Zonulin Level and Clinical and Laboratory Parameters of Childhood Obesity

Tuncay Küme[1], Sezer Acar[2], Hale Tuhan[2], Gönül Çatlı[2], Ahmet Anık[2], Özlem Gürsoy Çalan[1], Ece Böber[2], Ayhan Abacı[2]

[1]Dokuz Eylül University Faculty of Medicine, Department of Medical Biochemistry, İzmir, Turkey
[2]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey

What is already known on this topic?

Zonulin is a protein that increases the intestinal permeability in small intestinal system by modulating the intracellular tight junctions.

What this study adds?

The present study is the first study to compare the serum zonulin levels between obese and non-obese children. We demonstrated that serum zonulin levels were higher in obese children when compared to healthy children, which may play a role in the pathogenesis of obesity and related metabolic disturbances.

Abstract

Objective: To investigate the relationship between zonulin levels and clinical and laboratory parameters of childhood obesity.
Methods: The study included obese children with a body mass index (BMI) > 95th percentile and healthy children who were of similar age and gender distribution. Clinical (BMI, waist circumferences, mid-arm circumference, triceps skinfold, percentage of body fat, systolic blood pressure, diastolic blood pressure) and biochemical (glucose, insulin, lipid levels, thyroid function tests, cortisol, zonulin and leptin levels) parameters were measured.
Results: A total of 43 obese subjects (23 males, mean age: 11.1 ± 3.1 years) and 37 healthy subjects (18 males, mean age: 11.5 ± 3.5 years) were included in this study. Obese children had significantly higher insulin, homeostasis model assessment of insulin resistance, triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDL-C), zonulin and leptin levels than healthy children ($p < 0.05$), while glucose levels were not different ($p > 0.05$). Comparison of the obese children with and without insulin resistance showed no statistically significant differences for zonulin levels ($p > 0.05$). Zonulin levels were found to negatively correlate with HDL-C and positively correlate with leptin levels, after adjusting for age and BMI.
Conclusion: To the best of our knowledge, this is the first study investigating the relationship between circulating zonulin level (as a marker of intestinal permeability) and insulin resistance and leptin (as markers of metabolic disturbances associated with obesity) in childhood obesity. The results showed that zonulin was significantly higher in obese children when compared to healthy children, a finding indicating a potential role of zonulin in the etiopathogenesis of obesity and related disturbances.
Keywords: Tight junctions, zonulin, insulin resistance, obesity, childhood

Introduction

The prevalence of an overweight state, which is an important cause of morbidity and mortality in the world, has increased to pandemic proportions among children and adolescents. Imbalance between energy intake and expenditure as well as sedentary lifestyle are the leading causes of obesity (1). Obesity is associated with systemic microinflammation due to the release of proinflammatory peptides in visceral adipose tissue. Systemic microinflammation in obesity is the major cause in the pathogenesis of metabolic disorders such as insulin resistance (IR), dyslipidemia, type 2 diabetes (2). Recent evidence suggests a possible role of intestinal barrier dysfunction from releasing proinflammatory peptides in obesity (3).

The intestines have a function as a barrier to protect the body from infectious, toxic, allergic agents as well as from

Address for Correspondence: Ayhan Abacı MD,
Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
Phone: +90 232 412 60 76 **E-mail:** ayhanabaci@gmail.com

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antigenic loads and also from caloric loads. Recent studies which report an increase in intestinal permeability and absorption together with a decrease in intestinal motility in patients with metabolic disorders indicate that there is a link between intestinal barrier dysfunction and metabolic disorders (3).

The structure, function, and regulation of interepithelial zonula occludens [tight junction (TJ)] are important for intestinal barrier function. Firstly, the zonula occludens toxin (zot), an enterotoxin secreted by *Vibrio Cholera,* was reported to affect the TJs. Subsequently, human zonulin, a physiological mediator in 47 kDa protein structure which regulates the permeability of intestinal TJ and acts as an agent of innate immunity, was isolated as a homolog of zot. Intestinal TJ dysfunction and upregulation of zonulin were found to be the primary defects in these diseases (4). Circulating zonulin levels are considered to be a useful marker of intestinal permeability. Recently, higher circulating zonulin levels were reported in obese adult subjects as compared to the non-obese and also in adults with glucose intolerance as compared to those with normal glucose tolerance (3,5). Significantly higher serum zonulin levels were also reported in obese subjects with biopsy-confirmed nonalcoholic fatty liver disease (NAFLD) than in obese subjects without NAFLD (4).

Leptin, the protein product of the *ob* gene, was shown to be involved in the regulation of food intake, energy consumption, body weight, and glucose metabolism (6). It was shown that leptin concentrations change in nutritional states such as fasting followed by a subsequent increase in food intake (7). However, there are no studies which evaluate the association between serum zonulin and leptin levels.

In the present study, we aimed to investigate the relationship of circulating zonulin level as an intestinal permeability marker and that of leptin as a metabolic disorders marker in childhood obesity.

Methods

Forty-three obese children with a body mass index (BMI) greater than the 95th percentile according to the standards of the Centers for Disease Control and Prevention (CDC-2000) and 37 healthy children of similar age, gender, and pubertal stage distribution with a BMI between the 5th and 85th percentile were consecutively enrolled in the study (8). Patients and control subjects with chronic diseases (cardiovascular, gastrointestinal, and respiratory), a history of drug use (steroids and antipsychotics), endocrine pathology (Cushing syndrome and hypothyroidism), or suspected syndromes associated with obesity (Prader-Willi and Laurence-Moon-Biedl syndromes) were excluded from the study. Subjects with a recent history of upper airway infection, gastroenteritis, and use of antibiotics were also excluded.

All subjects underwent a thorough physical examination. A biochemical evaluation including thyroid function tests and serum cortisol measurement for probable endocrine pathology was performed in all obese subjects.

The study was initiated after the approval of the local ethics committee of Dokuz Eylül University Faculty of Medicine. A written informed consent of the parent(s) of each subject was also obtained before the study.

Height was measured using a Harpenden stadiometer with a sensitivity of 0.1 cm. Weight was measured using a SECA scale with a sensitivity of 0.1 kg, with all clothing removed except undergarments. BMI was calculated by dividing weight (kg) by height squared (m^2). Waist circumference (WC) and mid-upper arm circumferences (MAC) were measured using standard techniques. Triceps skinfold thickness (TSF) (in millimeters) was measured with a Harpenden skinfold caliper. The percentage of body fat (PBF) was measured using bioelectric impedance analysis (Tanita BC-418, Tokyo, Japan).

Findings for pubertal development were evaluated according to Tanner staging (9). A testicular volume of ≥4 mL in males and breast development of stage 2 and over in females were considered to be findings of puberty.

Blood pressure was measured by one of the investigators using a validated protocol. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice at the right arm after a 10-min rest in the supine position using a calibrated sphygmomanometer. Hypertension was defined as blood pressure values above the 95th percentile for height, age, and gender (10).

The venous blood samples were collected in plane tubes after 10-12 h of night fasting. The plane tubes were centrifuged at 1200xg for 10 minutes and serum samples were transferred into the Eppendorf tubes using plastic Pasteur pipettes. Routine parameters (glucose, insulin, lipids, thyroid function tests, cortisol) were analyzed on the same day. Samples to be analyzed for special parameters (zonulin, leptin) were stored at -80 °C until analysis.

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

CA, USA). IR was evaluated according to the homeostasis model assessment of IR (HOMA-IR) index. Different cut-off values for prepubertal and pubertal subjects were determined based on 85th percentile values of control cases (prepubertal >2.5, pubertal >4) (11).

Serum leptin levels (catalog no: EK0595 and EK0437, Boster Biological Technology Co Ltd, Wuhan, China) and zonulin levels (catalog no: CSB-EL028107HU and CSBEQ27649HU, CUSABIO Biotech Co Ltd, Wuhan, China) were measured by enzyme linked immunosorbent assay kit (ELISA) based on the principle of competitive enzyme immunoassay. In this assay, the microplate in the kit is pre-coated with antibody specific to the analyte. The standard is reconstituted and prepared by serial dilution with sample diluents. The serum samples are diluted 1:10 with sample diluents for leptin and adiponectin assays and 1:2000 for zonulin. Standards and samples are loaded into the appropriate microtiter plate wells and any analyte present is bound by immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific to the analyte is added to the wells. After washing, avidin-conjugated peroxidase is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added and color develops in proportion to the amount of analyte. The color development is stopped and the intensity of the color is measured spectrophotometrically at a wavelength of 450 nm. A standard curve of known concentration of analyte is established and the concentration of analyte in the samples is calculated accordingly. The ELISA assays for leptin and zonulin had a sensitivity of <10 pg/mL and 0.156 ng/mL, a detection range of 62.5-4000 pg/mL and 0.625-40 ng/mL, an intraassay coefficient of variation (CV) of <10% and <8%, as well as interassay CV of <10% and <10%, respectively.

Statistical Analysis

The analyses of the data were conducted with Statistical Package for the Social Sciences 16.0.1 (SPSS Inc., Chicago, IL, USA). The distribution of the data was evaluated with the Kolmogorov-Smirnov test. For numerical comparisons, the student's t-test or Mann-Whitney U-test were used according to the distribution of the measured parameters. Categorical variables were compared using the chi-square test. A correlation analysis was performed using Spearman's correlation analysis. Since zonulin levels were not normally distributed, they were transformed to logarithmic values for multivariate linear regression analysis. Variables with a p-value < 0.05 in the bivariate correlation analysis were included in a multivariate linear regression analysis model to assess the independent determinants of serum zonulin level. A partial correlation was also performed, with serum zonulin as a dependent variable, controlling for potential confounders such as age and BMI. The data were presented as mean ± standard deviation or median and interquartile range (IQR). In all statistical tests, p-values <0.05 were considered significant.

Results

A total of 43 obese subjects (23 males, 20 pubertal, mean age: 11.1±3.1 years) and 37 healthy subjects (14 males, 23 pubertal, mean age: 11.5±3.6 years) were included in the study. The groups were similar for age, gender, and pubertal status. There were significant differences between obese and healthy children in terms of BMI, BMI- standard deviation score (SDS), WC, MAC, TSF, fat mass, PBF, SBP, and DBP ($p<0.05$), as shown in Table 1. Obese children had significantly higher insulin, HOMA-IR, TG, TC, LDL-C, HDL-C, zonulin, and leptin levels than the healthy children ($p<0.05$), while glucose levels were similar in the two groups ($p>0.05$) (Figure 1, Table 2).

Comparison of the obese children regarding their findings on IR showed statistically significant differences for BMI, MAC, WC, fat mass, PBF, insulin, and HOMA-IR ($p<0.05$). Age, sex, BMI-SDS, TSF, SBP, DBP, serum glucose, TG, TC, LDL-C, HDL-C, zonulin, and leptin levels were similar in the insulin resistant and non-resistant obese children ($p>0.05$) (Table 3).

Spearman's correlation analysis revealed that serum zonulin levels negatively correlated with age and HDL-C, while positive correlated with BMI-SDS, PBF, LDL-C, HDL-C, and leptin levels in the entire cohort. Zonulin level were significantly associated with only HDL-C and leptin levels, after adjusting for age and BMI ($r=-0.348$, $p=0.026$; $r=0.417$, $p=0.007$, respectively) (Table 4). In the multivariate backward regression analysis ($r^2=0.503$, $p<0.001$), log-transformed zonulin was significantly

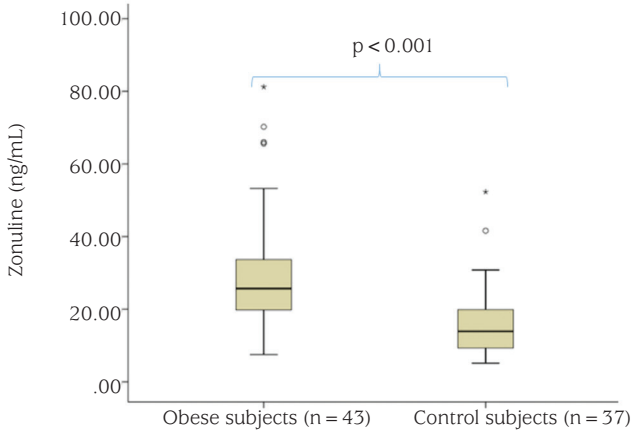

Figure 1. Zonulin levels in obese and healthy subjects

Table 1. Clinical characteristics in the obese and control groups

	Obese subjects (n = 43)		Control subjects (n = 37)		p-value
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	
Age (years)	11.1 ± 3.1	10.8 (4.5)	11.5 ± 3.6	12 (6.3)	0.635[a]
Sex (Male/Female)	23/20		14/23		0.823[c]
Pubertal/prepubertal	20/23		23/14		0.184[c]
BMI (kg/m^2)	28.9 ± 4.6	27.4 (6.2)	18.3 ± 2.8	18 (3.75)	< 0.001[a]
BMI SDS	2.2 ± 0.3	2.2 (0.3)	0.02 ± 0.8	0.12 (1.4)	< 0.001[b]
WC (cm)	96.1 ± 14.2	96.5 (20)	66.6 ± 9.7	67 (12.6)	< 0.001[a]
MAC (cm)	30.0 ± 4.2	29 (6.0)	20.9 ± 3.8	21 (6.2)	< 0.001[a]
TSF (mm)	29.7 ± 5.9	30 (11.5)	13.1 ± 4.9	11.6 (8.0)	< 0.001[a]
Fat mass (kg)	26.4 ± 11.8	24.2 (15.0)	8.1 ± 3.2	7.7 (3.1)	< 0.001[b]
PBF (%)	36.0 ± 6.4	35.2 (9.4)	18.3 ± 5.4	18.6 (6.5)	< 0.001[a]
SBP (mmHg)	122.2 ± 15.2	120 (20)	103.9 ± 13.0	100 (18.7)	< 0.001[b]
DBP (mmHg)	76.7 ± 11.7	75 (10)	66.9 ± 10.2	65 (15)	0.004[b]

[a]Student's t-test, [b]Mann-Whitney U-test, [c]Chi-square test,

IQR: interquartile range, BMI: body mass index, BMI-SDS: standard deviation score of body mass index, WC: waist circumferences, MAC: mid-arm circumference, TSF: triceps skinfold, PBF: percent body fat, SBP: systolic blood pressure, DBP: diastolic blood pressure, SD: standard deviation

Table 2. Laboratory findings in the obese and control groups

	Obese subjects (n = 43)		Control subjects (n = 37)		p-value
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	
Glucose (mg/dL)	82.8 ± 12.6	81 (17)	85.1 ± 8.2	86 (13.5)	0.339[a]
Insulin (uIU/mL)	19.9 ± 12.7	16.8 (18.3)	5.7 ± 3.2	5.3 (5.5)	< 0.001[b]
HOMA-IR	4.1 ± 2.7	3.3 (3.9)	1.2 ± 0.7	1.1 (1.2)	< 0.001
Triglyceride (mg/dL)	133.2 ± 56.8	123 (71)	74.1 ± 24.9	73 (40.5)	< 0.001[b]
TC (mg/dL)	180.3 ± 33.2	168 (43)	155.1 ± 21.9	150 (29)	0.001[b]
LDL-C (mg/dL)	108.0 ± 31.0	104 (39)	87.2 ± 19.5	90 (27)	0.001[b]
HDL-C (mg/dL)	45.7 ± 12.1	45 (9)	53.7 ± 13.3	53 (12)	< 0.001[b]
Zonulin (ng/mL)	30.1 ± 16.7	25.7 (15.8)	16.4 ± 9.8	13.9(10.8)	< 0.001[b]
Leptin (ng/mL)	10.7 ± 4.9	10.7 (7.1)	3.3 ± 1.8	2.9 (2.2)	< 0.001[b]

[a]Student's t-test, [b]Mann-Whitney U-test, [c]Chi-square test

IQR: interquartile range, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, SD: standard deviation

associated with BMI-SDS (β-coefficient 0.398, $p < 0.001$), and HDL-C (β-coefficient -0.379, $p < 0.001$), which explained 47.5% of the variance.

Discussion

Experimental studies have demonstrated a close association between intestinal permeability and the pathogenesis of obesity (12,13,14,15,16). The molecular transport between the intestinal lumen and the submucosa is regulated by dynamic TJ structures found between the intestinal cells (17). The recently described peptide, zonulin, is known to increase the intestinal permeability by altering the paracellular TJs. The weakening of the intestinal barrier leads to an increased exposure to pathogens and allergens (18,19). On the other hand, various organisms are known to have an effect on intestinal permeability and to live symbiotically in the intestinal system (20). This structure, referred to as the intestinal microbiota, is known to protect the body against various pathogens by creating a mechanic barrier on the surface mucosa of the intestinal system (20). Many studies have demonstrated that the microbiota is damaged and intestinal permeability increased in individuals exposed to a high-fat diet, leading to obesity and metabolic abnormalities (12,21). The mechanical barrier formed by the microbiota of the intestinal system is altered by changes in dietary habits

Table 3. Clinical and laboratory findings in obese patients with and without insulin resistance

	IR (-) Group (n = 22)	IR (+) Group (n = 21)	p[a]
Age (years)	10 (5.0)	12.2 (5.6)	0.063
Sex (Male/Female)	14/8	9/12	0.227[b]
BMI (kg/m^2)	26.8 (5.2)	31 (7.2)	0.003
BMI SDS	2.2 (0.4)	2.3 (0.4)	0.166
MAC (cm)	27.5 (4.2)	32 (6.0)	0.008
TSF (mm)	27.2 (9.4)	31.0 (10.5)	0.192
WC (cm)	88.5 (20.5)	105 (22.2)	0.006
Fat mass (kg)	18.6 (9.8)	30.1 (18.0)	0.001
PBF (%)	33.4 (6.6)	39.7 (7.5)	0.006
Glucose (mg/dL)	76.5 (18.5)	85 (16.5)	0.125
Insulin (uIU/mL)	10.6 (6.7)	28.7 (14.4)	< 0.001
Triglyceride (mg/dL)	113.5 (64.5)	128 (86)	0.211
TC (mg/dL)	165.5 (35.7)	177 (54)	0.258
LDL-C (mg/dL)	97.5 (40.5)	110 (40.2)	0.206
HDL-C (mg/dL)	47 (9.2)	42 (9.5)	0.058
HOMA-IR	2.1 (1.1)	5.9 (3.2)	< 0.001
SBP (mmHg)	120 (12.5)	122.5 (27.5)	0.564
DBP (mmHg)	75 (10)	77.5 (17.5)	0.815
Zonulin (ng/mL)	27.5 (17.6)	22.9 (13.2)	0.194
Leptin (ng/mL)	10.2 (8.0)	12.5 (5.7)	0.120

*Mann-Whitney U-test, [b]Chi-square test, Data are as given median (interquartile range)

BMI: body mass index, BMI-SDS: standard deviation score of body mass index, MAC: mid-arm circumference, TSF: triceps skin fold, WC: waist circumference, PBF: percentage of body fat, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, SBP: systolic blood pressure, DBP: diastolic blood pressure,

and by increased exposure of intestinal cells to antigens and pathogens. Consequently, the development of inflammation in the intestinal microbiota is suggested to increase zonulin expression (22,23,24). Furthermore, adipokines and cytokines [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and transforming growth factor-β], induced by fat tissues in obese individuals, trigger a metabolic imbalance (IR, type 2 diabetes mellitus, etc.) by causing subclinical systemic inflammation (25). Markers of subclinical inflammation, particularly IL-6, have been suggested to regulate the gene expression of haptoglobulin-2 (HP-2) known to encode zonulin protein (3,26). In conclusion, zonulin expression is thought to be regulated by subclinical systemic inflammation as well as by local intestinal inflammation.

Increased serum zonulin levels, by weakening the cytoskeletal structure between intestinal cells, are suggested to lead to IR and to also affect the other aspects of the metabolic syndrome in obese individuals through an increase in mucosal absorption surface (5). Studies investigating the relationship between zonulin, which has an effect on increased intestinal permeability, and IR, have yielded varying results (3,5,27). Moreno-Navarrete et al (3) were the first to investigate the association between zonulin and IR and demonstrated that serum zonulin levels were significantly higher in obese individuals when compared to non-obese individuals. In the same study, serum zonulin levels were found to correlate with anthropometric (BMI and waist-hip ratio), metabolic (fasting TG levels, uric acid, HDL-C levels, and insulin sensitivity index) and inflammatory parameters (IL-6). On the other hand, multi-regression analyses have shown that the fundamental relationship of zonulin levels is with IR index and have also demonstrated that this relationship is made possible by the subclinical inflammatory marker IL-6. In a study conducted by Zhang et al (5) on three adult patient groups with normal glucose tolerance, impaired glucose tolerance, and type 2 diabetes mellitus, zonulin was found to have a positive correlation with IR. However, Zak-Golab et al (27) could not demonstrate any relationship between zonulin levels and IR in obese adult patients and interpreted their results to be due to the small sample size and also to their inability to use oral glucose tolerance test (OGTT) instead of the HOMA-IR index in the evaluation of IR. Zonulin, which is considered a marker of intestinal permeability, is suggested to play a role in the development of the metabolic syndrome (IR, type 2 diabetes mellitus, and dyslipidemia) found in obese individuals. Similar to the findings reported by Moreno-Navarrete et al (3), our study demonstrated that serum zonulin levels were higher in obese children when compared to healthy children. This result suggests that zonulin, which is known to increase intestinal permeability, may play a role in the pathogenesis of obesity and related metabolic disturbances. Our findings, similar to those reported by Zak-Golab et al (27), also failed to show any relationship between serum zonulin levels and the IR index, HOMA-IR. However, this finding, namely, the absence of a relationship with IR index, may also be attributed to the small sample size in our study and also to the fact that metabolic syndrome was not as prominent in children as in adults.

Patients with IR have been reported to have a statistically significantly high level of TC and a low HDL-C level (28). Zhang et al (5) demonstrated that serum zonulin levels and IR have a positive correlation with TG and TC, but a negative correlation with HDL-C. In this study, zonulin was suggested to cause an increase in adipose tissue through the endocannabinoid pathway by increasing intestinal permeability, thereby leading to the development of dyslipidemia (5). However, in our study, serum zonulin levels

Table 4. Correlation coefficients and partial correlation coefficients between zonulin levels and anthropometrics and laboratory parameters

Parameters	Spearman's rho	*p-value	Partial correlation	**p-value
Age (year)	-0.287	**0.01**		
BMI (kg/m^2)	0.210	0.06		
BMI SDS	0.373	**0.001**	0.306	0.05
WC (cm)	0.192	0.094	0.202	0.206
MAC (cm)	0.164	0.154	-0.051	0.750
TSF (mm)	0.217	0.058	-0.073	0.651
Fat mass (kg)	0.217	0.105	-0.001	0.994
PBF (%)	0.423	**0.001**	0.120	0.456
Systolic BP (mmHg)	0.084	0.542	0.105	0.514
Diastolic BP (mmHg)	0.137	0.317	0.109	0.497
Glucose (mg/dL)	-0.147	0.193	-0.249	0.116
Insulin (uIU/mL)	0.174	0.122	0.007	0.965
HOMA-IR	0.176	0.117	-0.035	0.830
TG (mg/dL)	0.188	0.104	0.074	0.647
TC (mg/dL)	0.128	0.272	0.082	0.609
LDL-C (mg/dL)	0.246	0.032	0.197	0.216
HDL-C (mg/dL)	-0.422	**< 0.001**	-0.348	0.026
Leptin (pg/mL)	0.408	**< 0.001**	0.417	0.007

*Spearman's correlation analysis; Serum zonulin levels as dependent variable

**Partial correlation coefficient; controlling for age and body mass index

BMI: body mass index, BMI-SDS: standard deviation score of body mass index, WC: waist circumference, PBF: percentage of body fat, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAC: mid-arm circumference

and IR were not shown to have a relationship with LDL-C, TG, and TC, but were shown to have a negative correlation with HDL-C, which is in line with previous reports (5,28). Although the mechanism of the relationship of serum zonulin with HDL-C is not well understood, it is thought to be a precursor in the development of dyslipidemia and cardiometabolic imbalance.

Leptin is secreted by adipose tissue and its levels increase with the amount of adipose tissue (29). Zonulin is a peptide that increases intestinal permeability by altering the structure of TJs and is suggested to have a potential role in the etiopathogenesis of obesity (3). Hence, a close relationship between zonulin and leptin levels might be expected. However, to the best of our knowledge, there are no studies investigating the correlation between serum zonulin concentrations and serum leptin levels in children. In the present study, we found a significant positive correlation between serum zonulin concentrations and leptin levels in obese individuals.

Some limitations need to be acknowledged regarding the present study. Firstly, inflammatory markers (IL-6, TNF-α, C-reactive protein, etc.) were not measured and hence their relationship with serum zonulin could not be evaluated due to financial constraints. Moreover, the evaluation of IR was made using the HOMA-IR index, instead of the more sensitive OGGT. Although OGTT is known to be a more sensitive index than HOMA-IR in the evaluation of IR, a strong correlation was demonstrated between IR indices detected by HOMA-IR and OGTT (30). Another point is the relationship between serum zonulin levels and celiac disease. It has been claimed that celiac disease may lead to obesity by increasing serum zonulin levels (31). Our study did not include evaluation of the obese children in terms of celiac disease.

In conclusion, the results of the present study showed that zonulin and leptin were significantly higher in obese children when compared to healthy children. Furthermore, it was reported for the first time in obese children that zonulin did not correlate with any of the anthropometric and metabolic parameters, except with serum HDL-C levels. However, further studies identifying the zonulin receptor and/or other possible cofactors will be required to elucidate the exact role of zonulin in obesity and/or IR.

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Ethics

Ethics Committee Approval: Dokuz Eylül University; Date: 24.07.2014, Number: 2014/25-04. Informed Consent: A written informed consent of the parent(s) of each subject was also obtained before the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ayhan Abacı, Tuncay Küme, Ece Böber, Design: Ece Böber, Ayhan Abacı, Tuncay Küme, Gönül Çatlı, Hale Tuhan, Data Collection or Processing: Sezer Acar, Hale Tuhan, Ahmet Anık, Özlem Gürsoy Çalan, Analysis or Interpretation: Tuncay Küme, Ayhan Abacı, Özlem Gürsoy Çalan, Sezer Acar, Hale Tuhan, Literature Search: Ahmet Anık, Gönül Çatlı, Hale Tuhan, Sezer Acar, Writing: Ayhan Abacı, Sezer Acar, Hale Tuhan, Özlem Gürsoy Çalan.

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Comparison of Updated Weight and Height Percentiles with Previous References in 6-17-Year-Old Children in Kayseri, Turkey

Gökmen Zararsız[1], Betül Çiçek[2], Meda Kondolot[3], M. Mümtaz Mazıcıoğlu[4], Ahmet Öztürk[1], Selim Kurtoğlu[5]

[1]Erciyes University Faculty of Medicine, Department of Biostatistics, Kayseri, Turkey
[2]Erciyes University Faculty of Health Sciences, Department of Nutrition and Dietetics, Kayseri, Turkey
[3]Erciyes University Faculty of Medicine, Department of Pediatrics, Unit of Social Pediatrics, Kayseri, Turkey
[4]Erciyes University Faculty of Medicine, Department of Family Medicine, Kayseri, Turkey
[5]Erciyes University Faculty of Medicine, Department of Pediatric Endocrinology, Kayseri, Turkey

What is already known on this topic?

Among a number of indices for which reference standards are available; height and weight are the most useful in pediatric daily practice. Height and weight are the most easily obtained anthropometric indices. These indices have been used extensively in screening and monitoring of growth and have the advantages of simplicity and low cost for use in large-scale epidemiologic studies. National height and weight reference curves produced for Turkish children and adolescents living in Ankara (the capital city in Central Anatolia region), and İstanbul (the biggest and the crowded city in Marmara region), other than our data, are not comprehensive and has relatively big data set.

What this study adds?

The aims of the current study were; to present the updated reference data on height and weight for Turkish children and adolescents aged 6-17 years living in Kayseri, Turkey produced with generalized additive models for location, scale and shape (GAMLSS) and to compare these updated data with our previous study conducted three years earlier along with other recent references.

Abstract

Objective: To compare updated weight and height percentiles of 6-17-year-old children from all socio-economic levels in Kayseri with previous local references and other national/international data.

Methods: The second study "Determination of Anthropometric Measurements of Turkish Children and Adolescents study (DAMTCA II)" was conducted in Kayseri, between October 2007 and April 2008. Weight and height measurements from 4321 (1926 boys, 2395 girls) school children aged between 6 to 17 years were included in this cross-sectional study. Using these data, weight and height percentile curves were produced with generalized additive models for location, scale and shape (GAMLSS) and compared with the most recent references.

Results: Smoothed percentile curves including the 3rd, 5th, 10th, 15th, 25th, 50th, 75th, 85th, 90th, 95th, and 97th percentiles were obtained for boys and girls. These results were compared with DAMTCA I study and with two national (İstanbul and Ankara) and international data from Asia and from Europe.

Conclusion: This study provides updated weight and height references for Turkish school children aged between 6 and 17 years residing in Kayseri.

Keywords: Weight, height, percentile, children, adolescents, GAMLSS method

Address for Correspondence: Ahmet Öztürk MD,
Erciyes University Faculty of Medicine, Department of Biostatistics, Kayseri, Turkey
Phone: +90 352 437 49 37/23476 **E-mail:** ahmets67@hotmail.com

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Introduction

Growth and development of children are sensitive indicators of the general health and nutritional status of a population (1). The scarce studies on growth conducted in Turkey were based on children from large cities, representing relatively high socio-economic classes (2,3,4,5,6,7).

The concern about the worldwide increase in the prevalence of overweight and obesity in children is well known. The prevalence has increased substantially in children and adolescents in developed countries, and 23.8% (22.9-24.7%) of boys and 22.6% (21.7-23.6%) of girls were reported to be overweight or obese in 2013. The prevalence of overweight and obesity has also increased in children and adolescents in developing countries in recent years, from 8.1% (7.7-8.6%) to 12.9% (12.3-13.5%) in boys and from 8.4% (8.1-8.8%) to 13.4% (13.0-13.9%) in girls (8).

Turkey is a country with significant regional differences in socio-economic, demographic, and epidemiological features. This is partly due to its geography and also to past economic crises which have led to massive migratory movements of the population from rural to urban areas such as İstanbul (the biggest and most crowded metropolis) and Ankara (the capital city). Kayseri is one of the crowded cities located in Central Anatolia region of Turkey, where people used to migrate from eastern parts of the country, primarily for employment opportunities in industrial work areas.

Among a number of indices for which reference standards are available, height and weight, the most easily obtained anthropometric measurements, are also the most useful in pediatric daily practice. Height and weight are being used extensively in screening and monitoring growth. These measurements have the advantages of simplicity and low cost for use in large-scale epidemiologic studies (9).

In Turkey, national height and weight reference curves produced for Turkish children and adolescents living in Ankara (the capital city in Central Anatolia region) (10) and İstanbul (the biggest and the most crowded city in Marmara region) (6) have been reported. National height and weight reference curves have also been reported for Indian (11), Italian (12), Malaysian (13), and Polish children (14).

In our previous study [Determination of Anthropometric Measurements of Turkish Children and Adolescents I (DAMTCA I)], we reported height and weight reference values in Kayseri children and adolescents aged 6-18 years (15). After three years, during DAMTCA II study, we had the opportunity of obtaining weight and height measurements in children of the same region and to compare these two sets of data. The aims of the current study were to present the updated reference data on height and weight for Turkish children and adolescents aged 6-17 years living in Kayseri, Turkey, to produce generalized additive models for location, scale and shape (GAMLSS) (16,17) and also to compare these updated data with our previous study conducted three years earlier as well as with other recent references (6,15).

Methods

Data used in this study were obtained from the DAMTCA II, a cross-sectional study performed in the period between October 2007 and April 2008 for children aged between 6 and 17 years. This study was conducted in Kayseri, which is a Central Anatolian province with a population more than 1.2 million (18).

Multi-stage probability sampling was applied as the sampling method. Of the 708 schools in Kayseri, 17 (primary and secondary schools) were selected to randomly recruit children and adolescents aged between 6 and 17 years. Chronological age was calculated as the decimal age by subtracting the observation date from the birth date. The study protocol was approved by the Ethics Committee of Erciyes University and by the local educational authority. Children with any disorder affecting growth such as a known systemic or local disorder, metabolic, gastrointestinal or neurological condition, and using of any kind of medication were excluded. Parental written consent was obtained prior to the study, and the procedures were in accordance with those outlined in the Declaration of Helsinki (18).

Body weight was measured by bioelectrical impedance analysis (BIA), with Tanita BC-418 MA (Tanita Corporation, Tokyo, Japan) with correction for light indoor clothing. Height was measured with a portable stadiometer (SECA, Germany) sensitive to changes up to 1 cm. Daily calibration was performed to the portable devices. Height measurements were performed with the subject barefoot, the heels, hip and shoulders touching the stadiometer, and the head in neutral position with eyes gazing forward. The measurements were repeated twice, asynchronously, and the arithmetic mean was recorded for evaluation. All inter-observer correlation coefficients were calculated as 0.98.

Statistical Modeling

Age-related height and age-related weight z-score plots were checked and the discontinuities were examined to filter outliers. Liberal cut-off values were used as criteria to identify outliers (19). After filtering detected outliers, the remaining 4321 observations (1926 boys, 2395 girls) were split into training (70%) and test (30%) sets randomly. The training set was used to build models and the test set to select and validate models. GAMLSS were used to build the models, for each gender separately (20). For each gender and each measurement, LMS, LMST, and LMSP methods were applied to data. Box-Cox normal (BCN), Box-Cox *t*

(BCT), and Box-Cox power exponential (BCPE) distributions were applied for these methods, respectively. To estimate the distribution parameters, maximum penalized likelihood method was used with RS algorithm and Fisher scoring method. Cubic splines were used as smoothing functions. Analyses were applied using GAMLSS package (version 4.3-1) of R 3.1.1 software (www.r-project.org).

Model Building

In order to apply the LMSP method, we followed the optimization procedure of Rigby and Stasinopoulos (21). Here, Akaike's information criteria are used to select best models with parameter #3. At first, identity function was used as link functions for parameters that may relate to μ (median) and ν (skewness parameter), log-link function was used as link functions for σ (coefficient of variation) and τ (kurtosis parameter) (21). A grid search is applied for λ (power) between -2 to 2 in steps of 0.25, and an initial age transformation was optimized as $x = age^{\lambda}$. Next, initial degrees of freedom (df) of all four distribution parameters was taken as 1 and df (μ), λ and df (σ) values were optimized respectively. A grid search (between 1 to 20 in steps of 1 for df (μ) and df (σ); between -2 to 2 in steps of 0.05 for λ) were applied to optimize these parameters. Next, df (ν) and df (τ) parameters were optimized with a search ranging between 0 to 9 in steps of 1. Finally, fine tuning was conducted for the model with optimum parameters with changing values of df (σ), df (μ), df (ν), df (τ), and λ. Generalized Akaike Information Criteria (GAIC) was used for model comparisons. We followed the same procedure for LMST and LMS methods, considering the absence of kurtosis parameter τ in BCN distribution of LMS method. For each gender, each anthropometric measure, and each method, final models are given in Table 1.

A two-sided independent samples t test was applied for between gender comparisons (Table 2).

Results

Results given in Table 1 reveal that LMS was detected as the best method to fit age-related height in both genders and weight in boys. LMSP was detected as the best method to fit age-related weight in girls.

Table 2 shows mean ± standard deviation values for height (cm) and weight (kg) in boys and girls. Tables 3 and 4 show age-related percentiles of 6-17-year-old Turkish boys and girls for height (cm) and weight (kg), respectively.

Tables 5 and 6 compare the 50th percentile values of the current data for height and weight with other national and international studies according to gender.

Percentile curves of age-related weight measures of 6-17-year-old boys and girls are shown in Figures 1A and 1B, respectively. Percentile curves of age-related height measures of 6-17-year-old boys and girls are shown in Figures 2A and 2B, respectively.

Comparison of 3rd, 50th, and 97th percentiles of age-related weights in boys (Figure 3A) and girls (Figure 3B) among DAMTCA I, DAMTCA II, and İstanbul studies are shown in Figure 3. Comparison of 3rd, 50th, and 97th percentiles of age-related heights in boys (Figure 4A) and girls (Figure 4B) among DAMTCA I, DAMTCA II, and İstanbul studies are shown in Figure 4.

Figures 5A and 5B show differences (% values) in height (cm) and weight (kg) between DAMTCA I and DAMTCA II for 3rd, 50th, and 97th percentiles in boys and girls, respectively.

Discussion

In this study, we present cross-sectional reference percentiles and curves of weight and height in Turkish children and adolescents living in Kayseri, Turkey, produced with GAMLSS method. Cross-sectional studies can provide a record of the nutritional status for a precise period and for a specific population.

Table 1. Comparison of LMS, LMST, and LMSP methods in modeling age-related height and weights for each gender in 6-17 years old children

Method	Distribution parameters					GAIC (3)*
	dfμ	dfσ	dfν	dfτ	Λ	
Height (cm)						
Boys						
LMS	6	4	2	-	1.90	**12645.84**
LMST	6	3	1	1	2.00	12648.90
LMSP	6	4	2	2	1.95	12647.31
Girls						
LMS	4	3	1	-	1.70	**15287.39**
LMST	4	3	1	1	1.40	15291.55
LMSP	4	3	1	1	1.70	15292.94
Weight (kg)						
Boys						
LMS	4	2	1	-	1.75	**13159.54**
LMST	3	2	1	1	1.60	13169.88
LMSP	4	2	1	1	1.45	13161.40
Girls						
LMS	4	2	2	-	1.40	16076.60
LMST	4	3	1	1	1.65	16085.19
LMSP	4	2	3	3	1.25	**16074.92**

*Optimal model criteria with minimum GAIC are indicated as bold.
GAIC: Generalized Akaike Information Criteria

Table 2. Height (cm) and weight (kg) of the study sample

Age (years)	Boys			Girls		
	n	Height Mean (SD)	Weight Mean (SD)	n	Height Mean (SD)	Weight Mean (SD)
6	127	118.90 (4.73)*	23.01 (3.80)*	135	116.42 (5.34)	21.37 (3.44)
7	174	123.56 (5.76)	25.71 (4.53)	175	122.94 (5.35)	24.97 (4.37)
8	184	129.68 (5.61)	29.22 (5.45)*	190	128.78 (5.75)	27.96 (5.67)
9	154	134.89 (5.62)	32.92 (6.96)	161	134.07 (6.03)	31.49 (6.15)
10	178	139.94 (6.58)	35.45 (7.60)*	192	141.27 (7.31)	37.37 (8.71)
11	172	144.28 (6.89)*	38.69 (8.47)*	139	146.59 (6.63)	41.13 (8.66)
12	124	150.35 (7.42)*	43.44 (9.37)*	163	153.30 (6.21)	46.22 (8.72)
13	140	157.55 (8.55)	49.21 (10.35)	165	155.88 (5.33)	50.00 (9.97)
14	152	166.57 (8.84)*	56.03 (11.01)	150	159.76 (5.96)	55.15 (10.58)
15	218	172.08 (6.96)*	61.54 (10.53)*	378	160.67 (5.63)	54.66 (8.15)
16	219	174.09 (6.50)*	65.14 (12.34)*	414	160.90 (5.98)	56.08 (8.59)
17	84	175.26 (7.10)*	67.48 (10.65)*	133	160.54 (6.31)	54.62 (7.05)

Age indicates completed age group (e.g. 6.00-6.99 years, etc.). Independent samples t test is applied for between gender comparisons for each measure for each age. Significant results are displayed as *($p < 0.05$). SD: standard deviation

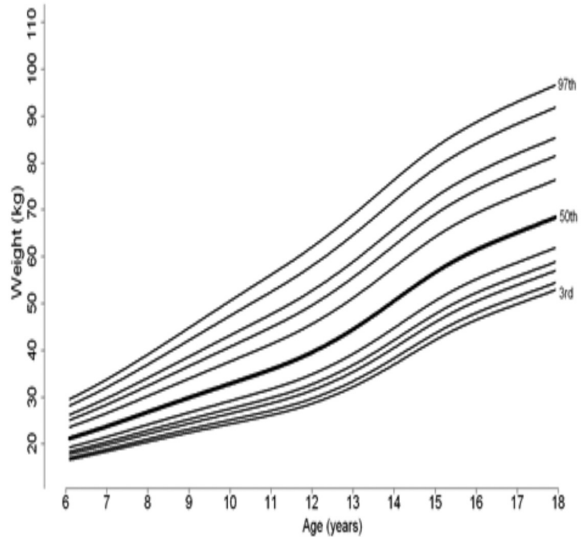

Figure 1A. Percentile curves of age-related weight measures of 6-17 year Turkish boys

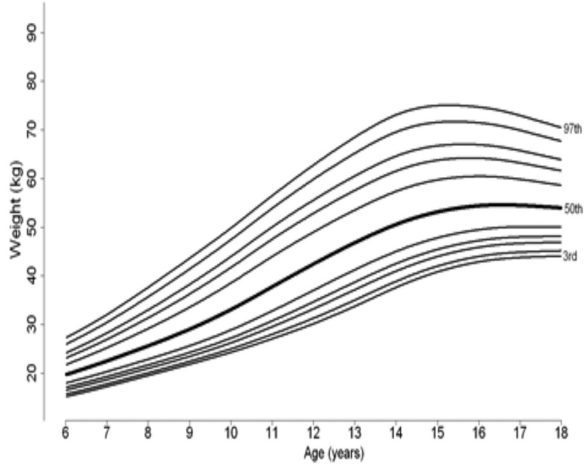

Figure 1B. Percentile curves of age-related weight measures of 6-17 year Turkish girls

Anthropometric indices, such as body weight and height, are the simplest, easiest to obtain, non-invasive, cheapest, and most widely accepted criteria for the evaluation of growth and body composition of children and adolescents. These indices also reflect, and thus are useful in the evaluation of nutritional status and health of both children and adolescents.

The reference percentile curves for weight and height of Turkish children have first been established by Neyzi et al (7) in 1970s and have been used since. The same authors have re-published the reference curves for Turkish children aged between 6 and 18 years of age by updating the curves (6). Both studies have enrolled school-aged boys and girls representing high socio-economic level and residing in İstanbul (Marmara region of Turkey) and the authors have presented their data as "predictive" reference values.

However, there are some points that can be explained by differences in methodology of the studies in the two regions. While the İstanbul study has been conducted via longitudinal follow-up of the same children, the current study has been conducted cross-sectionally in a mixed group. Also, age of onset of puberty in children in the Istanbul group appears to reflect an earlier onset due to a social improvement process in the past few decades. In addition, different nutritional habits and limited physical activity could affect the findings in pubertal children in the Istanbul group. Reflecting on our findings, it can be said that the findings of the children in the Kayseri group reflect the socio-economic improvement process in their pre-pubertal ages but not yet in their pubertal period. We can speculate that future studies on Kayseri children will reveal the expected reflection of socio-economic improvement on onset of puberty and on growth in puberty.

It is well known that height and weight differences in children can also be due to ethnic origin and geographic settlement. The differences between our values and the centers for

Table 3. Age-related height (cm) percentiles of the subjects

Age (years)	Percentiles										
	3rd	5th	10th	15th	25th	50th	75th	85th	90th	95th	97th
Boys											
6	107.03	108.23	110.04	111.23	112.94	116.02	118.95	120.47	121.49	122.96	123.90
7	111.79	113.03	114.91	116.16	117.98	121.28	124.48	126.16	127.28	128.92	129.97
8	117.01	118.28	120.22	121.52	123.43	126.95	130.42	132.27	133.51	135.35	136.53
9	122.04	123.32	125.31	126.65	128.63	132.34	136.08	138.09	139.46	141.48	142.80
10	126.65	127.98	130.04	131.44	133.53	137.49	141.54	143.75	145.27	147.53	149.01
11	130.40	131.80	133.98	135.48	137.73	142.03	146.48	148.93	150.62	153.16	154.84
12	134.29	135.82	138.22	139.86	142.33	147.07	152.00	154.73	156.61	159.44	161.32
13	139.39	141.11	143.80	145.64	148.39	153.63	159.03	161.99	164.02	167.07	169.08
14	146.68	148.57	151.49	153.46	156.37	161.80	167.25	170.18	172.16	175.10	177.02
15	155.08	156.95	159.80	161.69	164.46	169.52	174.47	177.08	178.83	181.39	183.04
16	160.32	162.05	164.66	166.40	168.94	173.54	178.00	180.34	181.91	184.20	185.67
17	161.83	163.53	166.11	167.84	170.36	174.97	179.48	181.86	183.46	185.80	187.32
Girls											
6	105.04	106.21	108.01	109.23	111.03	114.38	117.73	119.53	120.75	122.56	123.73
7	109.95	111.15	113.01	114.27	116.14	119.65	123.19	125.09	126.39	128.32	129.58
8	115.26	116.50	118.44	119.75	121.71	125.39	129.14	131.18	132.57	134.64	135.99
9	120.68	121.99	124.02	125.40	127.46	131.38	135.38	137.56	139.06	141.30	142.76
10	126.29	127.66	129.81	131.27	133.45	137.61	141.88	144.22	145.83	148.23	149.81
11	132.25	133.65	135.84	137.34	139.58	143.85	148.24	150.65	152.30	154.78	156.41
12	138.24	139.61	141.76	143.23	145.42	149.58	153.85	156.19	157.79	160.19	161.77
13	143.58	144.90	146.94	148.33	150.41	154.36	158.39	160.59	162.09	164.35	165.82
14	147.55	148.82	150.79	152.14	154.14	157.93	161.80	163.91	165.35	167.51	168.92
15	149.85	151.11	153.06	154.39	156.38	160.14	163.98	166.08	167.51	169.66	171.06
16	150.36	151.63	153.61	154.96	156.97	160.81	164.74	166.90	168.37	170.59	172.04
17	149.76	151.08	153.13	154.54	156.65	160.68	164.84	167.13	168.71	171.07	172.63

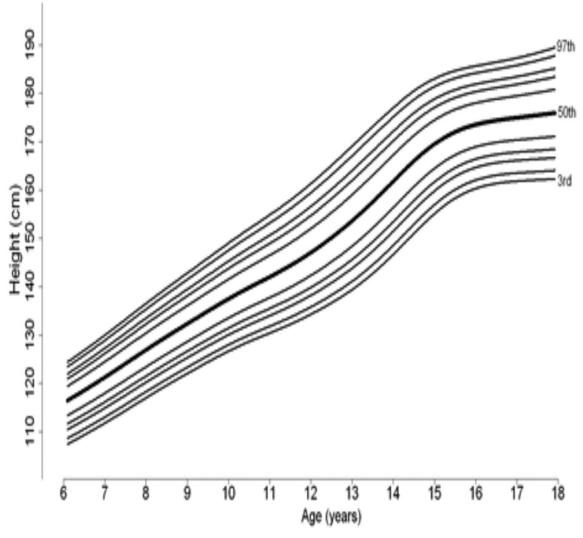

Figure 2A. Percentile curves of age-related height measures of 6-17 year Turkish boys

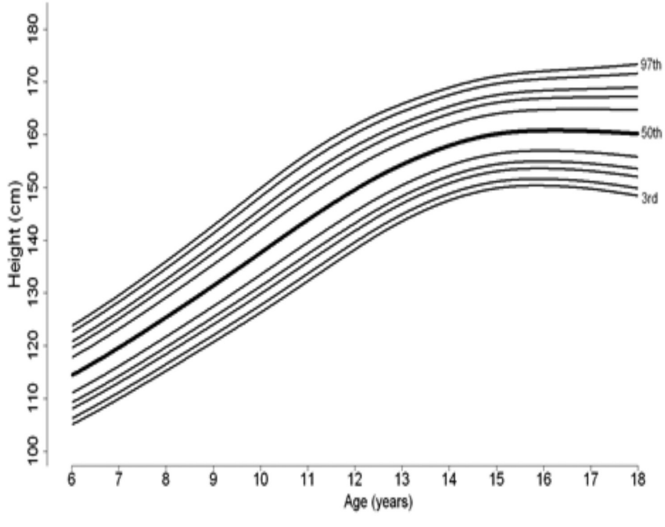

Figure 2B. Percentile curves of age-related height measures of 6-17 year Turkish girls

Table 4. Age-related weight (kg) percentiles of the subjects

Age (years)	Percentiles										
	3rd	5th	10th	15th	25th	50th	75th	85th	90th	95th	97th
Boys											
6	16.42	16.87	17.63	18.18	19.06	20.96	23.29	24.78	25.91	27.79	29.16
7	18.37	18.91	19.80	20.45	21.49	23.76	26.57	28.38	29.76	32.07	33.78
8	20.44	21.06	22.11	22.88	24.11	26.82	30.21	32.42	34.11	36.96	39.09
9	22.41	23.13	24.35	25.24	26.68	29.87	33.92	36.58	38.63	42.12	44.76
10	24.27	25.09	26.48	27.50	29.16	32.85	37.58	40.72	43.15	47.33	50.50
11	26.16	27.09	28.65	29.81	31.69	35.88	41.28	44.87	47.65	52.45	56.10
12	28.62	29.67	31.43	32.74	34.86	39.57	45.61	49.61	52.71	58.01	62.02
13	32.20	33.39	35.38	36.85	39.23	44.48	51.16	55.53	58.89	64.60	68.88
14	36.97	38.30	40.52	42.15	44.77	50.52	57.73	62.40	65.96	71.94	76.36
15	42.15	43.59	45.97	47.72	50.52	56.62	64.18	69.02	72.68	78.81	83.30
16	46.46	47.95	50.43	52.24	55.14	61.43	69.18	74.13	77.87	84.10	88.66
17	49.86	51.39	53.92	55.77	58.73	65.14	73.06	78.12	81.95	88.34	93.03
Girls											
6	15.17	15.68	16.51	17.09	17.97	19.70	21.75	23.10	24.15	25.92	27.24
7	17.27	17.80	18.67	19.31	20.33	22.53	25.23	26.96	28.27	30.45	32.05
8	19.54	20.08	21.01	21.71	22.88	25.63	29.17	31.38	33.02	35.71	37.66
9	21.85	22.43	23.46	24.25	25.62	29.04	33.54	36.27	38.24	41.39	43.61
10	24.29	25.00	26.24	27.20	28.88	33.09	38.54	41.72	43.96	47.44	49.81
11	27.09	28.00	29.56	30.76	32.81	37.77	43.97	47.54	50.04	53.89	56.49
12	30.18	31.31	33.23	34.67	37.04	42.46	49.03	52.87	55.60	59.87	62.80
13	33.76	35.03	37.16	38.73	41.26	46.83	53.54	57.59	60.53	65.22	68.51
14	37.63	38.89	40.98	42.53	45.04	50.59	57.38	61.55	64.62	69.58	73.11
15	40.83	42.00	43.98	45.44	47.83	53.16	59.74	63.78	66.76	71.59	75.03
16	42.89	44.01	45.89	47.27	49.50	54.43	60.48	64.22	66.99	71.49	74.72
17	43.79	44.89	46.71	48.02	50.10	54.53	59.87	63.24	65.77	69.95	72.99

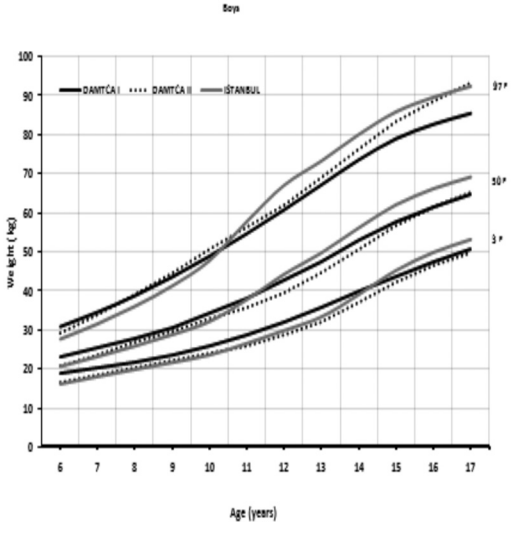

Figure 3A. Comparison of 3rd, 50th and 97th percentiles of age-related weights in boys among DAMTCA I, DAMTCA II and İstanbul studies

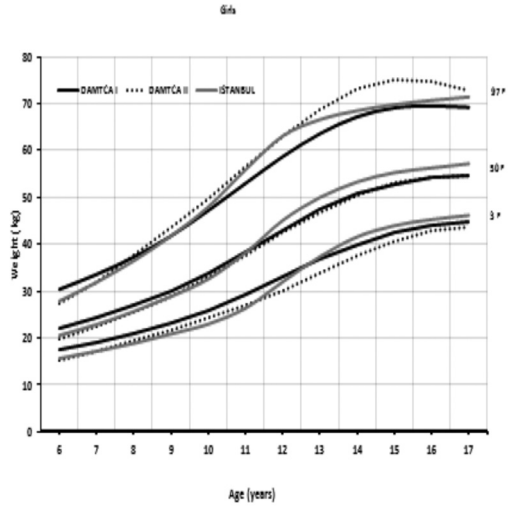

Figure 3B. Comparison of 3rd, 50th and 97th percentiles of age-related weights in girls among DAMTCA I, DAMTCA II and İstanbul studies

Table 5. Comparison of the 50th percentile of the current data with other national and international studies (height-cm) according to gender

Age (years)	Current data-DAMTCA-II (Kayseri, Turkey)		DAMTCA-I (Kayseri, Turkey)		Ankara (Turkey)		İstanbul (Turkey)		India		Italy		Malaysia		Poland	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
6	116.02	114.38	119.0	117.7	121.04	116.99	116.0	115.0	114.8	113.5	118.4	118.0	-	-	-	-
7	121.28	119.65	123.3	122.4	116.99	121.11	121.5	121.0	120.7	119.4	124.1	123.0	119.56	118.77	124.6	123.0
8	126.95	125.39	128.2	127.8	130.87	125.69	126.9	126.7	126.4	125.4	129.4	128.1	122.70	121.75	130.5	129.4
9	132.34	131.38	133.5	133.6	135.65	131.29	132.1	132.1	131.8	131.4	134.5	133.5	129.17	129.06	136.3	135.2
10	137.49	137.61	139.0	139.2	140.78	137.87	137.6	137.8	137.2	137.4	139.5	139.6	134.40	134.81	141.5	140.8
11	142.03	143.85	144.9	145.7	147.11	144.62	143.8	145.4	142.7	143.3	145.0	146.3	139.52	141.80	146.7	147.1
12	147.07	149.58	151.3	152.0	154.65	150.51	150.6	153.1	148.4	148.4	151.3	152.6	146.29	148.19	152.9	153.8
13	153.63	154.36	158.0	156.2	162.12	154.94	157.7	157.8	154.3	152.2	158.4	157.4	152.76	152.15	160.2	159.1
14	161.80	157.93	164.4	158.5	168.10	157.83	164.9	160.3	159.9	154.7	165.5	160.4	158.37	153.60	167.2	162.2
15	169.52	160.14	169.3	159.5	172.11	159.37	170.4	161.7	164.5	156.1	170.9	162.0	163.09	155.46	172.5	163.7
16	173.54	160.81	172.4	159.9	174.23	160.10	173.4	162.4	168.1	156.9	174.2	162.7	167.46	157.91	175.7	164.4
17	174.97	160.68	174.2	160.1	175.41	160.33	174.9	162.7	171.0	157.4	175.7	163.0	168.35	157.08	177.6	164.7

DAMTCA: Determination of Anthropometric Measurements of Turkish Children and Adolescents

Table 6. Comparison of the 50th percentile of the current data with other national and international studies (weight-kg) according to gender

Age (years)	Current data-DAMTCA-II (Kayseri, Turkey)		DAMTCA-I (Kayseri, Turkey)		Ankara (Turkey)		İstanbul (Turkey)		India		Italy		Malaysia		Poland	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
6	20.96	19.70	23.2	22.1	24.11	22.34	20.3	20.4	19.3	18.7	23.0	23.0	-	-	-	-
7	23.76	22.53	25.3	24.3	26.01	23.60	23.2	22.8	21.9	21.2	25.3	25.3	21.07	20.06	24.4	23.5
8	26.82	25.63	27.8	26.9	28.57	25.34	25.9	25.6	24.8	24.0	28.1	28.0	22.76	22.63	27.6	26.6
9	29.87	29.04	30.7	30.1	31.35	28.30	28.8	28.8	27.9	27.2	31.4	31.2	28.59	28.14	30.8	29.9
10	32.85	33.09	34.2	34.0	34.56	32.60	32.0	32.7	31.1	31.0	35.4	35.3	30.91	30.69	34.2	33.6
11	35.88	37.77	42.7	38.5	39.15	37.58	37.9	38.3	34.7	35.4	40.1	40.4	34.28	34.42	38.1	37.9
12	39.57	42.46	47.6	43.1	45.48	42.67	44.5	45.2	39.0	39.8	45.4	45.8	39.04	39.59	42.7	42.8
13	44.48	46.83	52.9	47.3	52.58	47.48	49.7	50.1	43.3	43.6	51.1	50.2	42.75	41.61	48.1	47.7
14	50.52	50.59	57.6	50.7	59.26	51.33	56.2	53.3	48.2	46.4	56.4	53.0	46.19	43.38	53.8	51.3
15	56.62	53.16	61.5	52.9	64.94	53.84	62.2	55.2	53.1	48.4	61.0	54.5	51.43	47.14	59.0	53.6
16	61.43	54.43	64.7	54.1	69.68	55.19	66.2	56.3	56.8	49.7	64.5	55.2	54.23	49.28	63.3	55.0
17	65.14	54.53	67.7	54.7	73.94	55.73	69.1	57.1	59.5	50.9	67.0	55.5	56.39	49.34	66.9	55.7

DAMTCA: Determination of Anthropometric Measurements of Turkish Children and Adolescents

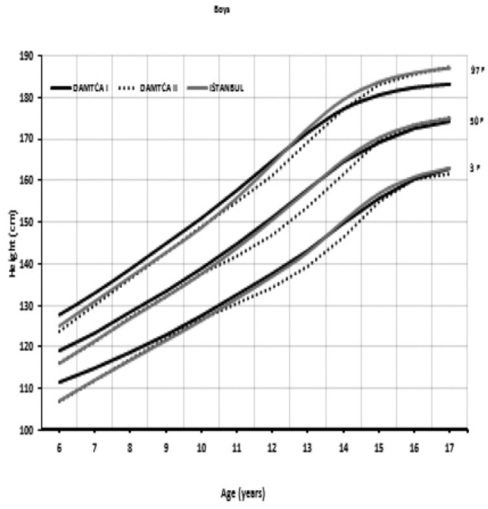

Figure 4A. Comparison of 3rd, 50th and 97th percentiles of age-related heights in boys among DAMTCA I and DAMTCA II and İstanbul studies

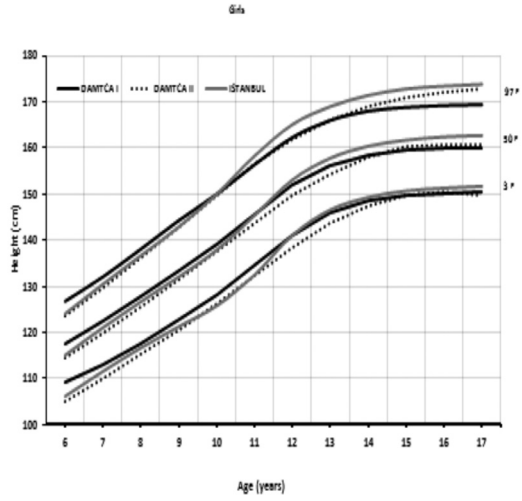

Figure 4B. Comparison of 3rd, 50th and 97th percentiles of age-related heights in girls among DAMTCA I, DAMTCA II and İstanbul studies

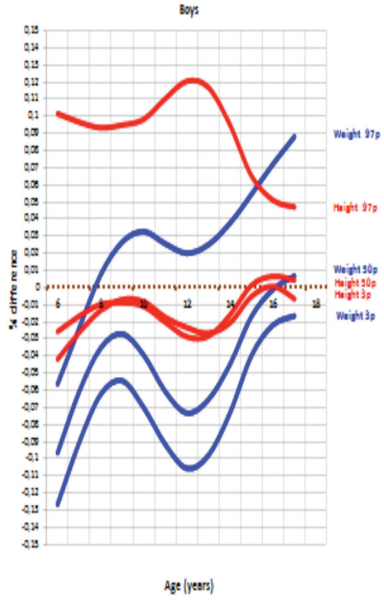

Figure 5A. Percent (%) differences of height (cm) and weight (kg) between DAMTCA I and DAMTCA II for 3rd, 50th and 97th percentiles in boys

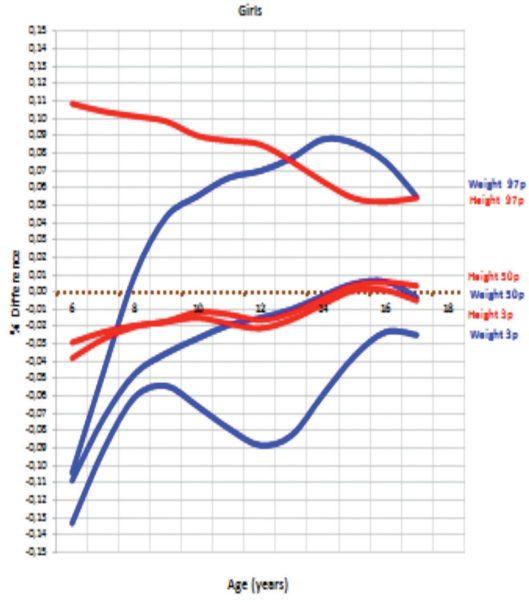

Figure 5B. Percent (%) differences of height (cm) and weight (kg) between DAMTCA I and DAMTCA II for 3rd, 50th and 97th percentiles in girls

disease control (22) or Iran data can be explained by both ethnic and geographical differences (23). With regard to differences from İstanbul data, we underline the impact of socio-economic factors to significantly influence growth, although the same ethnic origin is shared.

Our analysis indicates that DAMTCA-II height values are somewhat lower than those of the DAMTCA-I study, on an average of 2-3 cm between the ages of 6-14 years in both boys and girls. DAMTCA-II height values in other age groups and weight values in all age groups were similar to the DAMTCA-I study results for both girls and boys. DAMTCA-II height-for-age values in the boys were lower than the Ankara sample, but similar to the height-for-age values reported for İstanbul children. Weight measurements seemed to be similar in the DAMTCA-II and Istanbul studies for boys younger than 10 years old, but similar between boys older than 10 years. DAMTCA-II and Ankara studies revealed similar results in all age groups. When compared with international references, DAMTCA-II height and weight values for both boys and girls seemed to be higher than Indian and Malaysian children, but lower than Italian and Polish children.

In conclusion, we believe that the percentile values established in this group of boys and girls of 6-to-18 age group from Kayseri are representative and can be used in the monitoring of growth of children from all socio-economic levels residing in the region.

Ethics

Ethics Committee Approval: Erciyes University Faculty of Medicine Ethics Committee approval 03.04.2007, Informed Consent: Parental written consent was obtained prior to the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Gökmen Zararsız, M. Mümtaz Mazıcıoğlu, Ahmet Öztürk, Selim Kurtoğlu, Design: Gökmen Zararsız, M. Mümtaz Mazıcıoğlu, Ahmet Öztürk, Selim Kurtoğlu, Data Collection and Processing: M. Mümtaz Mazıcıoğlu, Ahmet Öztürk, Selim Kurtoğlu, Analysis and Interpretation: Gökmen Zararsız, Betül Çiçek, Ahmet Öztürk, Literature Research: Gökmen Zararsız, Betül Çiçek, Ahmet Öztürk, Writing: Gökmen Zararsız, Betül Çiçek, Meda Kondolot.

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ORIGINAL ARTICLE

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Association Between Endocrine Diseases and Serous Otitis Media in Children

Murat Koçyiğit[1], Taliye Çakabay[1], Safiye G. Örtekin[1], Teoman Akçay[2], Güven Özkaya[3], Selin Üstün Bezgin[1], Melek Yıldız[4], Mustafa Kemal Adalı[5]

[1]Kanuni Sultan Süleyman Training and Research Hospital, Clinic of Otolaryngology, İstanbul, Turkey
[2]Medical Park Gaziosmanpaşa Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
[3]Uludağ University Faculty of Medicine, Department of Biostatistics, Bursa, Turkey
[4]Kanuni Sultan Süleyman Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
[5]Bir Nefes Private Hospital, Clinic of Otolaryngology, Kırklareli, Turkey

What is already known on this topic?

Relationship between otitis media with effusion (OME) and endocrine diseases is not clear in pediatric population.

What this study adds?

Specific endocrine diseases such as metabolic syndrome, growth hormone deficiency, hypothyroidism, and idiopathic short stature may accompany OME.

Abstract

Objective: Otitis media with effusion (OME) is a condition in which fluid is retained in the middle ear cavity. The association between endocrine disorders and OME has not yet been determined. This study aimed to investigate the presence of OME in children diagnosed with an endocrine disease and the relationship between these two conditions.

Methods: The study was conducted on 918 pediatric patients (440 boys, 478 girls; mean age: 8.40, range 3-15 years) and 158 healthy controls (76 boys, 79 girls; mean age: 8.31, range 3-15 years). All children underwent an ear examination and a tympanometry performed by an otorhinolaryngologist. Tympanometry results were used to diagnose OME.

Results: OME was detected in 205 (22.3%) of 918 patients and in 19 (12.0%) of 158 subjects in the control group. The difference in frequency of OME between the two groups was statistically significant ($p = 0.003$).

Conclusion: The results of the study reveal that there may be a tendency towards the occurrence of OME in pediatric endocrinology patients.

Keywords: Otitis media with effusion, endocrine diseases, tympanometry

Introduction

Despite the development of antibiotics and advances in surgical techniques, the frequency of otitis media with effusion (OME) has been increasing (1). OME is a condition in which fluid is retained in the middle ear cavity but without otalgia, fever, or other symptoms (2). This condition has been shown to be caused by complex reactions involving the dysfunction of the Eustachian tubes, infection in the mucosa, immune deficiency, and allergy, among others (3). The incidence of OME varies widely, being reported as 50% in British children (4), 8.7% in Japanese children (5), 8% in Nigerian children (6) with differences according to surveyed regions. OME is a leading cause of hearing impairment in children, and its early and proper management can prevent hearing and speech impairment, which can cause developmental delay in children (7). The incidence of endocrine disorders has also been increasing, but the

Address for Correspondence: Teoman Akçay MD,
Medical Park Gaziosmanpaşa Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
Phone: +90 212 404 15 00 **E-mail:** akteoman@yahoo.com

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association between endocrine disorders and OME has not yet been determined. Although there are many studies in the literature about many diseases that are believed to be associated with OME, there are no comprehensive studies showing a relationship between endocrine diseases and OME. This study aimed to investigate the presence of OME in children diagnosed with an endocrine disease and the relationship of these conditions with one another.

Methods

Our study was conducted on 918 pediatric patients (440 boys, 478 girls; mean age: 8.40, range 3-15 years) who presented to the pediatric endocrinology outpatient clinic and on 158 children with no otolaryngologic or endocrine problems, constituting the healthy control group (76 boys, 79 girls; mean age: 8.31, range 3-15 years). All partipicants in the study group had at least one endocrine disease. Patients who had active otorhinolaryngological symptoms, ear wax, cleft palate repair history in the past, and cases with submucous cleft palate were excluded from the study. Children diagnosed with an endocrine or metabolic disease, who did not have any otorhinolaryngologic symptoms were compared to healthy children in the control group for presence of OME. The study was conducted from April to October 2015 due to the circulation of respiratory viruses during winter months. Informed consent was obtained from all individual participants included in the study or their parents.

A total of 918 patients in 24 different disease groups were evaluated. Those diseases with numbers of patients under 18 were excluded from the statistical evaluation; their OME rates were given only. The study was approved by the Institutional Review Board. Informed written consent was obtained from the parents of the children studied after explanation of the research purpose.

All children underwent a complete otolaryngologic examination. A flexible nasopharyngoscope was used to detect adenoid hypertrophy. Both ears were examined using an otoscope. Tympanometry was performed by an otolaryngologist using a MAICO m40, (Minneapolis, USA). Tympanometric measurement results were classified according to adjusted Jerger's classification as types A, As, B, or C (8). Types A and As curves were accepted as no effusion in the middle ear, while types B and type C were considered as predictive of OME. Tympanometry results were used to diagnose OME.

Statistical analysis were done by using Statistical Package for the Social Sciences 22.0 operating program (license no: 10240642). Pearson chi-square test and Fisher-Freeman-Halton test were used. Significance limit was set at $p < 0.05$.

Results

When the 918 patients and 158 healthy children who participated in the study were compared in terms of their ages, a statistically significant difference was not detected ($p = 0.086$). While 88 (9.6%) of the 918 patients had adenoid vegetation, 830 patients (90.4%) did not. However, when patients with adenoid vegetation were compared to those who did not have adenoid vegetation for presence of OME, a statistically significant difference was not observed ($p = 0.717$). On the other hand, a comparison of OME incidence in the two groups revealed a statistically significant difference (Table 1). Due to their small numbers, patients with hypophyseal insufficiency, adrenal insufficiency, hyperthyroidism, hyperinsulinemia, diabetes insipidus, MEN-1 (multiple endocrine neoplasia), Graves' disease, metabolic syndrome, macro-prolactinemia, hypoglycemia, vitamin D deficiency, hirsutism, congenital adrenal hyperplasia, hypogonadism, and primary amenorrhea were excluded from the statistical analysis (Table 2). OME ratios and the results of the comparisons in the remaining patients with the controls are shown in Table 3.

Discussion

OME is a leading cause of hearing impairment in children. Its early and proper management can avoid hearing and speech impairment and consequent developmental delay in children (7). Among the factors thought to influence the effects of OME are age, sex, race, season of the year, hereditary factors, number of family members, smoking status of parents, diseases experienced by children, and nursing methods. Factors reported to predispose to OME include upper respiratory tract infection, age, race, and attendance in day care centers, whereas factors that do not significantly influence OME include bronchitis, cystic fibrosis, socioeconomic status, smoking by parents, and antibiotic abuse (9). The association between endocrine disorders and OME has not yet been investigated. In our study, children diagnosed with an endocrine or metabolic disease and who did not have any otorhinolaryngologic

Table 1. Comparison between the patient and control groups for otitis media with effusion

	Rates of OME	p* value
Patient group (n = 918)	205 (22.3%)	**p = 0.003**
Control group (n = 158)	19 (12.0%)	

*: Chi-square test, $p < 0.05$
OME: otitis media with effusion

Table 2. The numbers (n) of patients and otitis media with effusion frequency in endocrine patients included in the study

Diseases	n	OME frequency	Diseases	n	OME frequency
Type 1 diabetes mellitus	181	16.6%	Hyperinsulinemia	5	0.0%
GH deficiency	179	30.2%	Diabetes insipidus	5	20.0%
Obesity	126	17.5%	MEN-1	5	20.0%
Idiopathic short stature	135	25.9%	Graves' disease	5	20.0%
Precocious puberty	55	16.3%	Macroprolactinemia	4	50.0%
Malnutrition	28	14.3%	Hypoglycemia	3	0.0%
Turner syndrome	18	27.8%	Vitamin D deficiency	3	100.0%
Metabolic syndrome	18	55.6%	Hirsutismus	3	36.0%
Pituitary insufficiency	10	60.0%	Congenital adrenal hyperplasia	3	0.0%
Adrenal insufficiency	8	12.5%	Hypogonadism	2	0.0%
Hyperthyroidism	7	16.7%	Primer amenorrhea	2	50.0%
			Total	**918**	**22.3%**

OME: otitis media with effusion, GH: growth hormone, MEN-1: multiple endocrine neoplasia-1

Table 3. Rates of otitis media with effusion and comparisons between controls and otitis media effusion patients with endocrine diseases

	Rates of OME	p* value
Type 1 diabetes mellitus	16.6%	p = 0.235
GH deficiency	30.2%	**p < 0.001**
Obesity	17.5%	p = 0.195
Idiopathic short stature	25.9%	**p = 0.046**
Hypothyroidism	36.6%	**p < 0.001**
Precocious puberty	16.3%	p = 0.665
Malnutrition	14.3%	p = 0.756
Turner syndrome	27.8%	p = 0.076
Metabolic syndrome	55.6%	**p < 0.001**

*: Chi-square test, p < 0.05

OME: otitis media with effusion, GH: growth hormone

symptoms were compared to healthy children in the control group for presence of OME.

In our study, a statistically significant difference was observed between the patient group and the controls in OME incidence, a finding suggesting that there may be a tendency towards the occurrence of OME in pediatric endocrine diseases.

In the relevant literature, while there is very little information about the importance of diabetes mellitus for ear diseases, the focus is on the impact of diabetes mellitus on patients with external otitis, malignant external otitis, otitis media, sudden sensorineural hearing loss and slowly progressive hearing loss (10).

Kim et al (11) assessed 140 children aged 2-7 years who underwent unilateral or bilateral ventilation tube insertion for treatment of OME (experimental group) and 190 children with no history of OME who underwent operations for conditions other than ear diseases during the same period and reported that childhood obesity was significantly higher in children with OME. This finding suggests that childhood obesity could have an effect on the development of OME. Kim et al (12) reported that pediatric obesity may have an effect on the development of OME, but pediatric overweight was not reported to be associated with occurrence of OME.

Middle ear problems are reported in rare genetic syndromes that cause short stature such as achondroplasia and

cartilage-hair hypoplasia (13,14,15). However, we did not find a study investigating incidence of middle ear diseases in children with idiopathic short stature.

There is a study showing that hearing loss may occur in patients with congenital hypothyroidism, even if they receive sufficient treatment (16). Although thyroid hormone replacement therapy was adequate in the patients in our study, OME incidence was higher than in the control group.

Micro- and macro-nutrition deficiencies can occur in malnourished children. It was reported that vitamin D and zinc deficiencies impair the function of the Eustachian tube and lead to middle ear problems and that this situation can be improved with treatment (17,18,19). We did not find a significant difference in frequency of OME between patients with nutritional deficiencies and the control group. The reason for this may be that our patients suffered mild or moderate malnutrition rather than severe malnutrition.

In the study conducted by Bergamaschi et al (20), persistent secretory otitis media was detected in 21.3% of 173 patients with Turner syndrome. In our study, the incidence of OME in patients with Turner syndrome was similar to this result.

The results of our study indicate that there may be a tendency towards the occurrence of OME in pediatric endocrine diseases. We believe further studies on the relationships particularly of metabolic syndrome, hypothyroidism, growth hormone deficiency, and idiopathic short stature with OME might be beneficial.

Ethics

Ethics Committee Approval: The study was approved by the Institutional Review Board. Informed Consent: Informed written consent was obtained from the parents of the children studied after explanation of the research purpose.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Murat Koçyiğit, Teoman Akçay, Mustafa Kemal Adalı, Design: Murat Koçyiğit, Data Collection or Processing: Taliye Çakabay, Safiye G. Örtekin, Selin Üstün Bezgin, Melek Yıldız, Analysis or Interpretation: Güven Özkaya, Literature Search: Murat Koçyiğit, Writing: Murat Koçyiğit.

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Clinical and Mutational Features of Three Chinese Children with Congenital Generalized Lipodystrophy

Xueying Su[1], Ruizhu Lin[1], Yonglan Huang[1], Huiying Sheng[1], Xiaofei Li[2], Tzer Hwu Ting[3], Li Liu[1], Xiuzhen Li[1]

[1]Guangzhou Women and Children's Medical Center, Department of Genetics and Endocrinology, Guangzhou, China
[2]Guangzhou Women and Children's Medical Center, Division of Medical Imaging, Guangzhou, China
[3]Univeristy Putra Malaysia, Department of Pediatrics, Selangor, Malaysia

What is already known on this topic?

Congenital generalized lipodystrophy (CGL) is a rare autosomal recessive disorder characterized by generalized lipodystrophy, hypertriglyceridemia, hyperinsulinemia, hepatomegaly, and acanthosis nigricans.

What this study adds?

This study revealed the clinical features, molecular characteristics, and treatments of three Chinese CGL patients in order to better understand the diagnosis, clinical treatment, and prognosis of this rare disease.

Abstract

Objective: To investigate the clinical and molecular features of congenital generalized lipodystrophy (CGL) in three Chinese patients with various typical manifestations. .

Methods: Data on clinical symptoms, results of laboratory analyses, and previous treatments in three Chinese patients were collected by a retrospective review of medical records. All coding regions and adjacent exon–intron junction regions of *AGPAT2* and *BSCL2* genes were amplified by polymerase chain reaction and sequenced.

Results: Generalized lipodystrophy, acanthosis nigricans, muscular hypertrophy, severe hypertriglyceridemia, and hepatomegaly were features in all three patients. Patient 1 developed diabetes mellitus at the early age of 2 months and he was the youngest CGL patient reported with overt diabetes. Patient 2 was found to have cardiomyopathy when she was aged 6 months. All of the patients were found to have mutations in the *BSCL2* gene, but none of these was a novel mutation. We did not find any AGPAT2 mutation in our patients.

Conclusion: All of our patients exhibited characteristic features of CGL due to mutations in the *BSCL2* gene.

Keywords: Lipodystrophy, hypertriglyceridemia, diabetes, cardiomyopathy

Introduction

Congenital generalized lipodystrophy (CGL), first described by Berardinelli in 1954 (1), is a rare autosomal recessive disorder characterized by near-complete absence of adipose tissue at birth (2,3). Affected individuals have severe hypertriglyceridemia, hyperinsulinemia, hepatomegaly, and often widespread acanthosis nigricans (4). Diabetes mellitus usually appears by the second decade or in adulthood (5,6,7,8). Additionally, hypertrophic cardiomyopathy is a classical late (second or third decade) complication (9,10) which has only been occasionally described in infancy or childhood (11,12,13,14). Currently, the disease is classified into four types according to clinical characteristics and type of mutations. Although four different CGL syndromes have been defined, 95% of reported patients correspond to CGL1 or CGL2 patients. CGL1 is caused by mutations in AGPAT2. The *AGPAT2* gene is located on chromosome 9q34 and encodes 1-acylglycerol-3-phosphate O-acyltransferase 2, which is involved in triglyceride biosynthesis (15). CGL2 is the most severe form of generalized lipodystrophy and is caused by mutations in *BSCL2* gene. The *BSCL2* gene,

Address for Correspondence: Xiuzhen Li MD, Guangzhou Women and Children's Medical Center, Department of Genetics and Endocrinology, Guangzhou, China **Phone:** +862038076073 **E-mail:** 13725100840@163.com

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located on chromosome 11q13, encodes a transmembrane protein called seipin (16). Seipin has been postulated to have an important role in lipid droplet assembly, in adipocyte differentiation, and in lipid droplets fusion. Patients with CGL2 show an almost complete lack of both metabolically active and mechanical adipose tissues. These patients have an increased prevalence of cardiomyopathy and mild mental retardation compared to healthy individuals (4). In this study, we examined the clinical features, molecular characteristics, and modes of treatment of three Chinese patients with CGL from independent families in order to better understand the diagnosis, clinical treatment, and prognosis of the condition.

Methods

Three Chinese patients aged 2 to 6 months with generalized absence of subcutaneous adipose tissues and with a clinical suspicion of CGL were studied. They were all born to healthy and non-consanguineous parents. All three patients are from unrelated families, and they are residents of three provinces, namely, Guangdong province in southern China, Jiangxi and Hubei provinces in central China. Clinical data were collected by retrospective review of medical records. The clinical diagnosis of CGL was established by presence of muscular hypertrophy, hepatomegaly, insulin resistance, and hypertriglyceridemia, which are features of generalized lipodystrophy. Informed consent was obtained from the parents of all patients. The study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center. The mutational analysis of *CGL* gene was performed at Guangzhou Women and Children's Medical Center from May 2012 to May 2015.

Blood glucose, cholesterol, and triglyceride levels were determined according to standard methods, using automated equipment. Serum insulin, follicle-stimulating hormone (FSH), and testosterone (T) levels were measured by chemiluminescence immunoassay (ADVIA Centaur XP Immunoassay Systems, Siemens, Erlangen, Germany). HbA1c was measured by immunoassay using the DCA Vantage Analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Presence of hepatomegaly was assessed by abdominal ultrasound, and cardiac function was assessed using echocardiography and electrocardiography.

Mutational Analysis

Genomic DNA was extracted from the peripheral leukocytes of the patients and their parents using a standard procedure. All exons and exon–intron splice junction regions of *AGPAT2* and *BSCL2* genes were amplified by PCR (Mastercycler Pro TM Gradient Thermal Cycler, Eppendorf, Hamburg, Germany). PCR products were purified and sent to BGI (Beijing, China) for direct DNA sequence analysis (DNA Analyzer3730, ABI, USA). Sequences were analyzed using Chromas software (V.2.01, Technelysium Pty Ltd, Tewantin QLD, Australia). Genetic variants were searched in the dbSNP, Clin Var, ExAC, and HGMD.

Treatment and follow-up

Patients 1 and 3 were fed with low-fat breast milk at diagnosis and patient 2 was fed with milk powder containing medium-chain fatty acid. For patient 1 who had overt diabetes, insulin was administered subcutaneously in doses 2 U/kg daily for 1 month and decreased to 1 U/kg daily in the following two months. Clinical follow-up started with diagnosis at 1 month and then 2–6 months intervals, subsequently. Height, weight, and laboratory evaluation were measured at every visit. For patient 1, the data of self-monitoring blood glucose were also recorded.

Results

The clinical and biochemical data on these patients are presented in Tables 1 and 2. Generalized lipodystrophy, acanthosis nigricans, muscular hypertrophy, hirsutism, hepatomegaly, and fatty liver were features in all three patients (Figure 1). All three patients had mild intellectual impairment with developmental language disorders.

Patient 1 was referred to our center at the age of 2 months for elevated glucose levels and poor weight gain. Physical examination was noteworthy for the generalized absence

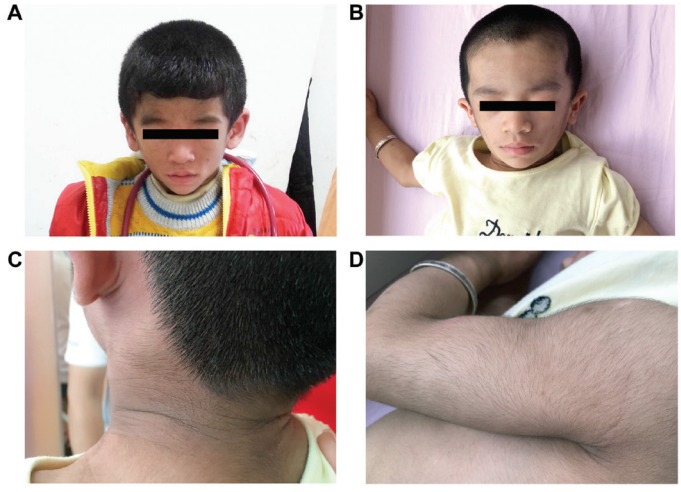

Figure 1. Clinical features of the three congenital generalized lipodystrophy patients indicate typical facial features with hollow cheeks and prominent masseters in patient 1 (A) and patient 3 (B); acanthosis nigricans (C), muscular hypertrophy and hirsutism (D) in patient 3.

Table 1. Clinical and biochemical data of the congenital generalized lipodystrophy patients at diagnosis

Patients	1	2	3	Normal range
Age (months)	2	6	3	/
Gender	Male	Female	Female	/
Birth weight (kg)	3.2	2.5	2.5	/
Clinical features Acanthosis nigricans	Yes	Yes	Yes	/
Hepatomegaly	Yes	Yes	Yes	/
Fatty liver	Yes	Yes	Yes	/
Biochemistry AST (U/L)	84	51	82	5-60
ALT (U/L)	219	74	109	7-40
Fasting glucose (mmol/L)	21.0	4.4	5.6	4.1-5.9
HbA1c (%/mmol/mol)	4.6/27	4.8/29	4.2/22	4.1-6.4/21-46
Fasting insulin (μIU/mL)	186	59	59	3-25
Fasting C-peptide (ng/mL)	14.18	5.05	6.81	1.10-4.40
TG (mmol/L)	22.17	16.17	12.10	0.23-1.70
T-Chol (mmol/L)	4.7	5.8	4.3	3.4-5.2
HDL (mmol/L)	0.52	0.76	0.73	0.88-1.80
LDL (mmol/L)	2.1	2.5	2.9	2.7-3.1
fT_3 (pmol/L)	7.5	7.3	6.8	2.3-6.3
fT_4 (pmol/L)	18.5	17.6	20.2	10.3-24.5
TSH (μIU/mL)	0.7	1.8	2.0	0.2-6.0
Testosterone (nmol/L)	1.1	1.0	0.7	0-0.7
Cortisol (nmol/L)	77	134	484	118-618

ALT: alanine aminotransferase, AST: aspartate aminotransferase, HbA1c: hemoglobin A1c, TG: triglyceride, T-Chol: total cholesterol, HDL: high-density lipoprotein, LDL: low-density lipoprotein, fT_3: free triiodothyronine, fT_4: free thyroxine, TSH: thyroid stimulating hormone

of subcutaneous adipose tissues; hypertrophy of all limb muscles; acanthosis nigricans in the neck; hirsutism in the neck, back and limbs; enlarged hands and feet. Additionally, he was noted to have emotional excitability and hyperactivity. The patient was diagnosed to have insulin resistance (fasting insulin 186 μIU/mL and fasting C-peptide 14.18 ng/mL) and diabetes mellitus [fasting blood glucose (FBG) 21.0 mmol/L] at the early age of 2 months. Laboratory examinations also indicated evidence of liver dysfunction [alanine aminotransferase (ALT) 219 U/L and aspartate aminotransferase (AST) 84 U/L] and dyslipidemia with markedly increased triglyceride levels and slightly decreased high-density lipoprotein (HDL) (22.17 mmol/L and 0.52 mmol/L, respectively). Additionally, ultrasonography revealed an enlarged liver with homogeneously increased echogenicity indicating steatosis. The boy was started on low-fat breast milk feedings, and insulin was administered subcutaneously in a dose of 2 U/kg daily. After one month, blood glucose was under control (FBG 3.9–8.3 mmol/L, postprandial blood glucose 5.0–15.0 mmol/L). At that time, the treatment was changed from insulin to glibenclamide. This fast reduction in insulin and switch of therapy to oral hypoglycemic drugs resulted in a rapid increase of glucose level. As a result, insulin treatment was resumed with a decreased dose of 1 U/kg daily. At the age of 6 months, he was weaned off insulin since his blood glucose was stable at 3.5–8.0 mmol/L. Feedings of low-fat breast milk led to a gradual decrease in serum lipid concentration (triglyceride 5.70 mmol/L). At the age of 1 year and 10 months, the boy returned to our center with a raised random blood glucose (17.2 mmol/L) and severe insulin resistance (insulin >300 μIU/mL and C-peptide 14.30 ng/mL).

Physical examination of patient 2 revealed a generalized and severe reduction of subcutaneous fat, prominent limb muscles, enlargement of the liver, and hirsutism in the neck, back, and limbs. Laboratory examination indicated that serum triglyceride level was raised (16.17 mmol/L). Her insulin and C-peptide levels were also high (59 μIU/mL and 5.05 ng/mL, respectively), but she had not developed diabetes until now. Echocardiography indicated an atrial septal defect, a left ventricular posterior wall thickness and a left ventricular outflow tract obstruction at 6 months of age. In addition to milk power containing medium-chain fatty acid, medical treatment with levocarnitine oral solution was given. After 1 month, her serum lipid concentration decreased dramatically (triglyceride 2.1 mmol/L). Now she is almost 3 years old and her triglyceride level is under control (Table 2).

The clinical and biochemical data of patient 3 are also given in Tables 1 and 2. In this patient, echocardiography revealed a patent foramen ovale. Ultrasonography demonstrated moderate hepatomegaly, fatty liver, and a small ovarian cyst (12 mm) when she was at an early age of 3 months. Three months later, the cyst was getting smaller (8 mm) and it disappeared at age 1 year.

Table 2. Clinical and biochemical data of the congenital generalized lipodystrophy patients at the most recent follow-up

Patients	1	2	3	Normal range
Age (years)	4	3	1.5	/
Body weight (kg)	21.0	16.0	11.5	/
Height (cm)	108	105	86	/
Clinical features				
Hepatomegaly	Yes	Yes	Yes	/
Fatty liver	Yes	Yes	Yes	/
Biochemistry				
AST (U/L)	38	52	103	5-60
ALT (U/L)	42	56	251	7-40
Fasting glucose (mmol/L)	4.8	4.5	3.8	4.1-5.9
HbA1c (%/mmol/mol)	/	/	5.0/31	4.1-6.4/21-46
Fasting insulin (μIU/mL)	/	60	23	3-25
TG (mmol/L)	2.30	3.99	3.45	0.23-1.70

ALT: alanine aminotransferase, AST: aspartate aminotransferase, HbA1c: hemoglobin A1c, TG: triglyceride

Genetic Analysis

Molecular alterations in the three patients are given in Table 3. No mutations were identified by sequencing all AGPAT2 exons and exon–intron junctions in our patients and their parents. Mutations in the *BSCL2* gene were found in all three patients (Table 3). All of these mutations have been previously reported. Our analysis showed that patient 1 and patient 2 carried the same compound heterozygous mutations, an insertion of a nucleotide, c.975insG in exon 7, resulting in a frameshift and truncated protein, G325fsX12 and a c.757G > T in exon 5, leading to a substitution of glutamic acid at codon 253 with a stop codon (Glu253X or E253X), respectively. Patient 3 had a homozygous c.545_546CGG trinucleotide insertion in exon 6.

Discussion

In this study, we examined the clinical and mutational features of three Chinese patients with CGL from unrelated families. Generalized lipodystrophy, acanthosis nigricans, muscular hypertrophy, hepatomegaly, insulin resistance, and hypertriglyceridemia were features present in all three patients. It is unusual to have diabetes mellitus early in life in patients with CGL. Though metabolic abnormalities of hyperinsulinemia and insulin resistance are evident early in life, overt diabetes generally develops during the pubertal years or adulthood (5,6,7,8,17). In a report by Van Maldergem et al (5), among 24 patients with BSCL2, 16 were diagnosed to have diabetes. However, the mean age of onset of clinical diabetes was 17.9 years. According to the report by Agarwal and his colleagues (6), patients with BSCL2 mutation had an onset of diabetes at a median age of 10 years. Recently, a nationwide study from Turkey showed that the mean age of onset of diabetes in these patients was 16.5 years (8). CGL patients who were diagnosed as insulin resistant in infancy and who developed diabetes mellitus at puberty have also been reported (7,17). Consistently, serum insulin levels of patient 2 and patient 3 in our study were high, but they had not developed diabetes so far. The unusual

Table 3. Molecular alterations in *BSCL2* in the three patients

Patients	Genotype	Type of mutation	Location	Base change	Amino acid change
1 and 2	Heterozygous	Insertion	Exon 7	c.975insG	p.Gly325 = fsX12
		Missense	Exon 5	c.757G > T	p.Glu253Ter
3	Homozygous	Insertion	Exon 6	c.545_546insCGG	p.Val184_Leu183delinsAspArg

feature of our patient 1 was the development of diabetes mellitus at the very early age of 2 months. There are only two patient reports of overt diabetes mellitus described at ages 4 and 5 months (12,18). Our patient is the youngest reported CGL patient with diabetes mellitus so far.

Hypertrophic cardiomyopathy is reported in 20–25% of CGL patients and is a significant cause of morbidity from cardiac failure and of early mortality (9,10,11,12). However, this life-threatening complication is usually reported in older patients. Bjornstad et al (9) reported 6 patients of BSCL presenting with myocardial hypertrophy. Rheuban et al (10) described 4 other similar patients. According to their results, the average age for diagnosis of hypertrophic cardiomyopathy in CGL was 20 years. Similarly, Lupsa et al (19) reported 44 patients with lipodystrophy and pointed out that the average age of developing cardiac abnormalities in these patients was about 20 years. There are at least four patients with an early onset of hypertrophic cardiomyopathy who have been reported. Bhayana et al (11) described a young girl with CGL harboring a mutation in BSCL2, who was found to have myocardial hypertrophy from 6 months of age. In a 4-month-old Chinese boy, Friguls et al (12) reported severe cardiomyopathy with cardiac failure and systemic hypertension. Debray et al (14) reported a young boy presenting at age 1 month with severe hypertrophic cardiomyopathy. Miranda et al (13) also reported a 2-month-old boy harboring cardiomyopathy due to a homozygous missense mutation in BSCL2. In this present study, patient 2 was diagnosed with hypertrophy cadiomyopathy when she was aged 6 months. Echocardiography indicated atrial septal defect, an asymmetric left ventricular posterior wall thickness and a left ventricular outflow tract obstruction. The mechanism causing hypertrophic cardiomyopathy in patients with CGL is unclear. Theoretically, the severe insulin resistance observed in these patients may prompt cardiomyocyte hypertrophy by activating IGF-1 receptors which are largely expressed in the myocardial tissue and stimulate cell growth (10). Further studies are needed to elucidate the exact mechanism.

As mentioned before, dyslipidemia, observed in all three patients, was characterized by an increase in triglyceride levels and a decrease in HDL. TG levels decreased dramatically after exposure to a strict low-fat and low-sugar diet. This finding indicates that diet control is beneficial for CGL patients.

Four different CGL syndromes have been defined. Type 1 is associated with AGPAT2 mutations, type 2 with BSCL2 mutations, type 3 with CAV1 mutations, and type 4 with PTRF mutations. AGPAT2 mutations were found predominantly in patients of African ancestry which means that CGL1 is the major type of CGL in populations of African origin. Type 2 CGL has been reported in patients of various ethnicities including patients from Norway, United Kingdom, and Mediterranean countries, as well as Middle Eastern Arabs (5). Racial differences in the pathogenesis of CGL may exist, but the specific mechanisms leading to these differences need to be further elucidated. Gene mutation analysis was performed in our patients. We did not find any AGPAT2 mutation in these patients, but all of them had mutations in the *BSCL2* gene. Patient 1 and patient 2 had the same compound heterozygous mutations, one mutation was inherited from the mothers [c.757G > T (p.Glu253Ter)] and the other from the fathers [c.975insG (p.Gly325 = fsX12)]. These two patients in our report had the same mutation of the same gene but revealed different clinical phenotypes. Patient 1 developed severe hypertriglyceridemia and diabetes mellitus at early infancy, while patient 2 had a much lower triglyceride level and no diabetes until now. Patient 2 had hypertrophic cardiomyopathy, which was absent in patient 1. These findings may suggest that the disease phenotype is not determined by the mutation alone and that other factors can contribute to the development of the clinical features in these patients. A homozygous mutation (c.545_546insCGG) of BSCL2 was found in patient 3. All of these mutations have been reported previously. Although the number of subjects we examined is small, these observations indicate that BSCL2 is a major causative gene for CGL in the Chinese people. According to previous reports, almost all Chinese CGL patients reported had mutations in BSCL2 (20,21,22,23), which is consistent with our results. The findings of this study may be helpful in expanding our knowledge of genotype-phenotype correlations in CGL patients.

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Ethics

Ethics Committee Approval: The study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center. Informed Consent: Informed consent was obtained from the parents of all patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Xiuzhen Li and Li Liu, Design: Xiuzhen Li and Li Liu, Data Collection or Processing: Ruizhu Lin, Yonglan Huang, Huiying Sheng, Xiaofei Li, and Tzer Hwu Ting, Analysis or Interpretation: Xueying Su, Xiuzhen Li and Li Liu, Literature Search: Xueying Su, Writing: Xueying Su, Xiuzhen Li and Li Liu.

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ORIGINAL ARTICLE

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Age-Specific Frequencies and Characteristics of Ovarian Cysts in Children and Adolescents

Hamdi Cihan Emeksiz[1], Okşan Derinöz[2], Esra Betül Akkoyun[2], Faruk Güçlü Pınarlı[3], Aysun Bideci[1]

[1]Gazi University Faculty of Medicine Hospital, Department of Pediatric Endocrinology, Ankara, Turkey
[2]Gazi University Faculty of Medicine Hospital, Department of Pediatric Emergency, Ankara, Turkey
[3]Gazi University Faculty of Medicine Hospital, Department of Pediatric Oncology, Ankara, Turkey

What is already known on this topic?

The discovery rate of ovarian cysts in children and adolescents has remarkably increased in parallel to the increased usage of ultrasonography in pediatric imaging. The vast majority of the ovarian cysts detected in children and adolescents are asymptomatic functional (physiologic) cysts with simple appearance and small size (1-3 cm). Although ovarian cysts are far more common and larger in adolescents than in children due to the increased hormonal stimulation of ovaries during puberty, there is still limited data available about the epidemiology and characteristics of ovarian cysts in the pediatric population.

What this study adds?

The present study documented the age-specific frequencies and characteristics of ovarian cysts in children and adolescents. The findings may contribute to the understanding of normal ovarian developmental process and may help improve the management of ovarian cysts in the pediatric population.

Abstract

Objective: The aim of the present study was to document ovarian cyst frequency and characteristics as well as distribution of these parameters with respect to age in children and adolescents.

Methods: We retrospectively analyzed the medical records of 1009 girls between the ages of 5-18 years who presented to our pediatric emergency department (PED) with pelvic pain and therefore underwent pelvic ultrasound examination between June 2011 and May 2014.

Results: In total, 132 of 1009 girls (13.1%) were identified as having ovarian cysts ≥1 cm in diameter. The frequency of ovarian cysts was found to be 1.8% (6/337) in children aged 5-9 years and 18.8% (126/672) in those aged 10-18 years. All the cysts detected in children aged 5-9 years were small (<3 cm) and simple with age-specific frequencies ranging between 1.5-2.7%. With the onset of adolescence, ovarian cyst frequency started to increase with age and ranged between 3.8-31.3% throughout adolescence. Age of peak ovarian cyst frequency was 15 years with a rate of 31.3%. Large ovarian cysts (>5 cm) were identified in 19 adolescents (15.1%) with most occurring during middle adolescence. Of the 19 adolescents, five were found to have cyst-related significant ovarian pathologies including cystadenoma (n = 3) and ovarian torsion (n = 2).

Conclusion: In children aged 5-9 years, ovarian cysts were infrequent and small (<3 cm). Peak ovarian cyst frequency was detected at the age of 15 years. All patients diagnosed with cyst-related significant ovarian pathologies were adolescents having a cyst >5 cm in diameter with a complex appearance in most.

Keywords: Ovarian cyst, frequency, characteristics, children, adolescents

Address for Correspondence: Hamdi Cihan Emeksiz MD, Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey
Phone: +90-462-3415656 11572 **E-mail:** hcemeksiz@gmail.com

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Introduction

Widespread use and availability of ultrasonography in pediatric imaging has led to an increase in the number of detected ovarian cysts in children suggesting that they are more common than considered in the pediatric population. The great majority of ovarian cysts are asymptomatic functional (physiologic) cysts with simple appearance and small size (1-3 cm), but sometimes they grow to larger sizes (>5 cm) and rarely become clinically evident by being ruptured or causing ovarian torsion (1,2). Although ovarian cysts are known to be more common and larger in adolescents compared to children as a result of increased gonadotropin stimulation of ovaries during puberty (3), very limited data exist about the epidemiology and characteristics of ovarian cysts in the pediatric population. There are only a few studies on the incidence and ultrasound findings of ovarian cysts in children and adolescents. Millar et al (4) found ovarian cysts in 2%-5% of females between the ages of 0-8 years, the majority of these cysts being insignificant and small, less than 1 cm in diameter. In adolescents, Porcu et al (5) reported that the frequency of ovarian cysts >3 cm was 12% in their series of 139 girls aged 10-19 years. Most of these cysts were considered to be functional cysts.

The aim of the present study was to document ovarian cyst frequency, size, and characteristics, as well as distribution of these parameters with respect to age in children and adolescents. Such information may contribute to the understanding of normal ovarian developmental process and may help improve the management of pediatric ovarian cysts.

Methods

We retrospectively reviewed the records of girls between the ages of 5-18 years who presented to our pediatric emergency department (PED) at Gazi University Faculty of Medicine Hospital, PED, Ankara, Turkey, between June 01, 2011 and May 31, 2014 because of pelvic pain and therefore underwent pelvic ultrasound examination. During that time interval, a total of 1157 girls (5-18 years of age) were subjected to pelvic ultrasound examination in PED. One hundred forty-eight girls who had chromosomal abnormalities, a history of chronic illness or cancer, and those whose one or both ovaries could not be seen on ultrasound were excluded from the study. Finally, 1009 girls who met the inclusion criteria were enrolled, and 132 of these 1009 girls were found to have ovarian cysts incidentally or purposely in ultrasound examination. Characteristics of the ovarian cysts including size, laterality, appearance, and frequency of ovarian cyst formation for each age and for growth stages [childhood (<10 years), early adolescence (10-14 years), middle adolescence (15-17 years), and late adolescence (18-19 years)] were analyzed in the present study. The ultrasound system used in the sonographic examination of the pelvic organs was a GE Logiq Ultrasound device (March 2009, Wisconsin, USA) with a 5-2-MHz convex transducer. All girls were scanned with a full bladder. A simple cyst was defined as having anechoic fluid, a smooth thin wall, posterior acoustic enhancement, and no solid component or septation. An ovarian cyst with a solid component and/or septation, and/or internal echoes and/or echogenic wall was considered as a complex ovarian cyst (1). The size of an ovarian cyst was defined as the maximum measurement in any dimension and was categorized into three groups: <3 cm, 3-5 cm, and >5 cm. In girls with bilateral ovarian cysts ≥1 cm, characteristics of the largest cyst were recorded. Cysts smaller than 1 cm in diameter (microcysts-ovarian follicles) were not included in the study. The study protocol was approved by Clinical Trials Ethics Committee of Gazi University Faculty of Medicine (#01262015-43).

Statistical Analysis

The statistical analysis was performed using the Package for Social Sciences (SPSS) software (version 16, SPSS Inc. Chicago, IL, USA). A descriptive analysis was used to determine the characterization of patients and ovarian cysts. Descriptive statistics were expressed as frequency and percentage for categorical variables, whereas quantitative data were expressed as mean standard deviation for normally distributed variables and as medians (minimum-maximum) for non-normally distributed data.

Results

During a 3-year time span, of 1009 girls (672 adolescents) aged 5-18 years, 132 (13.1%) were identified as having an ovarian cyst ≥1 cm. The frequency of ovarian cysts was found to be 1.8% (6/337) in children aged 5-9 years and 18.8% (126/672) in adolescents aged 10-18 years. Age-specific frequencies of ovarian cyst formation and their distribution with respect to cyst size are summarized in Table 1. Age-specific cyst frequencies in children aged 5-9 years varied between 1.5-2.7%. All the ovarian cysts detected in these children were small (<3 cm). With the onset of adolescence, ovarian cyst frequency started to rise and to reach ranges of 3.8-30.9% in early adolescence, 26.7-31.3% in middle adolescence, and 13.6% in late adolescence. Peak ovarian cyst frequency was at age 15 years with a rate of 31.3%. Of the 126 adolescents with an ovarian cyst, 19 (15.1%) were found to have large ovarian cysts (>5 cm) occurring mostly during middle adolescence.

Sonographic characteristics of ovarian cysts by age groups are given in Table 2. Most of the cysts were right-sided

(59.8%), simple (68.9%), and <3 cm in diameter (58.3%). Of the 19 large ovarian cysts, 11 (57.9%) were right-sided and 14 (73.7%) were complex. Seven adolescents were found to have a complicated ovarian cyst presenting with ovarian torsion (n=2) and ruptured cyst (n=5). The frequency of ovarian torsion was 10.5% (2/19) in adolescent girls with a cyst >5 cm in diameter. Of the two girls with ovarian torsion, one had a simple right-sided ovarian cyst with a size of 7 cm and presented with right lower quadrant pain and vomiting, while the other girl had a complex left-sided cyst with a size of 5.1 cm and presented with left lower quadrant pain. Both underwent cystectomy and detorsion. Girls with ruptured cysts were all hemodynamically stable.

Three adolescents had benign neoplastic ovarian cysts measuring >5 cm in diameter, identified as mucinous cystadenoma (n=2) and serous cystadenoma (n=1). All underwent salpingooophorectomy. Their tumor markers including alpha-fetoprotein (AFP), beta-human chorionic

Table 1. Ovarian cysts in children and adolescents

	Age	Number of girls who underwent pelvic USG	Number (%) of girls with a cyst <3 cm	Number (%) of girls with a cyst 3-5 cm	Number (%) of girls with a cyst >5 cm	Number (%) of girls with a cyst ≥1 cm
Childhood	5	64	1 (1.6)	-	-	1 (1.6)
(0-9 years)	6	66	1 (1.5)	-	-	1 (1.5)
	7	67	1 (1.5)	-	-	1 (1.5)
	8	67	1 (1.5)	-	-	1 (1.5)
	9	73	2 (2.7)	-	-	2 (2.7)
Early adolescence	10	78	2 (2.6)	-	1 (1.3)	3 (3.8)
(10-14 years)	11	78	5 (6.4)	-	-	5 (6.4)
	12	73	6 (8.2)	-	1 (1.4)	7 (9.6)
	13	63	6 (9.5)	3 (4.8)	2 (3.2)	11 (17.5)
	14	68	10 (14.7)	8 (11.8)	3 (4.4)	21 (30.9)
Middle adolescence	15	83	13 (15.7)	9 (10.8)	4 (4.8)	26 (31.3)
(15-17 yrs)	16	86	12 (13.9)	8 (9.3)	3 (3.5)	23 (26.7)
	17	77	11 (14.3)	6 (7.8)	4 (5.2)	21 (27.3)
Late adolescence (18-21 years)	18	66	6 (9.1)	2 (3)	1 (1.5)	9 (13.6)
Total		1009	77 (7.6)	36 (3.6)	19 (1.9)	132 (13.1)

USG: ultrasonography

Table 2. Characteristics of ovarian cysts by age groups

	Laterality		Size			Appearance	
	Right	Left	<3 cm	3-5 cm	>5 cm	Simple	Complex
Age group							
5-9 years	4	2	6	0	0	6	0
10-14 years	28	19	29	11	7	33	14
15-17 years	42	28	36	23	11	46	24
18 years	5	4	6	2	1	6	3

Table 3. Summary of patients with benign ovarian neoplasms

Patient no	Age (year)	Size (cm)	Appearance	Diagnosis	Operation	AFP (ng/mL)	β-HCG (mIU/L)	CA-125 (U/mL)	LDH (U/L)	CEA (ng/mL)
1	14	21	Complex	Mucinous cystadenoma	Right SO	1.0	0.1	16	242	0.6
2	17	15	Complex	Mucinous cystadenoma	Left SO	2.1	0.2	14	489	0.5
3	10	6.5	Complex	Serous cystadenoma	Right SO	1.3	0.1	10	152	0.5

SO: Salpingooophorectomy, AFP: alpha-fetoprotein, β-HCG: beta-human chorionic gonadotropin, CA-125: cancer antigen 125, LDH: lactate dehydrogenase, CEA: carcinoembryonic antigen

gonadotropin (β-HCG), cancer antigen 125 (CA-125), lactate dehydrogenase (LDH), and carcinoembryonic antigen (CEA) were all in normal ranges (Table 3). The patient with mucinous cystadenoma measuring 21 cm in diameter had an additional symptom of abdominal fullness. The two patients with mucinous cystadenoma underwent surgery soon after the sonographic examination due to the enormous size (≥15 cm) and appearance of the cysts, and the patient with serous cystadenoma, after 6 months of follow-up due to the persistence of the cyst.

Discussion

In our study, 132 girls (13.1%) aged between 5 and 18 years were found to have an ovarian cyst ≥1 cm in diameter. Age-specific frequencies of ovarian cysts were low and almost constant during childhood. With the onset of early adolescence, the frequency of ovarian cyst formation started to rise and made a peak by the age of 15 years and remained roughly elevated for all cyst size categories throughout the middle adolescence. All girls who were found to have a cyst-associated significant ovarian pathology including ovarian torsion or neoplasm were adolescents having a large ovarian cyst with a complex appearance in most.

In the present study, the frequency of ovarian cysts ≥1 cm was found to be 1.8% in children aged 5-9 years and age-specific cyst frequencies in this age interval varied between 1.5-2.7%. Thus far, the few studies documenting the frequency of ovarian cyst formation in children reported largely varying rates most probably due to the differences between the studies in terms of study design and sample size. Millar et al (4) retrospectively analyzed 1818 pelvic ultrasonography findings in prepubertal girls aged 0-8 years and found that the frequency of ovarian cysts < 1 cm in diameter was 2-5%, varying with respect to age group. Ovarian cysts > 2 cm were very rare in their series and detected in 0.9% of prepubertal girls and in only 0.2% of girls who were older than 2 years of age. However, the incidence of ovarian cysts between 1 cm and 2 cm in diameter was not reported in this study. We found age-specific frequencies of ovarian cysts of ≥1 cm in diameter almost constant and low from 5 years to 9 years of age reflecting the relatively dormant status of ovaries during childhood. All the cysts detected in children aged 5-9 years were < 3 cm in diameter, simple and uncomplicated.

The ovary is more active during puberty due to the increased gonadotropin secretion. Hence, finding an ovarian cyst is more common in adolescence than in any other stage of growth. However, very limited data are available on the incidence and characteristics of ovarian cysts in adolescents. Porcu et al (5) followed 139 adolescent girls aged between 10 and 19 years with serial ultrasound assessment in the follicular phase of the menstrual cycle and reported that the incidence of ovarian cyst formation > 3 cm was 12%. This was higher than the rate (8.2%) we found in adolescents for ovarian cysts > 3 cm. The difference in frequencies between the two studies may be related to their design, sample size, and ethnicity dissimilarities, as well as to the unmentioned age-specific distribution of adolescent girls in the study of Porcu et al (5), since clustering of their cases particularly at perimenarcheal ages would likely lead to the overestimation of the ovarian cyst incidence in adolescent girls. Kanizsai et al (6) studied the characteristics of ovarian cysts in 119 girls undergoing ultrasound examination due to irregular bleeding in most and reported that the majority of the cysts they detected in their obstetrics and gynecology clinic were simple and unilateral, findings which are similar to those in our study. They also categorized the cysts with respect to their size and found that most of the cysts (63%) were between 3-5 cm in diameter followed by 28% > 5 cm, and 9% between 1-3 cm. In contrast to these findings, more than half of the cysts (58%) in our series were found to be < 3 cm in diameter, 27% between 3-5 cm, and 15% > 5 cm. The frequency of ovarian cysts measured between 3-5 cm seems to be higher than normal in the series of Kanizsai et al (6) since most ovarian cysts detected in premenopausal women are functional cysts (follicular or corpus luteum) which are typically not larger than 3 cm in diameter (7). In addition, girls in the Kanizsai et al (6) study were evaluated for more typical complaints associated with ovarian disorders, as well as in a more competent division, both of which consequentially may have led to the overestimation of the frequency of ovarian cysts > 3 cm in their study.

The number and size of ovarian follicles vary depending on the stage of puberty (3). In the years just before menarche, some follicles exceed 1 cm in diameter and become ovarian cysts with a more prevalent fluid component. These cysts can reach larger sizes which then often spontaneously regress (6,8). Accordingly, in the present study, we found that the frequency of ovarian cyst formation began to rise by the age of 11 years which is nearly 1.7 years before the average age of menarche in Turkish girls (9). At perimenarcheal ages (within 3 years of menarche) the formation of ovarian cysts becomes more prominent because of considerably high number of antral follicles in this period of life and their increased sensitivity to mature gonadotropin stimulation as well (10,11,12). Moreover, the early period following menarche is commonly associated with anovulatory cycles. Therefore, ovulatory cysts are often seen as the result of aborted ovulations or corpus luteum persistence and this is the reason why both follicular and corpus luteal cysts are extremely common in this period of life (2). Consistent with these data, we found that the frequency of ovarian cyst formation made a remarkable peak by the age of 15

years (31.3%) and remained almost elevated for all cyst size categories throughout middle adolescence. However, with the onset of late adolescence, ovarian cyst frequency was found to follow a downward trend. The latter finding may be due to the completion of the ovarian maturation process in almost all girls by the end of middle adolescence.

Cysts greater than 5 cm pose a higher risk for torsion (2). Likewise, two adolescent girls who were identified to have ovarian torsion in our series had a cyst diameter >5 cm. The torsion rate was found to be 10.5% (2/19) in girls with an ovarian cyst >5 cm. Both girls were treated by cystectomy and detorsion. Five adolescents were sonographically detected to have ruptured ovarian cysts. All were hemodynamically stable.

After mature teratomas, cystadenomas are the second most common benign ovarian tumors in children and adolescents (13). They can be either serous or mucinous and can grow up to enormous sizes. Mucinous cystadenomas tend to be much larger than serous cystadenomas at presentation (14). Three girls with a cyst size >5 cm were found to have unilateral cystadenomas including 2 mucinous and 1 serous type. Consistent with the literature, both mucinous cystadenomas were larger than the serous one. Girls with mucinous cystadenoma underwent surgery soon after the sonographic examination due to the enormous size (≥15 cm) and appearance of the cysts. Surgery was performed in the girl with serous cystadenoma after 6-month follow-up due to the persistence of the cyst. Tumor markers studied were not helpful in the diagnosis of these tumors.

Our study has several limitations. It was retrospective in design, data about pubertal stage of the girls at presentation and menstruation history of the adolescents having periods were unavailable. Also, the ultrasonographic examinations were performed by different radiologists. Despite these handicaps, to our knowledge, this is the first study to report age-specific frequencies and characteristics of ovarian cysts in adolescence, and our findings may help to improve the management of future cases of adolescents with cyst-related disorders.

In conclusion, this study presented descriptive data on pediatric ovarian cyst frequency, size, and characteristics, as well as distribution of these parameters with respect to age in a relatively large cohort of girls from Turkey. In children aged 5-9 years; ovarian cysts were infrequent and small (<3 cm), suggesting that ovarian cyst-related urgent conditions seem to be rare in this period of life. With the onset of adolescence, ovarian cyst frequency increased with age and made a peak by the age of 15 years and remained roughly elevated for all cyst size categories throughout middle adolescence, presumably as a consequence of normal ovarian developmental process. All patients diagnosed with a cyst-related significant ovarian pathology were adolescents having a cyst >5 cm in diameter with a complex appearance in most.

Ethics

Ethics Committee Approval: The study protocol was approved by Clinical Trials Ethics Committee of Gazi University Faculty of Medicine (#01262015-43). Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Okşan Derinöz, Hamdi Cihan Emeksiz, Design: Hamdi Cihan Emeksiz, Okşan Derinöz, Data Collection or processing: Esra Betül Akkoyun, Hamdi Cihan Emeksiz, Okşan Derinöz, Analysis or Interpretation: Hamdi Cihan Emeksiz, Okşan Derinöz, Aysun Bideci, Faruk Güçlü Pınarlı, Literature Search: Hamdi Cihan Emeksiz, Writing: Hamdi Cihan Emeksiz, Aysun Bideci.

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ORIGINAL ARTICLE

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Safety and Efficacy of Stosstherapy in Nutritional Rickets

Daipayan Chatterjee, Mathad K. S. Swamy, Vikas Gupta, Vasu Sharma, Akshat Sharma, Krishti Chatterjee

Vardhaman Mahavir Medical College, Department of Orthopedics, New Delhi, India

What is already known on this topic?

Stosstherapy may be used for treating nutritional rickets.

What this study adds?

Single injection is sufficient enough for treating nutritional rickets. Alkaline phosphatase may be used as a secondary marker for monitoring. Vitamin D assay at 6 weeks and calcium, phosphate and alkaline phosphatase assay at 6 months is recommended to monitor the biochemical improvement.

Abstract

Objective: Stosstherapy has been used since early 19th century for treating nutritional rickets. However, there are no clear cut guidelines for the biochemical monitoring of this treatment. Repeated blood tests at short intervals increase the cost of therapy and noncompliance.

Methods: A prospective study was conducted on 191 cases of nutritional rickets below 10 years of age to evaluate the effectivity of stosstherapy. All cases were treated with a single intramuscular injection of vitamin D (600.000 IU) along with oral calcium (50 mg/kg) and vitamin D (400 IU per day) until radiological resolution. Dietary modifications and adequate sunlight exposure were also recommended.

Results: The mean age of presentation was 2 years 9 months. Mean sunlight exposure was 17 minutes/week with 90% having low sunlight exposure (<30 minutes/week). Prolonged breast feeding (>6 months) was found in 93.7% of the cases. With treatment, the clinical features started resolving by 1 month with complete resolution of most of the features over a period of 1 year. By 6 months, all the study subjects had complete radiological resolution. Serum levels of calcium and alkaline phosphatase (ALP) were restored by 6 months in most cases while phosphate and vitamin D levels normalized by 6 weeks.

Conclusion: Stosstherapy is a safe, cheap and effective method of treating nutritional rickets. Biochemical tests at initial presentation followed by vitamin D assay at 6 weeks and calcium, phosphate and ALP assays at 6 months is recommended in the monitoring of these patients. For regular monitoring, only ALP assay is recommended, provided one abstains from repeat injection of vitamin D based on high ALP levels.

Keywords: Stosstherapy, nutritional rickets, vitamin D, alkaline phosphatase

Introduction

Rickets is a metabolic bone disease characterized by deficiency of vitamin D and/or dietary calcium leading to impaired mineralization of newly formed bone matrix before epiphyseal closure (1). Nutritional rickets has undergone a recent surge in frequency during the last decade especially in developed countries where it was thought to have been eradicated (2). In developing countries, rickets has been ranked among the five most prevalent childhood diseases (3). Nutritional rickets can be treated by various regimes ranging from daily or weekly oral therapy to single high-dose (stosstherapy) oral or intramuscular therapy. The advantage of intramuscular high-dose therapy over other regimes is avoidance of daily dose, thereby increasing patient compliance and decreasing cost of therapy. On the other hand, the main disadvantages are injection site morbidity and potential complication of vitamin D toxicity. Stosstherapy has been used since early 19th century for treating rickets (4). However, there are no clear cut

Address for Correspondence: Daipayan Chatterjee MS, Vardhaman Mahavir Medical College, Department of Orthopedics, New Delhi, India
Phone: 9007930192 **E-mail:** daipayan27@yahoo.co.in

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guidelines as to which biochemical tests are needed and at what interval these tests are to be repeated for monitoring the effect of this treatment. Repeated blood tests at short intervals not only increase the cost of therapy but also increase noncompliance.

This study not only aims to evaluate the effectivity of stosstherapy in nutritional rickets but also addresses the above-mentioned problems.

Methods

A longitudinal prospective study was conducted over a 3-year period from September 2011 to September 2014 at Vardhaman Mahavir Medical College and Safdarjung Hospital (Central Institute of Orthopedics, New Delhi, India). Out of 212 cases of rickets attending the outpatient department, 196 untreated cases of nutritional rickets of ages below 10 years were selected on the basis of clinical, biochemical, and radiological features. Out of these cases, 5 were lost to follow-up. Thus, 191 patients were enrolled for the study after written informed consent was obtained from the parents. Cases with a history of prematurity, renal or hepatic disease, intestinal malabsorption, tumor, chronic diseases including tuberculosis and diseases of the skeletal system were excluded. Pretreatment biochemical investigations included estimation of serum calcium, phosphate, alkaline phosphatase (ALP), 25-hydroxyvitamin D (calcidiol), parathyroid hormone (PTH) levels along with urinary calcium and phosphate excretion. Rickets cases with normal PTH (hypophosphatemic rickets), phosphaturia (renal rickets-renal tubular acidosis type 1 and 2 and hypophosphatemic rickets), and high levels of calcidiol (vitamin D-dependent rickets types 1 and 2) were excluded. The cases so segregated were evaluated by taking a detailed history which included enquiry regarding average weekly sunlight exposure, duration of breast feeding, and average daily cow milk consumption in children more than 6 months (measured by determining the volume of the container used by the child to drink milk). Antero-posterior views of bilateral wrist and knee joints were used for radiological evaluation.

The selected cases were treated with a single high-dose (600.000 IU) intramuscular injection of cholecalciferol (Arachitol 6L, Abbott India) along with oral calcium (50 mg/kg) and vitamin D (400 IU per day) until radiological resolution. Dietary modifications (egg, milk, fish added to the diet) and adequate sunlight exposure (30 minutes/weekly) were also advised.

Calcidiol and PTH were estimated by chemiluminescent microparticle immunoassay (CMIA; Abbott Architect Plus i1000SR). Intra- and inter-assay coefficients of variation (CVs) for calcidiol were 1.4-3.7% and 2.7-4.6%, respectively. Calcium, phosphate, and ALP were estimated by spectrophotometry using arsenazo-3, ammonium molybdate, and para-nitrophenyl phosphate as reagents, respectively (Vitros 5.1 FS analyzer- Johnson and Johnson). Ethical clearance was obtained for the study from the hospital ethical clearance committee.

Follow-up was done at 3 weeks, 6 weeks, 3 months, 6 months, 9 months, and 1 year by estimation of serum calcidiol, calcium, phosphate, ALP levels and by radiographs of both wrist and knee joints (antero-posterior views bilaterally). Radiological resolution was quantified using Thacher's 10-point scoring system (5) where a score of '0' was considered to be complete radiological resolution. The cases were clinically examined by the corresponding author at all visits. All the radiographic films were reviewed and scored by the same radiologist (who was blinded to the study) and the corresponding author separately. All the cases were subjected to abdominal ultrasonography prior to treatment and at 6 months and 1 year posttreatment to check for nephrocalcinosis.

Pre- and post-treatment clinical, biochemical, and radiological parameters were compared and analyzed statistically by using paired t-test for quantitative data and chi-square test for qualitative data with the help of SPSS 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Data pertaining to sunlight exposure, initial vitamin D levels, and duration of breast feeding were compared.

Results

One-hundred and ninety-one cases (85 male, 106 female) were enrolled in the study. The mean age of presentation was 2 years 9 months (range 6 months-9 years). Mean sunlight exposure was 17 minutes/week (range 0 -2 hours/week) with 51% (97 cases) having no sunlight exposure and 90% (172 cases) having sunlight exposure of less than 30 minutes/week. Those exposed to sunlight were partially dressed (dress covering body and extremities up to mid-arm and mid-thigh) during the exposure. In 93.7% (179 cases) of the cases, there was a history of prolonged breast feeding (more than 6 months). Mean duration of breast feeding was 1 year 8 months (range 0-42 months). Average daily cow milk consumption was 60 mL (range 0-250 mL) with 48% (92 cases) having no milk consumption and 96.3% (184 cases) consuming 200 mL or less.

Table 1 enumerates the frequency and resolution of clinical features during the follow-up period. Swollen wrist and ankle was the most common clinical feature followed by

Table 1. Frequency and resolution of clinical features during a one-year follow-up period

	0 week	3 weeks	6 weeks	12 weeks	6 month	9 month	12 month
Swollen wrist and ankle	182 (95)	182 (95)	177 (93)	168 (88)	101 (53)	21 (11)	0
Genu varum	119 (62)	119 (62)	119 (62)	119 (62)	83 (43)	55 (29)	36 (19)
Frontal bossing	100 (52)	100 (52)	91 (48)	39 (20)	8 (4)	0	0
Pot belly	73 (38)	69 (36)	60 (31)	55 (29)	11 (6)	0	0
Genu valgum	52 (27)	52 (27)	52 (27)	52 (27)	52 (27)	52 (27)	52 (27)
Delayed teething/enamel hypoplasia	49 (26)	-	-	-	-	-	-
Wt $< 3^{rd}$ percentile	36 (19)	-	-	-	-	-	7 (4)
Rachitic rosary	21 (11)	21 (11)	17 (9)	17 (9)	6 (3)	0	0
Height $< 3^{rd}$ percentile	18 (9)	-	-	-	-	-	3 (2)
Repeat fractures	14 (7)	-	-	-	-	-	-
Harrison's sulcus	9 (5)	9 (5)	9 (5)	9 (5)	4 (2)	0	0
Windswept deformity	2 (1)	2 (1)	2 (1)	2 (1)	2 (1)	2 (1)	2 (1)

Table 2. Biochemical and radiological changes during follow-up

		Time of follow-up						
		0 week	3 weeks	6 weeks	3 month	6 month	9 month	12 month
Calcium (mg/dL)	Mean ± SD	8.8 ± 0.6	9.3 ± 0.5	9.4 ± 0.5	9.6 ± 0.5	9.7 ± 0.3	9.7 ± 0.2	9.7 ± 0.2
	Low (≤9.5)	167 (87)	126 (66)	110 (58)	74 (39)	18 (9)	0	0
	Normal (9.6-10.6)	24 (13)	61 (32)	98 (51)	116 (60)	173 (91)	191 (100)	191 (100)
	High (>10.6]	0	4 (2)	2 (1)	2 (1)	0	0	
Phosphate (mg/dL)	Mean ± SD	3.5 ± 0.8	4.4 ± 0.6	4.7 ± 0.6	5.1 ± 0.4	5.2 ± 0.2	5.2 ± 0.2	5.1 ± 0.2
	Low (≤3.6)	128 (67)	22 (17)	18 (9)	0	0	0	0
	Normal (3.7-5.4)	63 (33)	169 (83)	173 (91)	189 (99)	191 (100)	191 (100)	191 (100)
	High (>5.4)	0	0	0	2 (1)	0	0	0
ALP** (IU/L)	Mean ± SD	1315 ± 914	910 ± 625	676 ± 436	479 ± 251	356 ± 139	268 ± 86	234 ± 63
	Low	0	0	0	0	0	0	0
	Normal	17 (9)	65 (34)	104 (54)	138 (72)	166 (87)	191 (100)	191 (100)
	High	174 (91)	126 (66)	87 (46)	53 (28)	25 (13)	0	0
Calcidiol*** (ng/mL)	Mean ± SD	13 ± 8	47 ± 15	59 ± 13	61 ± 8	60 ± 10	53 ± 7	46 ± 7
	Low	188 (98)	8 (4)	0	0	0	0	0
	Normal	3 (2)	178 (93)	181 (95)	191 (100)	186 (97)	191 (100)	191 (100)
	High	0	5 (3)	10 (5)	0	5 (3)	0	0
Radiological score	Mean ± SD	6.8 ± 3.2	4.7 ± 3.2	2.4 ± 2	0.9 ± 1.2	0	0	0
	Number of patients with score "0"	0	21 (11)	41 (21)	90 (47)	191 (100)	191 (100)	191 (100)

*Numbers in parentheses ( ) represent percentage values.

**ALP: alkaline phosphatase. Values are adjusted for age and sex of the cases (6)

***Calcidiol levels are classified as per United State Endocrine Society classification.

SD: standard deviation

Table 3. Statistical significance of changes in mean serum levels of calcium, phosphate, alkaline phosphatase, and calcidiol between follow-up visits

Interval between follow-up visits	Statistical significance of the changes in mean (p-values)			
	Calcium	Phosphate	ALP	Vitamin D
0-3 weeks	0.000	0.000	0.000	0.000
3 weeks-6 weeks	0.010	0.000	0.000	0.000
6 weeks-3 months	0.000	0.000	0.000	0.046
3 months-6 months	0.000	0.000	0.000	0.056
6 months-9 months	0.466	0.722	0.000	0.000
9 months-1 year	0.765	0.000	0.000	0.000

ALP: alkaline phosphatase.

angular deformity of the lower limb. Clinical features started resolving by 1 month. The earliest sign of clinical improvement was increase in physical activity of the child as reported by the parents.

Biochemical findings are presented in Table 2. The mean serum calcium at initial presentation was 8.8 ± 0.6 mg/dL with 87% having hypocalcemia. Similarly, 67% cases presented with hypophosphatemia with a mean of 3.5 ± 0.8 mg/dL. High ALP levels were noted in 91% cases at initial presentation with a mean of 1315 ± 914 IU/L. By 6 months of treatment, the majority of the cases had achieved normal serum calcium (91%), phosphate (100%), and ALP (87%) levels. On the other hand, 98% had hypovitaminosis at presentation with a mean serum level of 13 ± 8 ng/mL. Normal vitamin D levels were restored in the majority (95%) of the cases by 6 weeks and in all the cases by 3 months. The change of mean serum calcium level from the previous visit was found to be statistically significant at every visit in the first 6 months of treatment (Table 3). Apart from the change of mean serum phosphate between the 6th and 9th months of treatment and the change in mean serum vitamin D level between the 3th and 6th months of the follow-up, all the changes in mean serum phosphate and vitamin D levels from the level at the previous visit were found to be statistically significant. Since the normal range of ALP is an age- and sex-dependent variable, data in Table 2 pertaining to ALP is adjusted for age and sex of the cases and classified as hyper, normal and hypo as per Turan et al (6) Vitamin D levels were defined as deficiency, insufficiency, sufficiency, excess, and toxicity as per the United States Endocrine Society classification (5,7).

The mean radiological score at initial presentation was 6.9 ± 3.1 (range, 1-10) with 45% (86 cases) having a score of 10. All the cases showed a healing line for rickets by 6 weeks. Around 47% (90 cases) had complete radiological resolution by 3 months. By 6 months, all the study subjects had complete radiological resolution. Serial abdominal ultrasonography did not reveal nephrocalcinosis in any of the cases.

Discussion

The word "stosstherapy" has been derived from the German word "stossen" meaning "to push". It involves the use of large doses of vitamin D to treat nutritional rickets. This treatment approach is based on the fact that vitamin D is efficiently stored in adipose tissue and muscles after a single large dose, following which continued conversion to the active metabolite 1,25-dihydroxy vitamin D helps to heal rickets. The main advantages of intramuscular stosstherapy over daily or weekly regimes or oral stosstherapy are compliance and cost. Only a single injection is to be administered which ensures good compliance. Each vial of 'Arachitol 6L' costs around 0.37 USD (United States Dollar), while the cost of other regimes is more than 10 times the cost of intramuscular stosstherapy.

Children with nutritional rickets were noted to have low sunlight exposure (90%), prolonged breast feeding (93.7%), and low consumption of cow milk (96.3%). There is very little research available to determine exactly how much sun exposure is necessary to maintain adequate vitamin D levels. Due to variations in age, skin color, latitude, time of day, and time of year, it is impractical to provide prescriptive advice to the population as a whole (8). Based on available research, it has been estimated that exposure of 40% of the body for around 30 minutes/week will result in generation of 1000 IU of vitamin D per day (9). Human milk has a low vitamin D (20 IU/liter) content (10,11). Assuming an average consumption of 750 mL/day, exclusive breast feeding provides only 11-38 IU/day of vitamin D, which is well below the recommended dietary allowance for an infant (12). Hence, as the vitamin D reserves are depleted (which takes around 6 months provided the mother was not vitamin D-deficient during pregnancy or postpartum), the infant starts developing signs of deficiency unless vitamin D is supplemented. In our study, we found a negative correlation between vitamin D at presentation and the duration of breast-feeding and a positive correlation between sunlight exposure and initial

calcidiol level implying that prolonged breast-feeding (>6 months) and low sunlight exposure (<30 minutes/week) lead to a decrease in vitamin D levels thus increasing the chances of rickets. Hence, we recommend exclusive breast feeding up to 6 months and sunlight exposure of at least 30 mins/week.

Swollen wrist and ankle were the most common clinical features (95%). They started improving by 6 weeks and resolved completely by 1 year (Figure 1). Angular deformity of the lower limb was the second most common presenting feature. In the majority of the cases, the varus deformity resolved by 1 year, while the remaining in the remaining minority, this finding neither improved nor deteriorated. It was noted that most of those who improved had mild deformities. Genu valgum was observed in children older than 4 years. Stosstherapy reduced the quantum of angulation in both genu valgum (Figure 2) and windswept deformity but failed to achieve complete resolution. These observations showed that the milder the deformity and the younger the child at initiation of treatment, the more the chances of resolution. The possible explanation for this observation is that with proper metabolic control, the lower limb bones resume a normal growth pattern. The initial varus deformity present in young children over time gradually changed to neutral or slight valgus (Figure 3). Other clinical signs such as frontal bossing (52%), pot belly (38%), rachitic rosary (11%), and Harrison sulcus (5%) all started improving by

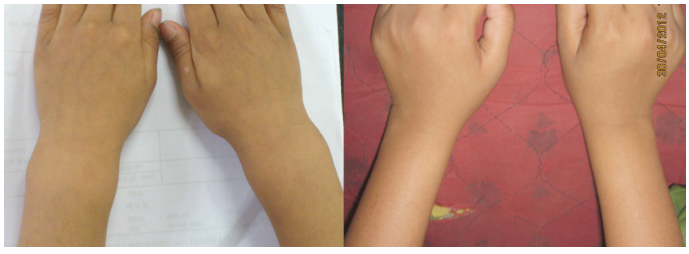

Figure 1. Resolution of swollen wrist at 1-year follow-up

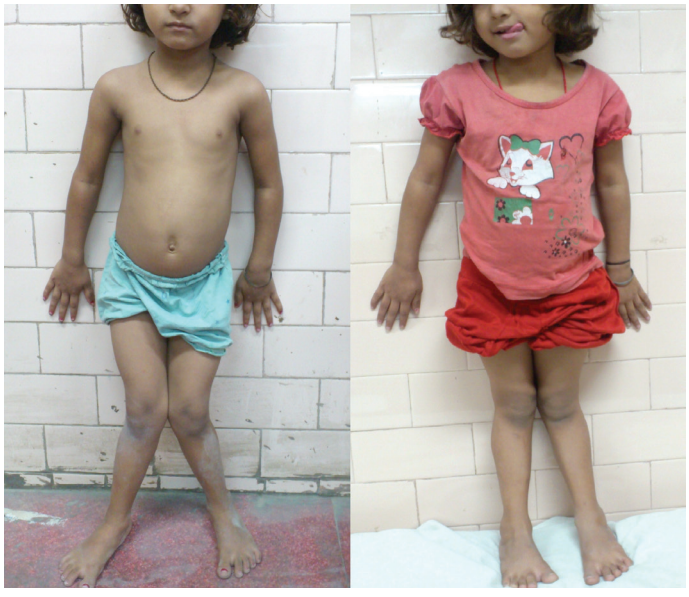

Figure 2. Reduction in the genu valgum deformity in a 9-year-old child at 1-year follow-up

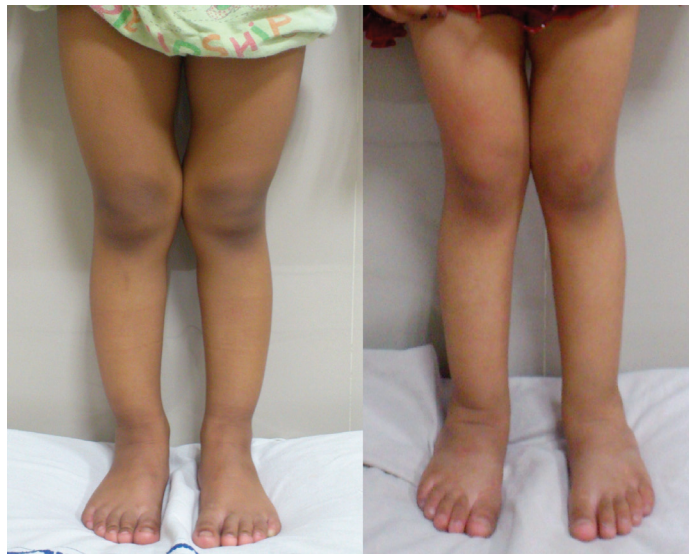

Figure 3. Resolution of varus angular deformity in the lower limb at 1-year follow-up

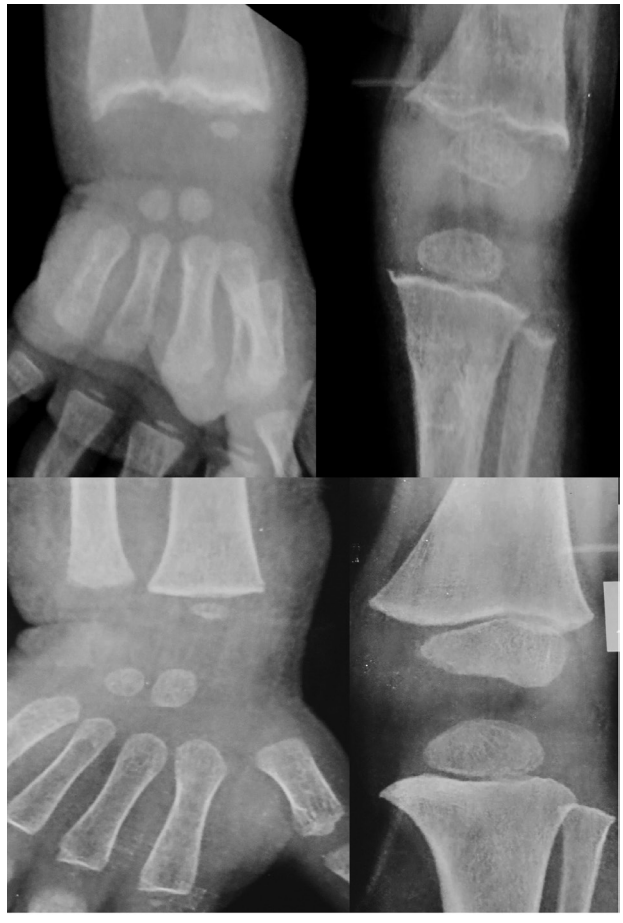

Figure 4. Radiogram showing healing at 6-month follow-up

the 6th week of treatment and achieved complete resolution by 9 months. Enamel hypoplasia (26%), repeated fractures (7%), and short stature (9%) were a few other less commonly observed features which also improved during follow-up. Thus, it can be concluded that stosstherapy led to clinical improvement in all the cases with complete resolution of most of the features over a period of 1 year. Our results are supported by studies conducted at Greater New Haven by DeLucia et al (13) on 43 cases and in Western Nigeria (14) on 26 cases with nutritional rickets.

Ozkan (15) has aptly enumerated the radiological changes noted during rickets. The earliest radiological findings are observed at the distal ulnar region in infants and the knee in older children. Expansion of the metaphysis (splaying), irregularity of the metaphyseal margin (fraying) giving it a brush-like appearance, cupping, and general osteopenia are the typical radiological findings in cases of nutritional rickets. In our study also, we came across these characteristic radiological changes. Cupping, splaying, and fraying noted in the initial radiographs were found to disappear gradually with treatment. Complete radiological resolution was achieved in all the cases by 6 months (Figure 4).

By 6 months, the majority of the cases had also achieved normal serum calcium (91%), phosphate (100%), and ALP (87%) levels. On the other hand, normal vitamin D levels were restored in the majority of the cases (95%) by 6 weeks and in all cases by 3 months. It was noted that there is no requirement for frequent biochemical tests during follow-up visits after stosstherapy. Thus, biochemical tests at initial presentation followed by a vitamin D assay at 6 weeks and calcium, phosphate, and ALP assays at 6 months are sufficient to monitor the biochemical improvement with treatment. This will not only reduce the cost of therapy but also help improve patient compliance by decreasing the number of needle pricks.

Hypercalcemia (2%), hyperphosphatemia (1%), and hypervitaminosis (5%) were noted in only a few cases during follow-up, but none of the cases were symptomatic, further ensuring the safety of the regime. Cesur et al (16) reported resolution of nutritional rickets with mega doses of vitamin D. Asymptomatic hypercalcemia was detected in 6 of their patients.

In the first year of follow-up, the mean ALP level at each visit was found to differ significantly from the level at the previous visit, a finding which implies that the ALP assay, if repeated at every visit, can monitor the biochemical improvement during follow-up. Thus, if the physician wants to monitor the biochemical improvement regularly, only ALP assay at follow-up visits is recommended as it is a reliable yet cost-effective alternative to the costly vitamin D assay. However, repeat injection of high-dose vitamin D should not be given based on high ALP levels during the follow-up period since ALP takes a long time to be restored (6-9 months). Injection of mega-dose vitamin D should only be repeated if the healing line of rickets does not appear in the radiograph of the wrist by 6 weeks and the patient's diagnosis is confirmed to be nutritional rickets (17).

Our findings indicate that stosstherapy leads to clinical, biochemical, and radiological resolution in nutritional rickets. A similar conclusion was also reported by Shah and Finberg (18) and Cesur et al (16). Thus, it can be concluded that stosstherapy is a safe, cheap yet effective method of treating nutritional rickets. A single injection of mega-dose vitamin D is sufficient for treating nutritional rickets. Stosstherapy also requires less frequent monitoring. Biochemical tests at initial presentation followed by a vitamin D assay at 6 weeks and calcium, phosphate, and ALP assays at 6 months is recommended to monitor the biochemical improvement with treatment. This will further reduce the cost of therapy and improve compliance of the patient. However, if the physician wants to monitor biochemical improvement regularly, serial assays of ALP only are recommended, provided one abstains from repeating injections of mega dose of vitamin D based on high ALP levels.

Ethics

Ethics Committee Approval: Ethical clearance was obtained for the study from the hospital ethical clearance committee. Informed Consent: The study after written informed consent was obtained from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Daipayan Chatterjee, Mathad K. S. Swamy, Vikas Gupta, Design: Daipayan Chatterjee, Vikas Gupta, Data Collection or Processing: Daipayan Chatterjee, Vasu Sharma, Analysis or Interpretation: Daipayan Chatterjee, Krishti Chatterjee, Literature Search: Daipayan Chatterjee, Vasu Sharma, Akshat Sharma, Krishti Chatterjee, Writing: Daipayan Chatterjee.

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CASE REPORT

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Uniparental Isodisomy of Chromosome 1 Unmasking an Autosomal Recessive 3-Beta Hydroxysteroid Dehydrogenase Type II-Related Congenital Adrenal Hyperplasia

Karin Panzer[1], Osayame A. Ekhaguere[2], Benjamin Darbro[1], Jennifer Cook[3], Oleg A. Shchelochkov[1*]

[1]University of Iowa Hospitals and Clinics, Stead Department of Pediatrics, Iowa, USA
[2]The Children's Hospital of Philadelphia, Division of Neonatal and Perinatal Medicine, Philadelphia, USA
[3]Blank Children's Hospital, Department of Pediatric Endocrinology, Iowa, USA
**Current Institution: National Human Genome Research Institute, Bethesda, Maryland, USA*

What is already known on this topic?

3-beta hydroxysteroid dehydrogenase type II (3β-HSD2) deficiency is a rare form of congenital adrenal hyperplasia (CAH) that is inherited in an autosomal recessive manner, typically with one gene variant inherited from each parent. Uniparental isodisomy (UPD) as the genetic basis of CAH has been reported in other forms of CAH.

What this study adds?

The first reported case of 3β-HSD2 deficiency arising from UPD of chromosome 1.

Abstract

Steroid 3-beta hydroxysteroid dehydrogenase type II (3β-HSD2) deficiency is a rare autosomal recessive form of congenital adrenal hyperplasia (CAH). We report the genetic basis of 3β-HSD2 deficiency arising from uniparental isodisomy (UPD) of chromosome 1. We describe a term undervirilized male whose newborn screen indicated borderline CAH. The patient presented on the 7th day of life in salt-wasting adrenal crisis. Steroid hormone testing revealed a complex pattern suggestive of 3β-HSD deficiency. Chromosomal microarray and single nucleotide polymorphism analysis revealed complete UPD of chromosome 1. Sanger sequencing of *HSD3B2* revealed a previously described missense mutation, c.424G>A (p.E142K) in homozygous state, thus confirming the diagnosis of 3β-HSD2 deficiency. We provide evidence of the existence of an uncommon mechanism for *HSD3B2* gene-related CAH arising from UPD of chromosome 1.

Keywords: Steroid 3β-HSD2 deficiency, *HSD3B2* gene, uniparental isodisomy

Introduction

Deficiency of 3-beta hydroxysteroid dehydrogenase type II (3β-HSD2) is a rare autosomal recessive form of congenital adrenal hyperplasia (CAH). In humans, 3β-HSD2 is predominantly expressed in the adrenal glands and gonads (1) and its encoding gene, *HSD3B2* (MIM*613890), is located on chromosome 1 (2,3). The enzyme 3β-HSD2 oxidizes and isomerizes Δ^5-steroids, namely, pregnenolone, 17-hydroxy-pregnenolone (17-OH Preg) and dehydroepiandrosterone into corresponding Δ^4-ketosteroids (4). Deficiency of the enzyme impacts the steroid hormone pathway by disrupting the biosynthesis of mineralocorticoid, corticosteroid, and sex hormones. Typically, 3β-HSD2 enzyme deficiency results in cortisol deficiency, salt wasting, and male undervirilization. However, depending on the degree of enzyme deficiency and activity of the 3-beta hydroxysteroid dehydrogenase type I (3β-HSD1) enzyme, a similar enzyme produced in the skin and placenta, milder presentation may occur (5). This variability in clinical presentation should

Address for Correspondence: Osayame A. Ekhaguere MD,
The Children's Hospital of Philadelphia, Division of Neonatal and Perinatal Medicine, Philadelphia, USA
Phone: +1 319 855-9093 **E-mail:** ekhaguereo@email.chop.edu

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prompt clinicians to seek clarification of the diagnosis using molecular methods.

Increased utilization of single nucleotide polymorphism (SNP) array in clinical practice has led to the recognition of uniparental isodisomy (UPD) that either disrupts imprinting patterns or unmasks autosomal recessive alleles (6). Reports on UPD as an underlying molecular mechanism of CAH are scarce (7). There are reported cases of UPD involving the two most common forms of CAH, steroid 21-hydroxylase (8) and 11-β-hydroxylase deficiency (9). Here, we report the first patient affected by *HSD3B2*-related CAH uncovered by UPD of chromosome 1. In general, UPD of chromosome 1 has been infrequently reported in other disease conditions (10).

Case Report

Our patient is a 7-day-old male infant born at 37 weeks 5 days gestation. He was taken to his primary care physician with complaints of poor weight gain and vomiting and was referred to our hospital.

The infant was the product of an uncomplicated pregnancy, born to Caucasian parents. His birth length was 50.8 cm (50th percentile), birth weight 3400 grams (50th percentile), and head circumference 38 cm (75th percentile). Physical examination was significant for hypotonia, non-specific dysmorphic facial findings including slight frontal bossing, hypotelorism, low nasal bridge and anteverted nares. Also noted was perineal hypospadias, bifid scrotum, penile chordee, and descended testes bilaterally. As part of the work-up for his nonspecific dysmorphic facies and hypotonia, an oligonucleotide microarray and a SNP analysis was sent. Also, at newborn screening, the patient was found to have a 17-hydroxyprogesterone (17-OHP) level of 33.2 ng/mL, borderline for his birthweight (normal limits <30, borderline is 30-74, and presumptive positive is ≥75). Laboratory evaluation revealed hyponatremia with a serum sodium level of 131 mEq/L (reference 135-145 mEq/L) with concurrent hyperkalemia and a potassium level of 7.9 mEq/L (reference 3.5-4.5 mEq/L). He was admitted to the pediatric intensive care unit and started on hydrocortisone, fludrocortisone, and sodium chloride supplementation. Steroid hormone testing obtained before treatment revealed a complex pattern suggestive of 3β-HSD deficiency. Steroid hormone determinations revealed the following abnormal values: 17-OH Preg 119.0 nmol/L (reference 0.3-26.2 nmol/L), 17-OHP 16.9 nmol/L (reference 1.3-6.4 nmol/L), dehydroepiandrosterone 95.4 nmol/L (reference 1.7-26.4 nmol/L), progesterone 1.8 nmol/L (reference <0.3-0.5 nmol/L), cortisol 1462.3 nmol/L (reference 77.3-303.5 nmol/L), 11-deoxycortisol 9.9 nmol/L (reference ≤5.9 nmol/L), 11-desoxycorticosterone 0.2 nmol/L (reference 1.0-2.7 nmol/L), and androstenedione 9.7 nmol/L (reference <1.8 nmol/L). Other laboratory results included: adrenocorticotropic hormone 8.4 pmol/L (reference 1.3-10.6 pmol/L) and testosterone 1.6 nmol/L (reference 0.7-1.7 nmol/L). Additional studies included chromosomal analysis, chromosomal microarray, and fluorescent *in situ* hybridization analysis (FISH) of sex-determining region Y (SRY).

The patient recovered from his acute illness, was discharged home on steroids and electrolyte replacements. He underwent urologic surgery to correct his urogenital anomalies.

Genetic Analysis

Chromosomal karyotype analysis was performed per standard technique. FISH studies were completed with Vysis SRY probe using standard technique. Chromosomal oligonucleotide microarray and SNP analysis was done using an Affymetrix CytoScanHD hg19 (NCBI build 37) whole genome array consisting of 1.9 million non-polymorphic markers and 750,000 SNP probes, with an average probe spacing of about 1.2 kb. Data were extracted and processed using Affymetrix ChAS software (Affymetrix, version 1.2.2) and Nexus Copy Number (BioDiscovery, version 7) software. Chromosomal analysis revealed normal male complement, 46,XY. SRY was present by FISH study. Chromosomal oligonucleotide microarray and SNP analysis revealed complete UPD of chromosome 1 (Figure 1), a 264 kb deletion of 11q14.1, and a 517 kb duplication of 17p13.2. The microdeletion of 11q14.1 and microduplication of 17p13.2 did not involve genes associated with known human disorders. Since UPD can clinically unmask mutations implicated in autosomal recessive disorders, gene content of chromosome 1 was reviewed for candidates associated with autosomal recessive syndromic ambiguous genitalia.

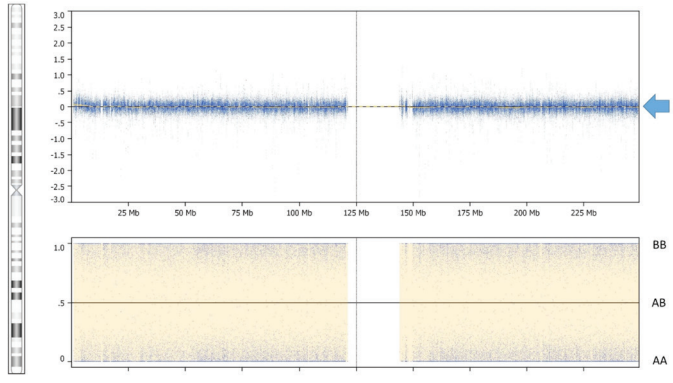

Figure 1. Uniparental isodisomy of chromosome 1. The figure demonstrates a normal copy number of chromosome 1 represented by probes in the top panel (blue arrow) averaging $\log_2 R = 0$ in the absence of AB alleles shown in the bottom panel

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Several genes met this criterion, including *HSD3B2*. Sanger sequencing of *HSD3B2* revealed a previously described missense mutation, c.424G > A (p.E142K) in a homozygous state, thus confirming the diagnosis of 3β-HSD2. Parental samples were not available for our review to establish the origin of mutation and the parental origin of UPD.

Discussion

This is the first report to demonstrate the role of UPD on chromosome 1 as the molecular basis for the rare *HSD3B2*-related CAH, unmasking a known homozygous missense mutation, c.424G > A (p.E142K). Mutation c.424G > A (p.E142K) was uncovered as an incidental finding during a work-up for non-endocrine indication of dysmorphic appearance and hypotonia. This mutation has previously been implicated in the development of classic and non-classic cases of *HSD3B2*-related CAH. Simard et al (11) reported an undervirilized male with salt-wasting CAH caused by compound heterozygosity of missense mutation p.E142K and a nonsense mutation p.W171X in HSD3B2. Pang et al (12) reported a female patient with non-classic presentation of 3β-HSD deficiency causing premature sexual hair growth and mild growth acceleration in childhood. Molecular analysis revealed compound heterozygous c.1022C > T (p.Pro341Leu) and c.424G > A (p.E142K) mutations in *HSD3B2*.

Steroid hormone testing in our patient revealed a complex pattern of abnormalities. In addition to an elevated 17-OH Preg (a 4.5-fold increase above the upper normal range) and dehydroepiandrosterone (a 3.6-fold increase above the upper normal range), the patient's profile revealed elevations of Δ^4-ketosteroids and their products (progesterone, 17-OHP, androstenedione, and cortisol). ACTH and testosterone were in the normal range. Elevations of Δ^4-ketosteroids and their downstream metabolites in patients with 3β-HSD2 deficiency have been reported in the literature (1). Increased levels of 17-OHP, androstenedione, and testosterone are thought to occur due to preserved activity of 3β-HSD1 encoded by a paralogue gene *HSD3B1* expressed postnatally in skin and placenta (13). Therefore, it has been suggested that an elevated ratio of Δ^5/Δ^4 -steroids could be a more informative biomarker to ascertain 3β-HSD2 deficiency (1). Genetic counseling for *HSD3B2*-related CAH involves a discussion of autosomal recessive inheritance and a 25% chance for parents to have another child with CAH. However, the recurrence risk for CAH arising from UPD of a complete chromosome is considered much smaller owing to the mechanism underlying isodisomy formation. UPD in humans, as first described by Engel (14) in 1980, is the presence of a chromosome pair or portions of a chromosome pair (15) that originate from a single parent, thus designated maternal or paternal. Mechanistic explanations of UPD involve gamete complementation, trisomic rescue, monosomic rescue, or post-fertilization mitotic error (16,17). The first two explanations would result in uniparental heterodisomy, while monosomic rescue and mitotic error and rescue may result in UPD. In all these cases, the original abnormality is thought to be a sporadic event without a significantly increased risk of recurrence, and parental chromosomal analysis would reveal normal results. Rarer mechanisms to generate complete UPD include correction of interchange trisomy or monosomy (in connection with a Robertsonian or reciprocal translocation), isochromosome formation, and correction of imbalance due to extra structurally abnormal chromosomes. In these rarer mechanisms, the parental karyotype should reveal a predisposing balanced or unbalanced chromosomal complement, and as such, may have an increased risk for recurrence. As parental samples were not unavailable, we could not establish the parental origin of UPD in this patient. However, the complete loss of heterozygosity of chromosome 1 in this patient suggests that monosomic rescue or mitotic error were the two most likely mechanisms of the observed UPD. In practice, since both of these mechanisms require the sequential occurrence of two abnormal events, UPD is usually a sporadic event with low risk of recurrence (18).

Follow-up and management of patients with *HSD3B2*-related CAH have been well established (19). However, in formulating the management of autosomal recessive disorders unmasked by UPD, one needs to consider the possibility of other clinically relevant autosomal recessive alleles that could be revealed through the UPD-mediated loss of heterozygosity (20). Additional copy number variants identified by microarray may cause additive effects or modify the clinical presentation for this patient over time. We speculate this mechanism may have contributed to the non-specific dysmorphic features and hypotonia in this patient. Finally, to date, most phenotypes associated with UPD of chromosome 1 have been linked to the autosomal recessive disorders without evidence for the existence of a possible imprinting disorder.

In conclusion, with this case report, we provide evidence for the existence of an uncommon mechanism of *HSD3B2*-related CAH arising from UPD of chromosome 1 that required the use of SNP-based array in the molecular evaluation of the patient, both from a diagnostic standpoint and recurrence risk assessment.

Ethics

Informed Consent: Consent to publish de-identified medical information has been provided by the patient's legal guardian.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Karin Panzer, Osayame A. Ekhaguere, Oleg A. Shchelochkov, Design: Oleg A. Shchelochkov, Data Collection or Processing: Karin Panzer, Osayame A. Ekhaguere, Jennifer Cook, Analysis or Interpretation: Benjamin Darbro, Oleg A. Shchelochkov, Literature Search: Karin Panzer, Osayame A. Ekhaguere, Oleg A. Shchelochkov, Writing: Karin Panzer, Osayame A. Ekhaguere, Oleg A. Shchelochkov.

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CASE REPORT

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Follow-up Findings in a Turkish Girl with Pseudohypoparathyroidism Type Ia Caused by a Novel Heterozygous Mutation in the *GNAS* Gene

Sezgin Şahin[1], Olaf Hiort[2], Susanne Thiele[2], Olcay Evliyaoğlu[3], Beyhan Tüysüz[4]

[1]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatric Rheumatology, İstanbul, Turkey
[2]University of Lübeck, Department of Pediatrics, Lübeck, Germany
[3]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
[4]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatric Genetics, İstanbul, Turkey

What is already known on this topic?

Pseudohypoparathyroidism type Ia (PHP-Ia) is characterized by multihormone resistance and Albright hereditary osteodystrophy. Heterozygous mutations in *GNAS* gene may result with decreased Gsα activity.

What this study adds?

Longitudinal follow-up of a PHP-Ia patient seems to be lacking in the literature. In our case, heterotopic ossification, subclinical hypothyroidism, and cerebral calcification developed late during the 8-year follow-up period. To our knowledge, this is the first report about bioinactive growth hormone associated with PHP-Ia. *GNAS* gene analysis revealed a novel mutation.

Abstract

Pseudohypoparathyroidism type Ia (PHP-Ia) is characterized by multihormone resistance and an Albright hereditary osteodystrophy (AHO) phenotype. It is caused by heterozygous mutations in *GNAS* gene. Clinical and biochemical findings of a female PHP-Ia patient were evaluated from age of diagnosis (6.5 years) to 14.5 years of age. The patient had short stature, brachydactyly, and subcutaneous heterotopic ossifications. Serum calcium and phosphorus levels were normal, but parathyroid hormone levels were high. Based on the typical clinical findings of AHO phenotype and biochemical findings, she was diagnosed as having PHP-Ia. A novel heterozygous mutation (c.128T > C) was found in the *GNAS* gene. Follow-up examinations revealed resistance to thyroid-stimulating hormone and a bioinactive growth hormone. Clinicians should take into consideration PHP-Ia in patients referred with short stature, and patients with an AHO phenotype must be further evaluated for hormone resistance, *GNAS* gene mutation, Gsα activity. To our knowledge, this is the first case report describing bioinactive growth hormone in PHP-Ia.

Keywords: Pseudohypoparathyroidism Ia, Albright hereditary osteodystrophy, ectopic ossification, *GNAS* gene, Gsα activity, short stature

Introduction

Pseudohypoparathyroidism (PHP) is defined as an end-organ resistance to parathormone (PTH) and is characterized by hypocalcemia, hyperphosphatemia, and increased PTH levels (1).

PHP-Ia is a subtype of PHP, caused by heterozygous inactivating mutations in *GNAS* which encodes Gsα. This gene is located on chromosome 20q13.11 and contains 13 exons and 12 introns. Gsα is essential for the actions of PTH and of many other hormones (2,3). PHP-Ia patients express resistance to hormones that act via Gs-coupled receptors, such as PTH, thyroid-stimulating hormone (TSH), gonadotropins, growth hormone-releasing hormone (GHRH). These patients, also known as cases of Albright hereditary osteodystrophy (AHO), show a constellation of features including short stature, short neck, round face,

Address for Correspondence: Beyhan Tüysüz MD,
İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatric Genetics, İstanbul, Turkey
Phone: +90 212 4143192 **E-mail:** beyhan@istanbul.edu.tr

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centripedal obesity, brachydactyly, ectopic ossifications, and mild mental retardation.

Low serum calcium, elevated serum phosphorus and high PTH are frequent findings in laboratory analyses. In some cases, calcium and phosphorus are within the normal range and only PTH is elevated (2,4).

The differential diagnosis among subtypes of PHP is very difficult. Table 1 shows subtypes of PHP with the classification based on the level of serum calcium, phosphorus, PTH, urinary cyclic AMP (cAMP), phosphaturia response to PTH, Gsα activity, and presence of AHO phenotype. PHP-I can be differentiated by the presence (PHP-Ia and PHP-Ic) or absence (PHP-Ib) of AHO. The only way to distinguish PHP-Ia from the much rarer PHP-Ic is to measure Gsα activity because of the identical clinical and biochemical features.

In this case report, we present the follow-up results of a longitudinal observation of clinical and biochemical profiles of a girl with typical phenotype of AHO and a novel mutation in *GNAS* gene.

Case Report

This 6.5-year-old girl patient was referred to our outpatient clinic for short stature and brachydactyly. She was born at term to non-consanguineous parents. Neither brachydactyly, short stature, nor any specific feature of AHO phenotype were present in either parent. The patient's birth weight was 2300 g (< 10 p) and her birth height was 45 cm (< 10 p). Developmental dysplasia of the hip was noted on neonatal examination. Her motor development was also delayed. She was reported to first smile to her mother at 3 months, to have acquired head control at 9 months, sitting at 24 months, walking at age 3 years. Physical examination revealed short stature [height: 109 cm, -1.75 standard deviation (SD)], a low body mass index (BMI = 13.5 kg/m^2, -1.59 SD), round face, full cheeks, depressed nasal bridge, short neck, brachydactyly of all digits of the hand (hand length: 9.5 cm, < 3th centile) and feet (Figure 1 A-1C and Table 2). Subcutaneous heterotopic ossification at the level of the right iliac crest (diameter 3x2 cm) was also noted.

The first laboratory examination revealed normal serum calcium (9.7 mg/dL) and phosphorus (5.3 mg/dL) levels, with an elevated serum PTH (138.1 pg/mL) level. The subsequent measurements of PTH concentrations were 98.5 pg/mL and 111.2 mg/mL, respectively. The serum levels of free triiodothyronine (fT_3), free thyroxine (fT_4), TSH, alkaline phosphatase (ALP), creatinine, and 25-hydroxyvitamin D (25-OHD) were normal. X-rays revealed marked shortness and thickness of metacarpal and metatarsal bones with cone-shaped epiphyses in all tubular bones of the hand (Figure 1E, 1F) and coxa valga with acetabular dysplasia. The patient's bone age was compatible with her chronological age. Cranial magnetic resonance imaging findings were normal. Chromosomal analysis revealed 46,XX karyotype. Audiological and ophthalmological examination showed bilateral minimal conductive hearing loss and retinitis pigmentosa, respectively.

Table 1. Clinical findings of our patient and differential diagnosis in cases of pseudohypoparathyroidism

	PHP-Ia	PHP-Ib	PHP-Ic	PHP-II	PPHP*	Present patient
AHO**	**Yes**	**No**	**Yes**	**No**	**Yes**	**Yes**
Hormone resistance	PTH, TSH, Gn***, GHRH****	PTH, sometimes TSH	PTH, TSH, Gn, GHRH	PTH	None	
Ca P↓	Mostly yes	Mostly yes	Mostly yes	Mostly yes	No	No
Serum PTH	Elevated	Elevated	Elevated	Elevated	Normal	Elevated
PTH infusion	Urine cAMP Phosphaturia	Urine cAMP Phosphaturia	Urine cAMP Phosphaturia	Urine cAMP Phosphaturia	Urine cAMP Phosphaturia	Not performed
GNAS gene defect	Inactivating mutations	Imprinting Dysregulation	No	No	Inactivating mutations	Yes
Gsα activity	Reduced	Normal	Normal	Normal	Reduced	Reduced

*PPHP: pseudopseudohypoparathyroidism,

**AHO: Albright hereditary osteodystrophy,

***Gn: gonadotropins,

****GHRH: growth hormone-releasing hormone.

PHP: Pseudohypoparathyroidism, PTH: parathormone, TSH: thyroid-stimulating hormone, Ca: calcium, P: phosphor

Gsα function was found as 48.1% of normal, suggesting impaired activity. Based on these clinical and biochemical findings, the most likely diagnosis was thought to be PHP-Ia. A heterozygous novel missense mutation (c.128T > C) was detected in exon 1 in the *GNAS* gene.

The patient was followed up until 14.5 years of age. At age 9 years, her height was 122 cm (-1.86 SD) and her BMI was 14.8 kg/m^2 (-0.86 SD) (Table 2). Borderline intellectual disability (IQ: 83) was detected in Stanford-Binet test at age 9 years. Pubertal development was Tanner stage II and appropriate to her age. Hormonal profile showed subclinical hypothyroidism (fT_3: 3.51 pg/mL, fT_4: 1.27 ng/dL, TSH: 5.97 mIU/L). [The diagnosis of subclinical hypothyroidism was based on a serum TSH value of > 4.2 μU/mL (reference interval = 0.27-4.2 μU/mL), while serum fT_3 (reference interval = 2-4.4 pg/mL) and fT_4 (reference interval = 0.93-1.7 ng/dL) levels were within the reference ranges].

Antithyroid antibodies were within normal limits and thyroid ultrasound was normal. Thus, increased TSH (6.02 mIU/L) with normal fT_3 and fT_4 levels in subsequent measurements suggested TSH resistance. Elevated PTH levels (138.1 pg/mL) were still evident, while serum calcium, phosphorous, and ALP concentrations were normal. Nephrocalcinosis was not detected in ultrasonography. Cranial computerized tomography (CT) scan did not reveal any basal ganglion calcification. L-T4 supplementation was initiated at a dose of 1.25 μg/kg/day.

The most remarkable findings of the physical examination at age 14.5 years were the increase in number of mobile subcutaneous heterotopic ossifications and bilateral calcification of the globus pallidus in cranial CT scan (Figure 1D). In addition to the initial lesion at the right iliac crest level, there were three new subcutaneous ossifications in the both hands and in the left foot. Her height was 143 cm (-2.80 SD) and her weight was 35 kg (-2.54 SD) (Table 2). While pubertal development was at Tanner stage IV, menarche had not occurred yet. During the 8 years of longitudinal follow-up, her pubertal development had been normal, indicating that there was no gonadotropin resistance. The patient's bone age was 13 years. Her height standard deviation score (SDS) regressed to -2.80 with a growth velocity of 5 cm/year. Growth hormone (GH) stimulation tests were performed. They revealed sufficient GH secretion (after clonidine stimulation GH peak: 14 ng/mL). Insulin-like growth factor 1 (IGF-1) stimulation test was performed to see if there was a response of IGF-1 increment to exogenous GH. This test revealed an increased response [baseline IGF-1 level: 262.1 ng/mL (-2.086 SD), peak IGF1 level: 393.5 ng/mL, ΔIGF1: 131.4 ng/mL, 50.1% increase] suggesting GH bioinactivity. [The IGF-1 generation test was performed as follows: Exogenous GH injections (100 μg/kg s.c. daily) were administered at 21:00 h for four days. Blood samples for IGF-1 were taken on day 0 before the first injection and 12 hours later after the last injection (a serum IGF-1 level increment greater than 20% was defined as GH bioinactivity). Baseline serum IGF-1 standard deviation calculation was performed (5)].

Molecular Methodology

The activity of Gsα protein from erythrocyte membranes of patients was investigated in heparinized blood samples.

Table 2. Results of anthropometry and hormone measurements during the follow-up of the patient

	Birth	Age 6.5 years	Age 9 years	Age 14.5 years
Weight, kg SDS	2.3	16 (-2.29)	22 (-1.75)	35 (-2.54)
Height, cm SDS	45	109 (-1.75)	122 (-1.86)	143 (-2.80)
BMI, kg/m^2 (z-score)	NA	13.5 (-1.59)	14.8 (-0.86)	17.1 (-1.03)
PTH, pg/mL	NA	138.1	138	101.3
TSH, mIU/L	NA	2.53	6.02	5.72
GH bioinactivity	NA	NA	NA	Present
Subcutaneous ossification level	ND	CI	CI	CI, RH, LH, LF
Intracranial calcification	NA	ND	ND	Globus pallidus

NA: not available, ND: not detected, CI: Crista iliaca, RH: Right hand, LH: left hand, LF: Left foot, SDS: standard deviation score, BMI: body mass index, PTH: parathormone, TSH: thyroid-stimulating hormone, GH: growth hormone

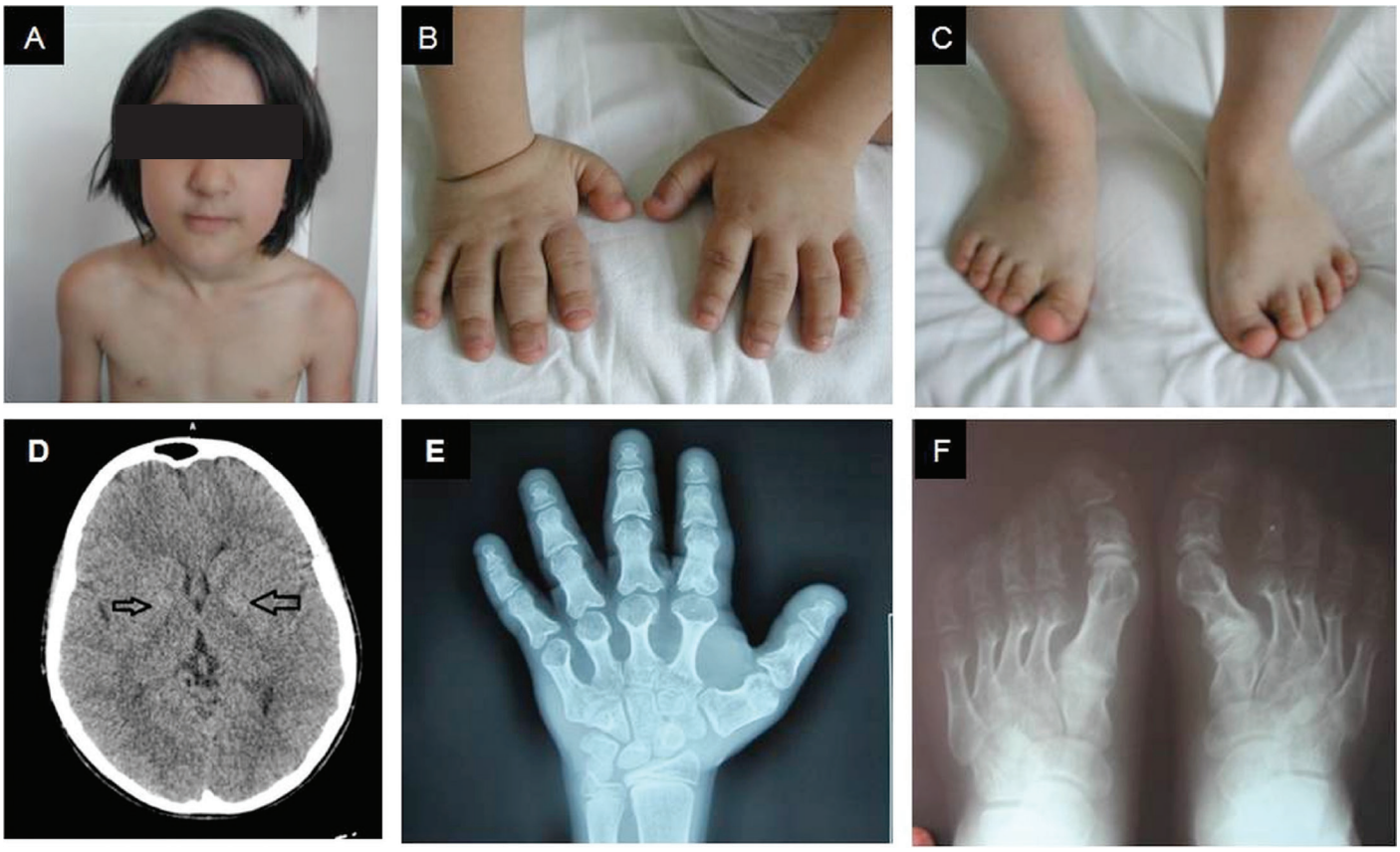

Figure 1. The photograph of the patient at age 7 years. Note round face, full cheeks, and short neck (A). Short hands and feet (B, C). Cranial computerized tomography showing bilateral calcification of globus pallidus (D). Roentgenogram of hands and feet. Marked shortness of metacarpals and metatarsals (especially 4th and 5th). (E, F) Cone-shaped epiphyses are visible in all tubular bones of hand

After solubilization, the Gsα protein from patient-derived erythrocyte membranes was incubated with GTPγS. Adenylyl cyclase from turkey red cell membranes were added, and the generated cAMP in the presence of ATP by RIA (Immuno Biological Laboratories, Hamburg, Germany) was measured. Results obtained in triplicate were expressed as percent of the mean of healthy controls (normal range: 85-115%).

For molecular genetic analysis, genomic DNA derived from peripheral leukocytes was isolated by standard procedures (Qiaquick DNA kit, Qiagen, Hilden, Germany). *GNAS* exon 1-13, (RefSeq NM_000516.4) including all intron/exon boundaries were amplified in 11 fragments by polymerase chain reaction (PCR) (primer sequences available upon request). PCR-amplified DNA was sequenced by direct cycle sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA).

Discussion

The hand and feet X-rays of this patient (clinical findings: short stature, short neck, round face, brachydactyly, and borderline intellectual disability) revealed marked shortness of metacarpals and metatarsals. The patient was also found to have high PTH levels, subclinical hypothyroidism, and heterotopic ossifications. While patients with AHO are usually obese, the BMI level of our patient was in the underweight group according to the World Health Organization guidelines. With these findings, the patient was diagnosed as a case of Albright PHP. Heterotopic ossifications and brachydactyly are the most unique features of AHO phenotype that distinguishes true AHO from a variety of clinical phenocopies (6,7). While brachydactyly was evident in the first evaluation of our patient, heterotopic ossification became apparent at the right iliac crest at 9 years of age. Moreover, the subcutaneous ossifications were observed to have increased in number at different parts of the body in the last follow-up examination.

Almost all features of PHP-Ia including hormone resistance, are also common in acrodysostosis syndrome. PHP-Ia can be differentiated from this syndrome only by the presence of GNAS mutation and of heterotopic ossifications (8). The differential diagnosis among subtypes of PHP is very difficult. In our patient, PHP-Ib and PHP-Ic were excluded

by decreased Gsα activity and PHP-II by both decreased Gsα activity and mutation. Gsα activity of our case was reduced to 48.1%, a finding that was consistent with PHP-Ia rather than PHP-Ib, -Ic, and -II.

The *GNAS* gene analysis revealed a novel heterozygous mutation (c.128T>C). This mutation results in the change of the aminoacid leucine at codon 43 with the aminoacid proline (p. Leu43Pro). There is a moderate physicochemical difference between Leu and Pro [Grantham dist.: 98 (0-215)] and the amino acid leucine at codon 43 is conserved between species up to C. elegans. It is possible that the mutation is pathogenic and causal for AHO in this patient. This mutation, as far as we know, has never been described before and was not detected in the *GNAS* gene. Thus, we have concluded that the mutation is meaningful.

Formerly, the term pseudopseudohypoparathyroidism (PPHP), was used for patients who display AHO features and carry heterozygous inactivating Gsα mutations without evidence of hormone resistance. Previous studies were reporting maternal inheritance of GNAS mutations results in AHO together with hormone resistance and named as PHP-Ia, while paternal inheritance of the same mutation was reported to lead only to AHO phenotype and was termed as PPHP (2,3). However, in a recent publication, mild PTH resistance besides AHO phenotype was reported in a PPHP patient, and ascertainment of the parental origin of the mutation was declared as the most effective diagnostic procedure in differentiating PPHP from PHP-Ia (9).

While Gsα is biallelically expressed in most tissues, it is predominantly maternally expressed in certain tissues such as renal proximal tubules, the thyroid, the gonads, and the pituitary. Paternal allele is suppressed in these tissues. Tissue-specific imprinting nature of *GNAS* gene is responsible for this difference (1,10). This might explain why the multi-hormone resistance in PHP-Ia patients primarily involves four hormones: PTH, TSH, gonadotropins, and GHRH (1,11).

While PTH resistance associated later with TSH resistance was apparent in our patient, resistance to gonadotropins or GHRH was not detected. The most important hormone resistance in PHP-Ia that results in clinically evident signs is renal resistance to PTH. Most patients present with hypocalcemia, hyperphosphatemia, and elevated levels of PTH. Despite presence of PTH resistance, some cases may have normal serum calcium and phosphorus levels. PHP-Ia patients have a reduced phosphatidic response to PTH, which leads to hyperphosphatemia. Besides hyperphosphatemia, proximal tubule resistance to PTH leads to decreased 1,25-dihydroxyvitamin D production, thus causing hypocalcemia. Unlike patients with primary hypoparathyroidism, PHP cases do not develop hypercalciuria, which shows that the anti-calciuric effect of PTH in the thick ascending limb remains intact (3). Some patients may show osteopenia and signs of rickets. Skeletal deformities like short ulna, genu varum-valgum, cubitus valgus may be seen (12).

TSH resistance becomes clinically apparent during the adolescence period (1). This resistance is mostly not severe, with TSH levels only slightly elevated or thyroid hormone levels slightly less than normal (11). Our patient developed subclinical hypothyroidism at 9 years of age.

Although short stature became apparent in the follow-up of our patient, she showed a normal GH response to clonidine stimulation test. Clonidine is a selective α-receptor agonist and causes GH release via GHRH. This normal response suggests that our patient did not have GHRH resistance. GH bioinactivity was diagnosed by detecting a 50.1% increment to IGF1 stimulation test. This diagnosis predicates that endogenously produced GH is inactive, probably due to disorders of GH gene. To our knowledge, this is the first report about the bioinactive GH associated with PHP-Ia. However, without GH gene analysis, this diagnosis is not precise. Reports on GH deficiency in patients with PHP-Ia are variable. While some authors reported GH deficiency in PHP-Ia (11,13,14,15), there are a few studies reporting patients without GH deficiency (16,17) as noted in the case of our patient.

However, our inability to perform a PTH infusion test and a *GNAS* gene analysis in the parents represent the main limitations of this case report, limitations which made the differential diagnosis more complicated in terms of PHP-Ia and PPHP. Also, we had no possibility to analyze neither the DNA samples of the parents nor the ribonucleic acid sample of the patient to be able to show the origin of the mutation, in other words, whether it was paternally or maternally expressed. Accordingly, the patient could also be classified as PPHP in the light of current literature and case reports.

In conclusion, clinicians should take into consideration PHP-Ia and PPHP in patients referred with short stature, and the subjects with AHO phenotype must be further evaluated for hormone resistance, *GNAS* gene mutation, Gsα activity. Repeated physical and laboratory examinations should be performed in order to detect changes which may occur in hormone resistance.

Ethics

Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Beyhan Tüysüz, Design: Beyhan Tüysüz, Data Collection or Processing: Beyhan Tüysüz, Analysis or Interpretation: Beyhan Tüysüz, Olcay Evliyaoğlu, Sezgin Şahin, Olaf Hiort, Susanne Thiele, Literature Search: Beyhan Tüysüz, Olcay Evliyaoğlu, Sezgin Şahin, Writing: Beyhan Tüysüz, Olcay Evliyaoğlu, Sezgin Şahin.

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CASE REPORT

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Clinical and Molecular Genetic Analysis in Three Children with Wolfram Syndrome: A Novel *WFS1* Mutation (c.2534T>A)

Gamze Çelmeli[1], Doğa Türkkahraman[2], Yusuf Çürek[1], Jayne Houghton[3], Sema Akçurin[1], İffet Bircan[1]

[1]Akdeniz University Faculty of Medicine, Department of Pediatric Endocrinology, Antalya, Turkey
[2]Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Turkey
[3]University of Exeter Medical School, Institute of Biomedical and Clinical Science, Exeter, United Kingdom

What is already known on this topic?

Wolfram syndrome is a rare, autosomal recessive disorder with heterogeneous clinical features including diabetes insipidus, diabetes mellitus, optic atrophy, deafness, and other manifestations. Over two hundred mutations have been reported in *WFS1* gene.

What this study adds?

This article presents clinical characteristics and mutation analysis results of 3 cases with Wolfram syndrome. Since Wolfram syndrome is a rare syndrome, the present article will expand the mutation database and help to understand the disease phenotype.

Abstract

Wolfram syndrome (WS) is an autosomal recessive disorder caused by mutations in *WFS1* gene. The clinical features include diabetes insipidus, diabetes mellitus (DM), optic atrophy, deafness, and other variable clinical manifestations. In this paper, we present the clinical and genetic characteristics of 3 WS patients from 3 unrelated Turkish families. Clinical characteristics of the patients and the age of onset of symptoms were quite different in each pedigree. The first two cases developed all symptoms of the disease in their first decade of life. The heterozygous father of case 2 was symptomatic with bilateral deafness. The first ocular finding of one patient (patient 3) was bilateral cataract which was accompanying DM as a first feature of the syndrome. In this patient's family, there were two members with features suggestive of WS. Previously known homozygous mutations, c.460+1G>A in intron 4 and c.1885C>T in exon 8, were identified in these cases. A novel homozygous c.2534T>A mutation was also detected in the exon 8 of *WFS1* gene. Because of the rarity and heterogeneity of WS, detection of specific and nonspecific clinical signs including ocular findings and family history in non-autoimmune, insulinopenic diabetes cases should lead to a tentative diagnosis of WS. Genetic testing is required to confirm the diagnosis.
Keywords: Wolfram syndrome, *WFS1* gene, genetic testing

Introduction

Wolfram syndrome (WS), also named diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) syndrome, is a rare autosomal recessive disorder. The main clinical features of the syndrome, namely, diabetes insipidus (DI), diabetes mellitus (DM), optic atrophy (OA) and deafness (D), constitute the elements of the acronym DIDMOAD. Other common manifestations of the syndrome are urinary tract abnormalities (neurogenic bladder, hydroureteronephrosis), hypogonadism, progressive neurodegenerative diseases (ataxia, dementia), and psychiatric problems (1). The prevalence of the disease was estimated to be 1 in 770.000 individuals and 1 in 500.000 children less than 15 years old. However, the prevalence is reported to be as high as 1 in 68.000 in the Lebanese population where consanguineous marriages are common (2).

The syndrome is caused by a loss-of-function mutation in the *WFS1* gene which is located on chromosome 4p16.1 and consists of eight exons (1). *WFS1* gene encodes wolframin, an endoglycosidase H-sensitive transmembrane glycoprotein localized in the endoplasmic reticulum (ER).

Address for Correspondence: Gamze Çelmeli MD,
Akdeniz University Faculty of Medicine, Department of Pediatric Endocrinology, Antalya, Turkey
Phone: +90 242 249 65 47 **E-mail:** gcelmeli@hotmail.com

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Wolframin has a hydrophilic amino-terminus in cytosol and a carboxyl-terminus in the ER lumen. Wolframin is mainly expressed in pancreas, brain, heart, and muscle tissues. It plays a crucial role in the ability of ER to process and fold new proteins properly by regulating intracellular Ca^{2+} homeostasis (3).

WS is a progressive neurodegenerative disorder with a high mortality rate. The median age of death is around 30 years (range 25-49 years) (4). Clinical suspicion at an early stage is important for prompt diagnosis and proper management.

The aim of this paper was to present clinical and genetic characteristics of the syndrome as observed in 3 WS cases from 3 unrelated Turkish families, of which one had a novel homozygous missense mutation (c.2534T > A) and the other two had a previously described mutation.

Case Reports

Case 1

A 7.8-year-old female patient first presented to our pediatric endocrinology clinic with a diagnosis of type 1 DM, bilateral sensorineural deafness, and bilateral optic atrophy. The patient had been on insulin therapy for 10 months. Her parents were first cousins. Pancreatic autoantibodies including anti-glutamic acid decarboxylase antibody, anti-insulin antibody and anti-islet cell antibody, as well as thyroid and celiac antibodies were all negative.

When she was 10.4 years old, the patient was diagnosed to have partial central DI, detected by the water deprivation and desmopressin challenge test (DCT). At this time, the anterior pituitary gland was found to be of normal size and structure on cranial magnetic resonance imaging (MRI). Genitourinary tract ultrasound (US) revealed bilateral mild pelvicalyceal dilatation and severe distension of bladder. Post-void residual urine was 73 mL (high for age), and there was no sensation of urination indicating neurogenic bladder. Sublingual desmopressin replacement therapy was initiated.

Sequence analysis of the *WFS1* gene was performed, and a previously known homozygous splicing mutation (c.460 + 1G > A) was found in intron 4 (5,6,7). Her parents were heterozygous for the same mutation.

Case 2

An 8-year-old female patient was referred to our pediatric endocrinology clinic with symptoms of diabetic ketoacidosis. Her mother and father were first cousins. Her father has been suffering from bilateral deafness.

In further evaluations, pancreatic, thyroid, and celiac antibodies were found to be negative. Due to the continuation of polyuria and polydipsia in a state of normoglycemia, DCT was performed. A diagnosis of central DI was considered. Urodynamic examinations showed incomplete emptying of the bladder. Bilateral mild pelvicalyceal dilatation was detected in urinary US.

At age 10 years, the patient presented with complaints of decreased visual acuity and loss of color vision. Optic nerve atrophy was detected on ophthalmoscopy and optical coherence tomography. Left-sided sensorial D was detected by audiometry.

Genetic analysis identified a previously known homozygous missense mutation (c.1885C > T) in exon 8 (7,8,9,10,11). Both her parents were heterozygous for the same missense mutation.

Case 3

A 12.4-year-old female patient presented to our pediatric endocrinology outpatient clinic with a diagnosis of type 1 DM and a left-sided cataract diagnosed at ages 3 years and 12 years, respectively. Her parents were first cousins. Her maternal cousin had type 1 DM, DI, ataxia, and end-stage chronic renal failure due to neurogenic bladder and hydronephrosis. Another cousin had also type 1 DM, DI, and neurogenic bladder.

Left- and right-sided cataract surgery was performed at ages 12.5 and 13 years, respectively.

At age 16 years, partial central DI was detected by DCT. Bright spot was not present on the imaging of neurohypophysis by MRI. Bilateral sensorineural D was detected by audiometry. At age 19 years, bilateral OA was detected by routine ophthalmoscopic examination. When she was 20 years old, gabapentin was prescribed because of bilateral neuropathic pain along the peroneal nerves. Antidepressant therapy was given for a state of minor depression. Grade 2 hydronephrosis was detected when the patient was 24 years old.

Genetic analysis of the *WFS1* gene revealed a novel homozygous missense mutation in exon 8. Homozygous T to A exchange at nucleotide position 2534 (c.2534T > A) leads to an isoleucine to asparagine exchange at codon 845 (p.I845N) (Figure 1). Her mother and father were heterozygous for the same mutation. Her maternal cousins did not agree to a genetic analysis.

Clinical features and genetic analysis of the patients are shown in Table 1.

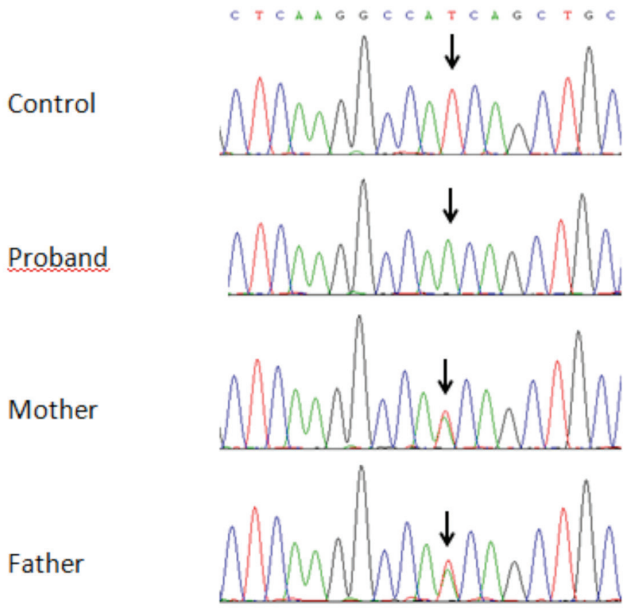

Figure 1. Electropherograms of mutant alleles in *WFS1* gene. The proband is homozygous for T to A transversion at nucleotide position 2534 (c.2534T > A) in exon 8 of *WFS1* causing isoleucine to be replaced by asparagine at codon 845 (p.I845N). Both parents are heterozygous for the same mutation (patient 3)

Discussion

WS is a rare neurodegenerative disorder. Juvenile-onset DM and OA are the prominent and earliest features of the disease in the pediatric age group (1). Only 14-58 % of the patients have all four components of DIDMOAD (4,12).

Insulin-dependent, non-autoimmune DM is often the first manifestation of WS which presents at an average age of 6 years (range from 3 weeks to 16 years) (1,4). It was shown that *WFS1* mutation increases ER stress, triggers the apoptotic pathway causing progressive β-cell loss, and impairs insulin secretion by disrupting intracellular Ca2 + homeostasis (13). When compared with type 1 DM, WS patients had a lower daily insulin requirement, lower hemoglobin A1c values, and a decreased tendency to develop ketoacidosis, findings attributed to the maintenance of some residual pancreatic β-cell mass (2,14). In the present cases, the age range at onset of diabetes was consistent with the literature. Only one of the patients (case 2) was admitted with ketoacidosis, which is a less common condition.

OA, a consistent finding in all patients, occurs at an average age of 11 years (range from 6 weeks to 19 years) with reduced visual acuity and loss of color vision (2). It is important to screen all patients with type 1 DM for OA to enable an early diagnosis of WS (1). Other less frequent ocular abnormalities reported are cataract (29.6-66.6 %), pigmentary retinopathy (30 %), diabetic retinopathy (7.6-34.6 %), pigmentary maculopathy, glaucoma, abnormal pupillary light reflexes, and nystagmus (1,14). It is striking that cataract can be the first ocular finding and that OA can develop later, as was the case in one of our patients (case 3). Although DM and OA association is the best diagnostic criterion for WS, WS should also be suspected in patients with non-autoimmune, insulin-deficient DM and in patients with atypical ocular abnormalities such as cataract.

In WS patients, central type DI becomes apparent in the second decade of life, at an average age of 14 years (range from 3 months to 40 years), with a frequency of 51-87 %. Previous studies have shown that gliosis, atrophy, and functional defects can be present in hypothalamic paraventricular and supraoptic nuclei (2).

Slowly progressive high-frequency sensorineural D is seen in 62 % of WS patients at an average age of 16 years (range 5-39 years) (4). Animal studies have shown wolframin expression involving ion homeostasis in inner ear cells (15). Audiometric testing enables an early diagnosis of sensorineural deafness.

Urinary tract abnormalities (neurogenic bladder, hydroureteronephrosis, and recurrent infections) are common findings (58 %) in WS, with a median age of onset of 20 years (range 10-44 years). Two of our patients developed neurogenic bladder and pelvicalyceal dilation in their first decade of life. Also, a maternal cousin of patient 3 was found to have end-stage chronic renal failure, secondary to neurogenic bladder and hydronephrosis, as a severe finding. Another cousin was reported to have neurogenic bladder. As renal failure is one of the important causes of death in WS, a careful assessment for urinary tract abnormalities and urinary infections are recommended (1,4).

In WS, neurological complications are reported to appear at a median age of 30 years (5-44 years) in 62 % of the cases. The most common symptom is truncal ataxia. Other common neurological signs are loss of gag reflex, loss of olfaction, myoclonus, epilepsy, nystagmus, and central apnea. The median age of death is 30 years (25-49 years) mostly due to neurological complications especially central respiratory apnea secondary to brain stem atrophy (2,4). MRI scans demonstrate generalized brain atrophy of visual pathways, cerebellum, brainstem, and cerebral cortex (1). One of our 3 patients (case 3) developed bilateral neuropathic pain in the legs at age 20 years. One of her maternal cousins who did not consent to molecular analysis had ataxia.

Psychiatric disease and behavioral disorders (severe depression, psychosis, organic brain syndrome, and impulsive verbal and physical aggression) are reported in

60% of WS patients (16). Because of the high incidence of suicidal behaviors, psychiatric consultation and follow-up is an essential part of the treatment of these patients (1). Minor depression was diagnosed in one of our three patients (case 3).

Other clinical findings, such as hypogonadism, pituitary hormone deficiency, gastrointestinal manifestations, and cardiac defects were not detected in our patients.

To date, over two hundred mutations, with a wide spectrum, have been reported in WS patients from different ethnic groups. In many patients, loss-of-function mutations such as stop, frame-shift, and splice site mutations were found. Missense mutations were reported in 35 % of the cases (8). Although there is no clear genotype/phenotype correlation, it was shown that harboring mutations other than missense ones lead to more severe disease and earlier onset of DM and OA (3).

In our second case, a previously reported homozygous missense mutation (c.1885C > T) in *WFS1* gene was found (7,8,9,10,11). In this pedigree, which was heterozygous for the mutation (p.R629W), bilateral D was present in the father. Kadayifci et al (9), who had first described this mutation, have reported that sensorineural D can be present in heterozygous carriers for *WFS1* gene mutation. It has been shown that heterozygous carriers in a WS family have an increased risk of the manifestations of WS, especially sensorineural deafness, psychiatric illness, and DM (9,10,17).

A novel homozygous missense mutation was identified in our third patient. The isoleucine residue at codon 845 is highly conserved across species and it is therefore likely that the p.I845N mutation is pathogenic. In silico analysis using sorting intolerant from tolerant and polymorphism phenotyping v2 predicted that the mutation affects the protein function and causes the damage.

In conclusion, by presenting these 3 patients, we would like to emphasize that WS is a clinically heterogeneous disease.

Table 1. Clinical and genetic features of the patients with Wolfram syndrome

	Case 1	Case 2	Case 3
Current age/Sex	10.8 years/female	12.6 years/female	28 years/female
Consanguineous marriage	Yes	Yes	Yes
Family history	Negative	Positive	Positive
DM/age of diagnosis	Type 1/7 years	Type 1/8 years	Type 1/3 years
OA/age of diagnosis	Bilateral/7 years	Bilateral/10 years	Bilateral/19 years
DI/age of diagnosis	Partial central/10.4 years	Complete central/8 years	Partial central/16 years
D/age of diagnosis	Bilateral sensorineural/7 years	Left-sided sensorineural/10 years	Bilateral sensorineural/16 years
Urinary tract abnormalities/age of diagnosis	Mild pelvicalyceal dilation, neurogenic bladder/10.4 years	Mild pelvicalyceal dilation, neurogenic bladder/8 years	Hydronephrosis/24 years
Neuropsychiatric abnormalities/ age of diagnosis	None	None	Bilateral neuropathic pain, minor depression/20 years
Other clinical features/age of diagnosis	None	None	Bilateral cataract/12 years
Type of mutation	Splice site	Missense	Missense
Position	Intron 4	Exon 8	Exon 8
Nucleotide change	c.460 + 1G > A	c.1885C > T	c.2534T > A
Amino acid change	-	p.R629W	p.I845N
Protein domain	-	Transmembrane	C-terminal

DM: diabetes mellitus, OA: optic atrophy, DI: diabetes insipidus, D: deafness

Its clinical signs can be seen at any age and its many features may escape from attention. As early diagnosis is important in order to handle the treatable complications of WS, routine ophthalmoscopic and urological evaluation is recommended in all patients with non-autoimmune, insulin-deficient DM at the time of diagnosis. A diagnosis of WS should be suspected in diabetic patients with uncommon associations such as D and ocular findings. Genetic mutation analysis plays a key role for definitive diagnosis and allows carrier detection.

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Ethics

Informed Consent: Written informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Gamze Çelmeli, Doğa Türkkahraman, Sema Akçurin, Design: Gamze Çelmeli, Doğa Türkkahraman, Yusuf Çürek, Sema Akçurin, İffet Bircan, Data Collection or Processing: Gamze Çelmeli, Doğa Türkkahraman, Yusuf Çürek, Analysis or Interpretation: Gamze Çelmeli, Jayne Houghton, Sema Akçurin, İffet Bircan, Literature Search: Gamze Çelmeli, Yusuf Çürek, Jayne Houghton, İffet Bircan, Writing: Gamze Çelmeli, Doğa Türkkahraman, Jayne Houghton, Sema Akçurin.

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CASE REPORT

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Testicular Adrenal Rest Tumor in Two Brothers with a Novel Mutation in the *3-Beta-Hydroxysteroid Dehydrogenase-2* Gene

Ayla Güven[1,2], Seher Polat[3]

[1]Göztepe Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
[2]Amasya University Faculty of Medicine, Department of Pediatrics, Amasya, Turkey
[3]Erciyes University Faculty of Medicine, Department of Medical Genetics, Kayseri, Turkey

What is already known on this topic?

Testicular adrenal rest tumors (TART) are usually seen in adolescents and adults with congenital adrenal hyperplasia (CAH) due to 21-hydroxlase deficiency.

What this study adds?

We presented two siblings with CAH due to novel mutation in 3β-*hydroxysteroid dehydrogenase* gene. To the best of our knowledge, TART development in children and infant with novel 3-Beta-Hydroxysteroid Dehydrogenase-2 mutation has not been reported previously.

Abstract

Testicular adrenal rest tumors (TART) occur frequently in adolescents and adults with 21-hydroxylase deficiency. There have been no reports of TART in children with 3β-hydroxysteroid dehydrogenase deficiency (HSD3β). Biopsy proven TART was diagnosed in a $3^{1/12}$-year-old male patient and also in his 22-month-old sibling. Hormonal and anthropometric measurements were performed during glucocorticoid and fludrocortisone treatment. The mutational analysis was performed by direct DNA sequencing of the complete coding region of the *HSD3β2* gene. Initially, both siblings were treated with high doses of hydrocortisone and fludrocortisone. TART regressed with dexamethasone treatment in both patients. However, growth velocity decreased and weight gain increased in both patients. Dexamethasone was changed to high-dose hydrocortisone (> 20 mg/m^2/d). Sequencing analyses revealed a novel homozygous p.W355R (c.763 T > C) mutation at exon 4 of the *HSD3β2* gene in both siblings. These two patients are, to our knowledge, the first known cases of TARTs with a novel mutation in the *HSD3β2* gene detected during childhood. High-dose hydrocortisone treatment is more reliable for TART in children.

Keywords: *HSD3β* gene, testicular adrenal rest tumor, congenital adrenal hyperplasia, 46,XY disorder of sex development

Introduction

Testicular adrenal rest tumors (TART) are frequently encountered in adult male patients with congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency (21-OHD). TART can also be detected in early childhood. The youngest CAH patient with TART reported in the literature was younger than 8 weeks old (1,2).

To the best of our knowledge, the two patients presented in this report are the youngest cases of 3-beta-hydroxysteroid dehydrogenase (HSD3β) deficiency associated with TART.

Case Reports

Case 1

A 17-day-old baby boy was referred to our Department from another clinic with hypospadias, left cryptorchidism, and bifid scrotum. His parents were second cousins. His birth weight was 3600 g and his length was 52 cm. Karyotype was 46,XY. Laboratory investigation was consistent with adrenal insufficiency: sodium (Na) 120 mEq/L, potassium (K) 6.9 mEq/L, chlorine (Cl) 96 mEq/L, adrenocorticotropic hormone (ACTH) 546 pg/mL, 17-hydroxyprogesterone

Address for Correspondence: Ayla Güven MD,
Göztepe Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
E-mail: aylaguven@yahoo.com

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(17-OHP) 29 ng/mL, and dehydroepiandrosterone sulfate (DHEAS) 1550 µg/dL. In addition to fluids and electrolytes, hydrocortisone (HC) (100 mg/m^2/d, divided into three doses, i.v.) and fludrocortisone (FC) (0.1 mgx2/d, p.o.) were added to the treatment. At age 6 months, HC and FC treatment was stopped for a week after which an ACTH-stimulation test was performed. The test results were consistent with HSD3β (Table 1). The parents were told to bring the patient for a follow-up visit every 3 months. During the follow-up period, the daily dose of HC was continued as higher than 13 mg/m^2 (p.o., divided into three doses, with the morning dose being highest) and the serum ACTH level was below 42 pg/mL. However, plasma renin activity (PRA) was higher for his age at 19 months old (77.6 ng/mL/hr, normal: 1.71-11.15). Despite strict treatment with FC, increased PRA levels for his age were found to persist.

The patient underwent a hypospadias and left orchidopexy operation at age 15 months. At 31/12 years of age, the right testicle volume was 4 mL. ACTH level was 42 pg/mL and PRA 29.1 pg/mL (1-6.5). Scrotal ultrasonography (USG) showed that the diameters for the right testicle were 33x15x13 mm (3.5 mL) and 11x11x6 mm (0.4 mL) for the left testicle. Heterogeneous parenchyma and multiple different-sized hypoechoic nodules associated with microcalcifications were noted in the right testicle. Although the increased right testicular volume was thought to be due to TART, the pediatric surgeon performed a biopsy to rule out a Leydig cell tumor. Pathologic examination of the right testicle revealed diffuse Leydig cell proliferation (Figure 1). Reinke crystalloids were not identified. Strong immunopositivity for inhibin B was detected in the tissue (Figure 2). TART was diagnosed, and HC was changed to dexamethasone (DEX) (0.5 mg/day, p.o. administered in a single late-evening dose). DEX was used to optimize medical treatment in a dosage equivalent to HC [20 mg of HC = 0.5 mg DEX (3)]. After 6 months of suppressive DEX treatment, growth velocity (GV) was 0.5 cm and the patient became obese [body mass index standard deviation score (BMI SDS) 2.77]. Therefore, DEX was changed to prednisolone (6 mg/m^2/day). Although GV of the patient improved with prednisolone treatment, the nodules failed to regress. During prednisolone treatment, ACTH and PRA were within normal ranges (19.2 pg/mL and 2.1 ng/mL/h, respectively). When he was 5 years old, although plasma ACTH was normal (8.8 pg/mL), PRA was increased (12.3 ng/mL/h, normal: 0.5-5.85) and high-dose HC (20 mg/m^2/day) was started again. To check the efficiency of the treatment, anthropometric measurements and physical examinations were performed and ACTH, 1.4 androstenedione, and testosterone levels were measured at three-month intervals. During the treatment, GV was calculated as 7.5 cm, 7.2 cm, and 5 cm for the first, second, and third years, respectively.

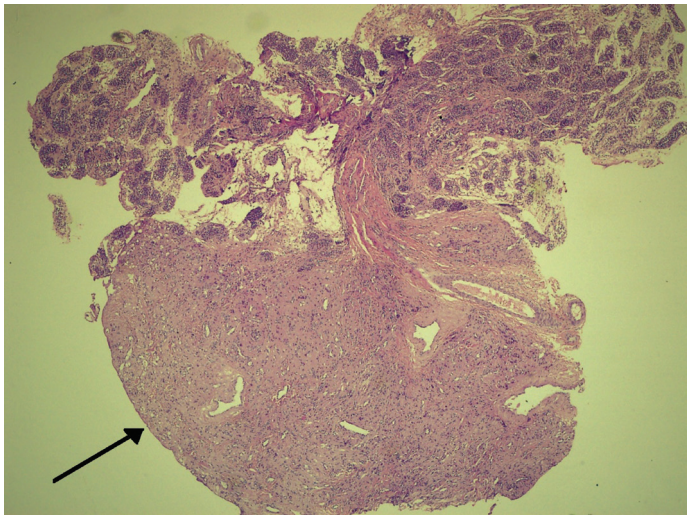

Figure 1. Leydig cell proliferation area (marked with a black arrow) and adjacent residual testicular parenchyma predominantly composed of Sertoli cells (HE x20)

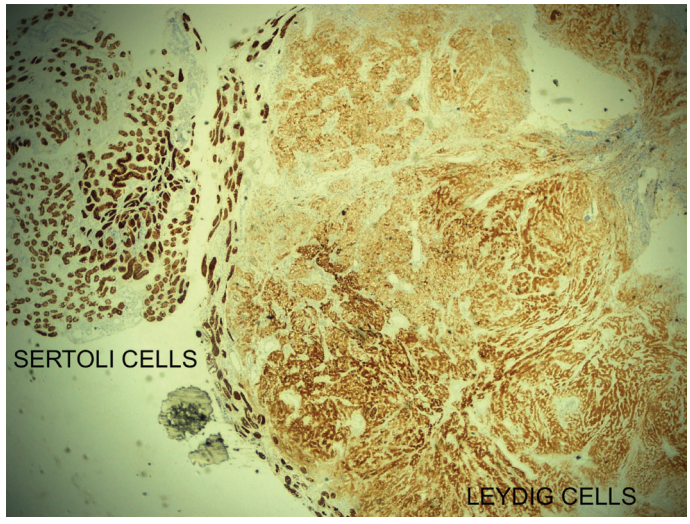

Figure 2. Leydig and Sertoli cells showed immunopositivity with inhibin B

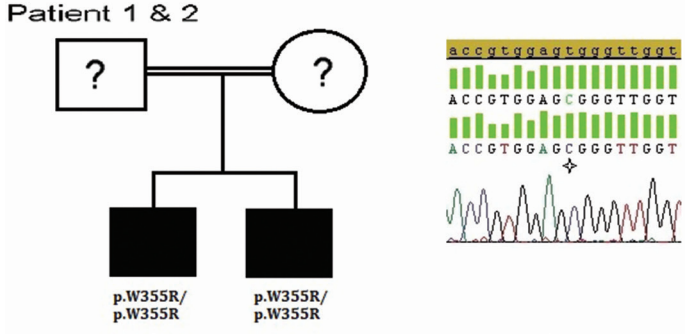

Figure 3. Molecular genetic analysis of the *HSD3β2* gene. Pedigree of the patients with electropherograms of the mutation [p.W355R (c.763 T > C)]. Star indicates mutated nucleotides. Question marks indicate individuals not available for genetic analysis

At 8 years of age, hypoechoic masses with irregular contour in both testicles were found in scrotal USG examination (34x14x9 mm in the right testicle and 13x8x4 mm in the left testicle). High-dose HC was switched to DEX. DEX and FC treatment was continued until the patient was 810/12-year-old, at which time, the DEX treatment was stopped and high-dose HC treatment was started again. At the last visit, he was $9^{4/12}$-year-old. Weight was 38.4 kg (1.22 SD), height was 137.2 cm (0.27 SD), BMI was 20.1 kg/m^2 (1.38 SD), and bone age was 10 years. The right testicle was 5 mL, left testicle was 2 mL. GV was 6.2 cm. A scrotal USG revealed regression of TART in the right testicle (hypoechoic mass 4x4x5 mm). The results of hormonal analyses at this time were: ACTH 49 pg/mL, androstenedione < 0.3 ng/mL, and PRA 3.1 ng/mL/h.

Case 2

This patient was the younger brother of the first patient. The patient was admitted to clinic for hypospadias at age 4 days. His birth weight was 3300 g and length was 51 cm. At admission, weight was 3000 g. The phallus was 2x1.5 cm on the dorsal side and 1.5x1.5 cm on the ventral side. Urethral meatus was opening to the base of the phallus. Bilateral testicles were detected in the bifid scrotum. The results of the ACTH-stimulation test performed at age 10 days revealed HSD3β deficiency (Table 1). The karyotype of the patient was 46,XY. HC and FC were started. His physical and laboratory examinations were repeated every 3 months. At age 22 months, both testes volumes were found to be increased for age (left 3 mL, right 5 mL). This finding was accompanied by a high plasma ACTH level (161 pg/mL, normal: 6-46) and a normal PRC level (16.8 ng/mL, normal: 1.71-11.15). Scrotal USG showed that the right testicle was 28x11x10 mm and the left one was 28x12x9 mm. Also, hypoechoic masses, 21x8x6 mm in diameter, were detected in the hilum in both testicles. TART was diagnosed, and the HC dose was increased to 20 mg/m^2/day. After 4 months, testicular size was reduced to 2 mL. The HC dose was decreased to 15 mg/m^2/day. The patient underwent a hypospadias operation at age 26/12 years. At age 211/12 years, both testicles were increased in size to 6 mL. HC was substituted with DEX in a dose of 0.5 mg/day (=30 mg/d HC). High-dose DEX treatment was given for three months, following which, the dose was decreased to 0.35 mg/d. DEX and FC treatment was maintained for 11 months, then, because the patient was becoming obese (BMI SD 3.87), the treatment was switched to high-dose HC (25 mg/m^2/day). During DEX treatment, GV was 5.4 cm/year. Bone age was 4 years. The efficiency of the treatment was also checked by measuring ACTH, 1.4 androstenedione, and testosterone levels at three-month intervals. On his last visit, the patient

Table 1. Adrenocorticotropic hormone-stimulation test results in the two patients (blood levels)

	ACTH pg/mL		Cortisol µg/dL		17-OHP ng/mL		1.4 AS ng/mL		Testosterone ng/mL		Progesterone ng/mL		DHEAS µg/dL	
	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2
Min														
0	> 1250	507	0.45	16.5	27.5	112	0.2	16.7	0.18	4.5	0.6	36.1	1550	3870
30		-	0.73	17.6	35.1	106	0.4	14.2	0.18	4.4	0.6	43.3	-	2985
Renin pg/mL	> 500	> 460												
Aldosterone pg/mL	-	466												

ACTH: adrenocorticotropic hormone, 17-OHP: 17-hydroxyprogesterone, AS: androstenedione, DHEAS: dehydroepiandrosterone sulfate

was $4^{8/12}$-year-old, his weight was 27 kg (2.73 SD), his height was 110.1 cm (0.69 SD), the volume of the right testicle was 3 mL, and that of the left testicle was 1 mL. During high-dose HC treatment, GV was 7.1 cm/ 9 months. Scrotal USG revealed a 2x2x1-mm hypoechoic mass in the right testicle. Hormonal analyses revealed an ACTH level of 7.3 pg/mL (< 46), serum 1.4 androstenedione level of < 0.3 ng/mL, and PRA was 8.06 ng/mL/h (0.5-5.85).

Hormonal Analysis

Morning blood samples were obtained from both siblings for basal androgens and precursor levels. ACTH-stimulation test (Synacten, 250 µg intravenously) was also performed. Total testosterone, DHEAS, cortisol, and ACTH were estimated using immunoenzymatic methods (Beckman Coulter, DXI 800 ,USA). 17-OH progesterone and 1,4 androstenedione, aldosterone, and PRA were measured using an Immunotech assay kit (Beckman Coulter) with radioimmunoassay method (ICN ISO DATA Gamma Counter).

Imaging Studies

The same investigator performed TART investigation by using ultrasonography and the Toshiba aplio XV 3.5 MHz convex probe. Measurements were performed separately for the right and left testicles. Testicular volume was calculated with the formula [(length X width (mm) × height (mm) × 0.523/1000 (mL)].

Mutational Analysis of *HSD3β2*

Blood samples for DNA analysis were obtained after having an informed consent from the parents. A standard protocol was followed for the preparation of genomic DNA from peripheral blood leukocytes. Exons II, III, and IV, and the exon-intron boundaries of the *HSD3β2* gene were amplified by polymerase chain reaction as described previously (4). The mutational analysis was performed by direct DNA sequencing of the complete coding region of the *HSD3β2* gene. The samples were electrophoresed on an automated sequencer (ABI3500) and analysed with the ABI SeqScape 3.7 software. Sequence variants were designated according to the recommendations of the Human Genome Variation Society (www.hgvs.org/rec.html) using the GenBank reference sequences. NC_000001.11 (HSD3β2 g.DNA), NM_000198.3 and (HSD3β2 c.DNA), NP_000189.1 (HSD3β2 p.protein).

Sequencing analyses revealed a novel homozygous p.W355R (c.763T > C) mutation (Figure 3) located at exon 4 of the *HSD3β2* gene in both siblings. Parents' DNA samples were not available for genetic studies.

Discussion

This report of two patients describes TART development in two brothers with an HSD3β2 homozygous mutation. Bilateral TART associated with large adrenal rest tumor located in the perirenal region was reported in an adult CAH patient with known HSD3β2 mutation (5). However, TART development in infants/children has not been reported previously.

TART is most frequently seen in adult males with CAH. Presence of adrenal gland masses in the testis in childhood was first demonstrated in 1940 in a 37/12-year-old child with CAH (6). The youngest patient reported in the literature was a 3-week-old infant who died due to Adrenogenital syndrome (7).

In almost all cases with TART, the tumor is associated with 21-OHD (1). Frequency of TART gradually increases during the pubertal period, and its prevalence in 21-OHD was reported as 28% in early puberty and as 100% at the end of puberty (8). On the other hand, TART is rarely reported in 11-hydroxylase-deficient patients (9). To the best of our knowledge, these two siblings presented here are the youngest cases of HSD3β2 mutation associated with TART.

The etiology and pathogenesis of TART in CAH patients are not completely understood. In the embryonic period, ectopic adrenocortical cells are neighbors of the testes. These cells, along with the testicles, can also be stimulated by ACTH during the descent of the testicles and are thought to have migrated into the scrotum during this early period (10).

It has been previously accepted that ACTH has the most important role in TART growth. Because these tumors usually develop in poorly-controlled patients, the increased ACTH is thought to stimulate the proliferation of these tumors. In fact, intensive glucocorticoid treatment may reduce the tumor size in most cases (10,11). However, occurrence of TART in well-controlled CAH patients and the observations that glucocorticoids in supra-physiologic doses cannot reduce these tumors in some patients are findings which indicate that other growth-promoting factors possibly play a role in the pathogenesis of TART (12).

It has been shown that CYP11B1 and CYP11B2's mRNAs involved in the synthesis of aldosterone are excessively expressed in TART (13,14). Also ACTH and mRNA expression of angiotensin II (AII) receptors in the adrenal rest tissues was detected (15). AII has a strong trophic effect on the zona glomerulosa and stimulates aldosterone synthesis (16). If efficient suppression of renin is not done, angiotensinogen I production could be stimulated and, in salt-wasting CAH

patients, AII synthesis could be increased. Consequently, ectopic steroidogenic cells in the testes would increase and could lead to TART.

Leydig cell tumor shows a similar histopathologic appearance with specific features of TART. Testicular tumor of CAH is frequently bilateral, but Leydig cell tumor is commonly unilateral.

Long-term and high-dose glucocorticoids (such as DEX) cause severe adverse effects such as obesity, osteoporosis, disorders in glucose metabolism, and growth retardation in children. On the other hand, high-dose glucocorticoids may suppress ACTH level and can cause TART regression (1,10). Single-dose DEX with or without additional HC doses associated with FC constitute the current treatment options for TART. Although some patients do not accept this treatment, still we need to use DEX to regress TART in some patients (10,11). On the other hand, these lesions may increase in size and number when glucocorticoid dose is decreased. Mineralocorticoid therapy is often underestimated in the management of TART, but the suppression of renin by adequate FC treatment, and thus contributing to reduced production of AII, is important in preventing TART development.

In both siblings, firstly, the HC dose was increased to more than 20 mg/m^2/d. However, the TART tissue did not show the expected regression, so HC was switched to DEX. Significant regression in TART tissue was obtained. During treatment with DEX, mild bone age advancement was noted in both patients. Growth rate decreased in both and they became obese. Therefore, DEX treatment was switched back to HC. Fortunately, normal GV was achieved with HC treatment in both siblings.

Although some authors recommend USG screening in all males with CAH from the age of 8, the age of TART screening in childhood is still unclear (17). We believe that regardless of age, all boys with CAH should undergo a careful physical examination, and when TART is suspected, scrotal USG examination should be performed. TART needs to be recognized at an early stage to prevent the adverse effects of aggressive glucocorticoid therapy.

The novel p.W355R mutation located in the C-terminal part of the protein and *in vitro* expression studies related with C-terminal part of the protein showed that the two truncated p.R335X, p.W355X mutant proteins yielded absent conversion of pregnenolone and dehydroepiandrosterone (DHEA), whereas the missense mutation p.P341L showed a residual DHEA conversion of 6% of wild-type activity. It was thus concluded that C-terminal mutations of the *HSD3β2* gene are responsible for classical *HSD3β2* deficiency due to putative structural alteration of the HSD3β2 protein and that this process is further aggravated by increased protein degradation (18). In our cases, two siblings with p.W355R mutation showed classical *HSD3β2* deficiency; therefore our study also shows that the C-terminal part of the protein must be important for correct enzymatic function.

Our observations in these two patients suggest that TART may develop at any age in CAH patients. In addition, these brothers are also interesting in that they are the youngest cases with novel HSD3β2 mutation reported in the literature.

Ethics

Informed Consent: Blood samples for DNA analysis were obtained after having an informed consent from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ayla Güven, Design: Ayla Güven, Data Collection or Processing: Ayla Güven, Analysis or Interpretation: Ayla Güven and Seher Polat, Literature Search: Ayla Güven, Writing: Ayla Güven and Seher Polat.

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CASE REPORT

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A Rare Cause of Short Stature: 3M Syndrome in a Patient with Novel Mutation in *OBSL1* Gene

Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya

Dr. Sami Ulus Obstetrics and Gynecology and Pediatrics Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey

What is already known on this topic?

The 3M syndrome is a rare autosomal disorder that can lead to short stature, dysmorphic features, and skeletal abnormalities with normal intelligence levels. The 3M syndrome is caused by loss-of-function mutations in the genes encoding cullin 7, obscurin-like 1, and coiled-coil domain containing protein 8.

What this study adds?

The 3M syndrome may be a significant cause of short stature with prenatal onset in geographical regions where kin marriage is practiced extensively. Implementing frequent genetic screening for possible cases may help to identify novel mutations.

Abstract

The Miller-McKusick-Malvaux (3M) syndrome is a rare autosomal disorder that can lead to short stature, dysmorphic features, and skeletal abnormalities with normal intelligence. A 16-month-old female patient had been referred to our clinic due to short stature. Case history revealed a birth weight of 1740 grams on the 39th week of gestation, with a birth length of 42 cm and no prior hereditary conditions of clinical significance in her family. On physical examination, her length was 67 cm [-3.6 standard deviation (SD) score], weight 7.2 kg (-2.9 SD score), and head circumference 42 cm (below 3rd percentile). She also had numerous characteristic physical features such as a triangular face, fleshy nose tip, a long philtrum, prominent mouth and lips, pointed chin, lumbar lordosis, and prominent heels. As her growth retardation had a prenatal onset and the physical examination results were suggestive of a characteristic profile, the diagnosis of 3M syndrome was strongly considered. Genetic assessment of the patient revealed a novel homozygous p.T45Nfs*40 mutation in the *OBSL1* gene. It is recommended that physicians pay further attention to this condition in the differential diagnosis of children with severe short stature.

Keywords: Childhood, short stature, genetic syndromes

Introduction

The Miller-McKusick-Malvaux (3M) syndrome is characterized by dysmorphic features, skeletal abnormalities, and severe prenatal as well as postnatal growth retardation with normal intelligence (1). The nomenclature 3M was derived from the initials of the surnames of three researchers who first identified the condition: namely, Miller, McKusick, and Malvaux (2). Although 3M syndrome is considered to be a relatively uncommon disorder, it is thought to possibly be an under-diagnosed condition (1).

The 3M syndrome is caused by loss-of-function mutations in the genes encoding cullin 7 (*CUL7*), obscurin-like 1 (*OBSL1*), and coiled-coil domain containing protein 8 (*CCDC8*). *CUL7* appears to be the major gene responsible for 77% of 3M syndrome, while *OBSL1* mutations account for a relatively small percentage of 16% (3). *CUL7* encodes for the *CUL7* protein that is a scaffold protein forming part of an E3 ubiquitin ligase enzyme responsible for cytoplasmic protein degradation. *OBSL1* encodes for a cytoskeletal adaptor protein which is localized within the prenuclear region. The function of *CCDC8*, on the other

Address for Correspondence: Melikşah Keskin MD, Dr. Sami Ulus Obstetrics and Gynecology and Pediatrics Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey **Phone:** +90 312 305 65 11 **E-mail:** meliksah.keskin@hotmail.com

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hand, is unknown. However, the protein it encodes for binds to *OBSL1* protein and is required for p54-mediated apoptosis in cells. Detailed mechanisms underlying the growth impairments seen in the 3M syndrome remain largely unclear. On the other hand, abnormalities in basic cellular growth as well as alterations in cellular response profiles to growth factor stimulations are likely candidates for causal processes (1). There is no specific treatment for 3M syndrome (3). However, variable responses to growth hormone (GH) affected by the genotype, showing a better outcome in patients with *CCDC8* mutations in contrast to *OBSL1* mutations, have been reported too (4). Our patient, who demonstrated a previously unidentified mutation on *OBSL1* gene, was a one year and four months old female patient with a diagnosis of 3M.

Case Report

A 16-month-old female patient was admitted to our clinic with a complaint of short stature. Her case history revealed that she was born as the first child of her consanguineous parents. The patient was born on the 39th week of gestation with a birth weight of 1740 grams and birth length of 42 cm. There is no known prior case of hereditary disease in her family. On physical examination, the patient's length was 67 cm [-3.6 standard deviation (SD) score], her body weight was 7.2 kg (-2.9 SD score), and head circumference 42 cm (below 3rd percentile). Her features consisted of a triangular face, a fleshy nose tip, a long philtrum, a prominent mouth and lips with a pointed chin alongside, lumbar lordosis, and prominent heels. Maternal body height was 164 cm and paternal height was 187 cm. Midparental height was estimated as 169 cm (+1 SD score). No noticeably short individual was reported in the family. The patient started forming syllables and walked approximately at one year of age. Laboratory analyses showed normal complete blood cell counts with normally functioning kidneys and liver. The patient was euthyroid and serological analyses for gluten-sensitive enteropathy were negative. Her bone age was in line with her chronological age. Her karyotype was 46,XX and bone X-rays revealed a lumbar lordosis (Figure 1). Echocardiographic findings were normal. Serum insulin-like growth factor 1 (IGF-1) level was 47 ng/mL (<-2 SD score) and serum IGF binding protein-3 (IGFBP-3) level was 2800 mg/mL (between +1/+2 SD score). L-dopa stimulation test scores revealed a peak GH response of 3.7 ng/mL.

Our patient had prenatal growth retardation and a significantly short stature in addition to triangular face, a long philtrum, a fleshy nose tip, prominent mouth and lips with pointed chin (Figure 2), as well as lumbar lordosis which collectively suggested a syndromic short stature. Drawing upon our prior experience with two sibling patients who were diagnosed with the 3M syndrome after a long period of undiagnosed clinical monitoring, we considered a 3M diagnosis to be appropriate for our current patient as well. The following genetic assessment, through the whole gene sequencing method, revealed a homozygous p.T45Nfs*40 (c.1273 dupA) mutation of *OBSL1* gene which led to diagnosis of 3M syndrome. Parental genetic analysis with same method also revealed that both of the parents had heterozygous mutations on *OBSL1*.

Clonidine stimulation test was planned as part of a secondary GH stimulation test. However, in view of the genetic profile of the patient (leading us to 3M diagnosis) and previous studies suggesting a degree of GH resistance as well as GH deficiency being possibly related with the 3M syndrome, and previous studies reporting that the 3M syndrome may be associated with the dysregulations of GH, IGF1, and IGF binding proteins, we refrained from applying the clonidine stimulation test to our patient (5).

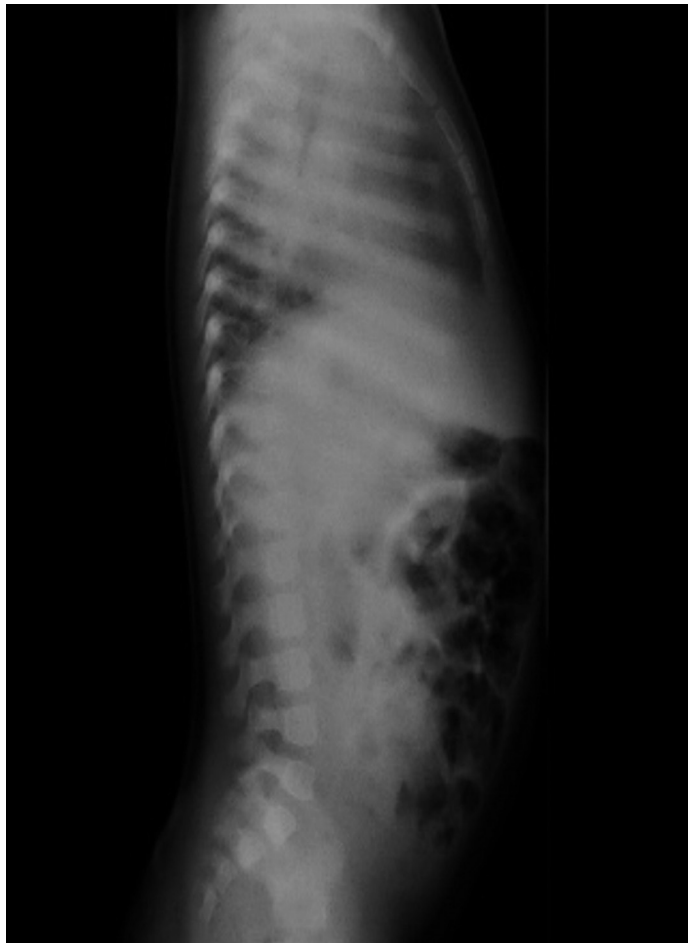

Figure 1. Radiography scan revealed mild reduction in thoracic vertebrae corpus height, with irregularities in upper and lower plateau as well as lordosis

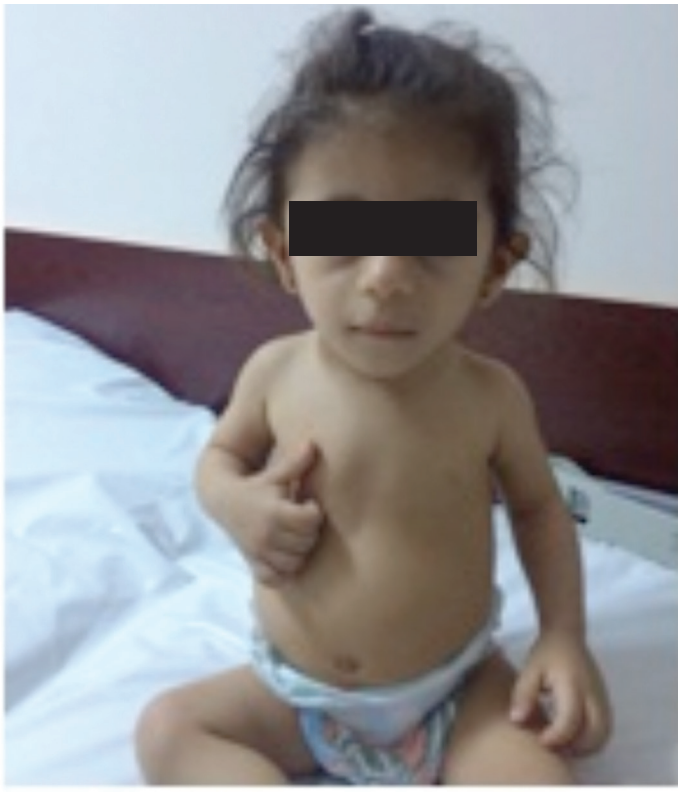

Figure 2. Our patient with her triangular face, long philtrum, fleshy nose tip, prominent mouth and lips and her pointed chin

Hence, following the consent of her parents, a 0.25 mg/kg/week dose of GH was initiated. At the end of the first 3 months after treatment initialization, the patient showed a 4.5 cm growth, and this was followed by an additional 2.5 cm growth in the following three months period. On the 6th month of treatment, serum IGF-1 level was 272 ng/mL (+1/+2 SD score) and IGFBP-3 level was 4.87 µg/mL (+1/+2 SD score). The patient is currently under clinical observation and is being treated by a 0.25 mg/kg/week dose of GH.

Discussion

The 3M syndrome is a clinical condition which is often not diagnosed during childhood (2). Here, we describe a 16-month-old female patient with the diagnosis of 3M syndrome. This individual applied to our clinic due to complaints of pre- and post-natal growth retardation. 3M diagnosis was considered following the clinical assessment for short stature. However, the 3M syndrome can appear with mild symptomatology and is a difficult condition to identify via differential diagnosis as short stature is known to have a wide variety of causal factors (3). We consider this to be the main reason for poor numbers of 3M diagnoses and significantly delayed diagnoses for those with the syndrome (5). This is a situation that can be harmful for prognosis, as an early diagnosis is crucial for genetic counselling since 3M syndrome is inherited as an autosomal recessive disorder (2).

The 3M syndrome is causally linked with the mutations on the genes *CUL7*, *OBSL1*, and *CCDC8*. A previous study showed that patients who were diagnosed with the 3M syndrome, having mutations on these genes, tended to be shorter to the degree of a -5.7 SD score for *CUL7*, a -4.7 SD score for *OBSL1*, and a -4.1 SD score for *CCDC8* (6). *OBSL1* gene mutations are the underlying causes for approximately 20% of the 3M syndrome patients (3). Mutation types reported include insertion, deletion, and substitution of nucleotides, all appearing on the first eight exons encoding for Ig domains of *OBSL1* proteins (7). The c.1273insA (p.T245fs*40) mutation had been identified as the prevalent mutation for the *OBSL1* gene in 12 of 23 families that had undergone screening (8). In our case, on the other hand, we observed that a novel frameshift mutation on *OBSL1* caused the 3M syndrome. Currently, little is known about the specific functions of *OBSL1*; yet, it was suggested that the *OBSL1* protein functions as a cytoskeletal adaptor protein linking the nuclear proteins to the cytoplasmic support network. Additionally, *OBSL1* was also found to be expressed in a wide variety of cell types, suggestive of its role as a scaffolding protein (9). In addition, alterations in IGFBP-2 and IGFB5 messenger ribonucleic acid levels were previously documented to be associated with *OBSL1* mutations in cases with 3M syndrome diagnoses (7).

There is no specific treatment for 3M syndrome (3). However, the use of recombinant human GH for the treatment of short stature was suggested (7). Previous studies suggested a degree of GH resistance as well as GH deficiency being possibly related with the 3M syndrome, and it was also reported that the 3M syndrome may be associated with the dysregulations of GH, IGF1, and IGF binding proteins (5). Significant individual variations were also reported in relation to GH responses and some studies also suggest that GH may be helpful in the treatment of the syndrome (10). On the other hand, according to various other reports, GH treatments have no effect on patients with the 3M syndrome (7,11). Even though GH treatment outcomes for the 3M syndrome appear controversial, we decided to initiate GH treatment since the expected final height of our patient appeared to be relatively short in view of previous literature about this syndrome. At the end of the first six months post-initiation with a 0.25 mg/kg/week dose, our patient demonstrated a 7-cm growth increment. Even though the duration of clinical observation was inadequate, the finding

of a sufficient rate in growth for that specific duration was satisfactory in deciding on a close clinical observation of serum IGF-1 and IGFBP-3 levels for the remaining duration of treatment.

The 3M syndrome may be more frequent than thought in countries such as Turkey, where kin marriage is a frequent practice. Pointing out this fact may help clinicians working in Turkey or other countries with similar practices to consider this syndrome in the diagnostic work-up of their future patients, hence revealing increasing numbers of cases in the near future. Our experience with previous 3M diagnoses is an example to this fact, as our case, which can be identified as a case of early diagnosis, could have been diagnosed mainly with respect to our prior knowledge regarding 3M syndrome. It was previously reported that final heights of the 3M syndrome patients range between 115 and 150 cm, which can lead to significant degrees of disadvantage in these individuals' lives (4). Since our patient is of a very young age, we postulate on the possibility that the initiation of GH treatment can, with a high chance, lead to a near-normal body height. Due to the fact that 3M syndrome is inherited via an autosomal recessive pattern, early genetic assessment leading to an early diagnosis can also aid in the genetic counselling for the rest of family members. In conclusion, 3M syndrome needs to be considered in the differential diagnosis of patients with growth failure, especially those with prenatal onset and characteristic symptoms.

Ethics

Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya, Design: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya, Data Collection or Processing: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya, Analysis or Interpretation: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya, Literature Search: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya, Writing: Melikşah Keskin, Nursel Muratoğlu Şahin, Erdal Kurnaz, Elvan Bayramoğlu, Şenay Savaş Erdeve, Zehra Aycan, Semra Çetinkaya.

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